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AMINO FUNCTION AND SIDE CHAIN MODIFICATIONS OF AMINO ACIDS, AND SYNTHESIS OF THROMBIN INHIBITING PEPTIDES.

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A.A. 2008

A Maria Luisa, Francesca e Monica....

a Domenico...

ai miei genitori...

...a Giorgia...la speranza nel futuro.

PART OF THIS THESIS PUBLISHED

A. Publications

1. Di Gioia, M. L.; Leggio, A.; Le Pera, A.; Liguori, A.; Perri, F.; Viscomi M.C. "Reduction of *N*-methoxy-*N*-methylamides to the corresponding amines with $AlCl_3/LiAlH_4$" *Letters in Organic Chemistry*, **2006**, 3, 468-469;
2. Belsito, E.; Di Gioia, M. L.; Greco, A.; Leggio, A.; Liguori, A.; Perri, F.; Siciliano, C.; Viscomi, M. C. "*N*-methyl-*N*-Nosyl-β^3-amino acids" *J. Org. Chem* **2007**, *72*, 4798-4802;
3. Belsito, E.; Carbone, C.; Di Gioia, M. L.; Leggio, A.; Liguori, A.; Perri, F.; Siciliano, C.; Viscomi, M. C. "Comparison of the volatile constituents in cold-pressed bergamot oil and volatile oil isolated by vacuum distillation" *J. Agric. Food Chem.* **2007**, *55,* 7847–7851;
4. Di Gioia, M. L.; Leggio, A.; Liguori, A.; Perri, F.; Siciliano, C.; Viscomi, M. C. "Preparation of *N*-Fmoc-*N*-methyl-α-amino acids and *N*-Nosyl-*N*-methyl-α-amino acids" *Amino Acids* **2008**, *0*; 000.

B. Poster presentations

1. Di Gioia, M. L.; Leggio, A.; Le Pera, A.; Liguori, A.; Napoli, A.; Siciliano, C.; Viscomi, M.C. "La preparazione dell'ornitina sulfamoilata e la sua incorporazione in catene oligopeptidiche con potenziale attività inibitoria della trombina" *XXIX Convegno Nazionale della Divisione di Chimica Organica della Società Chimica Italiana*, Potenza, 31 agosto-4 settembre **2004**;
2. Di Gioia, M. L.; Leggio, A.; Le Pera, A.; Liguori, A.; Napoli, A.; Siciliano, C.; Viscomi, M.C. "Un nuovo amminoacido non proteinogenico per la preparazione di inibitori oligopeptidici della trombina: l'ornitina sulfamoilata" *Itinerari di Chimica in Calabria 6*, Arcavacata di Rende, 21-22 ottobre **2004**;

3. Leggio, A.; Liguori, A.; Siciliano, C.; Viscomi, M.C. "L'impiego di ornitine modificate nella preparazione di sistemi tripeptidici analoghi del PPACK, potenziali inibitori diretti della trombina" *XXX Convegno Nazionale della Divisione di Chimica Organica della Società Chimica Italiana*, Siena, 19-23 settembre **2005**;
4. Liguori, A.; Di Gioia, M. L.; Leggio, A.; Greco, A.; Perri, F.; Siciliano, C.; Viscomi, M.C. "Omologazione di *N*-Nosil-α-amminoacidi ad *N*-metil-β^3-amminoacidi" *Convegno Congiunto Delle Sezioni Calabria e Sicilia della Societa' Chimica Italiana*, Palermo 4-5 dicembre, **2006**, pp. 40-41;
5. Di Gioia, M. L.; Leggio, A.; Liguori, A.; Viscomi, M. C. "Sintesi di *N*-metil-α-amminoacidi in soluzione" *XXXI Convegno Nazionale della divisione di Chimica Organica della Società Chimica Italiana*, Rende 10-14 Settembre **2007**;
6. Di Gioia, M. L.; La Monica, A.; Leggio, A.; Liguori, A.; Perri, F.; Siciliano, C, Viscomi, M.C. "Aldeidi tipo Garner ad elevata stabilità chinale ottenute da L- e D-Serina" *Convegno Congiunto Delle Sezioni Calabria e Sicilia della Societa' Chimica Italiana*, Messina 4-5 dicembre, **2007**.

C. Oral presentation

1. De Marco, R.; Di Gioia, M. L.; Leggio, A.; Liguori, A.; Perri, F.; Siciliano, C, Viscomi, M.C. "*N*-Fmoc-*N*-metil-a-amminoacidi per la sintesi peptidica" *Convegno Congiunto Delle Sezioni Calabria e Sicilia della Societa' Chimica Italiana*, Messina 4-5 dicembre, **2007**.

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SINTESI DEL LAVORO DI TESI

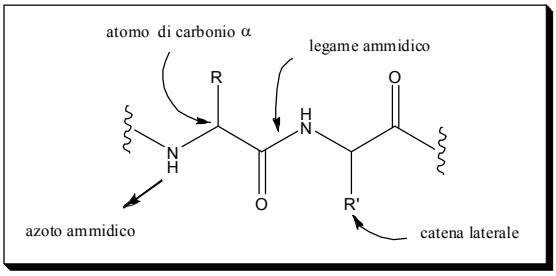
I peptidi regolatori costituiscono un'importante classe di molecole di natura non strutturale e non enzimatica, distribuite ubiquitariamente nell'organismo, che controllano varie funzioni cellulari, quali l'eccitabilità, la crescita, la permeabilità, l'espressione genica e il differenziamento cellulare.[1] Tali molecole inoltre, legandosi ai corrispondenti recettori, influenzano la comunicazione tra le cellule e regolano svariate funzioni vitali come il metabolismo, le difese immunitarie e la riproduzione.

Sebbene i peptidi naturali attivi abbiano un grande potenzialità di applicazione medica, essi richiedono spesso di essere modificati per bypassare i problemi inerenti i parametri farmacocinetici.[2] Tra le proprietà attese, ma spesso non presenti o non ottimizzate nel ligando naturale, assumono particolare importanza: la selettività accettore/recettore; l'elevata potenza; la stabilità contro l'inattivazione proteolitica, la biodistribuzione e la biodisponibilità.

Con questi presupposti la ricerca sui peptidomimetici nelle ultime due decadi ha visto un drammatico avanzamento nell'intento di incrementare l'attività biologica e farmacologica degli analoghi naturali, apportando appropriate modifiche strutturali sullo scheletro delle catene peptidiche e/o sui residui amminoacidici costituenti (Figura 1).

Figura 1.

Elementi di un peptide suscettibili di modifica

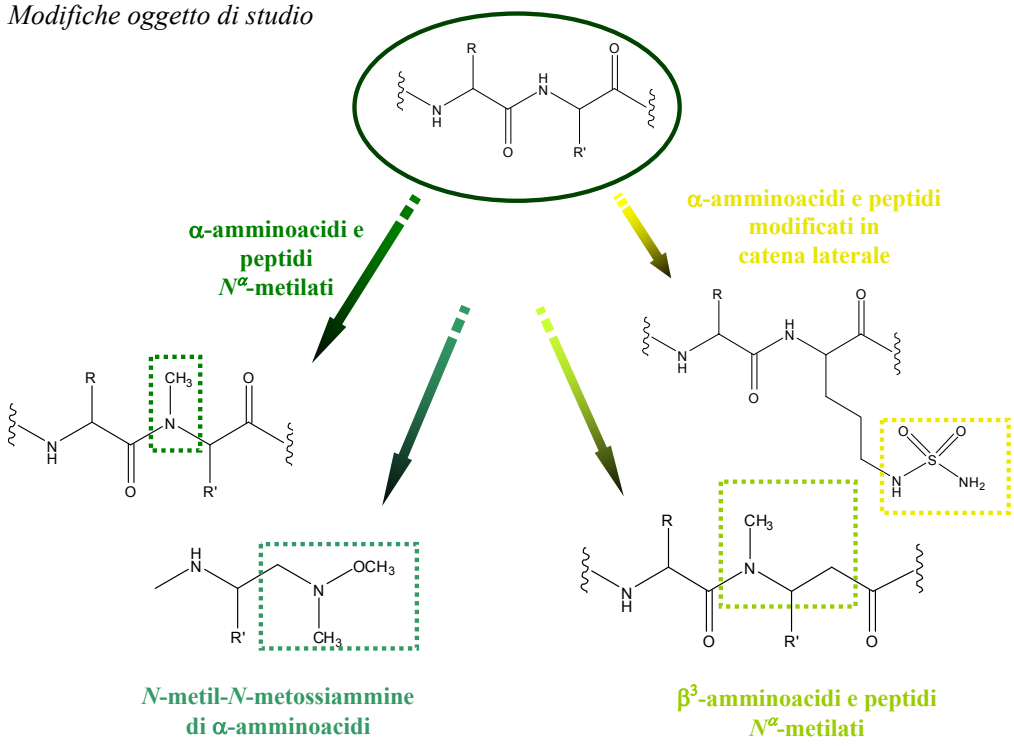


In questo contesto viene a collocarsi parte dell'attività di ricerca svolta nel triennio di dottorato. In particolare l'attenzione è stata focalizzata su tre punti (Figura 2):

1. sintesi di α-amminoacidi non naturali conformazionalmente ristretti (N^{α}-metilati) diversamente protetti sulla funzione amminica, da usare come building blocks nella sintesi peptidica;
2. sintesi di N^{α}-metil-β^3-amminoacidi da usare come building blocks nella sintesi peptidica;
3. trasformazione della funzione carbossi-terminale di amminoacidi in funzionalità *N*-metil-*N*-metossiamminica;
4. sintesi di amminoacidi modificati in catena laterale bioisosteri dell'arginina, per la sintesi di una nuova classe di inibitori della trombina.

Figura 2.

Modifiche oggetto di studio



L'incorporazione di amminoacidi *N*-sostituiti in catene peptidiche naturali permette di migliorare in maniera sostanziale l'attività biologica degli stessi peptidi. La sostituzione all'azoto blocca infatti i potenziali siti di legame ad idrogeno intramolecolari ed i siti di scissione di enzimi proteolitici, conferendo al peptide una minore flessibilità e modificandone di conseguenza l'intensità dell'azione biologica.[3]

Sistemi peptidici contenenti residui amminoacidici *N*-metilati hanno mostrato importanti attività farmacologiche di tipo antibiotiche,[4] anticancro[5] ed immunodepressive.[6]

Tuttavia, a causa della complessità della sintesi,[7] solo pochi *N*-metil amminoacidi sono disponibili in commercio, e la loro manipolazione introduce problematiche di racemizzazione che ad oggi non sono state pienamente risolte.

Tra le metodologie più utilizzate per l'ottenimento di amminoacidi e peptidi metilati all'azoto,[8] molte prevedono l'alchilazione di solfonammidi,[9] l'amminazione riduttiva[10], che comporta la riduzione di basi di Schiff intermedie utilizzando reazioni diverse. Tali metodiche sperimentate per la sintesi di *N*-metil amminoacidi otticamente attivi sono però spesso caratterizzate da condizioni drastiche di reazione e da assenza di generalità della procedura legata a bassa reattività o a racemizzazione.[11]

Recentemente nel laboratorio in cui è stato svolto il presente lavoro di ricerca, è stata realizzata una procedura estremamente vantaggiosa per la metilazione all'azoto di esteri metilici di α-amminoacidi[12] e peptidi,[13] in soluzione ed in fase solida,[14] basata sull'impiego del gruppo protettore *p-nitrobenzensolfonile* (Nosile) per la funzione amminica[15] e del *diazometano* come agente alchilante.[16]

La metilazione di amminoacidi e peptidi Nosil-protetti porta prevedibilmente alla metilazione dell'azoto solfonammidico come evento successivo alla formazione dell'estere metilico sulla funzione

carbossilica. La deprotezione della funzione metilesterea, necessaria per le procedure di sintesi peptidica, risulta però di difficile attuazione, e spesso è foriera di racemizzazione sullo stereocentro dell'amminoacido.[17]

La disponibilità di amminoacidi *N*-metil sostituiti, Nosile e Fmoc protetti, costituisce pertanto un notevole avanzamento per ciò che riguarda la disponibilità di tali substrati da impiegare in maniera agevole nella sintesi di catene peptidiche contenente amminoacidi *N*-metilati secondo le procedure della *Nosil-chemistry* e della *Fmoc-chemistry*, sia in soluzione che in fase solida.

Un primo obiettivo è stato l'ottenimento di *N*-metil-α-amminoacidi, Nosile e Fmoc protetti sulla funzione amminica, utilizzando la procedura di metilazione dell'azoto solfonammidico mediante diazometano. Il conseguimento di tale obiettivo ha richiesto una protezione preliminare, transiente e facilmente rimovibile della funzione carbossilica degli *N*-Nosil-α-amminoacidi. E' parsa opportuna la protezione della funzione acida come *estere difenilmetilico* (Benzidrile),[18] realizzata mediante difenildiazometano.[19] Il difenildiazometano, un solido cristallino dal caratteristico colore rosso, si ottiene facilmente attraverso una semplice reazione di ossidazione con Magtrieve™ dell'idrazone del benzofenone. La reazione del diazoalcano, con la funzione carbossilica è rapida e quantitativa, e visivamente si osserva la repentina decolorazione della soluzione. Un ulteriore vantaggio nell'impiego di tale gruppo protettore è la possibilità di lavorare con prodotti analizzabili mediante GC/MS ed agevolmente identificabili per la presenza di picchi caratteristici negli spettri di massa.

Gli amminoacidi Nosil-protetti **1a-f** sono stati convertiti quantitativamente nei corrispondenti esteri benzidrilici per trattamento con una soluzione in diclorometano di difenildiazometano (**2**), nel rapporto di 1.05:1 rispetto al substrato di partenza (Schema 1, Tabella 1). Gli amminoacidi totalmente protetti **3a-f** sono stati quindi sottoposti a

reazione di metilazione all'azoto per trattamento con diazometano: il semplice allontanamento del solvente e dei reagenti in eccesso mediante evaporatore rotante permette il recupero dei prodotti *N*-metilati **4a-f** con rese quantitative. Gli esteri **4a-f** sono i precursori chiave per l'ottenimento tanto degli *N*-metil-*N*-Nosil-α-amminoacidi che degli *N*-Fmoc-*N*-metil-α-amminoacidi.

Il trattamento acidolitico di **4a-f** con una soluzione di acido trifluoroacetico a temperatura ambiente, e le usuali operazioni di work-up hanno portato al recupero degli attesi building blocks **5a-f** con rese elevate (Tabella 1).

Schema 1.

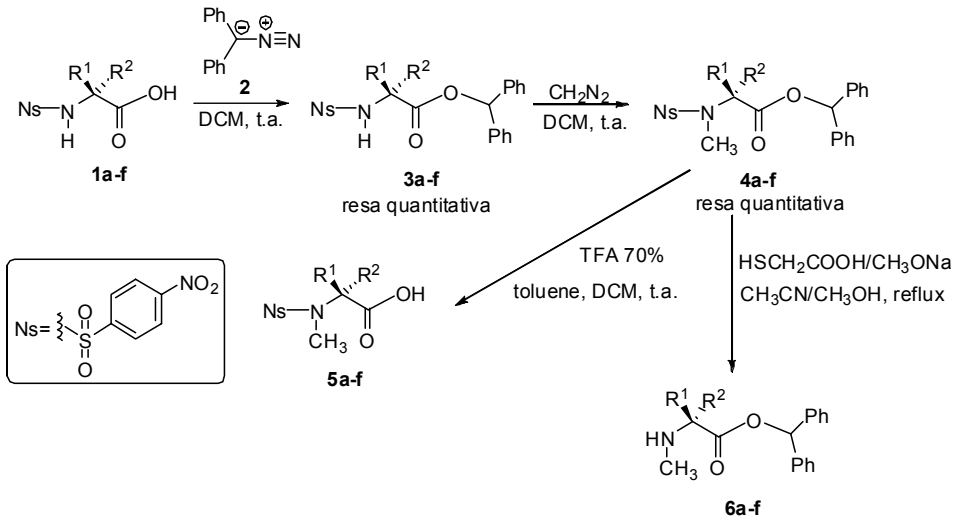


Tabella 1.

Sistemi impiegati

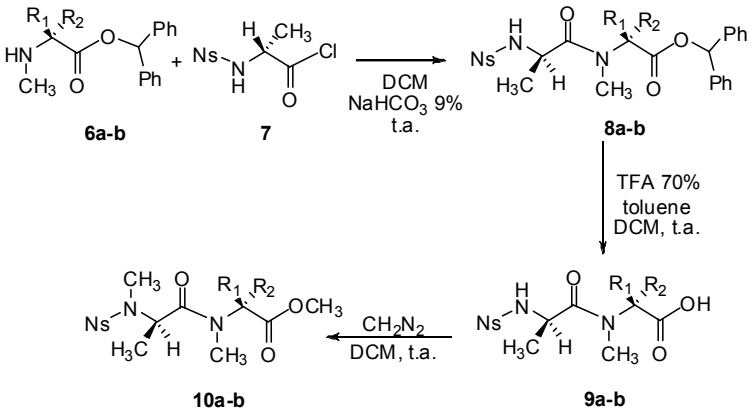
	R^1	R^2	5 resa (%)[a]	6 resa (%)[a]
a	$-CH(CH_3)_2$	-H	94	96
b	-H	$-CH(CH_3)_2$	94	96
c	$-CH_2CH(CH_3)_2$	-H	94	95
d	$-CH_2S(Bzl)$	-H	95	92
e	$-CH(CH_3)CH_2CH_3$	-H	98	95
f	$-CH_3$	-H	98	90

[a] composti isolati

Un cammino alternativo che parte dagli esteri **4a-f** è stato individuato per l'ottenimento di *N*-Fmoc-*N*-metil-α-amminoacidi. La nuova procedura prevede la formazione come intermedi di reazione degli *N*-metil-amminoacidi benzidrilesteri **6a-f** ottenuti per trattamento di **4a-f** con il sistema reagente acido mercaptoacetico/metossido di sodio in acetonitrile e metanolo alla temperatura di riflusso.

L'integrità stereochimica dei composti **6a-f** è stata ampiamente studiata convertendo gli esteri benzidrilici **6a-b** nei corrispondenti dipeptidi diastereoisomerici **8a-b**: i dipeptidi sono stati preparati attraverso reazione di accoppiamento con il cloruro della *N*-Nosil-D-alanina (**7**) nelle condizioni bifasiche dettate dalla reazione di Schotten-Baumann (Schema 2).[20] L'analisi ^{1}H-NMR effettuata su campioni dei grezzi di reazione dei dipeptidi **8a** e **8b**, conferma la presenza in ciascun campione di un solo epimero, dimostrando in maniera inequivocabile che la stereochimica dei centri chirali è mantenuta nel processo di metilazione e successiva deprotezione della funzione amminica.

Schema 2.



6a-b **7** **8a-b** **9a-b** **10a-b**

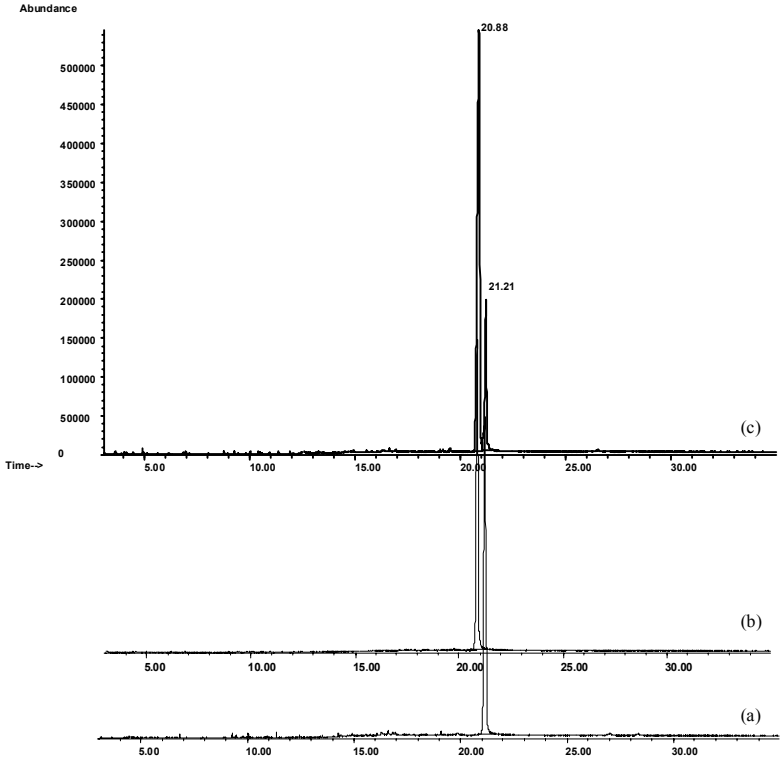
Al fine di effettuare una ulteriore verifica, i dipeptidi **8a** e **8b** sono stati sottoposti ad acidolisi nelle condizioni precedentemente descritte, e

successivamente derivatizzati mediante trattamento con diazometano che reagendo sia all'ossigeno della funzione carbossilica, che sull'azoto solfonammidico, consentendo il recupero quantitativo dei prodotti totalmente metilati **10a-b** (Schema 2).

Il cromatogramma di una miscela opportunamente preparata dei due composti, confrontato con quello dei grezzi dei singoli prodotti, conferma ancora la totale assenza di racemizzazione nella preparazione degli intermedi **6a-f** (Figura 3).

Figura 3.

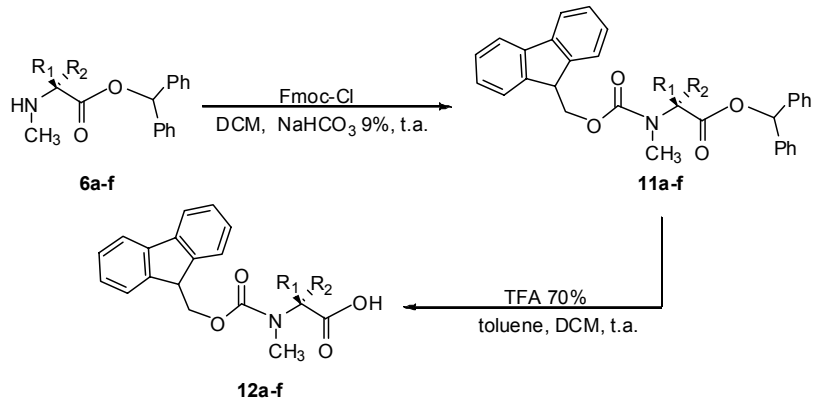
Analisi CG/MS dei dipeptides: (a) N-metil-N-Nosil-D-alanil-N-metil-L-valina metil estere (10a) (t.r. 21.21 min); (b) N-metil-N-Nosil-D-alanil-N-metil-D-valina metil estere (10b) (t.r. 20.88 min); (c) miscela di 10a e 10b.



L'obiettivo principale, costituito dall'ottenimento di *N*-Fmoc-*N*-metil-α-amminoacidi da poter impiegare come building blocks direttamente per la sintesi peptidica basata sulla Fmoc-Chemistry, era ancora da

raggiungere. A tal scopo gli intermedi **6a-f** liberi sulla funzione amminica sono stati trattati con Fmoc-cloruro in una soluzione bifasica di diclorometano e bicarbonato di sodio acquoso (Schema 3): le operazioni di work-up hanno consentito il recupero dei corrispondenti *N*-Fmoc-*N*-metil-α-amminoacidi esteri benzidrilici **11a-f**, con rese elevate (88-95%) (Tabella 2).

Schema 3.



6a-f **11a-f** **12a-f**

Tabella 2.

Sistemi impiegati

	R^1	R^2	11 resa (%)[a]	12 resa (%)[a]
a	$-CH(CH_3)_2$	-H	92	**96**
b	-H	$-CH(CH_3)_2$	92	**96**
c	$-CH_2CH(CH_3)_2$	-H	90	**98**
d	$-CH_2S(Bzl)$	-H	95	**94**
e	$-CH(CH_3)CH_2CH_3$	-H	94	**98**
f	$-CH_3$	-H	88	**96**

[a] **composto isolato**

I composti **11a-f** sono stati successivamente sottoposti a deprotezione della funzione carbossilica per trattamento con una soluzione di TFA in DCM: semplici operazioni di work-up hanno consentito il recupero degli

amminoacidi *N*-metilati, Fmoc-protetti **12a-f** con ottime rese (Schema 3, Tabella 2).

Da questi dati si evince che la strategia adottata tanto per la sintesi di *N*-metil-*N*-Nosil-α-amminoacidi che per quella di *N*-Fmoc-*N*-metil-α-amminoacidi è risultata vincente.

Il gruppo benzidrile scelto per la protezione della funzione carbossilica è ampiamente compatibile con le condizioni di metilazione mediante diazometano, e complessivamente sia con la *Nosil-Chemistry* che con la *Fmoc-Chemistry*. L'intera metodologia presentata risulta inoltre libera da processi di racemizzazione.

Il diazometano usato con brillanti risultati nel lavoro esposto ed in altri che lo hanno preceduto, manifesta tuttavia caratteristiche di pericolosità per la salute: è infatti in grado di metilare facilmente funzioni alcoliche, amminiche, tioliche e carbossiliche di strutture biologiche;[21] è esplosivo quando viene preparato e sottoposto a distillazione, e quando viene utilizzato in solventi idrocarburici che non lo solvatino.[14] Alternativamente lo si può preparare *in situ* a partire dal *trimetilsilildiazometano* (TMSDZ), un derivato sicuro e facile da preparare.[22] L'uso del TMSDZ, commercialmente disponibile, può essere esteso per la sicurezza del reagente e per l'assenza di rischi per la salute, anche ad ambiti non propriamente chimici.[23] Per tale ragione può costituire uno strumento molto generale per la metilazione di amminoacidi e peptidi.

Alla luce di queste considerazioni è stato affrontato uno studio accurato per valutare la reattività di questo reagente rispetto al ben conosciuto diazometano, nelle procedure di metilazione della funzione amminica di amminoacidi ottimizzate nei precedenti lavori.

Il meccanismo di reazione del trimetilsilildiazometano è ben diverso rispetto a quello del diazometano, necessita infatti della presenza di un nucleofilo protonato come il metanolo, che determina il "rilascio" del

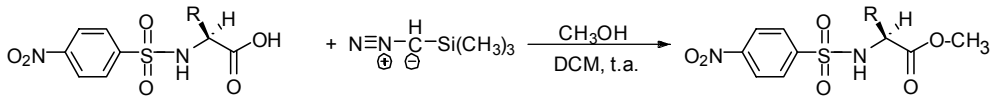
diazometano, ed in un momento successivo, fornendo un protone, consente la conversione del diazometano nello ione metildiazonio.

Le reazioni del trimetilsilildiazometano sono inoltre usualmente più lente, e richiedono un largo eccesso di reagente; ciò comporta spesso la formazione di prodotti di reazione collaterali.

Gli *N*-Nosil-α-amminoacidi in presenza di un eccesso di diazometano, reagiscono velocemente sia sulla funzione carbossilica che sulla funzione solfonammidica, fornendo i corrispondenti prodotti totalmente metilati. D'altra parte non è possibile indirizzare la reazione verso la mono-metilazione della funzione carbossilica: l'impiego di un difetto, così come un rapporto esatto del diazoalcano rispetto al *N*-Nosil-α-amminoacido, porta al recupero di una miscela di prodotti composta dall'estere metilico, dall'estere metilico *N*-metilato, e da parte del substrato non reagito.

La reazione di **1a**, sospeso in diclorometano, con trimetilsilildiazometano (**13**) e metanolo, nel rapporto di 1/5/7, porta esclusivamente alla metilazione chemospecifica della funzione carbossilica (Schema 4).

Schema 4.



1a,c,e-g **13** **14a,c,e-g**

Ns-aa/TMSDZ/MeOH= 1/5/7

Il semplice allontanamento del solvente di reazione e dei reagenti in eccesso, consente il recupero dell'estere metilico della *N*-Nosil-L-valina **14a** con rese quantitative ed in tempi brevissimi (2 minuti) (Schema 4, Tabella 3). La procedura è stata estesa con risultati di chemospecificità

paragonabili anche ad altri substrati lipofilici, ed i corrispondenti esteri **14c**, e **14e-g** sono stati recuperati quantitativamente (Tabella 3).

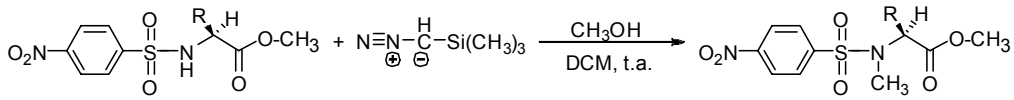
Tabella 3.

Sistemi impiegati, e tempi di reazione per la metilazione chemospecifica della funzione carbossilica

	R	Tempo (min.)
a	- $CH(CH_3)_2$	2
c	-$CH_2CH(CH_3)_2$	5
e	-$CH_3CH_2(CH_3)CH$	5
f	-CH_3	2
g	-$CH_2C_6H_5$	5

Gli esteri metilici **14** sono stati successivamente trattati con trimetilsilildiazometano e metanolo in diclorometano nel rapporto 1/9/13 (Schema 5).

Schema 5.



14a,c,e-g **13** **15a,c,e-g**

Ns-aa/TMSDZ/MeOH= 1/9/13

Tabella 4.

Sistemi impiegati, e tempi di reazione per la metilazione chemospecifica della funzione solfonammidica

	R	Tempo (h)
a	- $CH(CH_3)_2$	1
c	-$CH_2CH(CH_3)_2$	1.45
e	-$CH_3CH_2(CH_3)CH$	1.45
f	-CH_3	1
g	-$CH_2C_6H_5$	2

I composti **14** sono stati così metilati sulla funzione solfonammidica e convertiti rapidamente e totalmente nei corrispondenti *N*-metil-*N*-Nosil-α-amminoesteri **15a**, **15c**, e **15e-g** con rese quantitative (Tabella 4).

I tempi di reazioni relativi alla conversione dei *N*-Nosil-α-amminoesteri nei corrispondenti *N*-metil-*N*-Nosil-α-amminoesteri sono considerevolmente più lunghi di quelli necessari per la conversione degli amminoacidi nei corrispondenti esteri metilici.

Proprio la differente velocità delle due reazioni giustifica la chemospecificità osservata.

Complessivamente, il trimetilsilildiazometano rende possibile la metilazione chemospecifica della funzione carbossilica di *N*-Nosil-α-amminoacidi consentendo inoltre, e in un processo comunque successivo, il conseguimento anche della metilazione della funzione solfonammidica.

La metilazione di entrambe le funzioni nei substrati impiegati, è stata realizzata anche direttamente utilizzando un rapporto tra substrato, trimetilsilildiazometano e metanolo di 1/14/17, in diclorometano a temperatura ambiente.

Tutte le reazioni si sono completate in tempi prossimi alle due ore ed hanno portato con rese quantitative alla formazione degli *N*-metil-*N*-Nosil-α-amminometilesteri.

La procedura di metilazione della funzione solfomammidica mediante il sistema reagente TMSDZ/metanolo è stata estesa anche agli esteri benzidrilici dei Nosil-amminoacidi (Schema 6).

Gli esteri benzidrilici **3** solubilizzati in diclorometano, sono stati metilati sulla funzione solfonammidico impiegando un rapporto aa/TMSDZ/MeOH=1/12/17: l'andamento della reazione è agevolmente monitorato oltre che mediante cromatografia su strato sottile, anche mediante analisi GC/MS.

Schema 6.

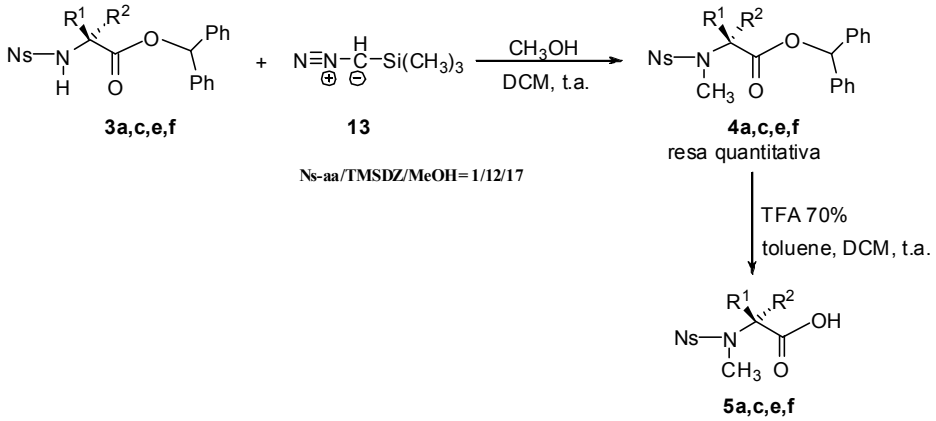


Tabella 5.

Sistemi impiegati, e tempi di reazione per la metilazione chemospecifica della funzione solfonammidica

	R	Resa[a] (%)
a	$(CH_3)_2CH$	98
c	$(CH_3)_2CHCH_2$	94
e	$CH_3CH_2(CH_3)CH$	94
f	CH_3	98

[a] composto isolato

Dal confronto tra l'uso del diazometano e quello del trimetilsilildiazometano nelle procedure presentate, è emerso che l'impiego del meno reattivo TMSDZ rende possibile la metilazione chemospecifica della sola funzione carbossilica degli amminoacidi Nosil-protetti in tempi estremamente rapidi.

L'approfondito studio effettuato per valutare l'efficacia del TMSDZ quale agente metilante dell'azoto solfonammidico di diversi *N*-Nosil-α-amminoacidi protetti sulla funzione carbossilica come esteri metilici e

come esteri benzidrilici, ha dimostrato l'applicabilità dello stesso, ma con dei limiti, considerando che i tempi risultano essere di gran lunga superiori rispetto a quelli richiesti dal diazometano. Pertanto il diazometano rimane il reagente d'elezione per una vantaggiosa preparazione di building blocks ad elevato valore aggiunto quali *N*-metil-*N*-Nosil-α-amminoacidi ed *N*-Fmoc-*N*-metil-α-amminoacidi.

Rimanendo nell'ambito degli amminoacidi *N*-metilati, è stata progettata e realizzata una strategia alternativa per la sintesi in soluzione di *N*-metil-β^3-amminoacidi chirali a partire dai corrispondenti α-amminoacidi, evitando l'uso di condizioni drastiche di reazione che caratterizzano le procedure riportate in letteratura.[24] Tale strategia, basata sull'utilizzo del diazometano come agente metilante e del gruppo *p*-nitrobenzensolfonile come gruppo protettore/attivante della funzione amminica, prevede l'omologazione diretta di α-amminoacidi ad *N*-metil-β^3-amminoacidi, mediante la ben nota procedura di Arndt-Eistert.

Recentemente i cloruri degli *N*-Fmoc-α-amminoacidi sono stati utilizzati con successo nella reazione di acilazione del diazometano: gli *N*-Fmoc-α-amminoacildiazometani ottenuti sono stati convertiti nei corrispondenti β^3-amminoacidi mediante riarrangiamento di Wolff .[25]

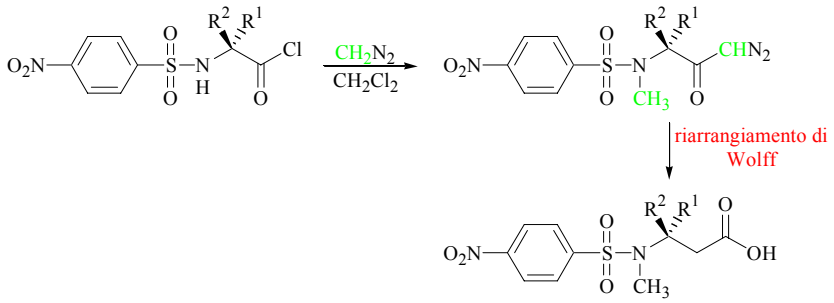
Con questi presupposti, si è ipotizzata una strategia basata sull'impiego Nosile per la protezione della funzione amminica degli α-amminoacidi: ciò poteva consentire di utilizzare come substrati di partenza nella sintesi di Arndt-Eistert i cloruri degli *N*-Nosil-α-amminoacidi che sono facili da preparare e piuttosto stabili (Figura 4).[13]

Per questa via si conseguirebbe in un unico stadio, non solo l'ottenimento dell'atteso α-diazochetone per addizione del diazometano al cloruro, ma anche la selettiva metilazione della funzione solfonammidica. Allo scopo, sono stati preparati diversi cloruri degli *N*-Nosil-α-amminoacidi (**16a-g**). Il passo successivo è stato il loro trattamento con diazometano al fine di ottenere in un unico passaggio i corrispondenti *N*-

metil-*N*-Nosil-α-amminoacildiazometani.

Figura 4.

Ipotesi di lavoro: cloruri degli N-Nosil-α-amminoacidi nella sintesi di Arndt-Eistert



Per verificare quest'ipotesi, il cloruro della *N*-Nosil-L-valina (**16a**), scelto come sistema modello, è stato trattato con una soluzione 0.66 M di diazometano in diclorometano: il lento gocciolamento del cloruro è indispensabile per evitare la formazione del corrispondente clorometil chetone. Si lascia la miscela di reazione sotto agitazione magnetica, in una miscela frigorifera di ghiaccio e cloruro di sodio per 50 minuti. In seguito a purificazione cromatografica del grezzo di reazione ottenuto per allontanamento del solvente in condizioni di pressione ridotta, si recupera l'*N*-metil-*N*-Nosil-L-valildiazometano **17a** con una resa del 73% (Schema 7, Tabella 6). Il composto **17a** è stato confermato e completamente caratterizzato mediante ^{1}H-NMR e ^{13}C-NMR. Il trattamento nelle stesse condizioni, dei cloruri degli *N*-Nosil-α-amminoacidi **16** (Schema 7, Tabella 6) ha portato alla formazione dei corrispondenti *N*-metil-*N*-Nosil-α-amminoacildiazometani **17** con rese comprese tra il 77% e il 89%.

Schema 7.

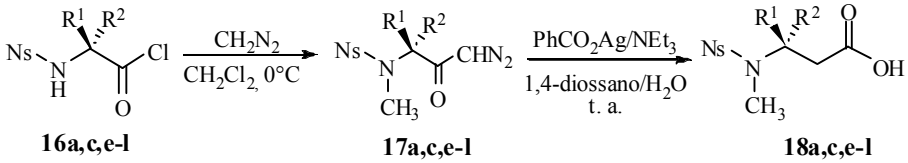


Tabella 6.

*Sintesi degli N-metil-N-Nosil-α-amminoacildiazometani **17** e dei corrispondenti prodotti di omologazione **18***

	R^1	R^2	17 resa (%)	18 resa (%)
a	$-CH(CH_3)_2$	-H	73	60
c	$-CH_2CH(CH_3)_2$	-H	77	74
e	$-CH(CH_3)CH_2CH_3$	-H	81	65
f	$-CH_3$	-H	78	83
g	$-CH_2(C_6H_5)$	-H	77	70
h	-H	$-CH_3$	81	82
i	-H	$-CH(CH_3)CH_2CH_3$	89	68
l	$-(CH_2)_4NHFmoc$	-H	69	62

Per verificare l'assenza di racemizzazione nel processo di formazione degli *N*-metil-*N*-Nosil-α-amminoacildiazometani, sono stati sintetizzati, con la metodologia già definita, gli *N*-metil-*N*-Nosil-α-diazochetoni della L-isoleucina (**17e**) e della D-*allo*-isoleucina (**17i**). Dal confronto tra gli spettri NMR protonici dei singoli campioni rispetto a quello derivante dall'analisi di un campione opportunamente preparato dei due, è emersa la totale assenza di processi di epimerizzazione.

Una volta ottenuti gli *N*-metil-*N*-Nosil-α-amminoacildiazometani, si è proceduto alla loro conversione nei corrispondenti *N*-metil-*N*-Nosil-β^3-omoamminoacidi, mediante la trasposizione di Wolff,[26] il passaggio principale nella reazione di omologazione di Arndt-Eistert degli acidi carbossilici.

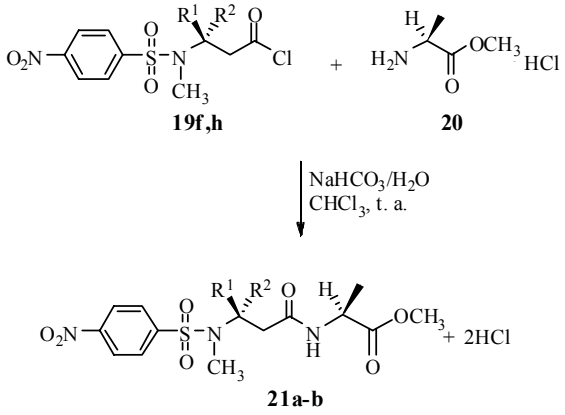
La reazione ottimizzata prevede l'aggiunta goccia a goccia di una soluzione di benzoato d'argento in trietilammina all'*N*-metil-*N*-Nosil-L-amminoacildiazometano **17** solubilizzato in acqua e diossano distillato. Entro 20 minuti le operazioni di work-up forniscono i corrispondenti prodotti omologati **18** in alte rese (Schema 7, Tabella 6).

Al fine di valutare le possibili applicazioni degli *N*-metil-*N*-Nosil-β^3-omoamminoacidi nella sintesi peptidica, sono stati preparati i cloruri, enantiomerici tra loro, degli *N*-metil-β^3-amminoacidi **19f** e **19h** usando la stessa procedura impiegata per gli α-analoghi (Schema 8).

I cloruri, recuperati dopo evaporazione del solvente con rese quantitative, e che risultano facili da preparare ed abbastanza stabili, sono stati impiegati per sintetizzare i dipeptidi diastereoisomerici **21a-b**.

I composti **19f** e **19h**, sono stati pertanto solubilizzati in cloroformio privo di etanolo, e sottoposti a reazione di accoppiamento con il cloridrato dell'estere metilico della L-alanina (**20**) solubilizzato in una soluzione acquosa al 5% di $NaHCO_3$. La reazione, condotta a temperatura ambiente per 25 minuti, ha portato alla formazione dei dipeptidi attesi con rese rispettivamente del 75% e del 64% (Schema 8).

Schema 8.



Al fine di verificare la differenziazione dei segnali ^{1}H-NMR dei due dipeptidi diastereoisomerici è stata predisposta una miscela dei grezzi di reazione di **21a** e **21b** da usare come termine di confronto per le analisi dei singoli. Il confronto dello spettro ^{1}H-NMR della miscela con gli spettri ^{1}H-NMR dei grezzi di reazione **21a** e **21b** esclude la presenza in ciascun grezzo dell'altro diastereoisomero.

In conclusione, è stata sviluppata una metodologia semplice e molto efficiente per l'omologazione di α-amminoacidi, protetti sulla funzione amminica con il gruppo Nosile, nei corrispondenti *N*-metil-β^3-amminoacidi.

In particolare, i risultati presentati, evidenziano i vantaggi relativi all'impiego del gruppo protettore Nosile nella preparazione in soluzione degli *N*-metil-β^3-omoamminoacidi che attraverso il facile ottenimento dei cloruri degli α-amminoacidi consente la formazione dell'α-diazochetone e la simultanea metilazione della funzione amminica durante il trattamento con diazometano. Gli *N*-metil-*N*-Nosil-α-amminoacildiazometani sono, poi, facilmente convertiti nei corrispondenti β^3-amminoacidi mediante il riarrangiamento di Wolff. Gli *N*-metil-*N*-Nosil-β^3-omoamminoacidi sono facilmente attivati sulla funzione carbossilica come cloruri: la stabilità di questi intermedi e la loro agevole disponibilità ne hanno reso possibile l'impiego nelle procedure sintetiche relative alla formazione di peptidi modificati in soluzione. È stato, altresì, dimostrato che tutta la procedura sintetica non causa alcuna evidente racemizzazione degli stereocentri presenti nei precursori.

Nell'ambito della sintesi di peptidi modificati l'interesse è stato rivolto, in un secondo momento, alla realizzazione di un'altra modifica dello scheletro peptidico e, in particolare, all'introduzione sull'estremità C-terminale di amminoacidi e peptidi, della funzionalità *N*-metil-*N*-metossiamminica.

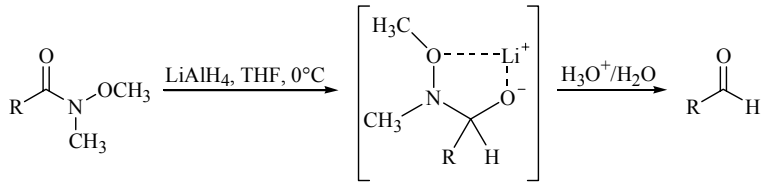
Le *N*-metil-*N*-metossiammine rappresentano utili intermedi di reazione nella sintesi organica;[27] diverse molecole biologicamente attive, o intermedi coinvolti nella loro sintesi,[28] sono caratterizzati dalla presenza di un gruppo funzionale *N*-metil-*N*-metossiamminico. In particolare, alcuni *N*-metil-*N*-metossiamminoacidi hanno mostrato un'interessante attività anticonvulsionante.[28b]

Al fine di realizzare una nuova procedura sintetica per la preparazione di *N*-metil-*N*-metossiammine, si è pensato che le *N*-metil-*N*-metossiammidi (ammidi di Weinreb) potessero rappresentare utili precursori.[29]

In letteratura è noto che la riduzione delle ammidi di Weinreb con un eccesso di $LiAlH_4$ porta alla formazione delle corrispondenti aldeidi.[30] La reazione procede attraverso la formazione di un intermedio ciclico molto stabile, che si origina in seguito all'addizione dell'idruro al carbonile ammidico e alla successiva coordinazione intramolecolare dello ione litio. La stabilità di quest'intermedio tetraedrico impedisce l'ulteriore addizione di idruro: soltanto il successivo trattamento idrolitico causa l'eliminazione della metilmetossiammina e la formazione della funzione aldeidica, senza ulteriore riduzione ad alcool (Figura 5).

Figura 5.

Conversione delle ammidi di Weinreb nelle corrispondenti aldeidi



Sono riportati solo pochi esempi in cui le *N*-metil-*N*-metossiammidi vengono convertite nelle corrispondenti ammine mediante riduzione.[31]

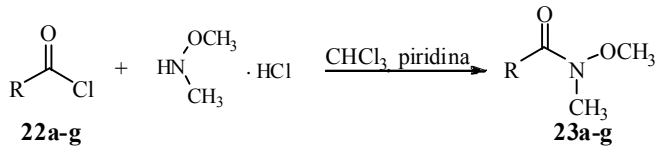
L'ipotesi di lavoro si è basata sull'idea che le ammidi di Weinreb potevano essere selettivamente convertite nelle corrispondenti *N*-metil-*N*-metossiammine scegliendo condizioni sperimentali opportune che impedissero la formazione del ciclo pentatomico metallo-chelato e che favorissero, allo stesso tempo, la trasformazione dell'ossigeno carbonilico in un buon gruppo uscente.[32]

Per raggiungere tale obiettivo si è pensato di utilizzare il tricloruro di

alluminio accoppiato all'agente riducente litio alluminio idruro.

I substrati di partenza, le *N*-metil-*N*-metossiammidi **23a-g**, sono state facilmente ottenute facendo reagire i corrispondenti cloruri **22a-g** con il cloridrato dell'*N*,*O*-dimetilidrossilammina in presenza di piridina (Schema 9).

Schema 9



22a-g **23a-g**

La reazione, condotta a temperatura ambiente per 50-60 minuti, ha prodotto, dopo trattamento idrolitico, le *N*-metil-*N*-metossiammidi **23a-g** con rese comprese tra l'84% e il 99% (Tabella 7).

Tabella 7

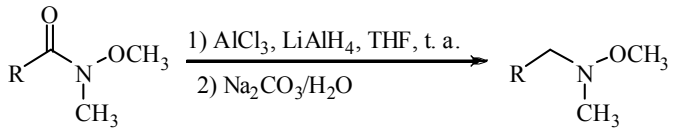
*Sintesi delle N-metil-N-metossiammidi **23a-g***

	R	resa (%)
a	C_6H_5	99
b	4-$CH_3C_6H_4$	93
c	4-$CH_3OC_6H_4$	91
d	4-ClC_6H_4	86
e	4-$NO_2C_6H_4$	84
f	$C_6H_5CH_2$	89
g	$CH_3(CH_2)_4$	90

I prodotti sono stati confermati e completamente caratterizzati mediante ^{1}H-NMR e GC-MS.

L'*N*-metil-*N*-metossibenzammide (**23a**), è stata scelta come substrato modello per testare la reazione di riduzione con il sistema reagente tricloruro di alluminio/litio alluminio idruro (Schema 10).

Schema 10



23a-g **24a-g**

Tabella 8

*Sintesi delle N-metil-N-metossiammine **24***

	R	resa (%)
a	C_6H_5	85
b	4-$CH_3C_6H_4$	84
c	4-$CH_3OC_6H_4$	100
d	4-ClC_6H_4	97
e	4-$NO_2C_6H_4$	95
f	$C_6H_5CH_2$	84
g	$CH_3(CH_2)_4$	84[a]

[a] *L'N-metil-N-metossi-1-esanammina (**24g**) è stata isolata come cloridrato*

In un tipico esperimento, l'ammide **23a** è stata solubilizzata in THF anidro e trattata con tricloruro di alluminio a temperatura ambiente; successivamente è stato aggiunto il litio alluminio idruro e l'*N*-metil-*N*-metossibenzilammina (**24a**) è stata ottenuta dopo 10 minuti con una resa dell'85%, in seguito ad un semplice work-up (Schema 10, Tabella 8).

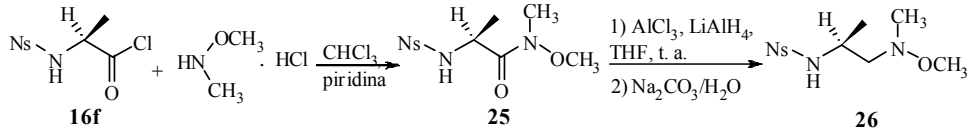
Alla luce degli eccellenti risultati ottenuti, la procedura è stata estesa ad altri sistemi alifatici ed aromatici (Tabella 8): la reazione procede a completezza in pochi minuti e a temperatura ambiente per tutte le ammidi di Weinreb sottoposte a riduzione.

In una fase successiva, al fine di sostituire la funzione carbossilica C-terminale di sistemi peptidici con la funzionalità *N*-metil-*N*-metossiamminica, si è pensato di utilizzare lo stesso sistema reagente per convertire le ammidi di Weinreb di α-amminoacidi opportunamente protetti sulla funzione amminica, nelle corrispondenti *N*-metil-*N*-

metossiammine.

A tale scopo il cloruro dell'*N*-Nosil-L-alanina (**16f**), solubilizzato in diclorometano anidro, è stato trattato con il cloridrato dell'*N*,*O*-dimetilidrossilammina, in presenza di piridina anidra, a temperatura ambiente per 1 ora; i trattamenti successivi hanno consentito il recupero della corrispondente *N*-metil-*N*-metossiammide **25** con una resa dell'80% (Schema 11). L'ammide **25** è stata solubilizzata in THF anidro e trattata con tricloruro di alluminio nel rapporto molare di 1:4; successivamente è stato aggiunto il litio alluminio idruro (**25**/$LiAlH_4$, 1:2) e il prodotto **26** è stato ottenuto dopo 20 minuti con una resa del 98%, in seguito ad un semplice work-up, senza necessità di ulteriori purificazioni.

Schema 11



Complessivamente lo studio effettuato, dimostra che le blande condizioni individuate per la riduzione delle ammidi di Weinreb consentono in maniera specifica l'ottenimento delle corrispondenti ammine con ottime rese. L'intera procedura ben si applica a sistemi amminoacidici *N*-Nosil protetti, consentendo di realizzare sistemi modificati sulla funzione *C*-terminale, di grande interesse per ciò che concerne l'applicazione nel campo dei peptidimimetici bioattivi.

Nel corso del triennio di ricerca, l'attenzione è stata rivolta anche nei riguardi della modifica delle catene laterali di amminoacidi inseriti in sequenze peptidiche avente attività biologica di tipo inibitoria diretta nei confronti della trombina, un'enzima diretta responsabile della regolazione del processo emostatico nell'uomo.[33]

La caratteristica tipica della varietà di piccole molecole peptidiche,

inibitori diretti della trombina, è la presenza di una residuo altamente basico quale quello guanidinico.[34] Tuttavia la presenza di residui fortemente polari ostacola l'assorbimento attraverso la parete gastrica di tali molecole, a seguito di somministrazione orale.[35]

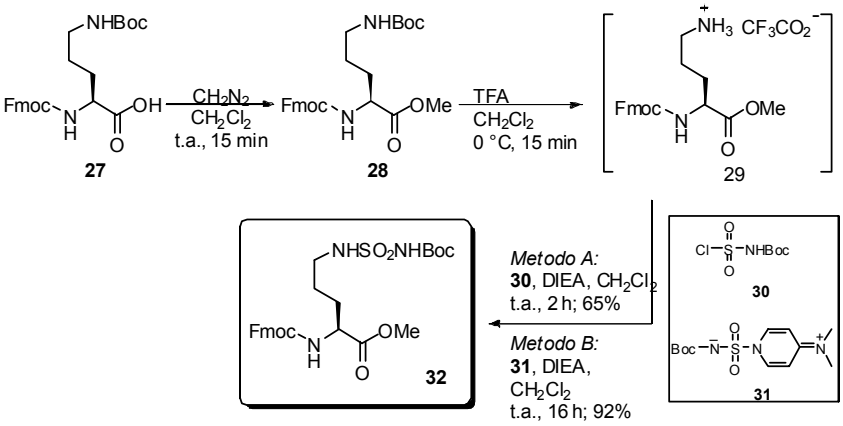
Motivati da tale ragione e dalla pletora di aspetti biologici collegati al ruolo di arginina e dei suoi isosteri modificati,[36] è stata sfruttata una via sintetica di semplice accesso e percorrenza, per la sintesi un nuovo derivato della L-ornitina (**32**), con l'obiettivo primario di sviluppare una nuova classe di inibitori tripeptidici della trombina umana, analoghi del ben noto PPACK (sequenza "core" del fibrinogeno).[37] Lo sviluppo di una serie importante di peptidomimetici in grado di influenzare l'attività della trombina è basato su composti analoghi del substrato naturale, il fibrinogeno. Questi inibitori occupano il sito attivo dell'enzima in competizione col fibrinogeno, bloccando l'attività. 88

Adottando la strategia "*ornitina→arginina*" si è arrivati alla sintesi dell'amminoacido **32** modificato in catena laterale, recante un gruppo sulfamoilico bioisostere del gruppo guanidinico dell'arginina, ma caratterizzato da una ridotta basicità. Il gruppo selezionato è un costituente di alcune sulfonamidi biologicamente attive, [38] ma non vi è in letteratura nessuna menzione riguardo la possibilità di funzionalizzare in tal modo la catena laterale della L-ornitina.[39] La parte -$NHSO_2NH_2$ nello scheletro della L-ornitina rappresenterebbe un gruppo farmacoforo a struttura tetraedrica, del gruppo della guanidinico dell'arginina, rimanendo al tempo stesso un efficiente sistema per la formazione di legami di idrogeno.[40] La ridotta basicità del raggruppamento sulfamoilico, pur mantenendo le caratteristiche di polarità necessarie per l'interazione enzima/substrato, dovrebbe concorrere ad implementare i parametri farmacocinetici richiesti da un ideale farmaco con attività inibitrice nei confronti della trombina, somministrabile per via orale.

La preparazione di **32** parte dalla N^{α}-Fmoc-N^{δ}-Boc-L-ornitina (**27**), e

segue una via costituita complessivamente da tre stadi sintetici (Schema 12). Il primo step prevede il trattamento dell'ornitina ortogonalmente protetta sulle due funzioni amminiche, con una soluzione di diazometano, al fine convertire la funzione carbossilica in metilesterea (**28**). Il trattamento acidolitico praticato su **28**, e le operazioni di work-up, consentono il recupero dell'intermedio libero sulla funzione -N^{δ}.

Schema 12



La catena laterale di **29** è stata dunque sottoposta a funzionalizzazione utilizzando il reagente **30** in presenza di diisopropiletilammina (DIPEA) (Schema 12, Metodo A). La resa poco soddisfacente calcolata nel recupero dell'amminoacido modificato, è imputabile alla limitata stabilità del reattivo **30** (preparato *in situ* a partire dal clorosulfonilisocianato e dal terbutanolo). Una interessante alternativa all'impiego del derivato **30** è l'azanide **31**: dal valore della resa di **32** risultato chiara la validità del reagente per la sulfamoilazione di **29** (Schema 12, Metodo B).

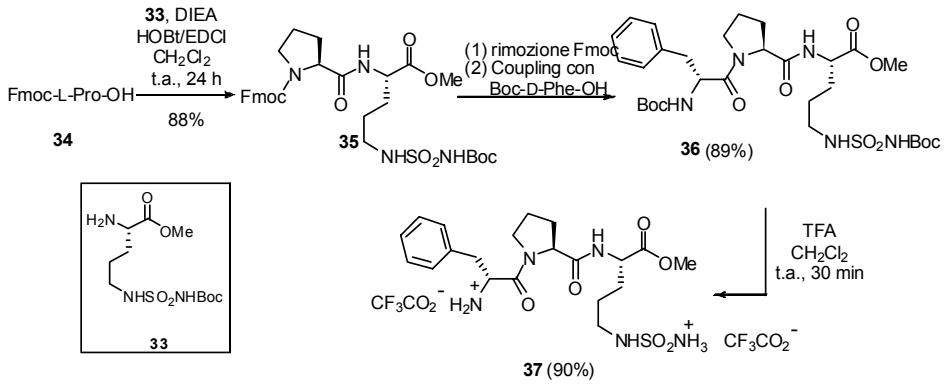
Il composto **32**, completamente caratterizzato mediante risonanza magnetica nucleare, risulta un solido cristallino stabile che può essere conservato anche per periodi di tempo prolungati.

Con l'obiettivo di valutare il ruolo biologico del gruppo solfamoile della catena laterale della L-ornitina, **32** è stato utilizzato come building block

nella preparazione dei tripeptidi **33** e **34**, analoghi del PPACK, regolatore analogo della sequenza chiave del fibrinogeno naturale della trombina (Schema 13).

La sintesi dei due tripeptidi è stata conseguita attraverso due consecutive reazioni di accoppiamento utilizzando il sistema EDCI/HOBt, in ambiente basico (DIEA).

Schema 13



L'ornitina modificata **33**, libera sulla funzione amminica N^{α}, è stata inizialmente sottoposta a reazione di accoppiamento con la *N*-Fmoc-L-prolina nelle condizioni precedentemente descritte. Il dipeptide **35**, recuperato con ottime rese, è stato sottoposto a procedura di rimozione del gruppo protettore Fmoc, e dunque a reazione di accoppiamento con la *N*-Boc-D-fenilalanina. Il tripeptide completamente protetto **36**, è stato recuperato con resa del 89%. Dopo il trattamento acidolitico finale mediante una soluzione di TFA in DCM), la precipitazione selettiva del tripeptide **37** utilizzando una miscela 1:2 MTBE/*n*-pentane e la successiva liofilizzazione, al fine di eliminare ogni traccia di terbutanolo (dal Boc), forniscono il prodotto atteso, recuperato con resa elevata. Entrambi i tripeptidi **36** e **37** sono stati sottoposti al test di coagulazione vitro al fine di valutare la loro potenza inibitoria nei confronti di trombina umana. Il tempo di trombina (TT), una misura della reazione in vitro fibrinogeno-

trombina, e il tempo di tromboplastina parziale attivata (APTT), il parametro utilizzato per valutare l'effetto anticoagulante sul trombina prodotta dal percorso intrinseco della cascata della coagulazione, sono stati determinati utilizzando un pool di plasma umano, ed usando diverse concentrazioni dei due dipeptidi. Lo studio ha evidenziato che solo il tripeptide **37** ha attività nei confronti dell'enzima: il tripeptide libero ha infatti mostrato attività forte e dose-dipendente.

Questo dato conferma la validità del gruppo sulfamoile (gruppo polare ma caratterizzato da contenuta basicità), e più in generale dell'ornitina sulfamoilata, come alternativa all'impiego dell'arginina nella preparazione di inibitori diretti della trombina, appartenenti alla serie PPACK. La procedura ottimizzata per la preparazione dell'intermedio modificato consente di poter disporre in maniera agevole di tale precursore, sfruttando reazioni semplici e veloci, che portano al recupero finale di prodotti puliti, che non necessitano di particolari procedure di purificazione.

Una ulteriore parte del lavoro svolto durante il triennio di dottorato, ha riguardato l'individuazione di una procedura efficiente per l'estrazione di olio di bergamotto che possedesse caratteristiche tali da renderlo un prodotto commercialmente appetibile.

L'olio essenziale di bergamotto è il costituente di base di molte preparazioni alimentari, cosmetiche e sanitarie.[41]

Gli oli di bergamotto ottenuti attraverso *cold pressed procedure* o altre metodologie estrattive contengono bergaptene, una sostanza fototossica e mutagenica.[42]

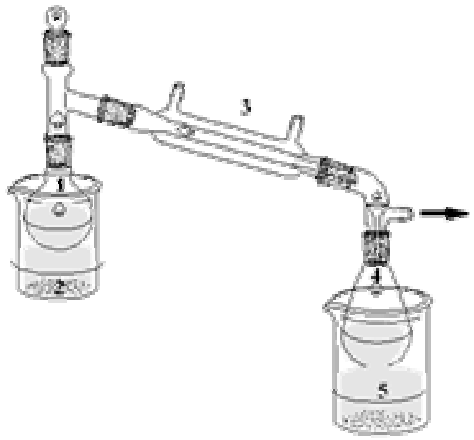
La qualità e la valenza commerciale di un olio essenziale di bergamotto è funzione inversa del contenuto di bergaptene, che in genere viene eliminato assieme alle altre furocumarine contenute nell'olio mediante distillazione frazionata sottovuoto o per trattamento con idrossido di sodio.[43]

Tali procedure comportano però perdita di prodotto e grave impoverimento nella composizione dell'olio finale ottenuto.

In questo studio è stato messo a punto un processo di distillazione sottovuoto applicato direttamente alla matrice vegetale in modo da migliorare la procedura di estrazione dell'olio, e la qualità dello stesso, ottenendo prodotti totalmente privi di bergaptene, e di elevata qualità (Figura 6).

Figura 6.

Illustrazione della strumentazione impiegata nella metodologia estrattiva



1. matrice
2. bagno d'olio
3. refrigerante
4. distillato
5. bagno freddo

La metodologia proposta prevede l'estrazione di oli aromatici e acque aromatiche da matrici vegetali senza l'uso di solventi.

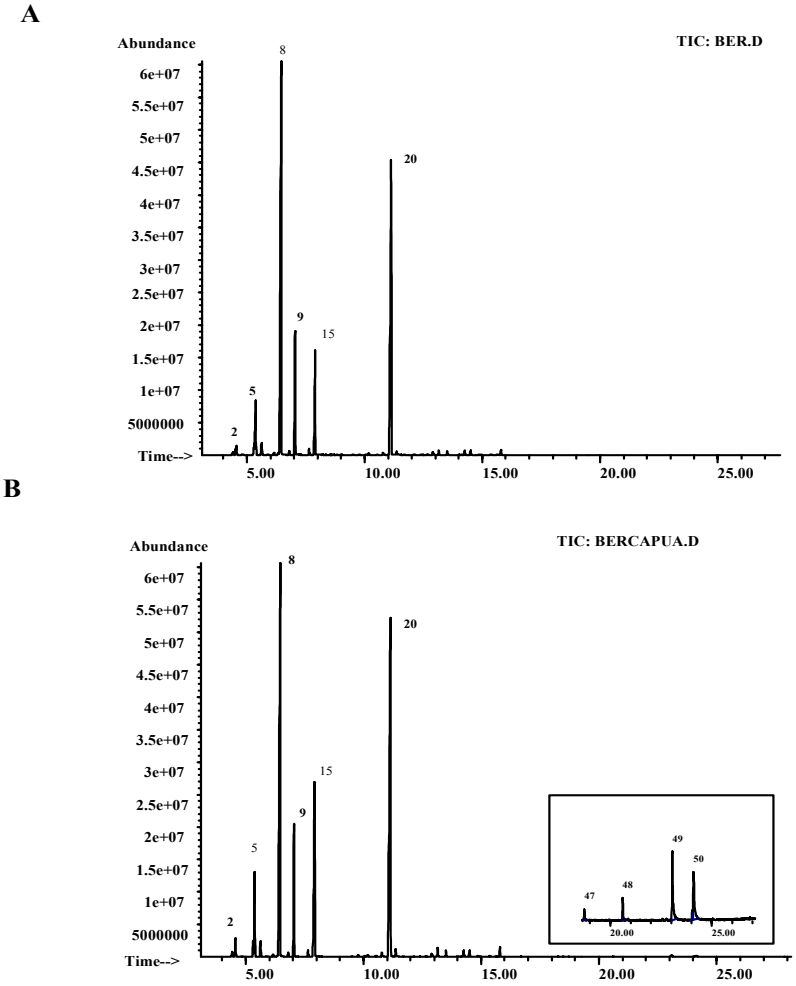
La composizione dell'olio essenziale di bergamotto ottenuto per distillazione in corrente di vapore delle bucce di bergamotto, valutata mediante analisi GC/MS appare totalmente simile a quello ottenuto da *cold-pressed procedure* (Figura 7).

L'olio essenziale ottenuto seguendo questa via, è caratterizzato da monoterpeni idrocarburici ossigenati e non, a catena aperta e ciclici. I componenti[44] più significativi a struttura monoterpenica, a catena aperta, sono: il β-mircene, il linalolo, il linalil acetato. L'aroma dell'olio è caratterizzato anche da molecole ossigenate a struttura aldeidica, quali il

nerale, il geraniale, il nonanale, il decanale.

Figura 7

A *Cromatogramma olio essenziale di bergamotto attraverso il metodo proposto.* ***B*** *Cromatogramma olio essenziale di bergamotto da cold-pressed*



Tra i monoterpeni ciclici a struttura idrocarburica i più rappresentati sono il thuiene l'α-pinene, il sabinene, il β-pinene , l'α-terpinene, il γ-terpinene e il terpinolene. Il limonene costituisce il componente a percentuale relativa maggiore nel cromatogramma (valore medio 43.67%).

Una caratteristica molto importante di questo olio di bergamotto è l'assenza di bergaptene e di altre furocumarine; questo composto risulta invece presente, sia pur in piccole quantità, nel campione di riferimento dell'olio derivante dalla metodologia *cold-pressed* (Figura 7).

Complessivamente questo olio possiede le caratteristiche ideali per la sua applicazione in ogni ambito in cui l'olio di bergamotto è prescritto.

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[44] I componenti sono stati determinati utilizzando campioni standard di riferimento che hanno mostrato lo stesso tempo di ritenzione e gli stessi spettri di massa dei componenti corrispondenti presenti nell'olio.

INTRODUCTION

Peptide foldamers constitute a growing class of nanomaterials with potential applications in a wide variety of chemical, medical and technological fields. In particular, their role as mediators of key biological functions and their unique intrinsic properties make them particularly attractive therapeutic agents: peptides show high biological activity associated with low toxicity and high specificity. The benefits conferred by these characteristics include little unspecific binding to molecular structures other than the desired target, minimisation of drug-drug interactions and less accumulation in tissues reducing risks of complications due to intermediate metabolites. Additionally, compared to small molecules, peptides offer valuable chemical and biological diversity on which intellectual property is still widely available.

For all this reason, the peptide therapeutics market is providing new commercial opportunities to biotechnology and pharmaceutical industries. To exploit these markets, biotechnology and pharmaceutical companies are actively pursuing the development of a variety of peptide-based technologies, peptide manufacturing technologies and drug delivery methods. They have faced a period of stagnancy in the decade of 90^{s} and have come a long way from their development to its acceptance as therapeutics. The naturally derived therapeutic peptides have been successful for a long time and with the advent of synthetic and biological peptides, many new drug candidates can be expected in the market. Biotechnology has enabled high through put screening and maintenance of peptide libraries, which increases the likelihood of developing new peptide based drug therapies. New peptide delivery technologies and improvement in clearance half-lives in vivo have further improved the

potentials of peptides in therapeutic applications. They are poised to play a significant role in the treatment of various debilitating diseases such as Alzheimer's disease and cancer. Therapeutic peptides are now viable alternatives to other biopharmaceuticals, such as antibodies especially in the case of cancer treatment due to their ability to penetrate tumours. Even the big pharmaceutical companies, traditionally focused on small molecules, are increasingly considering peptides in their pipelines.

On the other hand, low bioavailability of peptides following oral administrations attributed to their inactivation in the gastro–intestinal tract through enhanced enzymatic degradation in the gut wall by avariety of peptidases expressed at the enterocytes brush border, and to poor intestinal permeation.In addition, thein stability of peptides toward peptidases in the systemic blood circulation causes rapid elimination (i.e., short half-life). These factors limit the use of peptides as therapeutic agents in the clinical setting.

Several strategies have been used to reduce enzymatic cleavage and uptake into the systemic blood circulation, including *prodrug approaches*, *peptidomimetics*, and *structural modifications*, such as covalent attachment of polyethylene glycol (PEG), lipidation, and chemical modifications, for example, cyclization, D-amino acids or β-amino acids substitution, and *N*-alkylation. One of the techniques suggested to improve the enzymatic stability of peptides is *N*-methylation.

Having all this in mind, my interest during the three years of PhD was directed towards the synthesis of modified amino acids: particular attention was given to the modifications of side-chain, amino and carboxyl functions of α-amino acids.

The last part of my work was dedicated to the qualitative determination of organic analites in foods matrices by mass-spectrometry methodologies.

CHAPTER 1

Methods for the synthesis of *N*-methylamino acids

1. Introduction

Identification of peptide-based drugs with acceptable oral bioavailabilities will be a major challenge confronting pharmaceutical scientists in the future.

The rational design of orally bioavailable active-peptide will necessitate a compromise between structural features that optimize the pharmacological properties (e.g., receptor binding) and structural features that optimize the pharmacokinetics properties (e.g., membrane permeability, clearance, metabolism) of the molecule. The major biological barriers to the oral delivery of peptide-based drugs include the intestinal lumen, intestinal mucosa and the liver.[1]

Three broad approaches to the development of orally active peptide-drugs have been taken. The earliest such approach, and that best represented in today's pharmacy, is the identification of a non-peptidic natural product that mimics or antagonizes the biological activity of an endogenous peptide. At the other extreme is the identification of an appropriate protein and directly confronting bioavailability issues by using a novel route of delivery or the co-administration of an enzyme inhibitor.

The middle ground is the strategy of peptidomimetics that resemble a target protein but contain some synthetic element (aminoacidic residues) designed to reduce metabolism and to optimize the biological activity of the agent.

The range of tools available for limiting peptide bond hydrolysis in vivo extends from simple additions that chemically protect the targeted bond from attack, to its replacement altogether, to global changes that, instead, modify the peptide conformation in such a way that it is no longer recognized by the protease of concern. It is important to recognize that these two strategies are never completely unrelated, as even modest structural changes near the scissile peptide bond can result in significant conformational differences. For example, one straightforward chemical modification that can slow the addition of an enzymatic nucleophile to a peptide bond is the introduction of steric bulk in the form of an *N*-alkyl (usually methyl) group. Studies on *N*-methyl amino acids (NMA) containing peptides reveal that those residues increase proteolytic stability, increase membrane permeability (lipophilicity), and alter the conformational characteristics or properties of the amide bonds.[2]

Several reserch's groups have developed NMA containing peptides that show improved proteolytic resistance.[3] It was reported the site-specific substitution of certain peptides (e.g. the nonapeptide leuprolide), that are known luteinizing hormone-releasing hormone (LHRH) agonists, with the corresponding NMA.[4] Other analogues were found to be completely resistant to the action of chymotrypsin. This was attributed to the interruption of key hydrogen bonds in the chymotrypsin active site by *N*-methylation of the peptide substrate. Endothelin-1 is a peptidic constrictor of vascular smooth muscle cells. Starting from a hexapeptide antagonists of the receptor for Endothelin-1,[5] one potent hexapeptide inhibitor, site-specifically *N*-methylated, was synthesized: this analogue has significantly increased proteolytic resistance and enhanced antagonist activity.

A series of recent papers relating to Alzheimer's disease consider the use of small peptidic ligands bearing *N*-methyl amide bonds as a means

of interrupting or reversing amyloid protein aggregation into toxic fibrils or lumps.[6]

2. Methodologies for the synthesis of *N*-methylamino acids

Commercially available *N*-methylamino acids are still very expensive even if various methods have been developed for the synthesis of optically active *N*-methylamino acids. For these reasons many research groups are working to introduce and/or improve the procedures for the synthesis of those important building blocks.

2.1. *N*-methylation by alkylation

The procedures for direct *N*-methylation or *N*-alkylation originate with the work of Hinsberg,[7] who took a number of *N*-alkyl benzenesulfonamides and treated them with alcoholic potassium hydroxide and an alkylating agent, usually ethyl or methyl iodide, to obtain di-*N*-alkyl sulfonamides. These compounds were intermediates in the synthesis of secondary amines.

The extension to α-amino acids was was exploited by Fischer et al.,[8] the pioneer that opened up a line of research for NMA synthesis involving *N*-methylation of intermediate *N*-tosyl amino acids, extending Hinsberg's approach and also by nucleophilic substitution of α-bromo acids with methylamine.

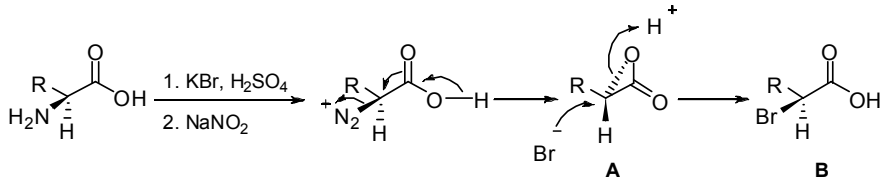
Izumiya and co-workers[9] combined the Fischer's methodologies to prepare a wide range of *N*-methylamino acids, helping to increase most of data available about NMAs.

2.1.1. Nucleophilic substitution of *a*-bromo acids

Following the work of Fischer and Mechel, NMAs were prepared by nucleophilic displacement of bromide from optically active α-bromo acids:[10] nucleophilic substitution of bromine by using an excess of methylamine at 0°C, provides the corresponding NMA with opposite configuration to the acids. Usually, starting α-bromo acids were prepared by diazotization of the amino acid:[11] the intermediate diazonium ion is attacked intramolecularly (S_N2), by the carboxylate group to form the labile three-membered lactone **A** (Figure 1).[12]

Figure 1.

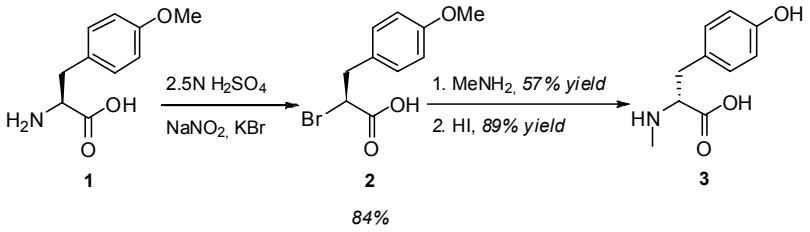
Mechanism of a-bromoacid formation via diazotization



The subsequent nucleophilic addition (again in S_N2 mode) by bromide ion provides the optically active α-bromo acids **B** with total retention of chirality respect to the original amino acid.

Izumiya and Nagamatsu[9] prepared *N*-methyl-D-tyrosine (D-surinamine) (**3**) by nucleophilic substitution on optically active α-bromo acid **4**, with methylamine at 100°C in a sealed tube (Scheme 1).

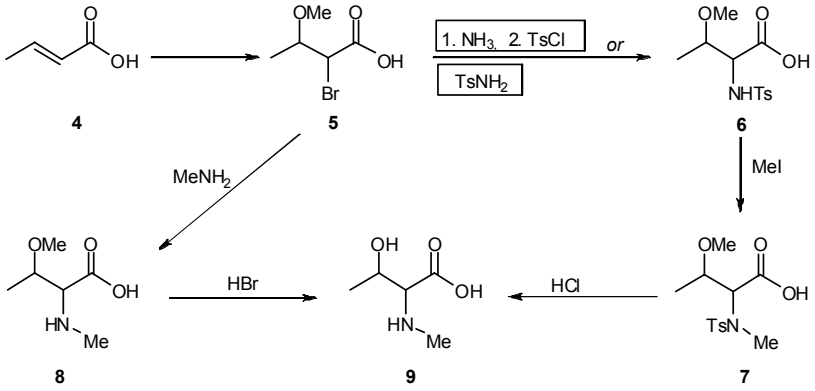
Scheme 1.



This methodology was extended to other amino acids, such as methionine, arginine, and ornithine, in synthesizing their corresponding *N*-methyl derivatives.[9] Izumiya has identified also an alternative way based on the preparation of hydroxyamino acids. Thus, in the synthesis of *N*-methylthreonine (**9**), 3-methoxy-2-bromoalkanoic acids (**5**) were prepared from alkenoic acids as precursors (Scheme 2). Are given two paths for preparation of **9**: the first involves amination with ammonia to generate *O*-methylthreonine; after sulfonylation with tosyl chloride, the subsequent *N*-methylation with methyl iodide gave the protected threonine **7**. The removal of *N*-sulfonyl and *O*-methyl groups were then achieved by treatment with hydrochloric acid to give the expected product (**9**). The second path requires the use of methylamine for the amination to make *N*,*O*-dimethylthreonine (**8**): the advantage of this way is the possibility to realize the *N*-methylation in a single step. *O*-demethylation with hydrobromic acid gave corresponding *N*-methylthreonine (**9**).

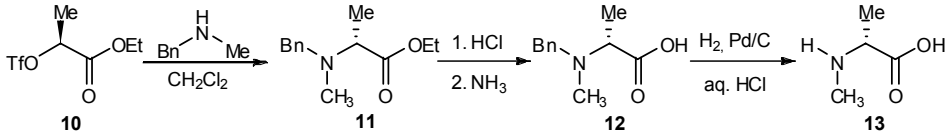
The sequences was applied to the synthesis of racemic serine, threonine, *allo*-threonine, β-hydroxyvaline, and also D-threonine and L-threonine. Alternatively, the sulfonyl sequence could be made more efficient by amination with *p*-toluenesulfonamide to give **6**.

Scheme 2.



Effenberger et al.[13] explored the use of activated α-hydroxy acid derivatives, as substitute for α-bromo acids. This technique was applied in the synthesis of *N*-methyl-D-alanine (**13**) (Scheme 3): the reaction of triflate **10** with *N*-methyl-*N*-benzylamine, provided the protected NMA **11** in good yields.

Scheme 3.



The advantages of this technique is that the goodness of trifluoromethanesulfonate as leaving group, make it possibile, even with weak amine nucleophiles, to operate at room temperature and below.

The synthesis of NMAs by nucleophilic substitution is a short and simple procedure, but it is also caracterized by low yields and racemization processes has not entirely been eliminated. For all these reasons, this approach to NMA synthesis has been basically abandoned. Only the Effenberger's approach, involving triflate displacement, remains a viable way: in fact was shown that provides optically pure derivatives.

2.1.2. *N*-methylation of sulfonamides

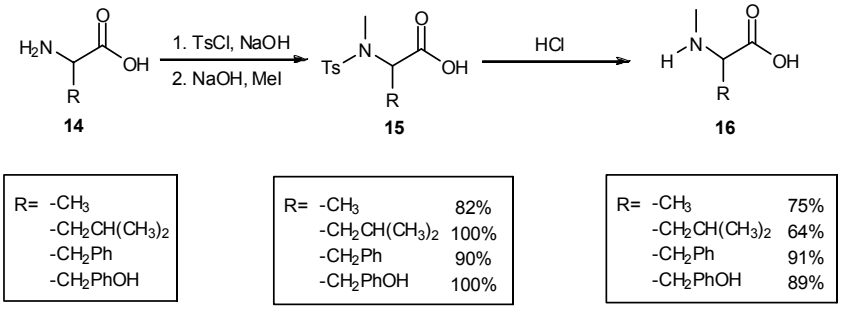
One of the frequently used methodologies for the methylation of nitrogen on amino acids and peptides, involves their conversion into the corresponding *N*-sulfonamidoamino acids. Sulfonamide protection greatly enhances the acidity of the sulfonamidic hydrogen, permitting deprotonation under basic conditions, and in the presence of an

alkylating reagent, furnishes the *N*-sulfonamide NMAs. On the other hand, *N*-sulfonamidoamino acids are the key intermediate also in the Mitsunobu protocol, for the synthesis of NMAs.

2.1.2.a. Base mediated alkylation

The earliest work of Fischer and Lipschitz[8] describes the preparation of NMAs by treatment of *N*-tosyl-α-amino acids **15** with NaOH, followed *N*-methylation with 2 equiv. of methyl iodide at 65-70°C (Scheme 4). A major limitation for this procedure is that his method does not proceed without racemization: loss of optical integrity happenes in the methylation step, in which sodium hydroxide was used at elevated temperatures.[14]

Scheme 4.

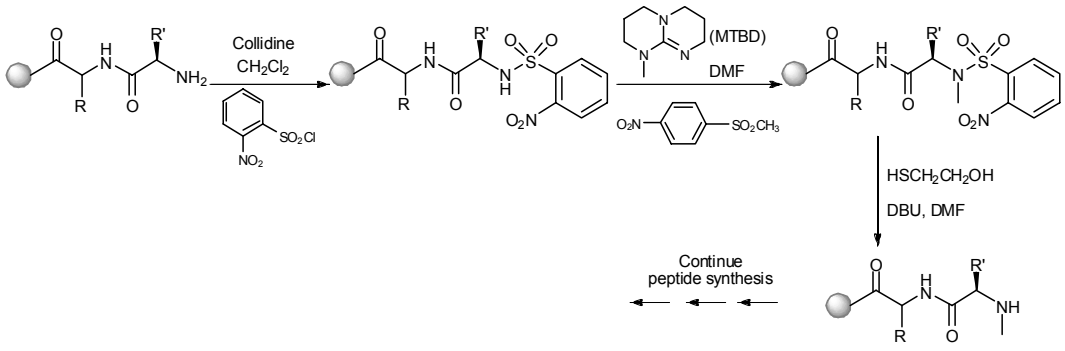


Hlavácek et al.[14] overcame this problem working on low temperature: *N*-methylation of *N*-tosylamino acid isopropyl and *tert*-butyl esters of alanine and valine, was realized using sodium hydroxide and dimethyl sulfate at 0°C. Pure *N*-methylamino acid derivatives of leucine, valine, phenylalanine, alanine, and ornithine were isolated during workup in near quantitative yields for the methylation step. Removal of tosyl-protecting group was achieved either with calcium metal in liquid ammonia or alternatively with hydrobromic acid at reflux in the presence

of phenol, to provide optically active NMAs.

Miller and Scanlan extended this protocol on solid support.[15] The sulfonamide was deprotonated with the nonionic guanidinium base, 7-methyl-1,5,7-triazabicyclo[4.4.0]dec-5-ene (MTBD), and alkylated with methyl *p*-nitrobenzenesulfonate (Figure 2). The removal of *o*-nitrobenzenesulfonamide (*o*-NBS) was performed in mild condition using *β*-mercaptoethanol/1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), by the formation of a Meisenheimer complex.[16]

Figure 2.

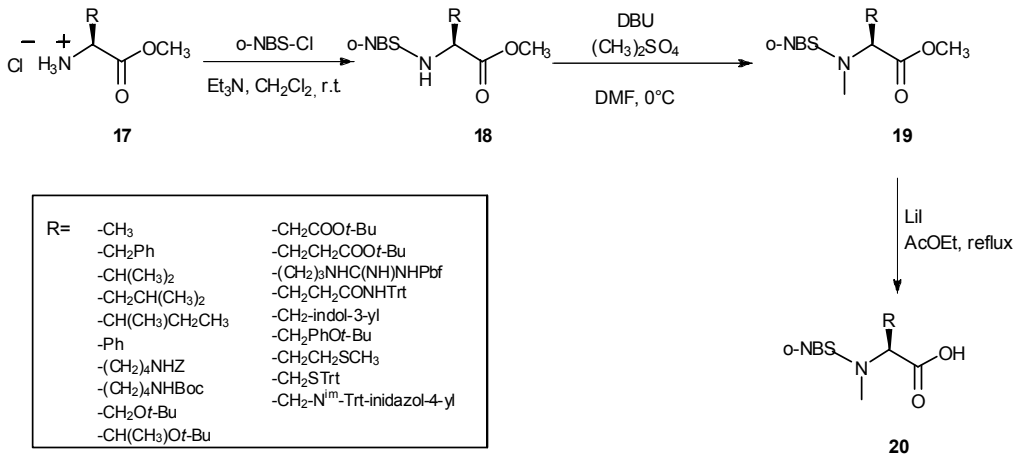


The method described allows the specific *N*-methylation of sulfonamide, without backbone methylation elsewhere in the growing peptide. The use of MTBD was critical to achive high yields and selectivity: in fact while weaker bases gave poor or no yields, the use of stronger bases couse methylation of the amide backbone.

An improvement of this protocol was realised by Biron et al.:[17] this procedure was chosen and transferred into solution. A first investigation of the reaction with *N*-*o*-NBS-phenylalanine methyl esters revealed that a minimum of 2 equiv. of MTBD is needed for full conversion to *N*-methyl-*N*-*o*-NBS-phenylalanine methyl esters, and is achieved in 5 min. in DMF, while *N*-methylation in DCM or THF was not achieved after 2 h. Unfortunately, MTBD is too expensive to be usedfor synthesis in gram

scale. Therefore, 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) and 1,8-diazabicyclo[5.4.0]undec-5-ene (BDU), two structurally similar hindered bases, were tested. Investigation of reaction revealed that both bases achieve complete conversion of a model substrate under same condition as MTBD. The less expensive and readily available DBU was chosen and tested on the *N*-*o*-NBS-amino acids methyl esters **17** (Scheme 5). *N*-methylation was then performed using DBU and dimethylsulfate in DMF at 0°C for 15 min., and gave de corresponding methylated compounds in 92-99% yields of isolated product without any purification. It is known that *N*-protected-*N*-methylamino acids methyl esters racemize more readily during saponification than the corresponding amino acid esters.[18] To overcome this problem, methyl ester cleavage by S_N2 dealkylation was tested.

Scheme 5.

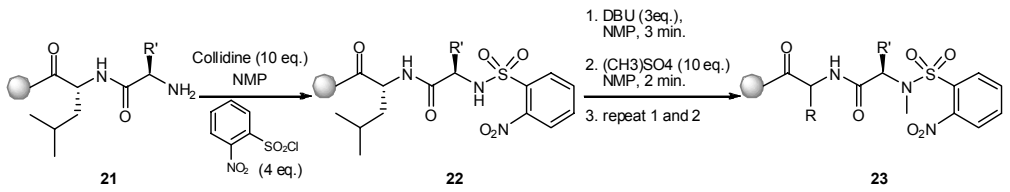


This mild and neutral method occurs with displacement of the carboxylate by using a powerful nucleophile such as iodide or cyanide in a dipolar aprotic solvent: in the performed conditions, the ethyl acetate was used as reaction solvent, with goods results. Compatibility with

Fmoc-SPPS was also investigated by coupling the synthesized building blocks to resins-bound leucine, using normal Fmoc-SPPS coupling conditions. The entire synthetic sequence is compatible with Fmoc-SPPS protecting groups, allowing a rapid synthesis of *N*-methyl peptide analogues.

Yet Biron and coworkers in a other work performed a three-step procedure for methylation of peptides in solid phase based on *N*-arylsulfonyl peptides.[19] Trityl resin (Tritylchloride polystyrene (TCP))-bound *N*-*o*-NBS-protected dipeptides **22** were prepared following the optimized procedure (Scheme 6), i.e. by treatment of the corresponding resin-bound amine-free dipeptides **21** with o-nitrobenzenesulfonyl chloride (4 eq) in the presence of collidine (10 eq) in *N*-methylpyrrolidone (NMP) for 15 min.

Scheme 6.



A first investigation of the reaction on the resin-bound *N*-*o*-NBS-dipeptide model with DBU and dimethylsulfate in DMF revealed a very important side reaction coming from the reaction of DBU with dimethylsulfate and full conversionof substrate into *N*-*o*-NBS-*N*-methyl dipeptide could not be achieved. To overcome this problem, DBU was first reacted with the resin-bound *N*-*o*-NBS-protected dipeptide **22** to ensure complete deprotonation of the sulfonamide and was characterized by the yellow coloration of the resin, then dimethylsulfate was added to the resin for *N*-methylation. Monitoring of the reaction showed that 91% of the *N*-*o*-NBS dipeptide **22** is *N*-methylated after 3 min of reaction with

DBU and 2 min with dimethylsulfate. Full conversion (>99%) into *N*-methyl-*N*-*o*-NBS dipeptide **23** is achieved when the procedure is repeated once more. One positive point about the *N*-methylation procedure with DBU is that no solvent changes have to be made, since the reaction is done in NMP.

Albanese et al. also worked with *o*-NBS protecting group of nitrogen atom of amino acids methyl esters, but in solution phase:[20] the *N*-methylated sulfonamide esters were prepared by treatment with alkyl halides in presence of solid potassium carbonate and triethylbenzylammonium chloride (TEBA) as phase transfer catalyst at 25 or 80°C. The use of TEBA enabled the non-nucleophilic base potassium carbonate to be utilized whereas, in the absence of TEBA, *N*-alkylation was considerably reduced. Removal of the *o*-nitrobenzenesulfonamide group is affected by thiophenol/potassium carbonate/acetonitrile at 80°C or potassium thiophenoxide/DMF at 25°C.

An interesting even milder approach for *N*-methylation of amino acid sulfonamides under neutral conditions was performed using diazomethane, by Liguori et al.[21]

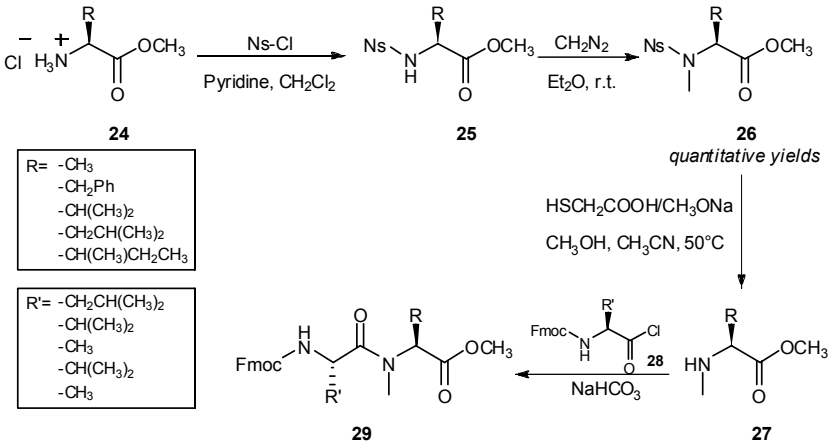
The initial attempt, involving the direct *N*-alkylation of amino acid methyl esters performed with a large excess of diazoalkane in the presence of aluminum trichloride, gave an intractable mixture of products, in wich and also starting material are present with the *N*-methylated and the *N,N*-dimethylated derivatives (in an approximate ratio of 4:2:4). In an attempt to increase the acidity of the proton on the nitrogen atom, various protecting group was evaluated. The reaction of diazomethane with *N*-acetylamino acids proceed in very low grade, and the starting material remained almost totally unchanged.

N-tosyl protection is inappropriate for inclusion in peptide synthesis, since the conditions for removal also cleave peptide bonds by acid

hydrolysis; the reductive method with sodium or calcium in liquid ammonia is a much milder process, yet when dealing with peptide chains, the workup is cumbersome and the reduction is not selective in protective group removal.

Nosyl (*p*-nitrobenzensulfonyl, Ns) protecting group was then selected to protect and activate the amino function of the amino acids derivatives: this strong electron-withdrawing masking group, enhances the reactivity of the N-H function toward diazomethane allowing the easy formation of the species responsible for methylation. NMAs methyl esters **26** are readily prepared starting from *N*-nosyl protected amino acid methyl esters **25**: the methylation reaction is performed by treatment of nosyl derivatives with un excess of diazomethane (Scheme 7). Finally, the protecting group is readily removed by tratment with 3 equiv. of mercaptoacetic acid in the presence 8 equiv. of sodium methoxide at 50°C, to give the free *N*-methyl-derivative **27** in >84% yields, and with totally retention of configuration on α-carbon.

Scheme 7.

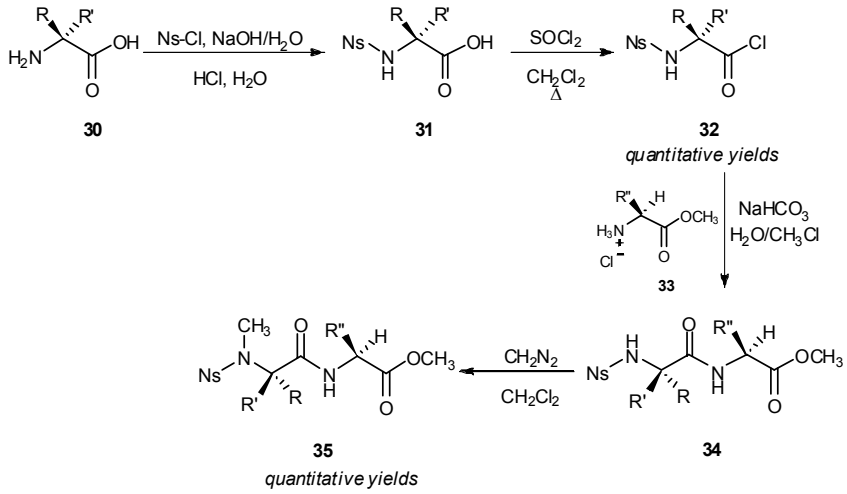


N-methyl-analogue are efficiently coupled to *N*-Fmoc amino acid chlorides **28** in aqueous $NaHCO_3$, to afford the corresponding dipeptides

29 in excellent total yields and with retention of the configuration of the carbon atom of the precursors.

A further interesting goal achived from this resercher team was the solution phase site-specific *N*-methylation of peptides.[22] To explore the direct methylation of oligopeptides with diazomethane, the synthesis of *N*-nosyl-protected peptides **35** was undertaken, starting from *N*-nosyl amino acids en their corresponding activated chlorides (Scheme 8).

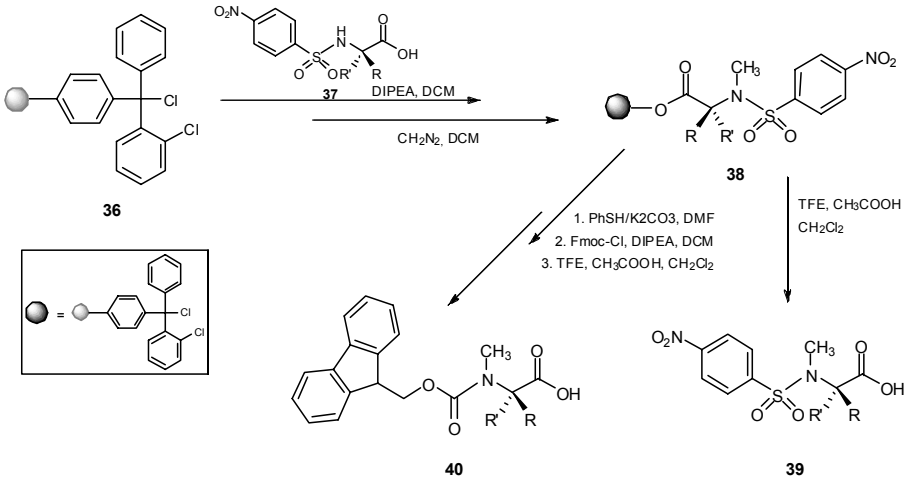
Scheme 8.



N-nosyldipeptides methyl esters were obtained without mesurable epimerisation, in high yields and purity. Methylation reaction of peptides was performed at room temperature by treatment with diazomethane in a molar ratio 1:8. The reaction was complete within 30 min., and the expected substrates were recovered in quantitative yields. The methylation is highly chemospecific: in fact, only products mono-methylated on the sulfonamide nitrogen atom were identified, and no traces of other methylated compounds were odserved (Scheme 8). In the light of the excellent results obtained, the procedure was then investigated with success also in the methylation of tripeptides.

This work was completed assessing the compatibility with standard Fmoc solution peptides synthesis, and in a more recent work the integral protocol was transferred to the synthesis on solid support.[23] The highly acid-labile 2-trichlorotrityl chloride resin (Barlos, **36**) was chosen, because it is compatible with the base-labile protecting group Nosyl and Fmoc, and its cleavage conditions are mild enough to retain all acid sensitive side-chain protection (Scheme 9).[24] The amino acids *N*-nosyl protected **37**, synthesized using Schotten-Baumann condition were loaded to the resin in the presence of diisopropylethylamine (DIPEA) in dichloromethane for 2h. *N*-methylation was then performed using 8 equiv. of diazomethane.

Scheme 9.



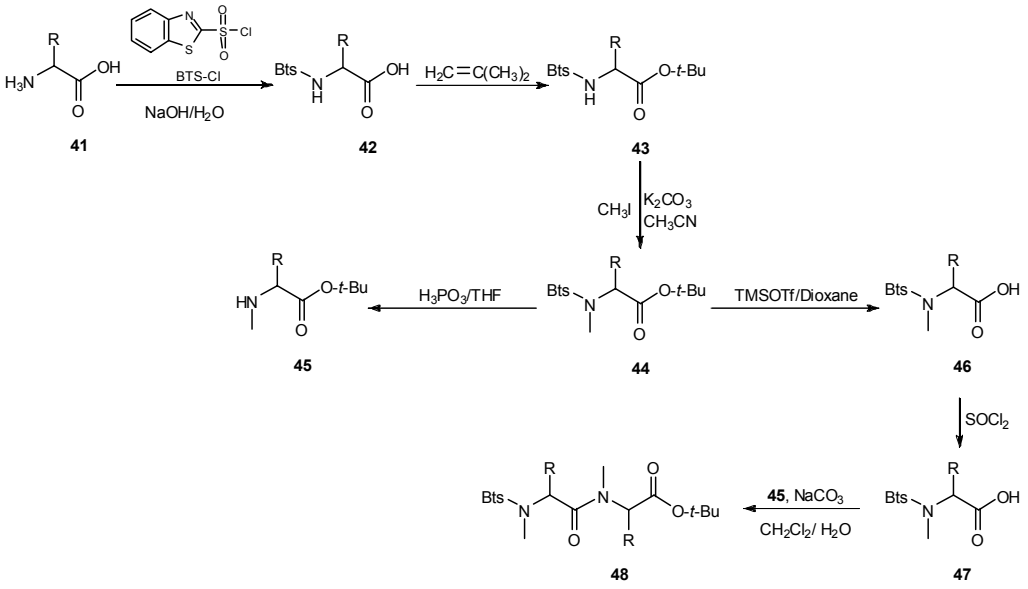
Clevage from the resin was, at this step, the most comprehensive method to test the efficacy of the methodology. Treatment with a solution of trifluoroethanol and acetic acid in dichloromethane, afforded *N*-methyl-*N*-Nosy-lamino acids in very goods yields **39**.

To obtain *N*-Fmoc-*N*-methyl-amino acids building blocks **40**, Nosyl protection was removed from the intermediates **38** via aromatic

nucleophylic substitution (SNAr), by treatment with thiophenol/potassium carbonate in DMF. The procedure was repeated once more to ensure complete deprotection in every case, then the Fmoc-protecting group was introduced by treatment with 4 equiv. of Fmoc-Cl and DIPEA (6 equiv.) in dichloromethane. The *N*-Fmoc-*N*-methylamino **40** acids were cleaved from resin under usual condition, and recovered in goods yields. The applicability of this method was tested for a set of lipophilic amino acids, and also for amino acids with acid-labile protecting groups (e.g., Boc, t-Bu, Trityl), to make the adopted procedure general. All the work shows that this is a very useful and general way for the synthesis of NMAs.

Benzothiazolesulfonamides is an interesting alternative to presented protecting groups for amino function:[25] Vadejs and co-workers from this way, realized a strategy for synthesis of *N*-methylated amino acids as shown in the subsequent scheme (Sheme 10).

Scheme 10.



An improvement in the procedure of deprotection of BTS-group, was realized by using mild condition:[26] benzothiazolesulfonamides of primary and secondary amines are efficiently cleaved by a nucleophilic aromatic substitution with a thiol (thiophenol) and a base such as potassium *t*-butoxide or diisopropylethyl amine in DMF.

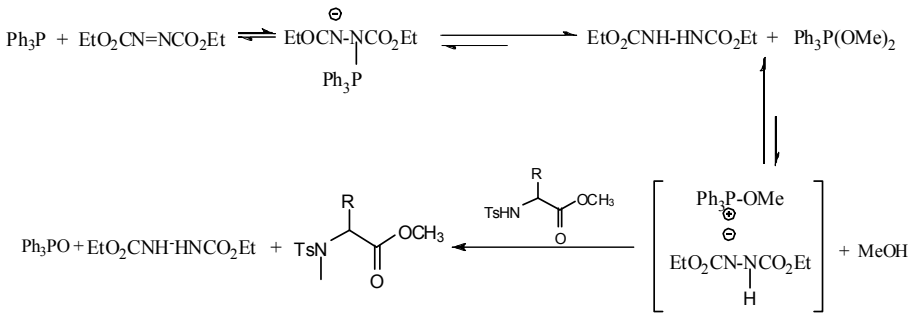
2.1.2.b. Mitsunobu protocol

Strong alkaline reagents can have an adverse effect on the optical purity of α-amino acids, especially when they are *N*- and *C*-protected, making the α-center the most acidic site and prone to enolization.

One alternative to this approach was the Mitsunobu protocol[27] (Figure 3): it is a versatile and widely used method that combine an oxidizing azo-reagent [most commonly diethyl azodicarboxylate (DEAD), or diisopropyl azodicarboxylate (DIAD)], a protonate co-reagent such as methanol or ethanol or isopropanol, and a reducing phosphine reagent, usually triphenylphosphine (TPP), under mild and virtually neutral reaction conditions.

Figure 3.

Mitsunobu protocol

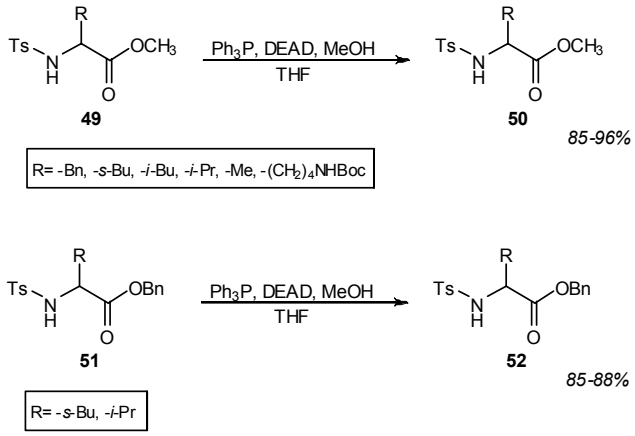


The most important advantages for this protocol is that the mild conditions are compatible with solid phase synthesis of *N*-alkylated

amino acids and peptides. Weinreb was the first that used this procedure especially for synthesis of NMAs.[28]

Papaioannou et al.[29] used the Mitsunobu protocol to effect the *N*-alkylation of *N*-tosyl protected amino acid methyl (**49**) and benzyl (**51**) esters and with retention of configuration and optical purity (Scheme 11). In their racemization's study about the ester hydrolysis of *N*-methyl-*N*-tosyl-L-valine methyl ester, it was found that deprotection with methanolic sodium hydroxide at room temperature produced up to 44% of the D-enantiomer. Alternatively, deprotection with iodotrimethylsilane effectively removed the methyl ester without racemization.

Scheme 11.

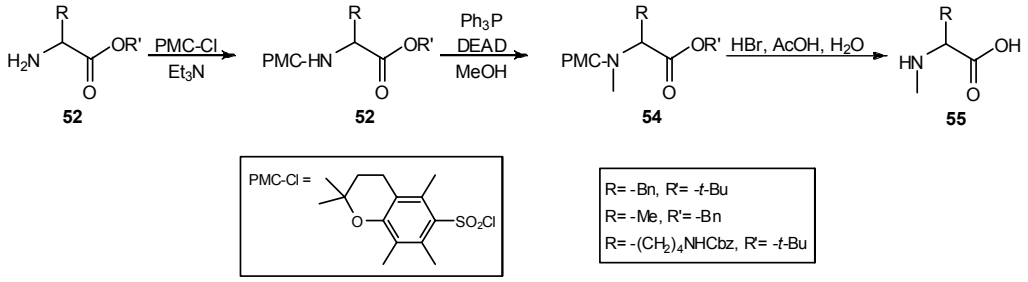


This reagent, however, is nonselective in that many other protecting groups are also removed.[30] Benzyl esters, which can be removed by catalytic hydrogenation, did not racemize, and therefore, they are the preferred choice for carboxyl protection in this case. The *N*-tosyl protection was reductively cleaved with sodium in liquid ammonia, providing optically active NMAs.

Wisniewski and Kolodziejczyk[31] addressed the problematic *N*-tosyl deprotection by employing the 2,2,5,7,8-pentamethylchroman-6-sulfonyl

or Pmc group, which has increased lability to acid conditions, to protect the nitrogen (Scheme 12).

Scheme 12.



The *N*-Pmc group is still sufficiently nucleophilic to participate in a Mitsunobu reaction: for this reason it is possible avoid the strongly basic conditions (*Fischer's approach*) associated with the methylation of *N*-toluenesulfonamides.

Yang and Chiu[32] applied a strategy similar to that of Miller and Scanlan to synthesize Fmoc-*N*-methylamino acid forms of alanine, valine, phenylalanine, tryptophan, lysine, serine, and aspartic acid (preloaded on 2-chlorotrityl resin), making the *N*-methylation step under Mitsunobu conditions, or with finely powdered potassium carbonate and methyl iodide. The sulfonamide group was removed with sodium thiophenoxide, carbamoylated with Fmoc-Cl/diisopropylethylamine, and then the cleavege from the resin provides the Fmoc-*N*-methylamino acids (>90% yields). The methylated amino acids thus isolated were found to be racemization free. The same conditions were applied for *N*-methylation of the resin-bound *N*-*o*-NBS-peptides: conversion in the corresponding methyl-derivatives was completed in 10 min. In the procedure described by Miller and Scanlan, removal of the *o*-NBS protecting group is achieved in 30 min with 10 equiv. of mercaptoethanol and 5 equiv. of DBU in DMF.[15,33] Time needed for complete deprotection was

investigated on resin-bound *N*-methyl-*N*-*o*-NBS-dipeptides in NMP and revealed that the reaction is completed in only 5 min. A great advantage in the use of this protectin group is that deprotection with mercaptoethanol is selective for *N*-methylated derivatives and does not proceed when the protected amine is not alkylated.

The Mitsunobu protocol for *N*-methylating *N*-sulfonylamino acids is an effective racemization free method for NMA synthesis. The use of *N*-nosyl protection over *N*-tosyl has provided a means for ready introduction and removal of sulfonamide type protection, and the neutral conditions of the Mitsunobu reaction permit a variety of protecting groups that can be included in an orthogonal protection scheme. This method, although mild and effective, can be expensive, and it would be preferable to limit this procedure to small scale and solid-phase synthetic schemes.

2.1.3. *N*-methylation of carbamates and amides

Among the identified methodologies for the synthesis of *N*-methylated amino acid, are commonly emploied those involving the conversion of the function of α-amino acids, in carbamates and amides derivatives.

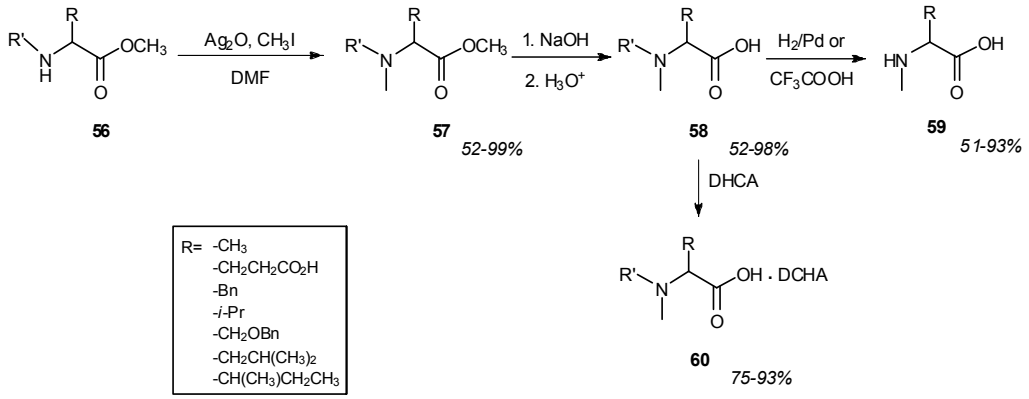
2.1.3.a. Silver oxide/methyl iodide

Das et al.[34] described the *N*-methylation of terminal and internal amide bond amino acid residues, or carbamate, when they permethylated peptides for use in mass spectrometry studies. Their intentions were to encrease the volatility of peptides for amino acid sequencing studies: in fact *N*-Methylation of peptide bonds would alleviate the volatility problem by removing the possibility of hydrogen bonding. Their

procedure involved treatment of substrate *N*-acyl peptides with excess methyl iodide and silver oxide in dimethyl formamide. The final methylated products showed higher volatility and allowed mass spectral analysis at lower temperatures in the ion source.

Olsen[35] has taken the studies of Das et al. to include α-amino acid *tert*-butyl (*N*-Boc) and benzyl (*N*-Cbz) carbamates. The yields of mono-*N*-methylamino acid methyl esters such as alanine and valine were routinely in the range 93-98% (Scheme 13). However, transformations of more reactive residues such as cysteine, arginine, methionine, aspartic acid, serine, and threonine were not successful.

Scheme 13.



Okamoto et al.[36] extended Olsen's procedure, and this time *N*-methyl analogues of glutamic acid and serine were synthesized with success (Scheme 13). Most of the *N*-methylamino acids **58** were isolated in crystalline form as their dicyclohexylamine (DCHA) salts **60** following ester saponification. However, it was found that the optical rotation data for *N*-methylserine and *N*-methylglutamic acid were lower than reported values. The silver oxide/methyl iodide method for *N*-methylation is a mild and racemization free process. However, the final NMAs are obtained as their methyl esters that are then saponified to give the

corresponding free acids and it been shown that this step compromises the chiral integrity of the NMAs. In addition, it is imperative that fresh[36] silver oxide be used in anhydrous conditions in the absence of light.

Alternatively, *N*-carbamoyl amino acid esters (i.e. *tert*-butyl or benzyl) should be employed in such a procedure to preclude methyl ester formation.[35] Tam et al.[37] did just that in the synthesis of *N*-methyl derivatives of α-*N*-Boc, side chain *N*-phthaloyl protected ornithine, and lysine. By blocking the carboxyl group as a benzyl ester, silver oxide/methyl iodide mediated *N*-methylation was achieved without transesterification to give the methyl ester. Removal of benzyl ester by hydrogenolysis afforded the free acids, in particular the *N*-methylornithine derivatives were used as precursors for *N*-methylarginine by guanidination with N^{im}-nitro-*S*-methylisothiourea on solid support.[37]

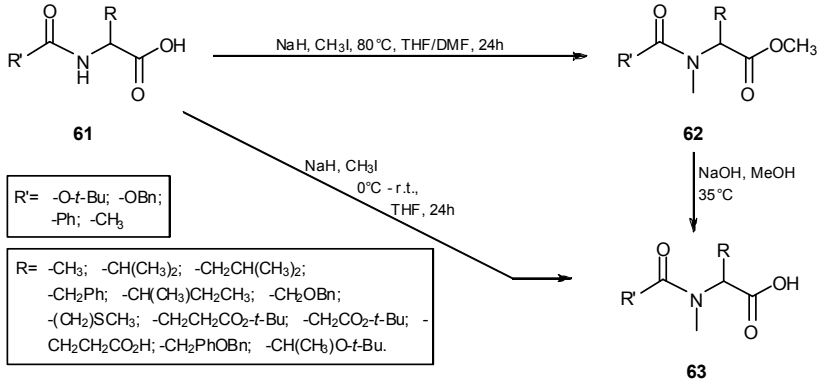
2.1.3.b. Sodium hydride, and methyl iodide or other methylating agent

The most broadly applied method for NMA synthesis is *N*-methylating *N*-acyl- and *N*-carbamoylamino acids with sodium hydride and methyl iodide as developed by Benoiton et al.[18b-c,38] It was reported that sodium hydride could remove the NH proton of secondary amides and urethanes.[38a] Benoiton exploited this in synthesizing a range of NMAs with different *N*-protection using excess sodium hydride and methyl iodide, and subsequently established the propensity of NMAs to racemize in basic, acidic, and various coupling reaction conditions.

The initially attempt of *N*-methylation regarded *N*-acyl-, *N*-tosyl-, and *N*-carbamoyl-α-amino acids by treating these *N*-protected amino acids with sodium hydride and methyl iodide in THF/DMF at 80°C for 24 h. Under these conditions, a large excess of methyl iodide (8 equiv) was required

for optimal conversion of substrates in the corresponding *N*-methylamino acids methyl esters **62** (Scheme 14). Removal of methyl ester protection was performed using warm sodium hydroxide in methanol/THF to give the free *N*-Cbz/*N*-acyl-*N*-methylamino acids **63** respectively. On the other hand, the use of harsh alkaline conditions in the deprotection of the carboxyl function causes varying degrees of undesired racemization at the α-carbon of the amino acids.[18b, 38b-c] A direct route to *N*-methylamino acids **63** was necessary so as to avoid hydrolysis of the ester **62** and therefore prevent racemization.

Scheme 14.



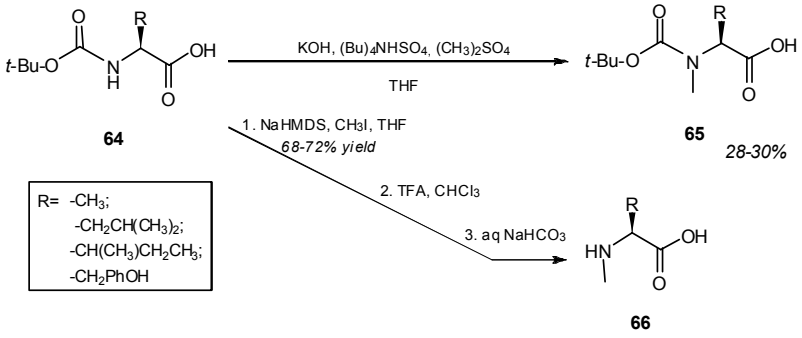
McDermott and Benoiton[39] found that reaction temperature was an important factor in avoiding methyl ester formation. In addiction, they identified acidic reaction conditions that caused racemization: they found the anhydrous HBr/acetic acid used for *N*-Cbz removal caused racemization. Analysis of the acid catalyzed racemization showed that anhydrous HBr/acetic acid caused racemization depending on acid strength, solvent polarity, and time. A decrease of each of these factors resulted in decreased racemization, and it was found that including water in the acidic mixtures suppressed racemization completely, as did hydrochloric acid mixtures in place of hydrobromic acid mixtures. Later,

Benoiton et al. documented the increased and variable susceptibility of NMAs to racemization in peptides during standard peptide coupling reactions. It was concluded that appreciable racemization occurred with aqueous hydroxide due to the absence of ionizable groups other than the α-center. The racemization studies were extended to include coupling reactions between NMA peptides via the mixed anhydride activation approach, and they identified factors such as ionic strength and solvent polarity as controlling racemization during peptide bond formation via the mixed anhydride activation/coupling procedure.

The use of *N*-Boc or *N*-Cbz protection causes difficulty in forming readily crystallizable NMA products. Coulton et al.[40] addressed these problems using diphenylphosphinamides as protection for the amino function, which are acid labile (95% TFA). The α-amino acid diphenylphosphinamides were methylated using the conditions of Benoiton.[41] A drawback of this procedure was that some racemization may have occurred.

Also Belagali et al.[42] utilized a similar approach to that of Benoiton but using as substrates *N*-Boc-L-amino acids (Scheme 15). However the *N*-Boc-L-amino acids **64** treated with sodium hydride/methyl iodide under the Benoiton conditions[30b] were transformed in the *N*-methyl derivatives **65** in low yields 30-40%.

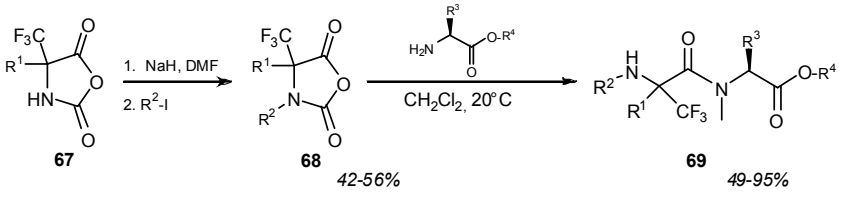
Scheme 15.



The harsher conditions depicted in Scheme 15, where **64** were treated with finely powdered potassium hydroxide, tetrabutylammonium hydrogen sulfate (phase transfer catalyst), and dimethyl sulfate, also produced the *N*-methyl-*N*-Boc-L-amino acids **65** in low yields. However, by switching to sodium hexamethyldisilazide (NaHMDS), the yields of the *N*-methylation step improved 2-fold and the NMAs were isolated as their methyl esters **66** after cleavage of the *N*-Boc group with TFA.

Burger and Hollweck,[43] produced another intresting variation on Benoiton's theme by alkylating *TFM Leuchs anhydrides* **67** [4-(trifluoromethyl)-1,3-oxazolidine-2,5-diones] (Scheme 16): the *N*-alkylated anhydrides **68** were used in dipeptide syntheses, since peptide bond formation of α,α-dialkylated residues at the carboxyl terminus is generally difficult.[44] It was found that alkyl bromides were ineffective in the alkylation step and that alkyl iodides were superior.

Scheme 16.



	R^1	R^2	R^3	R^4
a	-$CH_2CH(CH_3)_2$	-CH_3	-Bn	-$C(CH_3)_3$
b	-Bn	-CH_3	-Bn	-$C(CH_3)_3$
c	-Ph	-CH_3	-CH_3	-$C(CH_3)_3$
d	-Bn	-CH_3	-CH_3	-$C(CH_3)_3$
e	-Ph	-CH_3	-H	-CH_3

Prashad et al.[45] *N*-methylated *N*-Boc-dipeptides and amino acids esters treating the substrates with sodium hydride in THF and methylating the resulting anion with dimethyl sulfate. It was found that methylation's reaction requires catalytic amounts of water to provide the corresponding *N*-methylated derivatives in excellent yields. The authors postulate that

the addition of water produces dry sodium hydroxide that has better solubility in THF compared to sodium hydride.

N-methylation using powdered commercially available sodium hydroxide was also studied, and was found to be much slower (24h) compared to sodium hydride and wather condition (30 min.)

It was also noted that approximately 10% epimerization occurred at 30°C, but this could be ameliorated by ensuring that the reaction was conducted at a temperature in the range 17-20°C.

Stodulski and Mlynarski[46] recently reported their studies on the synthesis of *N*-alkyl-*N*-methylamino acids without need for protection of the carboxylic function.

The synthetic procedure is performed via consecutive alkylation of benzyloxycarbonyl (Cbz)-amino acids and smart one-pot Cbz-deprotection and methylation of amino function. Initially, starting from Benoiton's strategy, various bases was evalued as alternative to the use of NaH, such as Cs_2CO_3, or LiHMDS, in the syntesis of NMAs; but their application was not efficient.

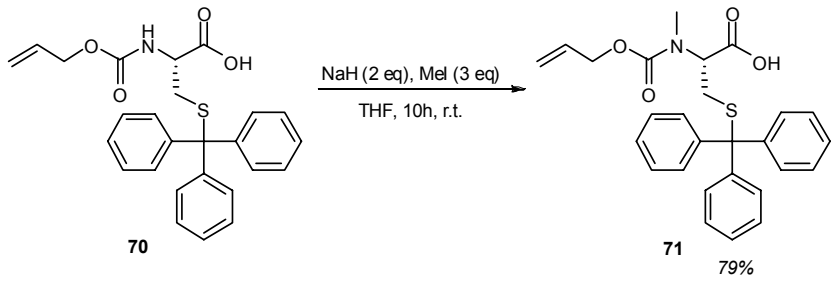
On the other hand, testing the same condition of Benotoin (CH_3I, NaH, THF, 0°C), but at elevated temperature (60°C), it resulted that *N*-methylated (and in general *N*-alkylated) Cbz-amino acids were recovered in good yields, and the mesured specific rotation values of compounds mach perfectly the literature data.

In a recent work, Albericio et al.[47] proposed a strategy based on the use of NaH/CH_3I for *N*-methylation of cysteine: in fact a robust method wich would allow the synthesis in solution of a broad range of *N*-Me-Cys derivatives that could respond to peptide synthetic requirements, has not yet been achived.

Since the major by-products from this way, was from a β-elimination reaction, *N*-Alloc-Cys(Trt)-OH (**70**), a more stable precursor to basic

medium, was chosen as starting material for *N*-methylation. **70** is subjected to standard procedure of methylation of *N*-Boc-amino acids wich use more MeI than used in *N*-Boc-Cys(Me)-OH.

Scheme 17.



The trityl group being much hindered, avoid the β-elimination reaction: the conversion of substrate is total, and no side-products were observed.

Starting from this intermediate [*N*-Alloc-*N*-Me-Cys(Trt)-OH], a number of *N*- and *S*-protected *N*-Me-Cys can be accessed easily by changing the protecting groups either in solution or in solid-phase.[47]

2.2. *N*-methylation by reductive amination

A promising method for installing the *N*-methyl function is reductive amination, which offers the possibility of placing alkyl groups other than methyl, covers the way of reductive amination. Methods devised for reducing the intermediate Schiff bases involve transition metal catalyzed hydrogenation, borohydride reduction, and Leuckart type reactions. Borane reduction has also been extended to *N*-formylamino acids with success.

2.2.1. Transition metal catalyzed reduction

N-Alkylation of amino acids by the Schiff base approach works well for aldehydes other than formaldehyde.[48] The simplicity of Schiff base reduction is extremely appealing, since the formation of this base is generally a mild, straightforward process performed by adding equivalent amounts of aldehyde and amine in an appropriate solvent and then reducing the intermediate.

The steric hindrance conferred by the alkyl group and amino acid side chains helps to minimize or prevent dialkylation, but not in the case of formaldehyde. In all cases reported, attempted mono-*N*-methylation of amino acids with formaldehyde results in a combination of *N,N*-dimethylation, *N*-monomethylation, and no reaction.[48-49] This is readily explained by the fact that the Schiff base intermediate when reduced to the *N*-methyl species is then a secondary amine that has greater nucleophilicity than the parent primary amino acid. Given that the methyl group is the smallest alkyl group, Schiff base formation with the secondary amine is favorable and occurs readily.

Keller-Schierlein and co-workers[49] synthesized N^{α}-methyl-N^{δ}-benzyloxycarbonyl-L-ornithine from N^{δ}-benzyloxycarbonyl-L-ornithine. N^{δ}-benzyloxycarbonyl-L-ornithine was treated with a solution of formaldehyde, and then reduced with sodium borohydride to give a mixture of di- and mono-*N*-methylamino acids and starting material. However, after cromatography purification, the expected compound was obtained in only 35% yield.

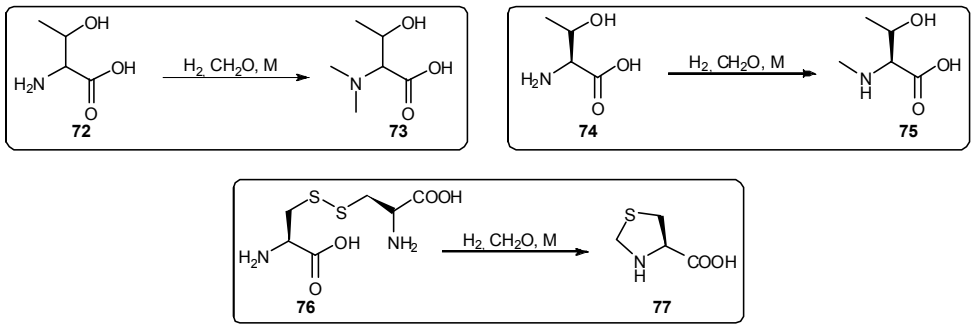
In a series of three papers, Bowman[50] describes the *N,N*-dimethylation of amino acid residues through the use of aqueous formaldehyde over Pd/C in a hydrogen atmosphere. This method provided quantitative yields of the *N,N*-dimethylamino acids of alanine, valine, leucine,

phenylalanine, tyrosine, cystine, aspartic acid, and glutamic acid, and it was noted that the *N,N*-dimethyl derivative of aspartic acid was racemized in aqueous solution at 100°C.[50a] In the second paper the researcher extends the methodology to the mono-*N*-alkylation of valine, leucine, and phenylglycine with various alkanals in ethanol or aqueous ethanol: working on the glycine, *N,N*-dialkylated amino acid was recovered.[50b] In the last work published, it is presented the reductive alkylation of di- and tripeptides as a means for the identification of the *N*-terminal amino acid, utilizing the same protocols as the two previous papers.[50c]

Ikutani[51] applied the Bowman methodology to synthesize *N,N*-dimethylamino acids of glycine, alanine, leucine, phenylalanine, and tyrosine and converted them to *N*-oxides by peroxide treatment. This was also the approach Poduska[52] used in dimethylating lysine derivatives.

The reductive amination of amino acids via palladium catalysis is a cheap, effective, and racemization free route to *N,N*-dimethylamino acids and mono-*N*-alkylamino acids, but selective monomethylation is impractical with this method. However, Suyama et al.[53] has been reported a case of selective monomethylation via transition metal catalyzed reduction of amino acids in the presence of formaldehyde with platinum, Raney nickel, and zinc activated with cobalt (Scheme 18).

Scheme 18.



They found that DL- and *allo*-DL-threonine (**72**) were easily dimethylated, but L- (**74**) and *threo*-D-threonine were mainly monomethylated. Under the same conditions, cystine **76** provided the thiazolidine carboxylic acid **77** (Scheme 18).

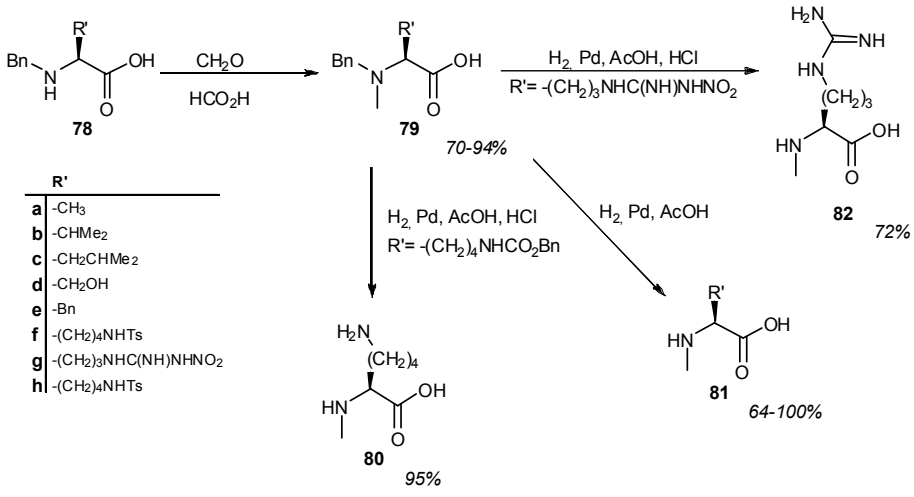
2.2.2. Leuckart reaction

The Leuckart reaction is a convenient one-step method for reductive amination of a wide variety of carbonyl compounds by their reaction with an amine in the presence of formic acid. The procedure involves heating *N*-benzylamino acids in formic acid solution in the presence of formaldehyde until effervescence due to carbon dioxide ceases.

This is the only type of reductive amination with formic acid/formaldehyde to produce NMAs; no other variations have been described in the literature.

Quitt et al.[54] reveal the functional group tolerance of this strategy for *N*-methylating *N*-benzylamino acids (Scheme 19).

Scheme 19.

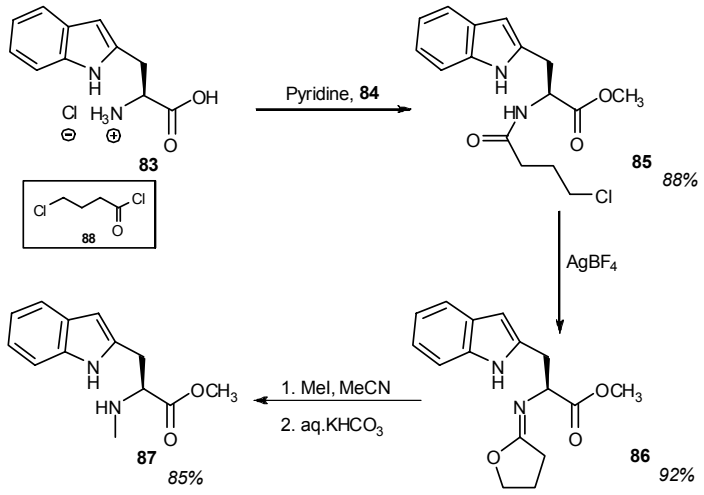


Two substrates that present difficulties for some other methods, lysine **78f** and **78h** and arginine **78g**, were successfully *N*-methylated via reductive amination with formaldehyde and formic acid to give structures **80** and **82**, respectively. The physical data obtained from these derivatives have provided a benchmark for comparison of NMAs due to the mildness of this racemization free method. The methodology was extended to other amino acids such as aspartic and glutamic acid, isoleucine, threonine, tyrosine, and glycine with success, as reported in a work of Ebata et al.;[55] albeit, the reactions were low yielding.

2.2.3. Quaternisation of imino species

Quaternization of imino species is a method rarely adopted for NMA preparation. However, this is an attractive approach for monomethylation of amino acids given that the imino group can only be alkylated once and this precludes possible dialkylation. Eschenmoser et al.[56] applied this novel procedure to the formation of *N*-methyltryptophan (L-abrine, **83**, Scheme 20).

Scheme 20.



In this case, *N*-chlorobutyroyltryptophan methyl ester (**85**) was treated with silver tetrafluoroborate, resulting in a cyclization to the iminolactone **86** limiting the valences of the nitrogen available for alkylation (Scheme 20). Quaternization of the imino species with methyl iodide was followed by hydrolysis with aqueous potassium carbonate to give **87**. The conversions of **85** to **86** and then to **87** can be done in one pot in 85% yield, and notably, the process was free of racemization.

O'Donnell and Polt[57] described the efficient preparation of Schiff base derivatives of amino acids by transimination from the reactive benzophenone Schiff base. Subsequently, the reaction with dimethylformamide dimethyl acetal that generated the amidine,[58] and the quaternization of the amidine with methyl sulfate or methyl triflate gives an iminium salt, which after hydrolysis give the *N*-methylamino acid.

2.2.4. Borohydride reduction

Borohydride reductions are alternative approaches to transition metal catalyzed reduction of Schiff base intermediates; however, they are seldom used to reduce Schiff bases, since chemical yields are compromised by competing side reactions.[57] Milder borohydrides such as sodium cyanoborohydride are more suited to this application, especially in the *N*-alkylation of amino acid esters with aldehydes.[59] Alternatively, triacetoxyborohydride has been recommended as a replacement reducing agent to sodium cyanoborohydride in that less toxic side products are formed and better yields and reproducibility of results can be obtained.[48d-e]

Jentoft and Dearborn[57] have described the reductive amination of proteins with formaldehyde in the presence of sodium cyanoborohydride, to produce *N*,*N*-dimethylated proteins. The reaction was described as

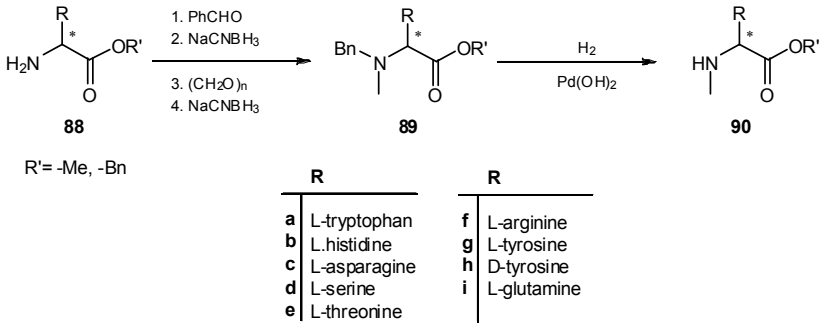
regiospecific, with methylation occurring only at the *N*-terminus and at lysyl side chains. They also discuss the superiority of sodium cyanoborohydride over sodium borohydride in its mildness and specificity in the reductive amination.

Polt et al.[48a] expanded on the utility of the ketimines by reducing them with sodium cyanoborohydride to give secondary amines. Condensation of a secondary amine with excess formaldehyde or other aldehydes in the presence of excess sodium cyanoborohydride gave *N*-(diphenylmethyl)-*N*-methylamino esters that were hydrogenolyzed over palladium catalyst to afford the *N*-methylamino acid esters.

Kaljuste and Undén[60] reported a novel small-scale approach to *N*-methylation via reductive alkylation on solid phase, effecting the mono-*N*-methylation of resin bound terminal amino acid residues. The authors describe the need for a readily removed *N*-protecting group as a means to prevent dialkylation, and to achieve this, the acid labile 4,4'-dimethoxydiphenylmethyl (4,4'-dimethoxydityl or Dod) group was employed.[61] *N*-Methylation of terminal solid-phase bound amines was performed with formaldehyde, acetic acid, and sodium cyanoborohydride in DMF. This reaction proceeded in yields in the range 56-99% for most common amino acids. Up to three methylation cycles for some amino acids were needed for complete reaction. It was noted that trifunctional amino acids required longer reaction times that could lead to undesirable side reactions. This could be avoided by decreasing reaction time, but consequently, incomplete methylation occurred.

Konopelski et al.[62] reported a facile two-pot synthesis on *N*-methyl amino acid esters by way of reductive amination (Scheme 21). The procedure was performed, without resorting to side-chain protection (**88**), via consecutive reductive amination reactions, first with benzaldehyde, then with paraformaldehyde.

Scheme 21.



Both sequences of imine formation/reduction are performedin the same flask, witout isolation. Following isolation of *N*-benzyl-*N*-methyl amino acid derivative (**89**), removal of the benzy protecting group affords desired material, and no trace of racemization are present.

2.2.5. Borane reduction

Borane is a mild reagent for the conversion of amides to the corresponding amines. The rate of reduction of amides by borane is dependent on several factors. In general, tertiary amides are more easily reduced than secondary amides, which are more easily reduced than primary amides.[63] It has also been shown that alpha-substituted amides tend to be more rapidly reduced than their unbranched counterparts. Both the electronic and steric nature of the substituent(s) on nitrogen also influence the rate of reduction. Brown and Heim report that for optimum reduction, 1.7 mmol of borane should be used per mol of tertiary amide, 2.0 mol per mol of secondary amide and 2.3 mol per mol of primary amide.

Krishnamurthy[64] achieved selective reduction of aniline formamide intermediates with a borane dimethyl sulfide complex ($BH_3 \cdot SMe_2$). A minimum of 2 equiv is required for the reduction, where 1 equiv is

involved in the reduction and the other equivalent is involved in complexation with the *N*-methylamine derivative formed. The two-step process provided high purity *N*-methylanilides in 80-100% yield.

Chu and co-workers[65] exploited the strategy of Krishnamurthy in the reduction of *N*-formyl-D-tryptophan methyl ester with a borane dimethyl sulfide complex. The reduction provided, after workup, *N*-methyl-D-tryptophan methyl ester in 56% yield.

Hall et al.[66] reduced amides in solution and on solid support with diborane. Iodine was employed in the reduction to promote oxidative cleavage of the borane-amine adducts. In this fashion, amino acid formates coupled to Wang resin were reduced with diborane in >72% yield and >75% purity for alanine, valine, serine, and phenylalanine.

Reductive amination is a very mild and racemization free process. Quitt's method[54] of reductive amination involving *N*-benzylamino acids is, to date, one of the recommended methods for the synthesis of NMAs and has been used frequently for comparison of physicochemical data. Amino acids such as lysine, serine, and arginine are readily *N*-methylated by this method.

A similar approach, adopting sodium cyanoborohydride reduction of *N*-(diphenylmethyl)amino acid ester Schiff base intermediates in solution and solid phase as described by Polt et al.[48a] and Kaljuste and Undén,[60] respectively, has revealed the efficacy of this approach as applied to a wide variety of amino acids, albeit on a small scale.

Although, in principle, this technique is similar to Quitt's method, there are more manipulations involved and the carboxyl terminus must be blocked. Another drawback is the excessive amounts of formaldehyde and sodium cyanoborohydride required for complete reaction that further limit this procedure to small-scale synthesis.

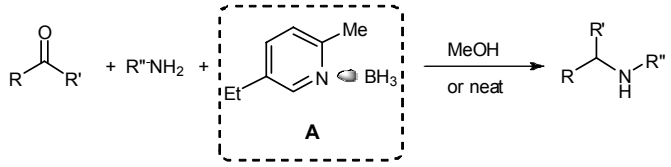
Reductive amination involving transition metal hydrogenolysis is

somewhat limited to dimethylation with formaldehyde, but monoalkylation with aldehydes other than formaldehyde is possible.[55-57] Even though there has been a case of selective monoalkylation of *threo* type threonines,[60] this selectivity is not conferred on other amino acids.

One seldom reported technique is the reduction of *N*-formylamino acids. This approach is an obvious one, since formylation of amino acids is readily achieved and, therefore, concerns of dialkylation and the need for multistep syntheses are eradicated. The only problem is the carboxyl group needs to be protected, since the use of borane can reduce acids to alcohols, and other amide groups present may also be reduced.

Sato et al.[67] introduces a new compound to carry out the synthesis of complex amine containing molecules: 5-ethyl-2-methylpyridine borane complex (PEMB). Amineboranes **A** are useful reducing reagents for carrying out such transformations (Scheme 22). Complexation of borane, BH_3, with a Lewis base, such as an amine, produces air and moisture stable amine borane adducts.

Scheme 22.



The strong interaction of the borane with the nitrogen of amine compounds leads to lower reactivity, compared to ether and sulfide complexes, allowing for enhanced stability, selective reactions and simplified handling. PEMB is a stable liquid and initial stability testing suggests a longer shelf life at ambient temperatures compared to pyridine borane, PyB. This includes: observed increases in color, density and viscosity which signal the progression of PyB polymerization that can

occur over time. The chemical reactivity of PEMB is equivalent to or greater than that of pyridine borane and 2-picoline borane for reductive amination. In addition, the reagent is effective at reductive amination in a variety of solvents including methanol, water or even neat conditions. The high yield, high through put methodology will increase the usefulness of the borane mediated reductive amination in industrial applications.

2.3. *N*-methylation by novel methods

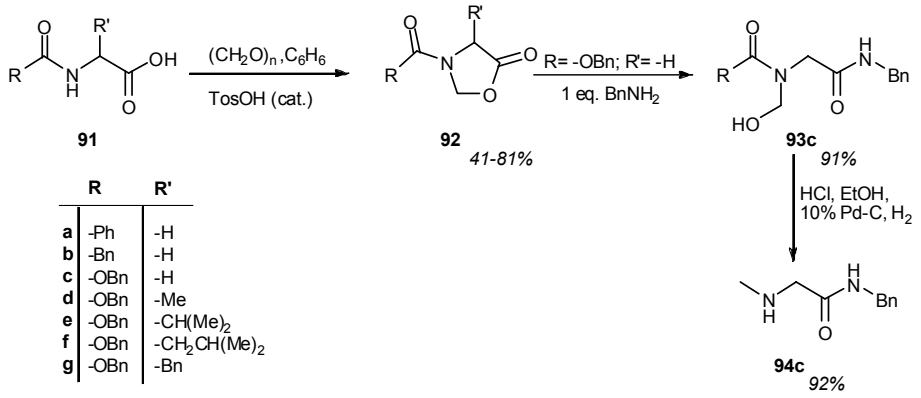
The following section is dedicated to the novel and sometimes-elaborate methodologies for *N*-methylamino acid synthesis. Although some of the methods employ aspects of previous sections for installing the *N*-methyl moiety, techniques were devised to prepare especially unusual NMAs required for natural product synthesis and other studies. The techniques devised for unusual NMA syntheses in most cases catered for particular NMA derivatives and are often not applicable to all or even some other amino acids.

2.3.1. 5-oxazolidinones

Ben-Ishai[68] noted that, when combining *N*-Cbz-amino acids with paraformaldehyde under acid catalysis, the reaction assumes an intramolecular mode and so 5-oxazolidinones **92** are prepared (Scheme 23). The 5-oxazolidinone ring is susceptible to nucleophilic attack and is easily opened by amines to form amides.[69] Ben-Ishai demonstrated this in the treatment of the 5-oxazolidinone **92c** with 1 equiv of benzylamine to afford the *N*-hydroxymethyl amide **93c**. Hydrogenation then gave an

N-methylglycine (sarcosine) derivative **94c**. It was noted that the *N*-hydroxymethyl amide **93c** could be treated with 1 eq. of benzylamine that removed the *N*-hydroxymethyl moiety, providing *N*-Cbz-glycine benzylamide.

Scheme 23.



Auerbach et al.[70] synthesized *N*-hydroxymethyl (or *N*-methylol) amides analogous to structure **93** from primary and secondary amides. They then demonstrated that the *N*-methyl amide could be generated by treatment with triethylsilane/trifluoroacetic acid in chloroform. They inferred the reduction proceeds by hydride transfer from the silane to an acyliminium ion derived from the *N*-hydroxymethyl amide. The author also describe the palladium catalyzed hydrogenation of *N*-hydroxymethyl amides to *N*-methyl amides in the presence of trifluoroacetic acid.

Several chemists have recognized the efficacy of 5-oxazolidinones, and so improvements have been made to their preparation and utility in conversion to other synthetically useful intermediates.

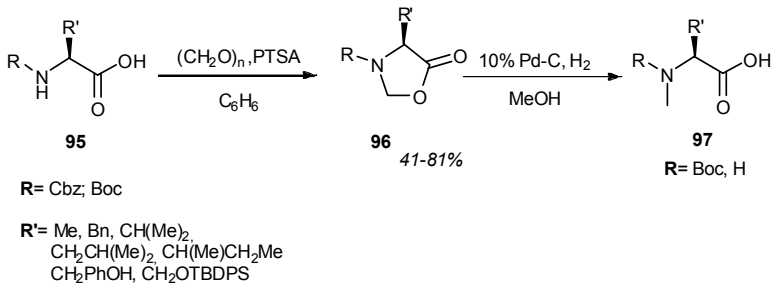
Freidinger et al.[71] extended the range of substrates that can be converted to 5-oxazolidinones through the use of Fmoc-protected amino acids and alkanals including paraformaldehyde. Using the conditions of Auerbach et al., triethylsilane/trifluoroacetic acid reductive cleavage

gave the expected *N*-Fmoc-*N*-methylamino acids and the *N*-alkyl derivatives. This sequence was applied to Fmoc-alanine, valine, methionine, phenylalanine, lysine, serine, and histidine.

Chipens et al.[72] applied the same methodology to the *N*-Cbz-5-oxazolidinones of glycine and phenylalanine and reduced them with triethylsilane/trifluoroacetic acid to the corresponding *N*-methyl derivatives, as did Aurelio et al.[73]

Reddy et al.[74] further extended the technique by preparing 5-oxazolidinones **95** with *N*-Cbz and *N*-Boc protection (Scheme 24) and then converted the *N*-Cbz compounds to NMAs with concomitant removal of the benzyl carbamate by hydrogenation over palladium catalyst. This was the first report of success in the use of hydrogenation of 5-oxazolidinones as a means of producing the *N*-methyl group directly. Reddy et al. also reports reducing *N*-Boc-5-oxazolidinones over palladium catalyst to generate *N*-Boc-*N*-methylamino acids **96**.

Scheme 24.

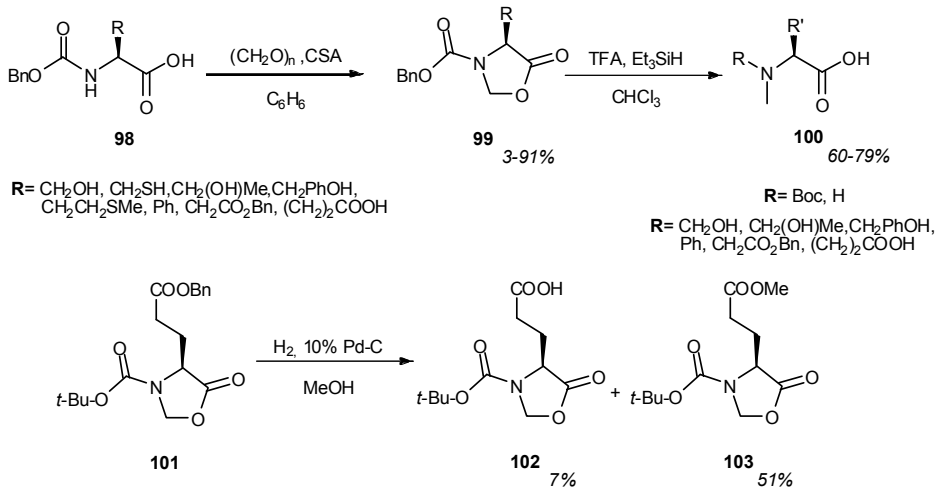


Aurelio et al. used the conditions described by Reddy et al. to prepare the *N*-Cbz-5-oxazolidinones **99** of numerous α-amino acids (Scheme 25). Several substrates with reactive side chains were attempted, with varying degrees of success. Threonine and serine, in particular, were prone to oxazolidinine formation by reaction with the side chain hydroxyl, and side chain protection was necessary for 5-oxazolidinone syntheses of α-

amino acids with basic side chains in order for the intermediates to form.

Other α-amino acids such as tyrosine, glutamic acid, and methionine were converted to the corresponding 5-oxazolidinone. Reduction of several of these substrates by catalytic hydrogenation gave varying amounts of the free α-amino acid in accord with Itoh. The authors resorted to the triethylsilane/trifluoroacetic acid reductive cleavage applied by Freidinger et al. to effect formation of NMAs **100**. Aurelio et al. also attempted the hydrogenolysis of the *N*-Boc-5-oxazolidinone **101** but did not isolate any of the expected NMA. Instead, were reconered two products **102** and **103** from reaction of the side chain.

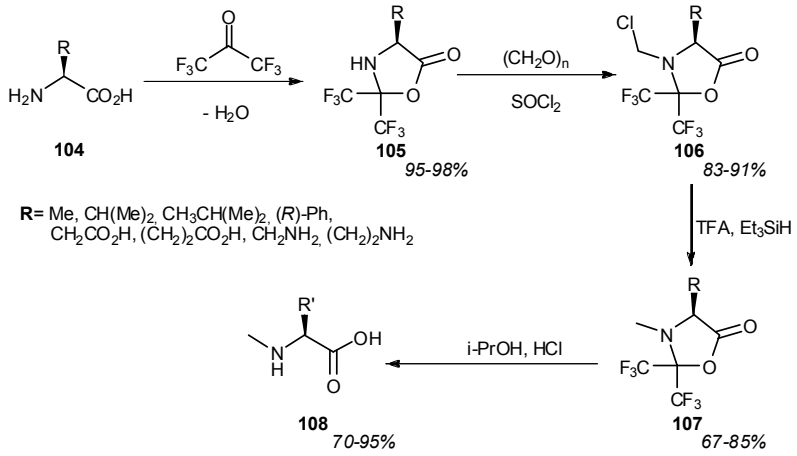
Scheme 25.



Spengler and Burger[75] employing 5-oxazolidinones not as the source of the *N*-methyl carbon but as a means of forming a cyclic aminal **105** (Scheme 26). The 2,2-bis(trifluoromethyl)-1,3-oxazolidin-5-ones were formed by reaction of the α-amino acids with hexafluoroacetone. The product aminals have the carboxylic function removed from the possibility of reaction and have only one nitrogen valence left for reaction, ensuring the mono-*N*-methylamino acid forms. The aminal **105**

was chloromethylated, and then the chloromethylaminal **106** was converted to the *N*-methyl-5-oxazolidinone **107** with triethylsilane and trifluoroacetic acid. Finally, acidolysis with 2-propanol or methanol allows for the isolation of the NMA **108**.

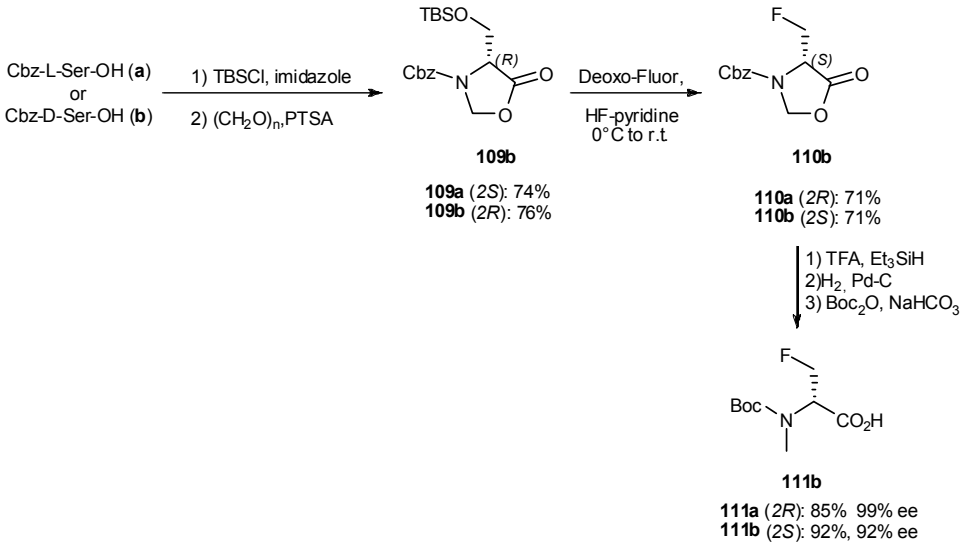
Scheme 26.



Recently, Hoveyda and Pinault,[76] discovered a convergent route for the synthesis of both enantiomers of *N*-Me analogues of 3-fluoroalanine through the use of oxazolidinones as synthetic intermediates (Scheme 27). Starting from Cbz-D-Ser-OH, or Cbz-D-Ser-OH, the TBS protection of the side chain alcohol, followed by the formation of the oxazolidinone with paraformaldehyde, was effected through standard conditions to obtain intermediates **109a** and **109b**, respectively. The fluorinated oxazolidinones (**109a**, **109b**) were then reduced by using the triethylsilane/TFA conditions reported by Freidinger and co-workers, although rather sluggishly, requiring 2 days for its completion. During this prolonged acidolytic reductive ring-opening, the Cbz protective group was partially cleaved. As we needed an acid labile protective group, such as Boc, in the final product, we simply proceeded to the hydrogenolytic deprotection of the remaining Cbz, followed by

protection of the secondary amine by the Boc protective group to obtain both antipodes of *N*-methyl-3-fluoroalanine (Scheme 27, **111a,b**).

Scheme 27.

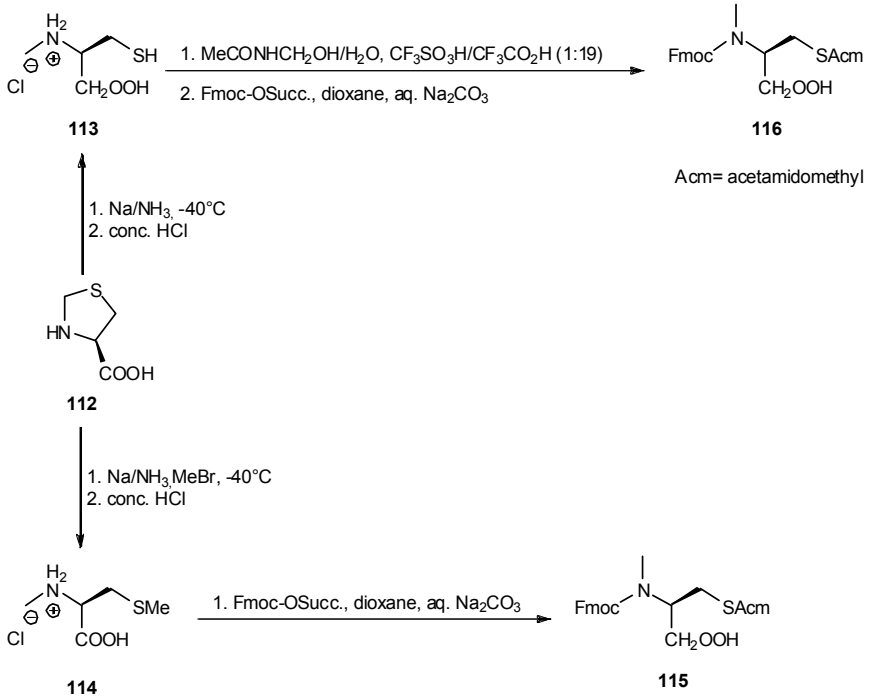


Another variation was described by Yamashiro et al.,[77] who utilized the thiazolidine as intermediate in the synthesis of [1-(*N*-methylhemi-L-cystine)]oxytocin by treating cysteine with formaldehyde solution. Sodium in liquid ammonia reduction of the thiazolidine intermediate thus obtained with an equivalent amount of water provides the *N*-methylcysteine, which was treated in the same pot with an equivalent amount of benzyl chloride, providing the *N*-methyl-*S*-benzyl-L-cysteine in 90% yield (the addition of water is crucial in suppressing dimerization). Carbamoylation with Cbz-Cl furnished *N*-Cbz-*S*-benzyl-L-cysteine in 84% yield.

Liu et al.[78] employed the same reductive protocol as Yamashiro et al.[77] in synthesizing Fmoc derivatives of *N*-methyl-L-cysteine (Scheme 28). The thiazolidine **112**[79] was reduced in the usual manner to provide *N*-methyl-L-cysteine **113**. In situ treatment with methyl bromide provides

the *S*-methyl derivative **114**, which was treated with Fmoc-succinimide to afford *N*-Fmoc-*N*,*S*-dimethyl-L-cysteine **115**. Alternatively, treatment of **113** with *N*-hydroxymethylacetamide and an organic acid provided an *S*-acetamidomethyl intermediate that was converted to the Fmoc derivative **116**.

Scheme 28.

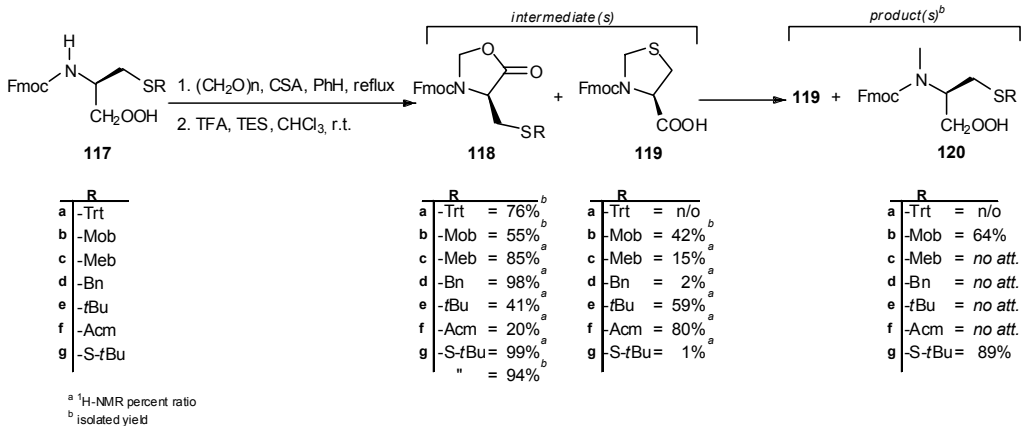


Reduction of the thiazolidine intermediate in the synthesis of *N*-methylcysteine is probably the most cost-effective and scalable procedure for synthesizing this derivative. The chemical manipulations involved are trivial, and the added advantage is the fact that regioselective alkylation of the thiol group enables a variety of cysteine derivatives to be synthesized.

The work of Ruggles et al.[80] focuses on the generation of *N*-MeCys and its subsequent use in Fmoc solid-phase peptide synthesis for the generation of *N*-methyl-cystine containing peptides. A number of

cysteine derivatives, varying in sulfhydryl protection, were subjected to *N*-methylation via an oxazolidinone intermediate. The Mob-substituent was chosen first as a result of its ease of removal, as well as its stability to acidic conditions (Scheme 29). Unfortunately, upon oxazolidinone formation not only was the desired intermediate **118b** formed but also thiazolidine **119b**. In the case of Cys(Trt), **119b** was not observed until acidic ring opening of oxazolidinone **118b**. However, here the Mob-protected sulfur-atom captures the iminium ion prior to capture by the carboxyl during the oxazolidinone forming step.

Scheme 29.



After isolation, treatment of **118b** with Freidinger's conditions did lead to the formation of *N*-Fmoc-*N*-Me-Cys(Mob)-OH. However, thiazolidine was formed again, but while the production of oxazolidinone was satisfactory, the generation of thiazolidine during both steps diminished its overall appeal. Both Meb- and Bn-protected cysteines also formed thiazolidine (**119c** and **119d** respectively) however, in much lower amounts. The degrees of thiazolidine formation between Mob, Meb, and Bn are attributed to the electrondonating character of the para-substituent on the benzene ring. While both Meb and Bn did provide the desired

oxazolidinones, the eventual difficult sulfhydryl deprotection of these groups led to investigating other sulfur-protected cysteines such as tBu and Acm. Regrettably both Cys(tBu) and Cys(Acm) derivatives formed significant quantities of thiazolidine. In the case of the Cys(tBu) derivative, the electrondonating *t*Bu-substituent and 3°-cation produced upon *t*-Bu cleavage are responsible for thiazolidine formation. This was not observed with Trt-protected due to the enhanced bulky character of the Trt-protecting group making sulfur-cyclization difficult. The pronounced formation of thiazolidine with the Cys(Acm) derivative is a result of the Acm protecting group's lability to TFA in the presence of hydride donors such as was used in this procedure. Clearly, the electronics and acid lability of the sulfhydryl protecting group play a dominant role in the formation of undesired thiazolidine. A suitable protecting group, one that is either electronwithdrawing or electronically neutral coupled with TFA stability and ease of deprotection postsynthesis, was still needed. This led to the investigation of *S*-*t*Bu-protected cysteine since this disulfide protecting group is primarily electronically neutral, stable to TFA, and can be removed easily by thiolysis. Oxazolidinone formation was superb and **118g** was isolated in excellent yield. Satisfyingly, acidic cleavage of oxazolidinone **118g** provided MeCys(StBu) **120g** in excellent isolated yield.

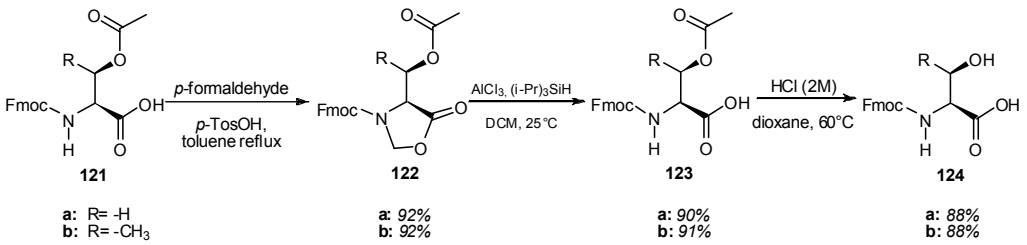
Use of Cys(StBu) for the generation of MeCys completes the study of Aurelio and coworkers[73c] since thiazolidine formation is circumvented and the difficulties with decomposition observed in their study with sulfur-containing amino acids was not observed with this derivative. In their report about the synthesis of *N*-benzyloxycarbonyl protected *N*-methyl serine and threonine *via* 5-oxazolidinone reduction, the side chain *β*-hydroxyl group was protected by O-acetylation. Furthermore, reductive cleavage with TFA gave the benzyloxycarbonyl protected *N*-methyl

serine/threonine. This method has been highly successful in generating novel *N*-benzyloxycarbonyl protected *N*-methylamino acid derivatives. However, reductive cleavage with TFA again required a long reaction time.

As a result of these limitations, Zhang et al. developed a more efficient method for the reduction of the intermediate Fmoc-oxazolidinones of non-functionalized and functionalized side chain-containing amino acids, using a Lewis acid catalyst such as $AlCl_3$ or $ZnBr_2$.[81] Further, it was reported that the $AlCl_3$ reaction needed 4 h for completion whilst that with $ZnBr_2$ required 22 h, with negligible side product formation. However, when we attempted this reaction for the synthesis of Fmoc-*N*-methyl-Ser(OH)-OH and Fmoc-*N*-methyl-Thr(OH)-OH without side chain protection, no *N*-methylated product was obtained using either $AlCl_3$ or $ZnBr_2$. Chruma reported the synthesis of *N*-methyl amino acids, including Ser and Thr, by reductive amination of O'Donnell's Schiff base amino esters with $NaBH_3CN$ and the appropriate aldehyde in THF.[48a] This method was found to be inadequate for the large scale production of *N*-methylated Ser and Thr, due to the diffculty in preparing the Schiff bases of the parent amino acids.

Bahekar et al.[82] reported an efficient method for the synthesis of Fmoc-*N*-methyl serine and threonine (Scheme 30).

Scheme 30.



In general, *N*-methylation of *β*-hydroxy amino acids is dificult, mainly due to *β*-elimination or dehydration. As shown in Scheme 30, the *N*-

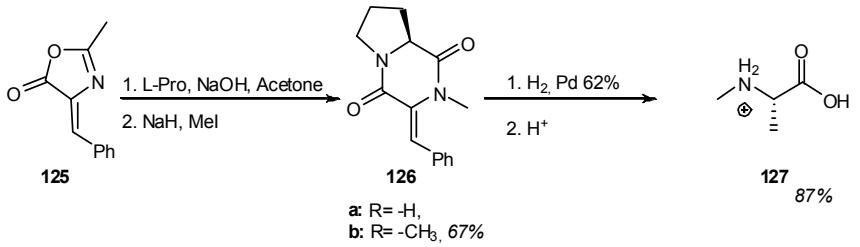
Fmoc-protected *O*-acetylamino acids **121a-b** were converted in the corresponding oxazolidinones **122a-b** in very good yields.

Reduction of the oxazolidinone was achieved using $AlCl_3$ and triisopropylsilane ($(iPr)_3$-SiH) giving Fmoc-*N*-methyl-*O*-acetyl amino acids **123a-b**. Finally, deprotection of the hydroxyl group was achieved using 2 M HCl giving Fmoc-*N*-methylated-*β*-hydroxyl amino acids **124a-b** in very good yields. Typically, the reaction time for the formation and subsequent reduction of the corresponding oxazolidinone was less than 1 h and the crude product obtained after work-up was sufficiently pure.

2.3.2. Asymmetric synthesis

One of the earliest study about the asymmetric synthesis of NMAs was reported by Poisel and Schmidt.[83] In this work, proline was used as an auxiliary in the asymmetric synthesis of amino acid derivatives (Scheme 31).

Scheme 31.

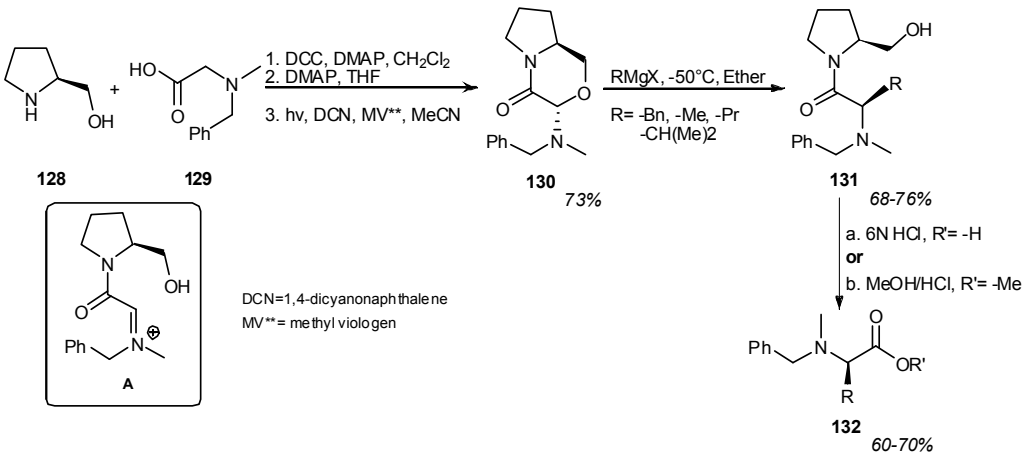


The azlactone **125** prepared from *N*-acetylglycine and benzaldehyde was treated with L-proline under basic conditions to form an arylidenedioxopiperazine **126a**. Methylation with sodium hydride/methyl iodide provided the chiral piperazine **126b**. Simple hydrogenation conditions using palladium metal provided *N*-methyl-L-phenylalanine-L-

proline diketopiperazine in 90% ee, which was cleaved under acidic hydrolysis, affording the free *N*-methylphenylalanine **127**.

Pandey et al.[84] utilized a sarcosine derived chiral precursor based on a recyclable L-prolinol auxiliary as a masked iminium ion equivalent **81** (Scheme 32). *N*-Benzylsarcosine **129** and L-prolinol **128** were condensed to form the chiral auxiliary **A**, and this was then treated with Grignard reagents to yield *N*-methylamino acid-L-prolinol dipeptides **131** with good stereoselectivity. Hydrolysis of the dipeptides with either aqueous HCl or methanolic HCl provided the corresponding *N*-benzyl-*N*-methylamino acids or esters **132**, respectively, and L-prolinol **128**, which was recycled (96% recovery).

Scheme 32.

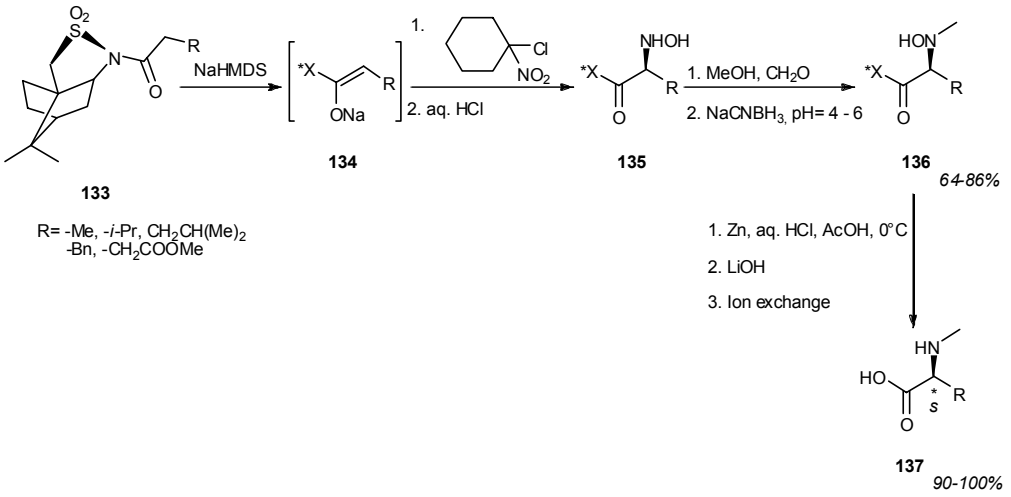


Agami et al.[85] have devised an elaborate method for construction of NMAs using the *"asymmetric derivatization of glycine cation equivalents*" to construct various NMAs. The first step involves a three-component condensation between *N*-methyl-D-phenylglycinol, glyoxal, and thiophenol to make the chiral intermediate[85a] that can be treated with an organozinc reagent (giving retention of configuration) or alternatively with a cuprate reagent (giving inversion of configuration), to generate the

required α-center with excellent control (>98% in most cases).

Oppolzer and co-workers[86] have a long and distinguished record of stereoselective transformations with camphorsultams and have used the *π*-face selective hydroxyamination of the camphorsultam enolate **134** to construct the α-amino acid skeleton as a hydroxylamine **135** (Scheme 33). The hydroxyamination was highly stereoselective, and the crystalline hydroxyamino products had enantiomeric excesses > 99%. Reductive alkylation of the hydroxylamine in methanolic formaldehyde with sodium cyanoborohydride followed by *N*,*O*-hydrogenolysis with zinc dust afforded the (*N*-alkylamino)acylsultam that was then cleaved by base hydrolysis to give the NMA **137**. The advantages of this multistep sequence are twofold: First, by the simple expedient of changing the camphorsultam auxiliary to the other enantiomer, preparation of the *R*-configured *N*-alkyl-α-amino acid is allowed equally efficiently.

Scheme 33.

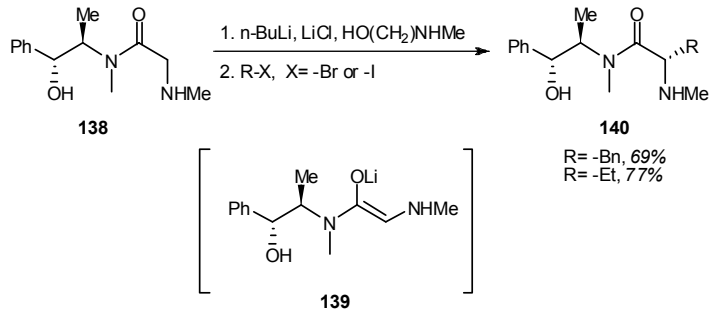


And second, the acyl function that was first appended to the auxiliary comprises the side chain of the final α-amino acid, and so modified α-

amino acids can be constructed with extreme structural variation. This last point demonstrates the versatility of the camphorsultam auxiliary, which has the potential to be applied in the development of *N*-methylamino acid libraries with high structural diversity.

Myers et al.[87] have made use of pseudoephedrine as a chiral auxiliary in the asymmetric synthesis of amino acids and *N*-methylamino acids. (*R,R*)-Pseudoephedrine was coupled to sarcosine to provide pseudoephedrine sarcosinamide **138** (Scheme 34). Treatment with *n*-butyllithium produced the enolate **139,** which was quenched with an alkyl halide to provide the *N*-methylamino acid pseudoephedrine dimer **140**, with excellent stereocontrol. Where R = Bn, the alkylation product **140** (*N*-methylphenylalanine) was isolated in 93% yield and 88% de in the crude state. Recrystallization afforded **140** in 69% yield and 99% de. Where R=-Et, **140** was isolated in 77% yield and 94% de after purification.

Scheme 34.



Radical based chemistry is one approach that has found limited use in NMA synthesis.

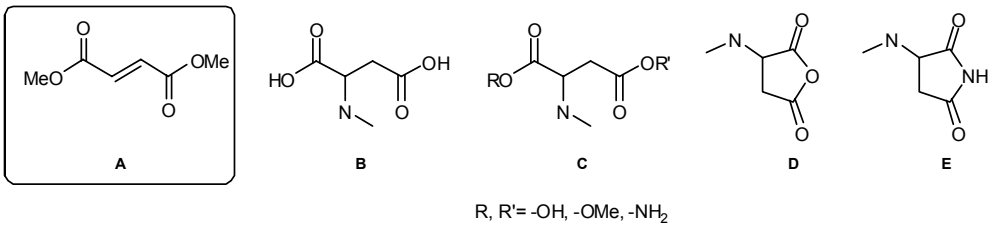
Laplante and Hall[88] have devised an ingenious solid supported *N*-methylation with pinacol chloromethylboronic ester. The process is based on Matteson's 1,2-carbon-to-nitrogen migration of boron in α-

aminoalkylboronic esters. This is basically the only procedure in which monomethylation of an *N*-unprotected amino acid is achieved in high yields with relative ease.

A practical method for the synthesis of *N*-methyl-DL-aspartic acid and new syntheses for *N*-methyl-aspartic acid derivatives are described, by Boros et al..[89] Since the early preparations of **B**, several methods aimed at the synthesis of NMA derivatives **C** (Figure 4). A shortcoming of these methods is, however, the propensity of asparagine, aspartic acid and their derivatives to form succinic-anhydride **D** or succinimide **E** cyclic intermediate products, leading to aspartyl and isoaspartyl residues, due to spontaneous ring opening.

Figure 4.

Structures of NMA derivatives

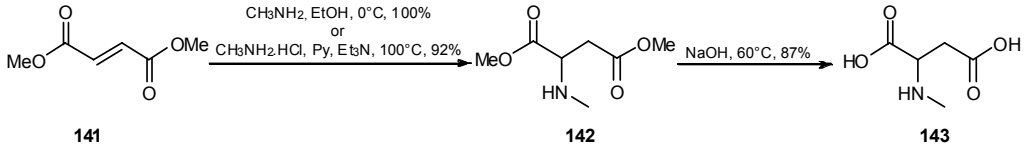


The authors report the syntheses of NMA's derivatives that were accomplished using fairly common strategies, including Michael addition of methylamine to substituted acrylates, by using **A** as starting material.

They have found that reaction of equimolar dimethyl fumarate **141** (**A**) and methylamine in ethanol solution at 0°C in 1 h results in rapid formation of *N*-methyl-DL-aspartic acid dimethyl ester **142** in quantitative yield (Scheme 35).

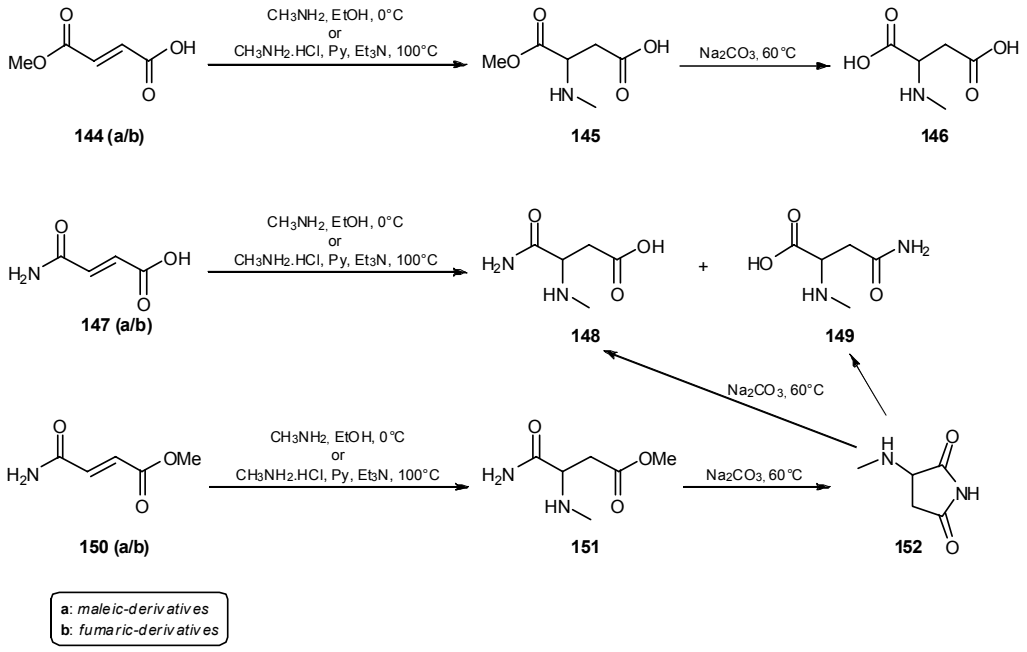
Under similar conditions, methyl maleate and methyl fumarate could be converted to *N*-methyl-DL-aspartic acid β-methyl ester.

Scheme 35.



In the cases of maleamic **150a** or fumaramic esters **150b**, the α-amide derivative **148** was formed, but hydrolysis of the product provided *N*-methyl-DL-asparagine **149** via base catalyzed ring closure to DL-α-methylamino-succinimide **152**, followed by selective ring opening (Scheme 36).

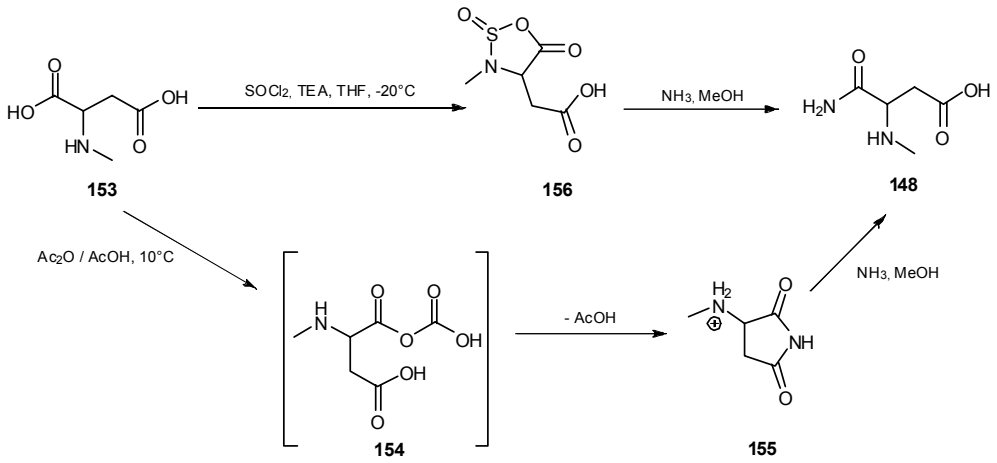
Scheme 36.



Efficient alternative methods for the preparation of NMA-α-amide **148** from unprotected NMA were developed, *via* sulphinamide anhydride **156** and aspartic anhydride **155** intermediate products (Scheme 37). The five-

membered sulphinamide anhydride **155** ring structure of the intermediate product, was formed in the reaction of **153** with thionylchloride. In the next step, the thio-analogue of cyclic Leuchs-anhydride **155**, a well known sensitive agent against nucleophylic reagents, reacts with ammonia. Ring opening and elimination of sulphur dioxide take place after nucleophylic attack of ammonia, leading exclusively to the α-amide, **148**.

Scheme 37.



In small scale it was reported that the reaction gave the expected product in good yield, but as a consequence of the unstable nature of the intermediate and the product, degradation occurred in larger scale before completion of the reaction.

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CHAPTER 2

Results and discussion

1. Introduction

Peptides are molecules of paramount importance in the fields of health care and nutrition. Several technologies for their production are now available, among which chemical and enzymatic synthesis are especially relevant.

Different technologies are now available for the production of peptides and proteins: the extraction from natural sources,[1] the production by recombinant DNA technology,[2] the production in cell-free expression systems,[3] the production in transgenic animals[4] and plants,[5] the production by chemical synthesis[6] and by enzyme technology using proteolytic enzymes under conditions of displacement of the equilibrium of the reaction towards the formation of peptide bond.[7]

The size of the molecule determines the technology most suitable for its production. Recombinant DNA technology is particularly suitable for the synthesis of large peptides and proteins, as illustrated by the case of insulin and other hormones.[8]

Chemical synthesis is a viable technology for the production of small and medium size peptides ranging from about 5 to 80 residues.[9]

Enzymatic synthesis is more restricted and has been hardly applied for the synthesis of peptides exceeding residues. Its potential relies on the synthesis of very small peptides and, in fact, most of the cases reported

correspond to dipeptides and tripeptides.[10] In this sense, the technologies for peptide production are not competitive with each other in most of the cases. Chemical and enzymatic approaches do not exclude each other and a recent trend is the combination of both.[11]

The application of native peptides for pharmacological use may have some restrictions because of the degradation by endogenous proteases, hepatic clearance, undesired side effects by interaction with different receptors, and low membrane permeability due to their hydrophilic character.[12] Low bioavailability of peptides following oral administration, attributed to their inactivation in the gastro-intestinal tract through, poor intestinal permeation, short half-life, limit their use as therapeutic agents in the clinical setting.[12c]

Chemically modified peptides with improved bioavailability and metabolic stability may be directly used as drugs and many efforts have been made to develop peptide-based, pharmacologically active compounds.

The most straightforward approach for peptide modification is to introduce changes into the side chains of single amino acids. The introduction of such functional groups that do not occur naturally in peptides restricts its conformational flexibility and enhances its metabolic stability.

Other option is the modification of the peptide chain (*backbone*), which can be accomplished in different ways: its exchange by an amide analogue, the alkylation of the -NH group of the peptide bond,[13] the reduction of the carbonyl group of the peptide bond,[14] or the exchange of the NH group of a peptide bond by an oxygen atom (depsipeptide),[15] a sulfur atom (thioester),[16] or a -CH_2 group (ketomethylene isostere).[17] Notable among these modified peptides are depsipeptides that are compounds having sequences of amino and hydroxy carboxylic acid

residues regularly alternating; they can also form cyclic structures named cyclodepsipeptides, where the residues are connected in a ring. There is great interest in this class of products due to their wide range of biological properties such as immunosuppressant, antibiotic, antifungi, antiinflammatory and antitumoral activities.[18]

Another approach is the construction of constrained structure peptidomimetics in which a region of a defined structure in the natural peptide is synthesized and bound by its amino and carboxy terminals through a non protein ligand.[19]

The synthesis of *β*-peptides (peptides constructed from non-natural *β*-amino acids) is a new promising area of peptide research. Short chains of *β*-amino acids usually form more stable secondary structures than the natural α counterparts and have proven to be endowed with interesting biological properties, for instance: inhibition of cholesterol and fat intestinal absorption.[20] These non-natural peptides are resistant to enzymatic degradation which can be a relevant characteristic for their therapeutic application.[21] Small *β*-peptides can be synthesized using conventional Fmoc/tBu SPPS strategy,[22] but the protocols have to be modified for longer peptides because of the high folding propensity of *β*-peptides, which increases coupling times and prevents complete deprotection. However, the replacement of piperidine by stronger bases has circumvented the problem of incomplete deprotection in Fmoc/tBu solid phase synthesis of *β*-peptides[23] and longer *β*-peptides and mixed type *α*-*β*-peptides have been successfully synthesized by chemical ligation.[9] *β*-Amino acids are, however, very expensive building blocks so that the usual practice in SPPS of using excess reagents has to be avoided. This problem has not been fully addressed and remains to be solved from a technological point of view. Methodologies for *β*-peptide synthesis have been recently reviewed.[24]

Identification of peptide-based drugs with acceptable oral bioavailabilities will be a major challenge confronting pharmaceutical scientists in the future. The rational design of orally bioavailable active-peptide will necessitate a compromise between structural features that optimize the pharmacological properties (e.g., receptor binding) and structural features that optimize the pharmacokinetics properties (e.g., membrane permeability, clearance, metabolism) of the molecule.

The major biological barriers to the oral delivery of peptide-based drugs include the intestinal lumen, intestinal mucosa and the liver.[25]

Three broad approaches to the development of orally active peptide-drugs have been taken:

1. The earliest such approach, and that best represented in today's pharmacy, is the identification of a non-peptidic natural product that mimics or antagonizes the biological activity of an endogenous peptide.
2. At the other extreme is the identification of an appropriate protein and directly confronting bioavailability issues by using a novel route of delivery or the co-administration of an enzyme inhibitor.
3. The middle ground is the strategy of peptidomimetics that resemble a target protein but contain some synthetic element (aminoacidic residues) designed to reduce metabolism and to optimize the biological activity of the agent.

The range of tools available for limiting peptide bond hydrolysis in vivo extends from simple additions that chemically protect the targeted bond from attack, to its replacement altogether, to global changes that, instead, modify the peptide conformation in such a way that it is no longer recognized by the protease of concern.

It is important to recognize that these two strategies are never completely unrelated, as even modest structural changes near the scissile peptide bond can result in significant conformational differences. For example, one straightforward chemical modification that can slow the addition of an enzymatic nucleophile to a peptide bond is the introduction of steric bulk in the form of an *N*-alkyl (usually methyl) group. Studies on *N*-methyl amino acids (NMA) containing peptides reveal that those residues increase proteolytic stability, increase membrane permeability (lipophilicity), and alter the conformational characteristics or properties of the amide bonds.[26]

Several research's groups have developed NMA containing peptides that show improved proteolytic resistance.[27] It was reported the site-specific substitution of certain peptides (e.g. the nonapeptide leuprolide), that are known luteinizing hormone-releasing hormone (LHRH) agonists, with the corresponding NMA.[28] Other analogues were found to be completely resistant to the action of chymotrypsin. This was attributed to the interruption of key hydrogen bonds in the chymotrypsin active site by *N*-methylation of the peptide substrate. Endothelin-1 is a peptidic constrictor of vascular smooth muscle cells. Starting from a hexapeptide antagonists of the receptor for Endothelin-1,[29] one potent hexapeptide inhibitor, site-specifically *N*-methylated, was synthesized: this analogue has significantly increased proteolytic resistance and enhanced antagonist activity. A series of recent papers relating to Alzheimer's disease consider the use of small peptidic ligands bearing *N*-methyl amide bonds as a means of interrupting or reversing amyloid protein aggregation into toxic fibrils or lumps.[30]

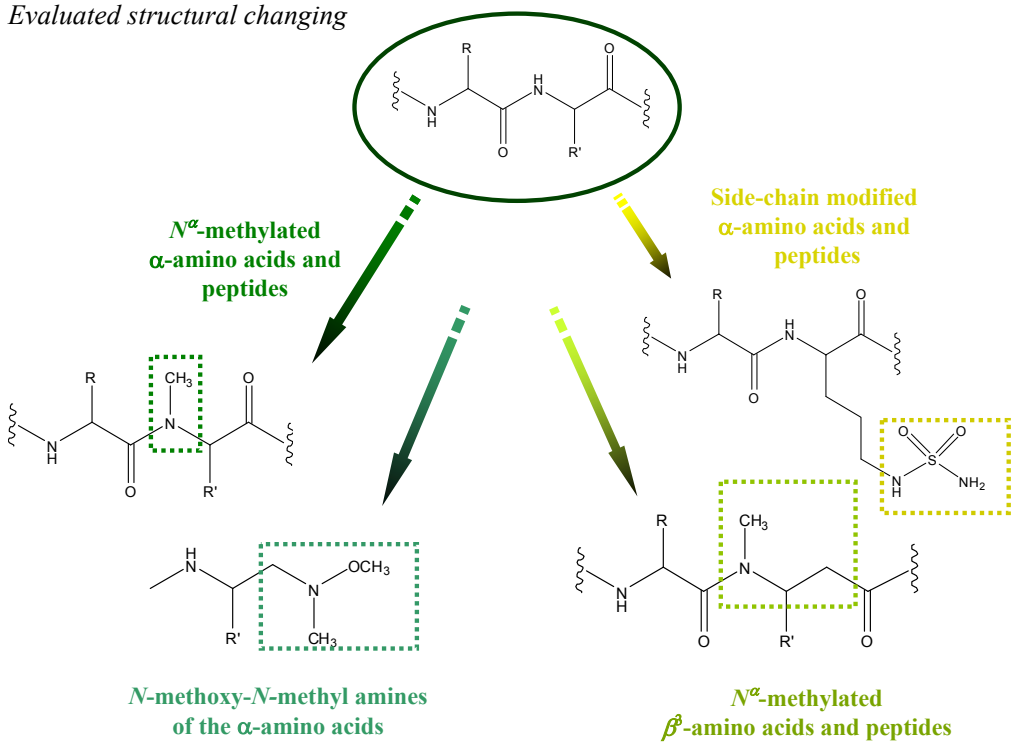
Several strategies have been adopted to reduce their enzymatic cleavage and to improve their systemic uptake, including structural modifications of the peptide chain such as β-amino acid substitution, and

N-methylation of aminoacidic residues.

Having all this in mind, my interest during the three years of PhD was directed towards the synthesis of modified amino acids: particular attention was given to the modifications of side-chain, amino and carboxyl functions of α-amino acids (Figure 1).

Figure 1.

Evaluated structural changing



The last part of my work was dedicated to the qualitative determination of organic analites in foods matrices by mass-spectrometry methodologies.

2. *N*-Fmoc-*N*-methyl and *N*-methyl-*N*-Nosyl α-amino acids

Amino acids, which are essentially incorporated in proteins, peptides, enzymes, and large numbers of bio-active phenomena have recently gained much interest in synthetic organic chemistry as extremely desirable building blocks, chiral auxiliaries, catalysts, and ligands. While Nature's creativity and precision in the construction of various structures is impressive, the development of practical and efficient synthetic methodologies for the construction of amino acids is still in its infancy.

N-Methyl amino acids are a group of important modified amino acids, which have been widely used in medicinal chemistry and biochemistry to change the conformation, restrict the flexibility, and enhance the potency of the molecule.[31] Incorporation of *N*-alkyl amino acids into peptides often improves the biological profile by increasing the proteolytic stability, conformational rigidity, and lipophilicity.

Various protocols have been developed for the synthesis of optically active *N*-methyl amino acids,[32] but the methods for installing the *N*-methyl moiety in the full range of amino acids still remain difficult.

We recently developed a simplified method which allows fast and efficient *N*-methylation of short peptide sequences protected on the carboxyl function as methyl esters, that involves the protection of the terminal amino function with the *p*-nitrobenzensulfonyl group (*Nosyl* group, Ns) and the subsequent *N*-methylation of the resulting peptides with an ethereal solution of diazomethane.[33] The role of Nosyl is dual: in fact this group protects and activates the amino function of the amino acid derivatives allowing the use of softer conditions for the *N*-methylation step. The methylation of *N*-Nosyl-protected amino acids and peptides with diazomethane affords the expected methylation of the

sulfonamide nitrogen atom afterwards the formation of the methyl ester at the carboxyl function. In peptide synthesis the methyl ester cleavage is normally performed with difficulty and could cause racemization at the chiral centres of amino acids.

The availability of Nosyl and Fmoc-protected *N*-methylated amino acids certainly represents a significant advancement for the incorporation of *N*-methylated amino acids into peptide chains according to the Nosyl- and Fmoc-chemistry procedures both in solution and solid phase.[34]
The aim of this part of my work is the development of a methodology to prepare *N*-Nosyl and *N*-Fmoc protected *N*-methylated amino acids using diazomethane as methylating reagent.

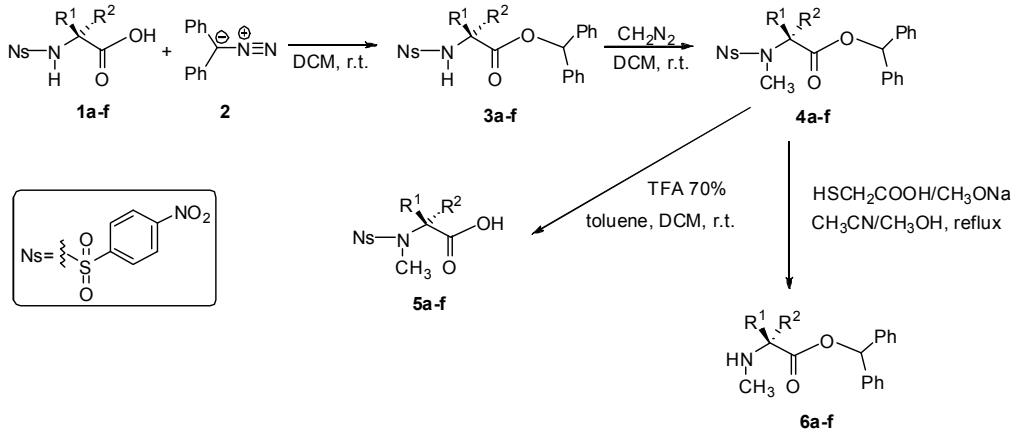
It is plain that for achieving this goal the α-amino acid carboxyl function needs a preliminary protection with a transient and easily removable blocking group. Protection and deprotection steps are critical in many synthesis schemes: appropriate choice of protector for the carboxyl group of the amino acid is crucial, because it must tolerate the reaction conditions and also be removable without affecting the rest of the molecule. Amino acid methyl esters are easy to prepare[35] and are commercially available. However, in spite of its stability, the methyl function has not been used as carboxylic acid protecting group because of the deprotection conditions: epimerization at the α-carbon of the amino acid moiety during the saponification step by basic hydrolysis is in fact a well-known problem.[36]

It seemed to be very attractive blocking the carboxyl moiety as diphenylmethyl (benzhydryl) ester.[37] This protecting group can be readily cleaved by hydrogenolysis[38] or acidolysis,[39] reaction conditions that usually do not affect peptides adversely; furthermore it is easily prepared starting from diphenyldiazomethane under neutral conditions.[40] Diphenyldiazomethane is easily obtained by the oxidation of

benzophenone hydrazone with Magtrieve^{TM} under mild and non-toxic reaction conditions. Magtrieve^{TM} is a selective, heterogeneous form of CrO_2, whose reduced form stays on the crystal surface.[41] It may be easily removed after the reaction by a simple magnetic separation, or by filtration on Celite.^{TM} This has significant environmental and cost advantages over traditional chromium reagents that require aqueous work-up and consequent appropriate disposal of the chromium waste. In addition, the reduced chromium surface can be simply reconverted to CrO^2 by heating in air, thus adding to its recyclability and cost-effectiveness.[39]

The *N*-Nosyl-α-amino acid benzhydryl esters **3a-f** were prepared in quantitative yields by treating the corresponding *N*-Nosyl-α-amino acids **1a-f** with diphenyldiazomethane **2**, prepared *in situ* by oxidation of benzophenone hydrazone with Magtrieve^{TM} in dichloromethane at room temperature for 15 min (Scheme 1, Table 1).

Scheme 1.



The resulting mixture was maintained under an inert atmosphere (N_2)

and stirred at room temperature. TLC analysis (EtOAc/hexane, 1:5 v/v) showed complete conversion of the starting compounds within 40 min. In addition, all reactions were also monitored by GC/MS analysis.

Table 1.

*Result of the synthesis of N-methyl-N-Nosyl-α-amino acid (**5a-f**) and N-methyl-α-amino acid benzhydryl esters (**6a-f**).*

entry	R^1	R^2	5 yield (%)[a]	6 yield (%)[a]
a	$-CH(CH_3)_2$	-H	94	96
b	-H	$-CH(CH_3)_2$	94	96
c	$-CH_2CH(CH_3)_2$	-H	94	95
d	$-CH_2S(Bzl)$	-H	95	92
e	$-CH(CH_3)CH_2CH_3$	-H	98	95
f	$-CH_3$	-H	98	90

[a] Isolated yield

After the reaction was finished, Magtrieve™ was retrieved by filtration through Celite™ and the solution was concentrated under reduced pressure to afford the corresponding benzhydryl esters **3a-f** in quantitative yields. Benzhydryl esters **3a-f** was fully caracterized by GC/MS, and NMR spettroscopy.

Characteristic of the Nosyl-derivatives is the presence in their mass spectra (EI) of the typical peaks at m/z 186 and m/z 122 deriving from the fragmentation of the Nosyl-group; in addition to these peaks, Benzhydryl esters derivatives, show as a tipical peak at m/z 167, the fragment that originates from loss of RCOO- radical.

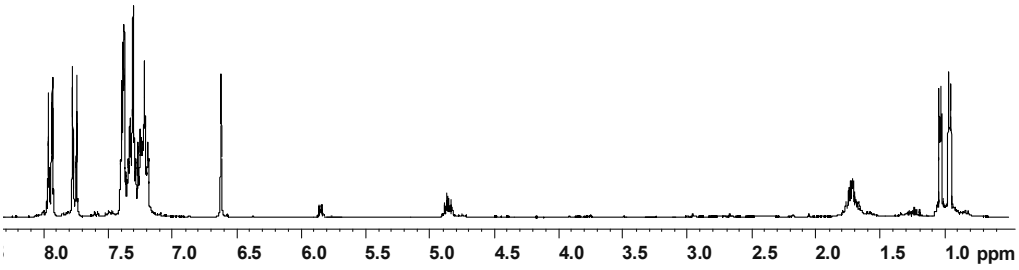
For example, the mass spectrum of the *N*-Nosyl-L-valine benzhydryl ester (**3a**) is characterized by the peak deriving from the loss of isopropyl side-chain radical (m/z 273), but the base peak is at m/z 167 that is the Benzhydryl cation; finally, the fragments at m/z 186 and m/z 122, are structurally correlated to the presence of the Nosyl-protecting group.

Figure 2 shows the ^{1}H-NMR spectrum of the same compound (**3a**): in

particular it is possible to distinguish two doublets at 0.82 ppm and 1.02 ppm characteristic of the two diastereotopic methyl groups of valine; the signals of the benzydryl protecting group are the singulet at 6.65 ppm (methinic proton), and the multiplet at 7.18-7.39 ppm corresponding to the aromatic protons.

Figure 2.

*N-Nosyl-L-valine benzhydryl ester (**3a**): ^{1}H-NMR (300 MHz)*



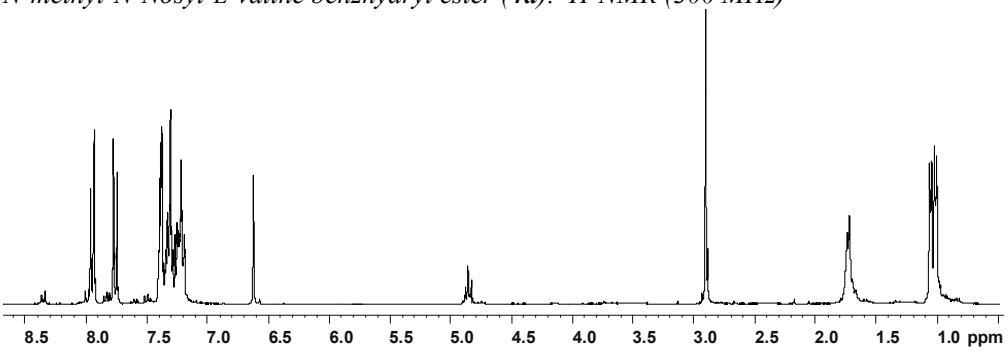
3a-f were then treated with diazomethane: a 0.66 M solution of diazomethane in dry dichloromethane (8 mmol) was added dropwise to a magnetically stirred solution of the *N*-Nosyl-α-amino acid benzhydryl esters **3a-f** (1 mmol) in dry dichloromethane at room temperature (Scheme 1). Within 1.5h, by simple evaporation of the solvent under reduced pressure, the corresponding *N*-methylated derivatives **4a-f** were recovered in quantitative yields and high purity.

Figure 3 shows the ^{1}H-NMR analysis performed on a sample of *N*-methyl-*N*-Nosyl-L-valine benzhydryl ester (**4a**).

A comparison between this spectrum with that reported in Figure 2 (the starting material), it is possible to observe the disappearance of the doublet signal at 5.88 ppm relative to –NH, and the appearance of a singulet at 2.94 corresponding to the protons of methyl group on the sulfonamidic function.

Figure 3.

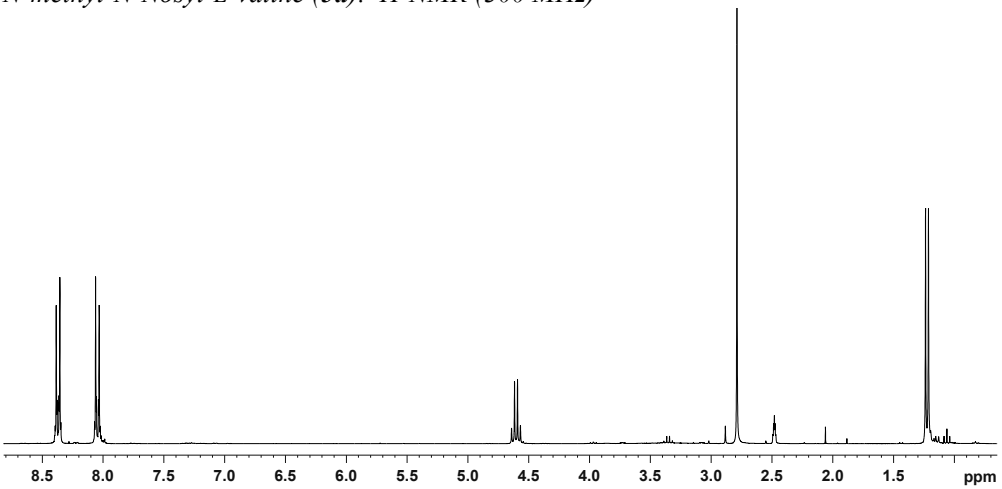
*N-methyl-N-Nosyl-L-valine benzhydryl ester (**4a**): ^{1}H-NMR (300 MHz)*



The *N*-methylated benzhydryl esters **4a-f** are the key precursors of both *N*-methyl-*N*-Nosyl-amino acids and *N*-methyl-*N*-Fmoc-amino acids. The treatment of **4a-f** with 70% trifluoroacetic acid in dichloromethane in the presence of toluene as a scavenger, afforded the corresponding *N*-Nosyl-*N*-methylated amino acids **5a-f** in high yields (94-98 %) (Scheme 1, Table 1).

Figure 4.

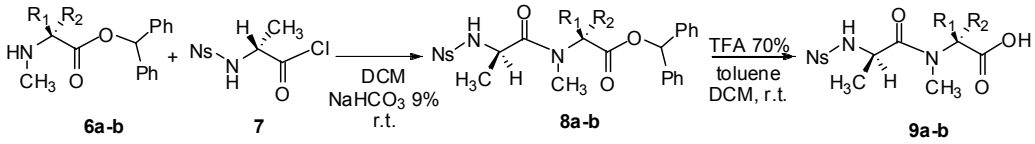
*N-methyl-N-Nosyl-L-valine (**5a**): ^{1}H-NMR (300 MHz)*



An alternative reaction path was developed to obtain starting from the

N-methylated benzhydryl esters **4a-f** the corresponding *N*-methyl-*N*-Fmoc-amino acids. This approach involved the formation, as synthetic intermediates, of the *N*-methyl amino acid benzhydryl esters **6a-f** (Scheme 1, Table 1), obtained by deprotection of amino function: for this pourpose mercaptoacetic acid (3 mmol) was added to a solution of **4a-f** (1 mmol) in dry acetonitrile (10 mL) under N_2 at 50 °C. Sodium methoxide (7 mmol) was then gradually added to the solution with a variable amount of methanol to facilitate the sodium methoxide solubilization. The resulting mixture was stirred for 40 min monitoring the conversion of the precursors **4a-f** by TLC (EtOAc/hexane, 1:5 v/v) and the formation of the *N*-deprotected products **6a-f** by ninhydrin test. After work-up, the combined organic extracts, dried over Na_2SO_4, were evaporated under vacuum to afford the *N*-Methyl-α-amino acid benzhydryl esters **6a-f** in 90-96% overall yields. The stereochemical integrity of the *N*-methylated products **6a-f** was investigated by converting **6a-b** into the corresponding diastereomeric dipeptides **8a-b**. The dipeptides **8a-b** were synthesized by coupling of **6a-b** with *N*-Nosyl-D-alanine chloride **7** under Schotten-Baumann conditions (Scheme 2).[42]

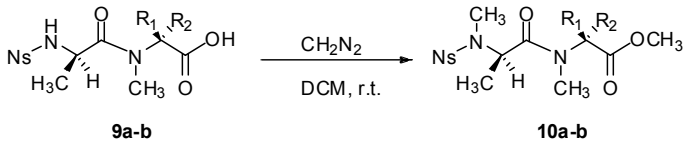
Scheme 2.



The products **8a-b** were recovered in good overall yield (89 and 91%, respectively) and isolated in high purity grade, without need of chromatographic purification. ^{1}H-NMR spectra of **8a** and **8b** were different for some signals and showed the presence of a single

diastereomer in both samples. The presence of only one diastereoisomeric dipeptide in both crude products proved distinctly that the stereochemistry of the chiral centres is retained throughout the methylation process and the subsequent deprotection of the amino function. Furthermore, in order to exclude any detectable racemization process, the dipeptides **8a** and **8b** were also analysed by GC/MS after conversion into the corresponding more volatile methylated dipeptides **10a-b** (Scheme 3).

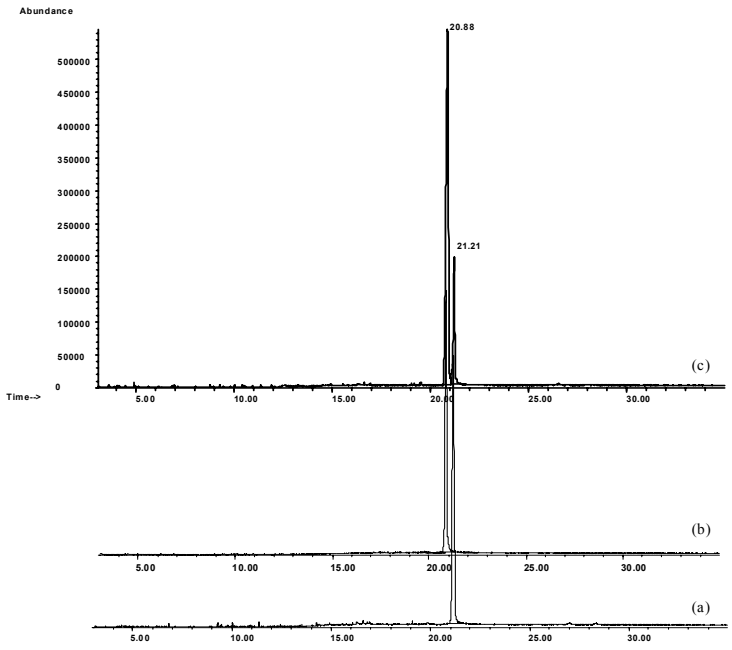
Scheme 3.



9a-b **10a-b**

Figure 5.

*CG/MS analyses of N-Nosyl dipeptides: (a) N-methyl-N-Nosyl-D-alanyl-N-methyl-L-valine methyl ester (**10a**) (r.t. 21.21 min); (b) N-methyl-N-Nosyl-D-alanyl-N-methyl-D-valine methyl ester (**10b**) (r.t. 20.88 min); (c) a mixture of **10a** and **10b**.*

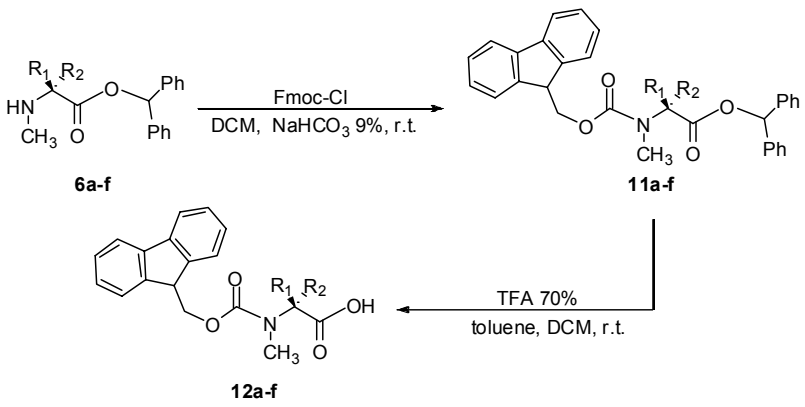


GC/MS analysis performed on an appropriately prepared mixture containing 28 mg of the crude dipeptide **10a** and 70 mg of the crude dipeptide **10b** was compared with those obtained from the single products **10a** and **10b** (Figure 5).

Hence every step of the adopted procedure, from the *N*-methylation of the *N*-Nosyl-α-amino acid benzhydryl esters up to the deprotection of amino function, does not cause any loss of the chiral integrity of the asymmetric α-carbon atoms of the precursors.

The main goal to achieve was the obtainment of *N*-methyl-*N*-Fmoc-α-amino acids to employ directly as building blocks for the construction of peptide chains based on Fmoc strategy. To this purpose, the synthetic intermediates **6a-f** (1 mmol) were suspended in a $NaHCO_3$ solution (6 mL, pH = 8), then a solution of FmocCl (1 mmol) in dichloromethane was added gradually and the resulting mixture was stirred at room temperature for 1h (Scheme 4). After complete conversion of the starting reactants, the workup of the reaction mixture allowed the recovery of the corresponding *N*-methyl-*N*-Fmoc-amino acid benzhydryl esters **11a-f** in high overall yields (88-95%) and with high purity grade (Table 2).

Scheme 4.



To remove the carboxyl protecting group, trifluoroacetic acid (5-7mL

$mmol^{-1}$) and toluene (2 nnmol) were added to a solution of the *N*-Methyl-*N*-Fmoc-α-amino acid benzhydryl esters **11a-f** in dichloromethane (Scheme 4, Table 2). The resulting mixture was stirred at room temperature and under N_2 for 1 h. After complete conversion, work-up procedures afforded the corresponding *N*-methyl-*N*-Fmoc-amino acids **12a-f** in excellent yields (94-98 %).

Table 2.

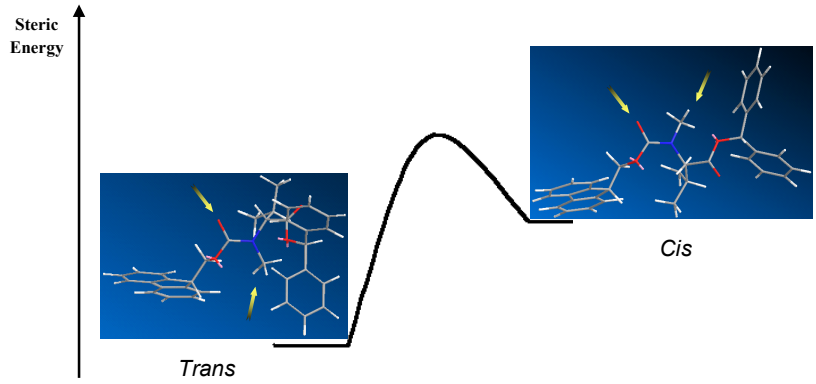
*Result of the synthesis of N-Fmoc-N-methyl-α-amino acid benzhydryl esters (**11a-f**) and N-Fmoc-N-methyl-α-amino acid (**12a-f**).*

entry	R^1	R^2	11 yield (%)[a]	12 yield (%)[a]
a	$-CH(CH_3)_2$	-H	92	96
b	-H	$-CH(CH_3)_2$	92	96
c	$-CH_2CH(CH_3)_2$	-H	90	98
d	$-CH_2S(Bzl)$	-H	95	94
e	$-CH(CH_3)CH_2CH_3$	-H	94	98
f	$-CH_3$	-H	88	96

[a] Isolated yield

As an example, the ^{1}H-NMR spectrum of *N*-Fmoc-*N*-methyl-L-valine is given in the Figure 7. The character of double bond in the amide linkage imposes a barrier rotational that originates two rotamers, clearly visible in this experiment done at 19 °C (Figure 6).

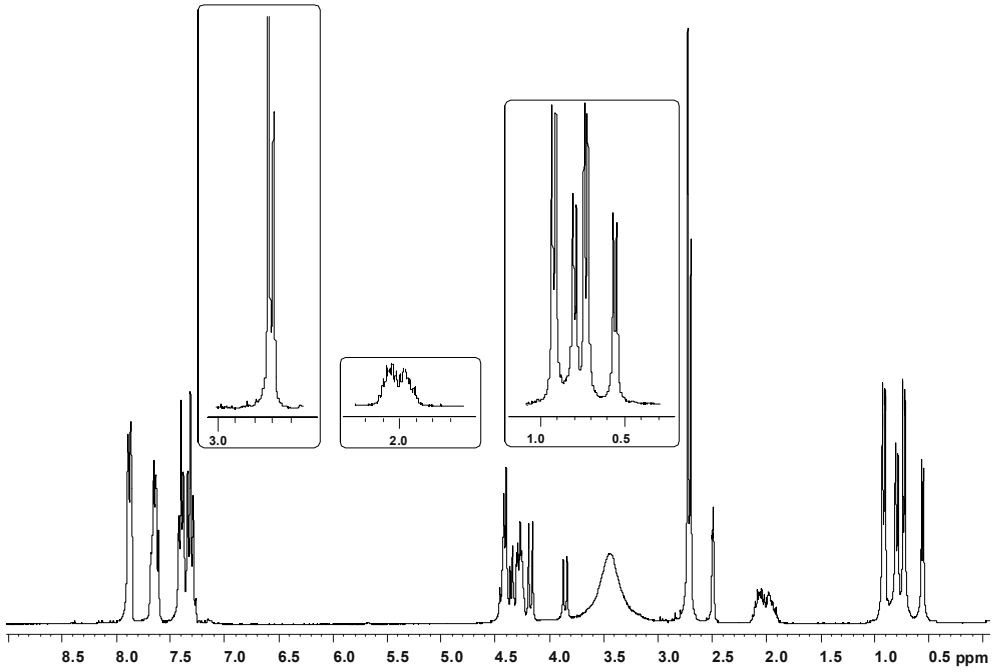
Figure 6.



In particular, in the spectrum it is appreciable the splitting of the signals at 0.60-0.94 ppm deriving from the two diastereotopic methyl groups of valine, of the singulet related to the protons of methyl on sulfonamidic nitrogen (2.78 ppm) and finally, the multiplet related to the proton of β-CH (1.95-2.05 ppm).

Figure 7.

*N-Fmoc-N-methyl-L-valine (**12a**): ^{1}H-NMR (300 MHz)*



The obtained results demonstrate that the synthesis of *N*-methylated *N*-Fmoc and *N*-Nosyl protected α-amino acids occurs in a simple way and in high yields.

In conclusion it was reported a simple and convenient methodology for the preparation of *N*-Nosyl- and *N*-Fmoc-*N*-methyl-α-amino acids.

The benzhydryl group represents an useful carboxyl protecting group for its easy introduction, stability to ongoing methylation reaction, and

selective removal under mild conditions.

An additional advantage of this protecting group consists in the possibility to work with products that, when are protected on the amino function with the nosyl group, are easily analyzed by GC/MS.

The *N*-methylation with diazomethane of *N*-Nosyl-α-amino acid benzhydryl esters provides the key structural precursors of both Nosyl- and Fmoc-*N*-methylated α-amino acids.

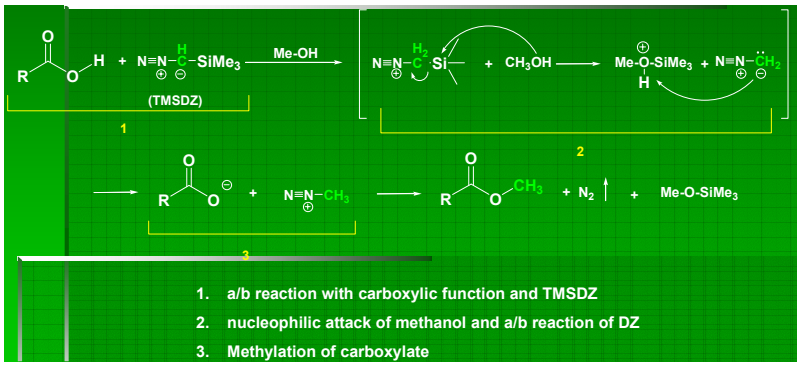
The entire synthetic process, do not cause any detectable racemization of the amino acid chiral centres.

3. Methylation of α-amino acids and derivatives using trimethylsilyldiazomethane

Diazomethane has been used for the *N*-methylation of amino acids and peptides.[33, 43] However, this reagent is explosive when it is prepared by distillation and used in hydrocarbon solvents which cannot solvate it.[44] Furthermore it is dangerous because the methyl diazonium ion could methylate the alcoholic hydroxyl, amino, thiol and carboxyl functions of biological structures. Alternatively, diazomethane can be prepared *in situ* from trimethylsilyldiazomethane, a safe and easy to prepare precursor.[45] Trimethylsilyldiazomethane (TMSDZ) must be used in the presence of a protic nucleophile such as methanol which determines the "release" of diazomethane (Figure 7). Subsequently, it provides a proton that converts diazomethane into methyl diazonium ion.
Trimethylsilyldiazomethane usually reacts much slower than diazomethane.

Figure 7.

Reaction mechanism of reagent system TMSDZ/Methanol with carboxylic acids

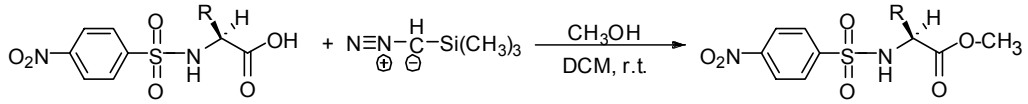


The use of commercially available trimethylsilyldiazomethane can be

extended to not exclusively chemical fields, because of the safety of the reagent and the absence of risks for the health. In fact, trimethylsilyldiazomethane can be easily handled by users that operate in biological and medical contexts also for routine procedures. Thus, this reagent could represent a general tool for the methylation of amino acids and peptides.

N-Nosyl-α-amino acids react instantaneously with an excess of diazomethane both on the sulfonamidic function and the carboxyl group, providing the corresponding *N*-methyl-*N*-Nosyl-α-amino acid methyl esters. On the other hand it is not possible to realize exclusively the methylation of carboxyl group: the use of a small amount of diazomethane led to a mixture of products. In fact, treatment of *N*-Nosyl-L-alanine (**1f**) with 1 equiv. of diazomethane provided a mixture of *N*-Nosyl-L-alanine methyl ester (60% yield), *N*-methyl-*N*-Nosyl-L-alanine methyl ester (10% yield) and the unchanged starting material. Reaction of **1f**, dissolved in dry dichloromethane, with a 2.0 M ethereal solution of trimethylsilyldiazomethane (**13**) and dry methanol, in the molar ratio 1:5:7 respectively, gave the chemospecific methylation of carboxyl function. After 2 min, evaporation of the solvent and the excess of trimethylsilyldiazomethane under vacuum afforded *N*-Nosyl-L-alanine methyl ester (**14f**) in quantitative yield (Scheme 5).

Scheme 5.



Ns-aa/TMSDZ/MeOH= 1/5/7

In the light of the result obtained, the reaction was then extended to the

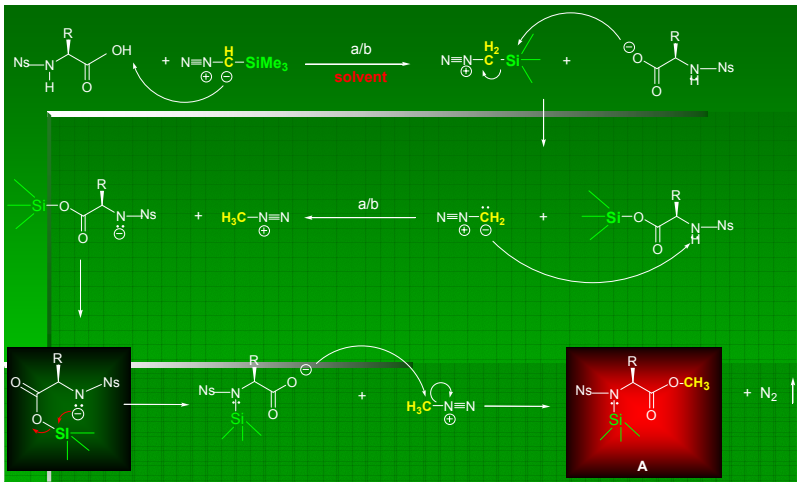
other *N*-Nosyl-α-amino acids **1a-g** (Table 3). In all considered cases, methylation performed at room temperature was chemospecific and went to completion in such a few minutes. The corresponding *N*-Nosyl-α-amino acid methyl esters **14a-g** were recovered in quantitative yields within 5 min.

Table 3.

entry	R	time (min.)
a	- $CH(CH_3)_2$	2
c	$-CH_2CH(CH_3)_2$	5
e	$-CH_3CH_2(CH_3)CH$	5
f	$-CH_3$	2
g	$-CH_2C_6H_5$	5

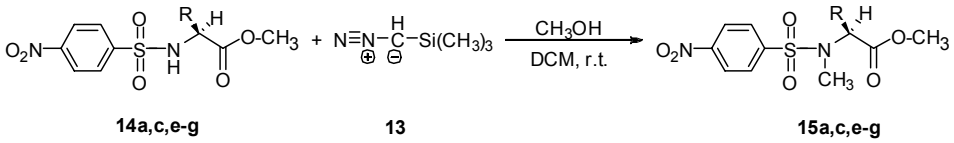
The small excess of methanol is extremely important to avoid the formation of by-products in the reaction mixture: in fact using TMSDZ/Methanol in equal ratio, it has been observed the formation of a product silylated on sulphonamidic nitrogen (**A**), which presumably derives from migration of the trimethylsilyl group, according to the mechanism reported in the Figure 8.

Figure 8.



Methyl esters **14a-g** were treated with a 2.0 M ethereal solution of trimethylsilyldiazomethane (**13**) and dry methanol, in the molar ratio 1:9:13 respectively, to give the corresponding *N*-methyl-*N*-Nosyl-α-amino acid methyl esters **15a-g** in quantitative yields (Scheme 2).

Scheme 6.



14a,c,e-g **13** **15a,c,e-g**

Ns-aa/TMSDZ/MeOH= 1/9/13

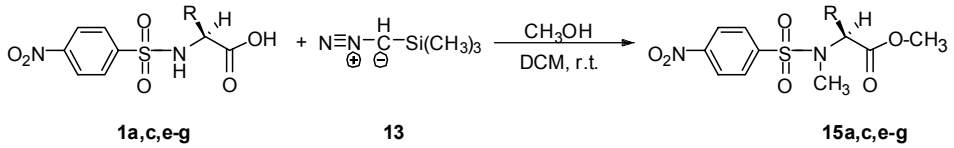
Table 4.

entry	R	time (h)
a	- $CH(CH_3)_2$	1
c	-$CH_2CH(CH_3)_2$	1.45
e	-$CH_3CH_2(CH_3)CH$	1.45
f	-CH_3	1
g	-$CH_2C_6H_5$	2

N-methylation required longer reaction time if compared to the conversion of *N*-Nosyl-α-amino acids into the corresponding methyl esters: this difference justifies the observed chemospecificity.

Methylation of **1a-g** both on the sulfonamidic and the carboxyl group was accomplished by using *N*-Nosyl-α-amino acids, trimethylsilyldiazomethane and methanol in the molar ratio 1:14:17 respectively (Scheme 7).

Scheme 7.



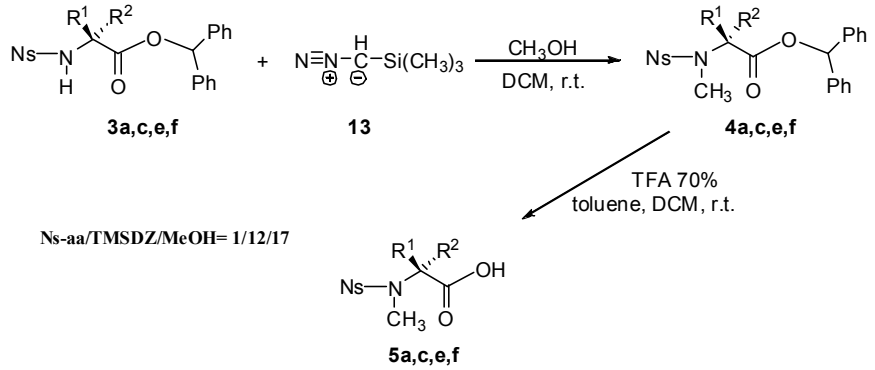
1a,c,e-g **13** **15a,c,e-g**

Ns-aa/TMSDZ/MeOH= 1/12/17

In all cases, the reaction was performed at room temperature and was complete in 1-2 hours, and the corresponding *N*-methylated compounds **15a-g** were recovered in quantitative yields.

In order to study the reaction of benzhydryl esters with TMSDZ, the model compound *N*-Nosyl-L-alanine benzhydryl ester (**3f**, 1 mmol) dissolved in dry dichloromethane was treated with a 2.0 M ethereal solution of trimethylsilyldiazomethane (12 mmol) in the presence of dry methanol (17 mmol) (Scheme 8).

Scheme 8.



After 3 hours, evaporation of the solvent and the excess of trimethylsilyldiazomethane under vacuum afforded the corresponding *N*-methyl-*N*-Nosyl-L-alanine benzhydryl ester (**4f**) in quantitative yield. The benzhydryl esters **3a,c,e** were quantitatively converted into the corresponding *N*-methyl derivatives **4a,c,e** by treatment with trimethylsilyldiazomethane, using the same conditions adopted for **3f**. The subsequent deprotection of the carboxyl function of **4a,c,e,f** by acidolysis (acidolysis by treatment with a 70% dichloromethane solution of trifluoroacetic acid, in the presence of toluene as scavenger), afforded the corresponding *N*-methyl-*N*-Nosyl-α-amino acids **5a-d** in excellent yields (Scheme 8, Table 5).

Table 5.

entry	R	yield[a] (%)
a	$(CH_3)_2CH$	98
c	$(CH_3)_2CHCH_2$	94
e	$CH_3CH_2(CH_3)CH$	94
f	CH_3	98

[a] Isolated yield

In conclusion, this part of the study regarding the reactivity trimethylsilyldiazomethane as methylating agent, provided an improvement in the *N*-alkylation procedure of *N*-Nosyl-α-amino acids and derivatives.

Trimethylsilyldiazomethane is less hazardous than the diazomethane, easier to handle, and commercially available.

With respect to diazomethane, the use of trimethylsilyldiazomethane offers considerable advantages in terms of selectivity. In fact, the choice of appropriate reaction conditions allows to perform the chemospecific methylation of the carboxyl function of *N*-Nosyl-α-amino acids. *N*-Nosyl-α-amino acid methyl esters can be methylated on the sulfonamidic function by an additional treatment with trimethylsilyldiazomethane.

Furthermore, this reagent allows the methylation of *N*-Nosyl-α-amino acids both on the sulfonamidic function and the carboxyl group.

The methodology can also be applied to *N*-Nosyl-α-amino acids protected on the carboxyl function as benzhydryl esters.

In all cases, the methylation products are recovered in quantitative yields and high purity without need of chromatography.

However, the derivatization reactions with trimethylsilyldiazomethane occur at much longer reaction times compared to the methylation with diazomethane, and often it is possible to recover by-products.

4. *N*-Methyl-*N*-Nosyl-β^3-amino acids

β-Peptides differ from their natural counterparts, the α-peptides, by having CH_2 groups inserted into every amino acid residue, either between the C=O groups and the α-carbon atoms (β^3) or between the α-carbon and nitrogen atoms (β^2).

β^3-Amino acids are widely encountered in nature, especially in compound having marine origin, and they are representative building blocks in the design of biologically active substances having peptidic morphology.

Pharmaceutical target molecules, such as peptide structure based drugs, many natural products,[46] and a vast array of metabolites,[47] contain optically active β-amino acid residues. Furthermore, β^3-amino acids are considered as the ideal precursors of β-lactam antibiotics.[48]

Numerous studies dealing with peptides and other biologically active substances containing a β^3-amino acid framework are reported in the literature.[49] These molecules, the β-peptides, are able to fold into well defined three-dimensional structures.[50]

β-Peptides have shown increased stability against the degrading action of mammalian proteases,[51] due to the inability of proteolytic enzymes to cleave the amide bonds adjacent to the β^3-amino acid.[52] Another peculiar aspect lies in the fact that β^3-amino acids are non-mutagenic.

All these features confer to β-peptides valuable characteristics to be promising candidates in pharmaceutical applications as peptidomimetics.

Due to the importance of β^3-amino acids, their *N*-methyl derivatives are potentially useful amino acid surrogates for incorporation in lead therapeutic agents having β-peptide structure and characterized by an increased lipophilicity and bioavailability. The resulting conformational rigidity of *N*-methylated peptides[53] may produce compounds with

improved binding characteristics and new receptor subtype selectivity.[54]

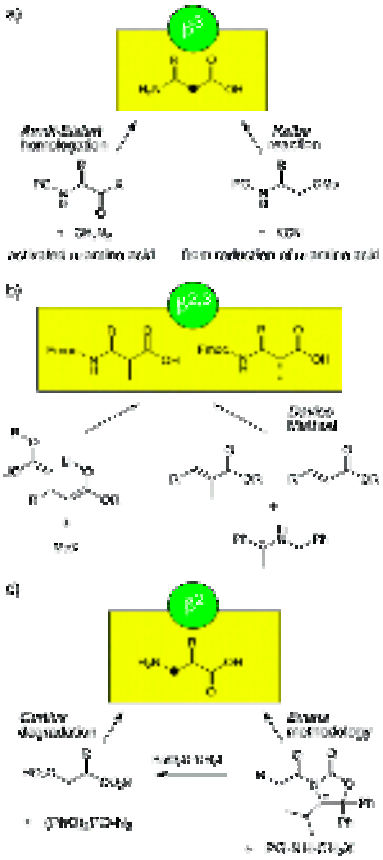
Furthermore, *N*-methylation of optically pure β^3-amino acids could be straightforward in making available monomers useful for the developments of new foldamers,[55] as well as in the synthesis of biologically active peptidomimetic drugs.[56] For example, it is known that introduction of an *N*-methyl-β^3-amino acid at the 4^{th} position of tetrapeptide analogs of dermorphin, a potent natural analgesic with long-lasting opioid-like activity, provides molecules stable to proteolytic enzymes.[57]

Substitution also improves receptor affinity, selectivity and analgesic activity. *N*-Methyl-β^3-amino acids could also generate appropriate modified β-peptide scaffolds useful to enlighten the mechanisms regulating the gastrointestinal high affinity carrier system of β^3-amino acids in human and mammalian organisms.[58] Consequentially, it is crucial to provide optically pure *N*-methyl-β^3-amino acids with a large supply.

Although several methods are reported in the literature for the synthesis of β^3-amino acids (Figure 9), only few procedures have been developed for the synthesis of *N*-methyl-β^3-amino acids, and[59] some of these approaches are characterized by harsh reaction conditions or lots of synthetic steps.

Recently, in the laboratory in which three years of PhD research has made, it was developed a very simple and efficient approach for preparing *N*-methylated-α-amino acid methyl esters using the nosyl group to protect the starting α-amino acid methyl esters.[33a] The methylation reaction simply requires the treatment of nosyl derivatives with an ethereal solution of diazomethane. In addition, this procedure has been successfully employed for site-specific *N*-methylation of the terminal amino function of *N*-Nosyl peptides.[33b]

Figure 9.



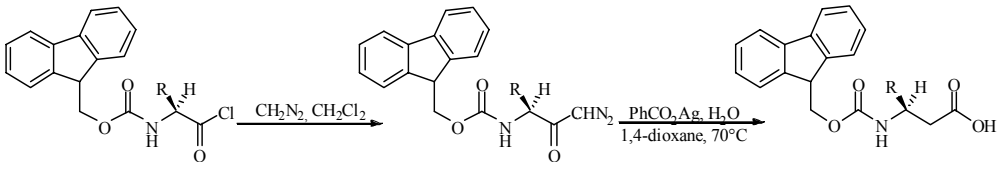
(a) stereospecific classical homologation of α-amino acids (H-Xaa-OH) by insertion or addition of a one-carbon moiety to give β^3-amino acids (H-β^3hXaa-OH);

(b) α-methylation of a β-amino acid derivative or overall enantioselective Michael addition of NH_2/H or NH_2/CH_3 to an acrylate ester for preparation of $\beta^{2,3}$-amino acids;

(c) overall enantioselective amino-methylation of a "des-amino" acid, directly or through a succinic acid half-ester, to provide β^2-amino acids (H-β^2hXaa-OH).

Furthermore, in a previous work, it was also realized the direct homologation of *N*-Fmoc-α-amino acids into the corresponding β^3-amino acids, according to the Arndt-Eistert procedure and using Fmoc-α-amino acid chlorides as starting materials (Figure10).[60]

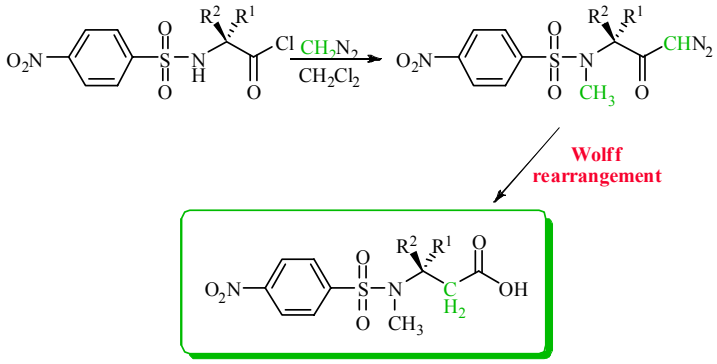
Figure 10.



On the other hand, the stability of the Nosyl group towards acids allows the activation of carboxyl function as chloride;[33b,33c] therefore, the *N*-Nosyl-α-aminoacyl chlorides could be the ideal starting substrates in the Arndt-Eistert reaction. Nosyl group enhances the acidity of the NH function, so that *N*-Nosyl-α-aminoacyl chlorides should react with diazomethane both on the NH moiety and the carbonyl group, achieving in only one step the formation of the *N*-methyl diazoketones; the next step of omologation (Wolff rearrangement[61]) would provide the expected *N*-methyl-omologated derivatives (Figure 11).

Figure 11.

Working hypotheses: N-Nosil-α-aminoacyl chloride in the Arndt-Eistert procedure



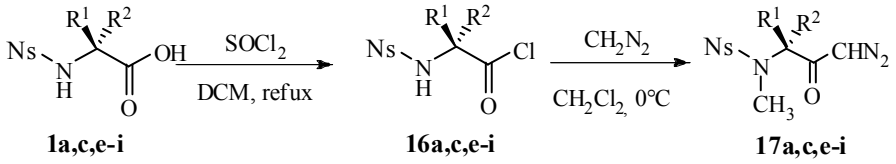
By this way, applying our recent improvement in the methylation of amino acids *N*-Nosyl masked[33a-c] it was performed an appealing pathway for the synthesis of *N*-methyl-β[3]-amino acids building-bloks.[43]

Using previously published procedures,[33c] the *N*-Nosyl-α-amino acids **1a-i** were prepared from the corresponding α-amino acids and *p*-nitrobenzenesulfonyl chloride.

N-Nosyl-α-amino acids **1a-i**, dissolved in dry ethanol free methylene chloride were then converted into the corresponding *N*-Nosyl-α-aminoacyl chlorides **16a-i** by treatement with thionyl chloride (Scheme

9, Table 6). The resulting mixture was stirred under reflux for 20-30 min, until complete conversion of the precursor. Evaporation of the solvent under vacuum afforded the respective chlorides **16a-i** in quantitative yield (Scheme 9).

Scheme 9.



1a,c,e-i **16a,c,e-i** **17a,c,e-i**

Table 6.

entry	R^1	R^2	17 yield (%)
a	$-CH(CH_3)_2$	-H	73
c	$-CH_2CH(CH_3)_2$	-H	77
e	$-CH(CH_3)CH_2CH_3$	-H	81
f	$-CH_3$	-H	78
g	$-CH_2(C_6H_5)$	-H	77
h	-H	$-CH_3$	81
i	-H	$-CH(CH_3)CH_2CH_3$	89

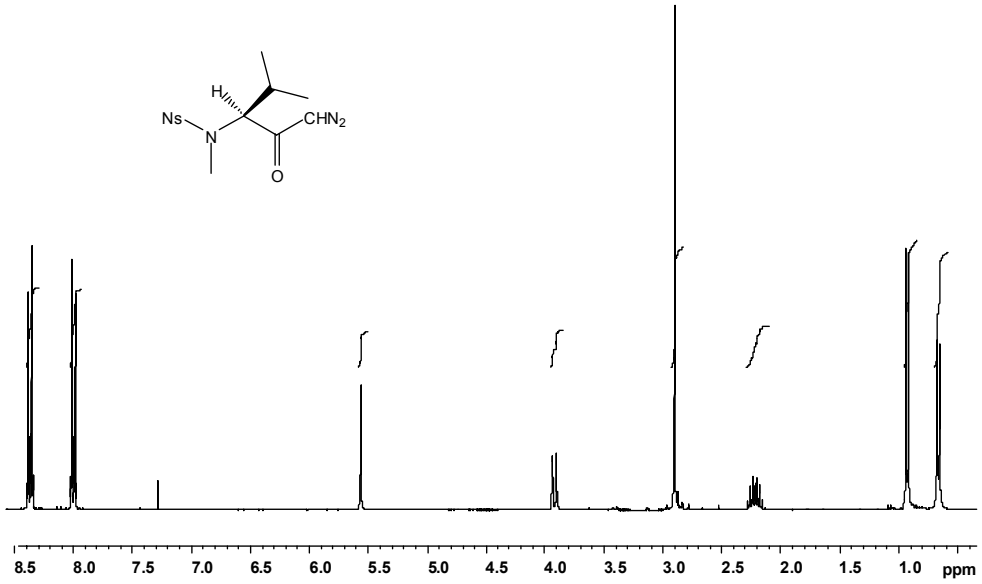
The *N*-Nosyl-α-aminoacyl chlorides **16a-i** were transformed into the *N*-methyl-*N*-Nosyl-α-aminoacyldiazomethanes **17a-i**: then, a solution of the *N*-Nosyl-α-aminoacyl chloride **16a-i** (1 mmol) in dry methylene chloride was added dropwise to a stirred 0.66 M methylene chloride solution of diazomethane (10 mmol) at 0 °C. The mixture was maintained under stirring for about 50-60 min. After complete conversion of the precursor into the corresponding *N*-methyl diazoketone, the organic solvent was removed under vacuum and the oily residue was purified by column chromatography to afford the respective *N*-methyl-*N*-Nosyl-α-aminoacyldiazomethane **17a-i** in 69%-89% yields.

In ^{1}H-NMR spectrum of the *N*-methyl-*N*-Nosyl-α-valinyldiazomethane **17a** (Figure 12) we can distinguish two doublets at 0.66 ppm and 0.93

ppm characteristic of the two groups of diastereotopic methyl groups of valine. The methyn proton group of isopropyl side chain is represented by a multiplet that echoes a 2.15-2.29 ppm. We can also distinguish a singlet at about 2.90 ppm of -NCH_3 protons group, confirming that the methylation was occurred and at the same time, a singlet at about 5.57 ppm corresponding to hydrogen of -CHN_2 group confirm the presence of diazoketone.

Figure 12.

*N-methyl-N-Nosyl-L-valinyldiazomethane (**17a**): ^{1}H-NMR (300 MHz)*

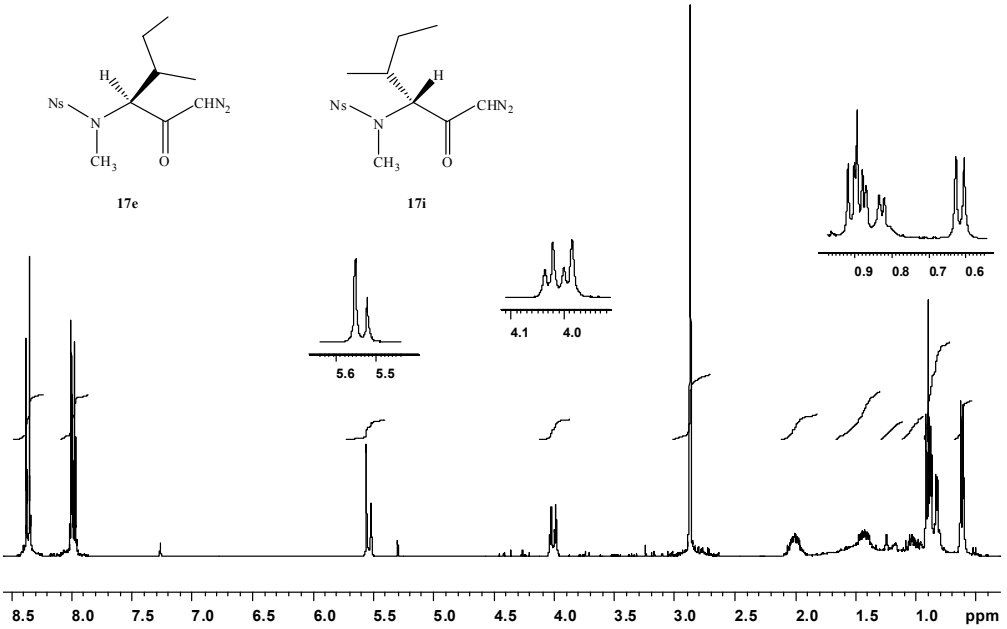


To verify the absence of racemization in the process of formation of *N*-methyl-*N*-Nosyl-α-aminoacyldiazomethanes, a couple of diasteromeric compounds were prepared by the methodology already defined: the *N*-methyl-*N*-Nosyl-α-diazoketons of L-isoleucine (**17e**) and D-*allo*-isoleucine (**17i**) were obtained in 81% and 89% yields, respectively (Table 6). In order to exploit the stereochemical features of the novel synthetic approach, it was synthesized the couple of diastereomers *N*-

methyl-*N*-nosyl-L-isoleucyldiazomethane (**17e**) and *N*-methyl-*N*-nosyl-D-*allo*-isoleucyldiazomethane (**17i**). To observ the differentiation of ^{1}H-NMR signals of two diastereoisomers, a mixture of the two compounds was prepared (Figure 13), and this spectrum was compared with the spectra of single compound **17e** (Figure 14) and **17i** (Figure 15).

Figure 13.

*Mixture of N-methyl-N-Nosyl-α-diazoketons of L-isoleucine (**17e**) and D-allo-isoleucine (**17i**): ^{1}H-NMR (300 MHz)*



By observing the spectrum of the mixture (Figure 13) you can see that the two diastereoisomers differ significantly in some signal:

- chemical shift of two methyl groups in side chain;
- chemical shift of the α-CH proton, for which it is possible to distinguish a doublet at 4.03 ppm for **17e**, and a doublet at 4.01 ppm for **17i**;

- chemical shift of the proton on CHN_2 group, which is represented by a singlet at 5.50 ppm for the **17e** and a singlet at 5.55 ppm for **17i**.

Figure 14.

*N-methyl-N-Nosyl-α-diazoketons of L-isoleucine (**17e**)): ^{1}H-NMR (300 MHz)*

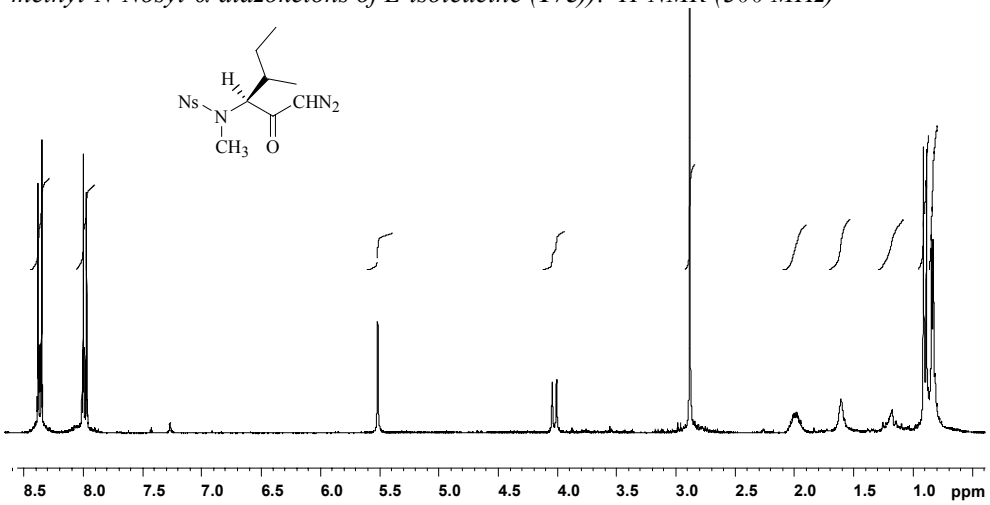
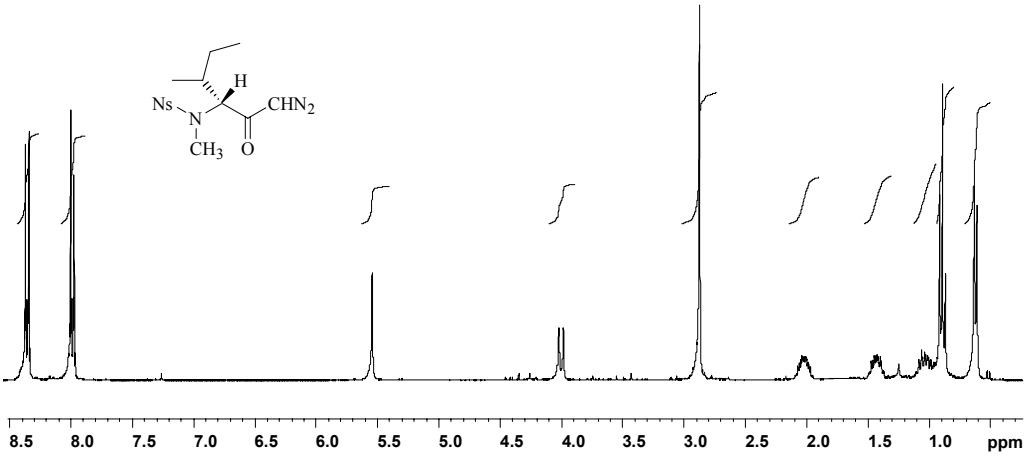


Figure 15.

*N-methyl-N-Nosyl-α-diazoketons of D-allo-isoleucine (**17i**): ^{1}H-NMR (300 MHz)*



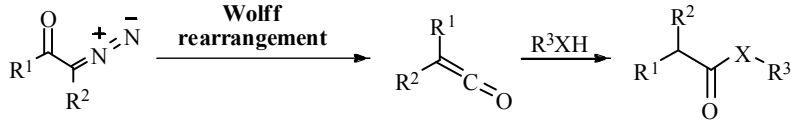
The ^{1}H- and ^{13}C-NMR analysis of each compound showed resonances attributable to only one diastereomer, demonstrating that the

stereochemistry of the original chiral carbon atom is totally retained during the preparation of diazoketones **17a-i**.

Compounds **17a-i** were successively converted into the corresponding *N*-methyl-*N*-Nosyl-β^3-homoamino acids by Wolf rearrangement.

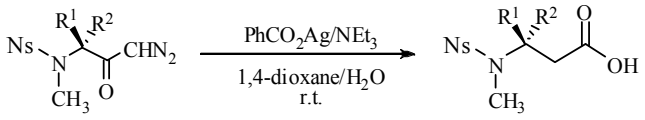
The Wolff rearrangement[62] of compounds α-diazocarbonylic-derivatives is the main passage in the Arndt-Eistert omologation reaction of carboxylic acid (Figure 16).

Figure 16.



The homologation reaction was initially performed using experimental conditions already reported in the literature.[57] In particular, 1 mmol of the *N*-methyl-*N*-Nosyl-alanyldiazomethane **17f**, chosen as model system, was dissolved in a 1,4-dioxane/water mixture and subjected to the treatment with catalytic amounts of silver benzoate, at 70 °C. After 5 hours, the catalyst was filtered off and the solvent removed. Hydrolytic work-up of the crude mixture afforded *N*-methyl-*N*-Nosyl-β^3-homo-L-alanine (**18f**) only in a 30% total yield. Unexpectedly, the main reaction product was the *N*-methyl-4-nitrobenzenesulfonamide. The homologation were significantly improved when silver benzoate is dissolved in triethylamine and acts as a homogeneous catalyst:[63] the resulting homogeneous solution was added to a 1,4-dioxane/water system containing the *N*-methyl-*N*-Nosyl-alanyldiazomethane (**17f**) (Scheme 10). The use of an excess of triethylamine with catalytic amounts of silver benzoate at room temperature for 20 min, led to the rearrangement product **18a** in higher yield (78%).

Scheme 10.



17a,c,e-i **18a,c,e-i**

Table 7.

entry	R^1	R^2	18 yield (%)
a	$-CH(CH_3)_2$	-H	60
c	$-CH_2CH(CH_3)_2$	-H	74
e	$-CH(CH_3)CH_2CH_3$	-H	65
f	$-CH_3$	-H	83
g	$-CH_2(C_6H_5)$	-H	70
h	-H	$-CH_3$	82
i	-H	$-CH(CH_3)CH_2CH_3$	68

It is recently reported that triethylamine facilitates the formation of silver nanoclusters[64] which are the species responsible for the catalysis the Wolff rearrangement of diazoketones.[65] The catalyst system triethylamine/silver benzoate produces only traces of the sulfonamide in the reaction mixture.

In the light of this excellent results, the procedure was then extended to the other *N*-methyl diazoketones **17b-i**. In all cases, reaction performed at room temperature was complete in a few minutes and the corresponding *N*-methyl-*N*-Nosyl-β^3-amino acids **18b-i** were recovered pure without need of chromatography, and in yields variable between 65% and 83% (Table 7).

Similarly to what has been done for the preparing step of diazomethanes, even for this omologation step, it has been verified the stereochemical integrity of the present chiral centers. For this reason the diastereomeric omologated amino acids **18e** and **18i** were synthetized with the methodology already defined. The spectrum of a mixture of two

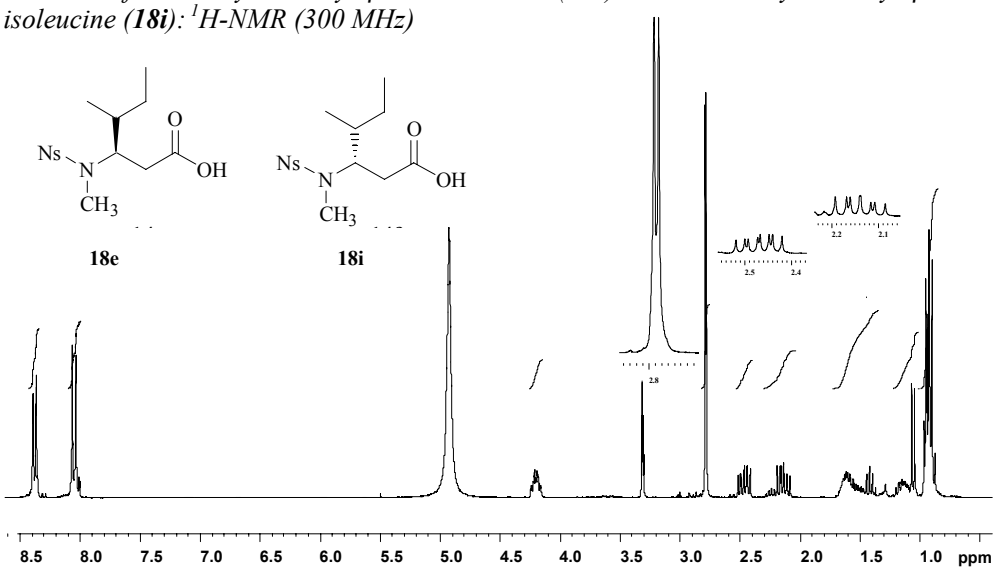
compounds, compared with the spectra of the single substrates, reveals the absence of any racemization trace.

^{1}H-NMR spectrum of mixture (Figure 17), shows that **18e** and **18i** are cleary different for the subsequent signals:

- chemical shift of the β-CH_2 protons (**18e**: 2.13 ppm; 2.46 ppm; **18i**: 2.15 ppm; 2.48 ppm);
- chemical shift of protons of -NCH_3, (**18e**: 2.78 ppm; **18i**: 2.79 ppm).

Figure 17.

*Mixture of N-methyl-N-Nosyl-β^3-L-isoleucine (**18e**) and N-methyl-N-Nosyl-β^3-D-allo-isoleucine (**18i**): ^{1}H-NMR (300 MHz)*



^{1}H- and ^{13}C-NMR spectroscopy applied to the samples of *N*-methyl-*N*-Nosyl-β^3-homo-L-isoleucine (**18e**) and its epimer **18i** led to the conclusion that also homologation is not affected by racemization.

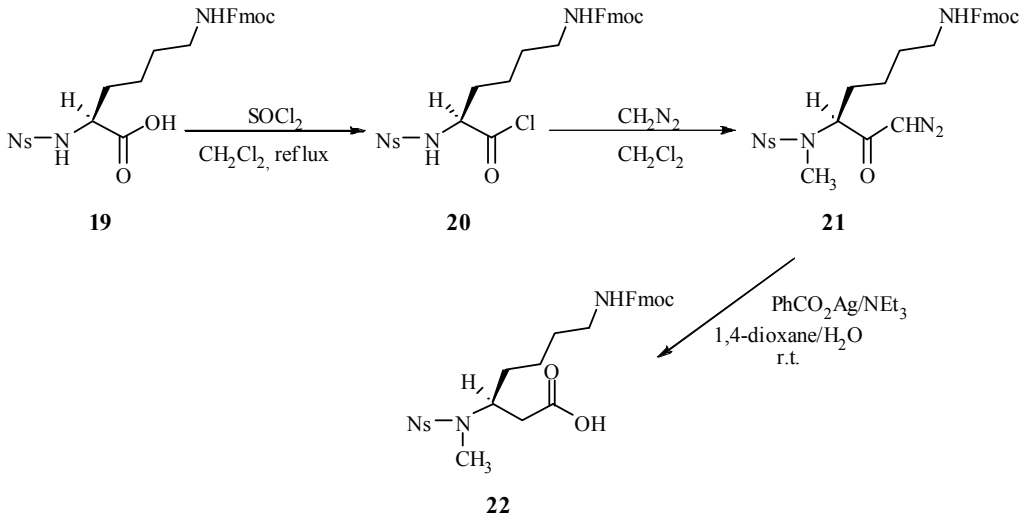
For completeness, we investigated the application of the described procedure to *N*-Nosyl-L-lysine protected on the ε-amino function with the Fmoc group. The choice of the base-labile masking group in the side-chain of lysine was dictated by the necessity for the use of a protection

compatible with the developed methodology.

The starting N^{α}-Nosyl-N^{ε}-Fmoc-L-lysine (**19**) was prepared by treatment of the commercial N^{ε}-Fmoc-α-L-amino acid with *p*-nitrobenzensulfonyl chloride in a 1,4-dioxane/water solution containing triethylamine. Reaction of **19** with thionyl chloride furnished **20** (Scheme 11), which was in turn converted into the corresponding *N*-methyl diazoketone **21** under experimental conditions similar to those used for the preparation of **17a-g**.

Column chromatography was necessary to recover pure **21** in 69% yield.

Scheme 11.



Homologation of **21** under the catalytic conditions optimized as previously described, proceeded at room temperature for 20 min, giving the desired N^{α}-methyl-N^{α}-Nosyl-N^{ζ}-Fmoc-β^3-homo-L-lysine (**22**) in a 62% yield, without need of chromatography (Scheme 11).

Finally, to verify the possible applications of *N*-methyl-β^3-amino acids as building blocks in the synthesis of modified peptides, **18f,h** were

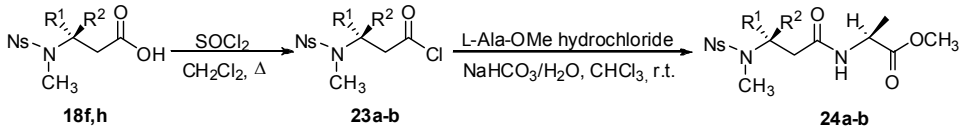
quantitatively converted into the corresponding acyl chlorides **23a-b** by treatment with thionyl chloride, using the same conditions adopted for the α-analogues (Scheme 13).

Dipeptides **24a-b** were then obtained by coupling of the *N*-methyl-*N*-Nosyl-β^3-homoamino acid chlorides **23a-b**, dissolved in ethanol free methylene chloride, with L-alanine methyl ester hydrochloride dissolved in an aqueous basic solution (Scheme 12).

The reactions proceeded at room temperature and went to completeness in about 25-30 min.

Dipeptides **24a-b** were obtained in goods yields (Table 7), and in high purity without need of chromatography.

Scheme 12.



18f,h **23a-b** **24a-b**

Table 7.

*Synthesis of N-methyl-N-Nosyl-dipeptides **24f,h**.*

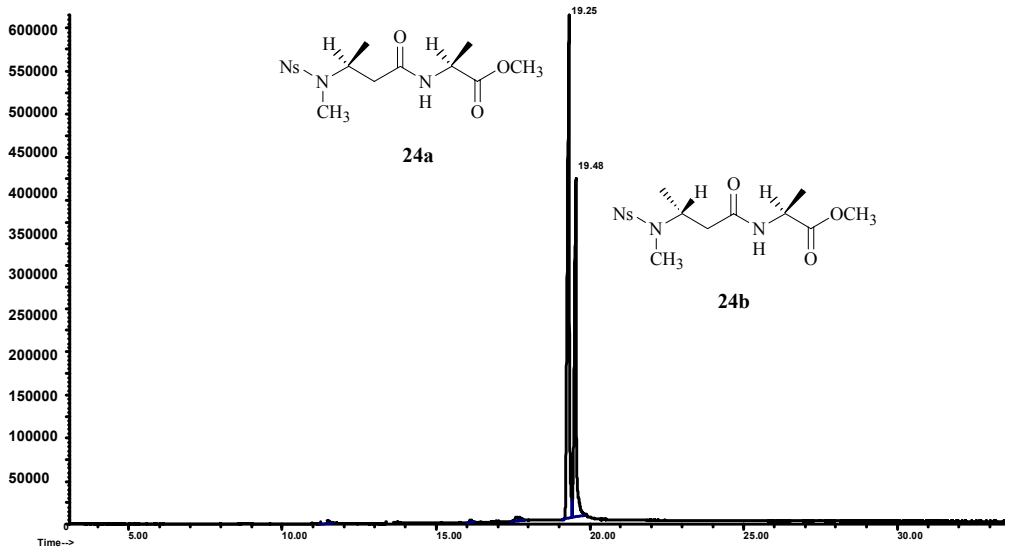
entry	R^1	R^2	Yield (%)
24a	H	CH_3	75
24b	CH_3	H	64

The single diastereomeric dipeptides **24a-b**, and then a mixture of the two compounds, were characterized by GC-MS analysis.

Each diastereomer showed a unique peak, while the mixture presented two well resolved peaks (Figure 18).

Figure 18.

*GC-MS analysis of dipeptides mixture **24a** e **24b***



H^1-NMR spectroscopy definitively excluded the formation of epimerized products in the coupling reaction: in fact, the comparison of ^{1}H-NMR spectrum of the mixture (Figure 20) with ^{1}H-NMR spectra of the crude reaction product **24a** (Figure 19) and **24b** (Figure 21) precluded the presence in each sample of the other diastereoisomer.

^{1}H-NMR spectrum of the mixture (Figure 21) shows the presence of two compounds that differ in the signals:

- side chain protons of *N*-methyl-β^3-alanine (**24a**: 0.94 ppm; **24b**: 0.96 ppm);
- side chain protons of L-alanine alanine (**24a**: 1.22 ppm; **24b**: 1.19 ppm).

Figure 19.

*Dipeptide **24a**: ^{1}H-NMR (300 MHz)*

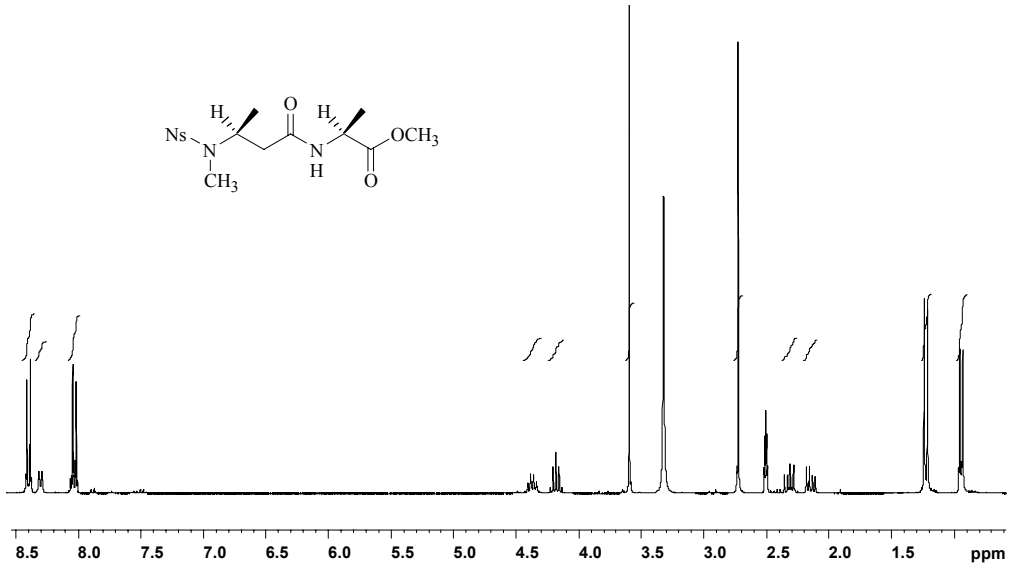


Figure 20.

*Dipeptide **24b**: ^{1}H-NMR (300 MHz)*

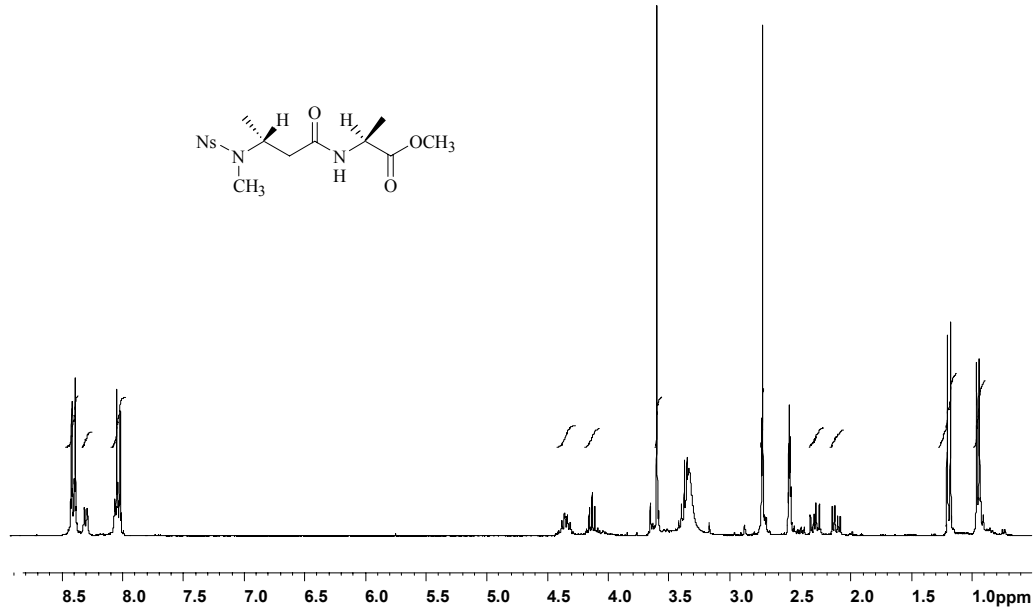
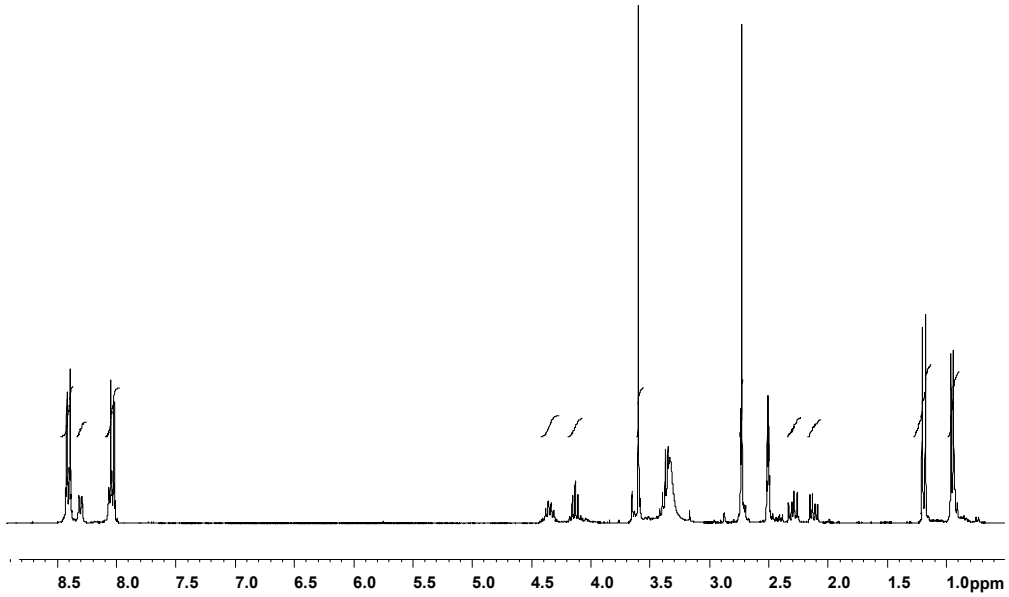


Figure 21.

*Mixture of dipeptides **24a** and **24b**: ^{1}H-NMR (300 MHz)*



In conclusion it was developed a highly efficient and simple methodology for the homologation of *N*-Nosyl-protected α-amino acids into the corresponding *N*-methyl-β^3-amino acids.

The nosyl protecting group offers great advantages in the preparation of *N*-methyl-β^3-amino acids: in fact, the sulfonamide masking group enhances the NH proton acidity, allowing the formation of diazoketones and the simultaneous methylation of the amino function during the treatment of *N*-Nosyl-α-aminoacyl chlorides with diazomethane.

N-Methyl-*N*-Nosyl-α-aminoacyldiazomethanes are then smoothly converted into the corresponding β^3-amino acids by Wolff rearrangement.

With respect to the other procedures already reported in the literature, our methodology allows the easily obtainment of *N*-methyl-β^3-amino acids in few synthetic steps and under very mild conditions.

5. Reduction of *N*-methoxy-*N*-methylamides to the corresponding amines with $AlCl_3/LiAlH_4$

N-Methoxy-*N*-methylamines are useful reaction intermediates in organic synthesis.[66] Furthermore some biologically active molecules, or intermediates involved in their synthesis, are characterized by the presence of an *N*-methoxy-*N*-methylamino functional group. In particular, some *N*-methoxy-*N*-methyl amino acids display significant anticonvulsant activity in mice and rats.[67]

To establish a new procedure for the preparation of *N*-methoxy-*N*-methylamino, it was thought that the *N*-methoxy-*N*-methylamides (Weinrebe amides) could be useful precursors.[68]

Weinreb amides usually undergo partial reduction to the corresponding aldehydes.[69] There are only few reports in the literature in which *N*-methoxy-*N*-methylamides are converted into the corresponding amines by reduction.[70] A particular resin-bound hydroxamate has been reduced to the corresponding tertiary hydroxylamine resin using monochloroalane.[71] The same reagent has been used for the conversion of 1-(benzyloxy)-2-azetidinones to the corresponding 1-(benzyloxy)-2-azetidines.[72] Nevertheless, these approaches require heating and long times.

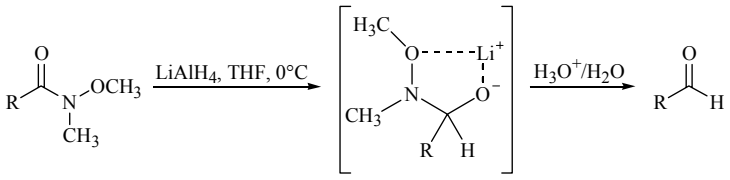
For this purpose we prepared *N*-methoxy-*N*-methylamides (Weinreb amides)[73] and studied their reduction to *N*-methoxy-*N*-methylamines by using aluminium trichloride together with lithium aluminium hydride to obtain the *N*-methoxy-*N*-methylamines.

The conversion of *N*-methoxy-*N*-methylamides into the corresponding amines using lithium aluminium hydride as the only reagent requires vigorous reaction conditions and long times. The proceeding of this

reduction reaction gives a strong indication that the metal-chelated tetrahedral intermediate **A** (Figure 22) is involved both in the formation of the aldehyde that is recovered after hydrolysis and in the formation under reflux of the amine. We believed that Weinreb amides might be easily converted into the corresponding *N*-methoxy-*N*-methylamines by choosing suitable experimental conditions that prevent the formation of the metal-chelated tetrahedral intermediate **A** by transforming the carbonyl oxygen into a good leaving group.

Figure 22.

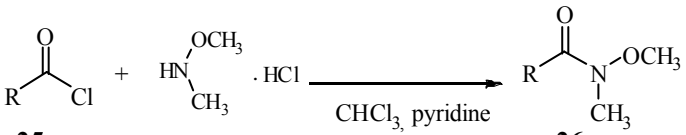
Metal-chelated tetrahedral intermediate of N-methoxy-N-methylamides/ Li^+



A

The *N*-methoxy-*N*-methylamides **26a-g** were prepared following the procedure reported in literature,[73] by treating the corresponding chlorides **25a-g** with *N,O*-dimethylhydroxylamine hydrochloride in the presence of pyridine (Scheme 13, Table 8).

Scheme 13.



25a-g **26a-g**

The reaction, conducted at room temperature for 50-60 minutes, furnished, after hydrolytic treatment, the expected substrates in good overall yields (Table 8). The products were characterized by ^{1}H-NMR

and GC-MS.

Table 8.

entry	R	Yield (%)
a	C_6H_5	99
b	4-$CH_3C_6H_4$	93
c	4-$CH_3OC_6H_4$	91
d	4-ClC_6H_4	86
e	4-$NO_2C_6H_4$	84
f	$C_6H_5CH_2$	89
g	$CH_3(CH_2)_4$	90

N-Methoxy-*N*-methylbenzamide, (**26a**) was chosen as model system.

Initially, we attempted the direct reduction of *N*-methoxy-*N*-methylamides using lithium aluminium hydride using the conditions reported in the literature.[70] *N*-Methoxy-*N*-methyl-2-phenylacetamide (**26a**) dissolved in dry tetrahydrofuran was added dropwise over two hours to a suspension of lithium aluminium hydride in dry ether at room temperature and subsequently the reaction mixture was refluxed.

In order to monitor the reduction reaction, samples from the reaction mixture were treated with saturated aqueous sodium carbonate solution and extracted with ether; the organic phase was then analysed by GC-MS.

After thirty minutes the GC-MS analysis of the first sample showed the absence of the starting amide and the formation of 2-phenylacetaldehyde, traces of 2-phenylethanol and a small amount of *N*-methoxy-*N*-methyl-2-phenylethanamine. The following analyses showed a progressive diminution of the aldehyde and an increase in the concentration of amine. After twelve hours, the reaction mixture contained only the amine and a small amount of 2-phenylacetaldehyde. Chromatographic purification of the crude reaction product afforded *N*-methoxy-*N*-methyl-2-

phenylethanamine and 2-phenylacetaldehyde in 62% and 12% yields, respectively.

The conversion of *N*-methoxy-*N*-methylamides into the corresponding amines using lithium aluminium hydride as the only reagent requires vigorous reaction conditions and long times.

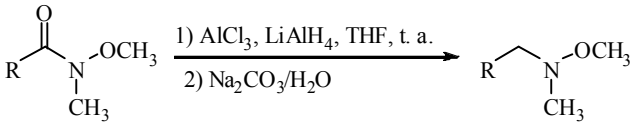
The proceeding of this reduction reaction gives a strong indication that the metal-chelated tetrahedral intermediate **A** (Figure 22) is involved both in the formation of the aldehyde that is recovered after hydrolysis and in the formation under reflux of the amine.

The assumptions to be checked were that the Weinreb amides might be easily converted into the corresponding *N*-methoxy-*N*-methylamines by choosing suitable experimental conditions that prevent the formation of the metal-chelated tetrahedral intermediate **A** by transforming the carbonyl oxygen into a good leaving group.

For this purpose it was investigated the use of aluminium trichloride together with lithium aluminium hydride to obtain the *N*-methoxy-*N*-methylamines.

N-Methoxy-*N*-methylbenzamide (**26a**) was chosen as model system to test the reduction reaction with the aluminium trichloride/lithium aluminium hydride reagent system. Amide **26a** (1 mmol) was dissolved in dry tetrahydrofuran and treated with aluminium chloride (2 mmol) at room temperature (Scheme 14).

Scheme 14.



26a-g **27a-g**

Subsequently lithium aluminium hydride (1 mmol) was added and *N*-methoxy-*N*-methylbenzylamine **26a** was obtained in ten minutes in excellent yield (85%)

after a simple work-up and without any further purification.

Table 9.

*Conversion of Amides **26** into Amines **27** ([a]The reactions were run for 10 min. [b]**(27g)** was isolated as the hydrochloride salt.)*

entry	R	Yield (%)[a]
a	C_6H_5	85
b	4-$CH_3C_6H_4$	84
c	4-$CH_3OC_6H_4$	100
d	4-ClC_6H_4	97
e	4-$NO_2C_6H_4$	95
f	$C_6H_5CH_2$	84
g	$CH_3(CH_2)_4$	84 [b]

In the light of the excellent result obtained with the amide **25a** the reduction reaction was extended to other aliphatic and aromatic systems (Table 9). The reactions were complete within a few minutes with all the Weinreb amides used. Both aromatic substrates **26b-e** and aliphatic ones **26f-g** were treated under the same conditions as **26a** and the corresponding amines **27b-g** were obtained in 84%-100% yields (Table 9).

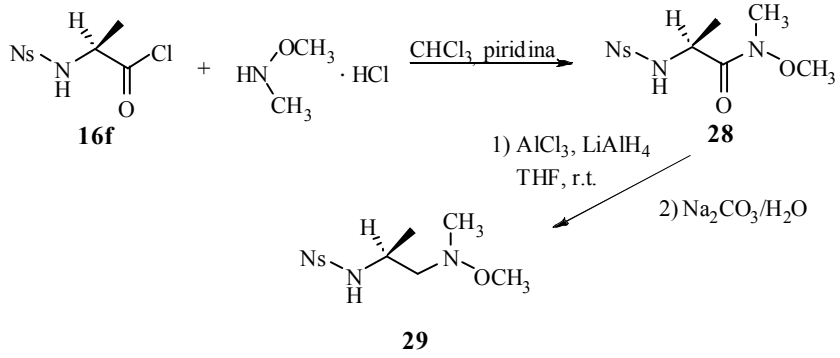
The reduction reaction performed using $AlCl_3/LiAlH_4$ as the reagent system constitutes an excellent approach to convert in a very short time and at room temperature Weinreb amides in their corresponding amines. The most important aspect of the proposed methodology could be the coordination of $AlCl_3$ to the carbonyl oxygen of the Weinreb amide that makes difficult the formation of chelate **A** and the subsequent obtainment of the aldehyde. Consequently the *N*-methoxy-*N*-methylamine is the sole reduction product obtained quickly and under mild conditions.

In a subsequent time, in order to replace the carboxylic acid C-terminal peptide systems with the *N*-methoxy-*N*-methylamine functionality, it was thought to use the same reagent system to convert the Weinreb amides of

of α-amino acids, properly protected on amino function, in the corresponding *N*-methoxy-*N*-methylamine.

To that end, the *N*-Nosyl-L-alanine chloride (**16f**), dissolved in anhydrous dichloromethane, was treated with *N,O*-dimethylhydroxylamine hydrochloride, in the presence of anhydrous pyridine, at room temperature for 1 hour; subsequent work-up processes allowed the recovery of the corresponding Weinreb amide in 80% yield (Scheme 15).

Scheme 15.

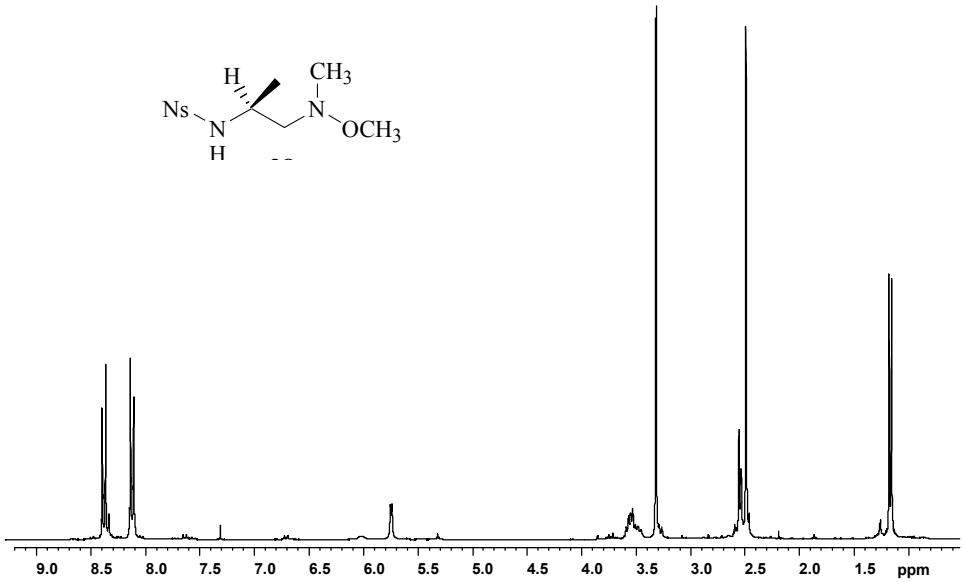


The reduction reaction was performed by using a different ratio of the reagent system ($AlCl_3/LiAlH_4$) respect to that required in the previous cases: amide **28** dissolved in anhydrous THF, was treated with aluminum trichloride in the molar ratio of 1:4; then lithium aluminum hydride (**28**/$LiAlH_4$, 1:2) was added and the product **29** was obtained after 20 minutes in high yield (98%).

29 was completly caracterized by NMR spectroscopy (Figure 23). The analysis of ^{1}H-NMR spectrum of the compound **29**, exclude the presence of signals attributable to by-products. Indeed, in the spectrum are observed only signals attributable to the structure of **29**.

Figure 23.

*Product of reducion **29**: ^{1}H-NMR (300 MHz)*



Reduction of Nosyl-amino acid-Weinreb amide performed using $AlCl_3/LiAlH_4$ as the reagent system constitutes an excellent approach to realise in a very short time and at room temperature a very useful modification of the carboxylic function of amino acids.

6. A new non-natural arginine-like amino acid derivative with a sulfamoyl group in the side-chain

L-Arginine is the most basic (pK_a 12.48) of the naturally occurring common amino acids and plays a unique role in a number of important physiological and pathophysiological processes.[74] Positively charged at neutral pH, the guanidinium group of the L-arginine side chain serves as an ion-pairing moiety in several biologically relevant molecular interactions. Proteases such as trypsin, thrombin, and Factor Xa preferentially cleave substrates containing an L-arginine residue at the P1 position.[75] L-Arginine is also a component of the conserved integrin receptor-binding RGD peptide motif,[76] analogues of which have been used to modulate cell adhesion.[77] Cell-penetrating agents have also been developed based largely upon a 9 amino acid sequence containing 6 L-arginine residues derived from the HIV-1 Tat protein.[78]

The typical feature of the variety of small-molecule direct thrombin inhibitors is the presence of a highly basic guanidine or amidine moiety. However, strongly basic groups will tend to hinder absorption across the gut wall.[79] Replacement of arginine by any other amino acid in homologous peptide sequences, modification of its side-chain[80] or guanidine moiety, by introducing non-polar and/or neutral groups of reduced basicity, and elimination of the cationic site from the side-chain[81] represent valuable approaches to afford a series of pharmaceutically relevant synthetic peptides[82] of greater protease specificity, bioavailability, inhibition potency, and stability against proteolysis together with improved binding affinities for many biologically relevant receptors.[83] The growing demand for an ideal protease inhibitor has increased the need for assorted libraries of

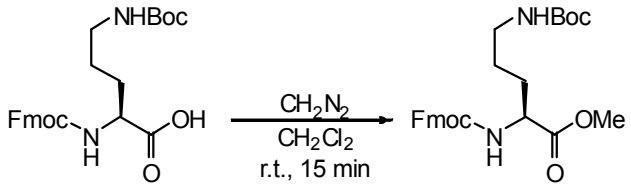
synthetic compounds. In this context, non-proteinogenic amino acids are of considerable interest.[83]

Motivated by pharmacological interest, and by the plethora of biological aspects related to the role of arginine and of its modified isosteres, we have exploited a facile synthetic access to a new L-ornithine derivative (**35**) with the aim to develop a new class of human thrombin inhibitors.

Compound **35** featuring a masked sulfamoyl group on the δ-carbon atom of the side-chain in substitution of the highly basic guanidine residue of natural arginine. The selected group is a constituent of many biologically active sulfonamides[84] and sulfamoyl carbamates,[85] but among the different moieties proposed for the chemical functionalization of the L-ornithine side-chain,[86] no mention can be found in the literature about the employment of the sulfamoyl group. The -$NHSO_2NH_2$ moiety in the L-ornithine skeleton could define a particular tetrahedral pharmacophore, surrogate of the guanidine group of arginine: it is an efficient H-bonding acceptor,[87] and confers to compound **35** a weakly acidic character, due to the possible loss of the SO_2NHR proton.[88]

The preparation of **35** started from N^{α}-Fmoc-N^{δ}-Boc-L-ornithine (**30**), and involved a three-steps procedure. Methyl ester **31** was obtained by treatment of the starting precursor **30** with a dichloromethane solution of diazomethane at room temperature (Scheme 16).

Scheme 16.

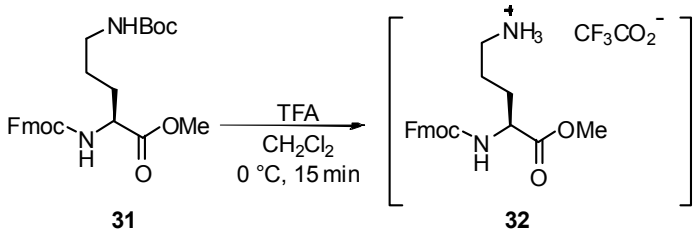


30 **31**

Methylation reaction afforded **31** in yields of 96%, and pure enough to be subjected to the next synthetic step without need for chromatography.

Removal of the Boc group from **31** was performed by acidolysis at 0°C, using TFA (Scheme 17). Conversion of **31** was complete and the resulting salt **32**, not isolated, was immediately subjected to sulfamoylation.

Scheme 17.

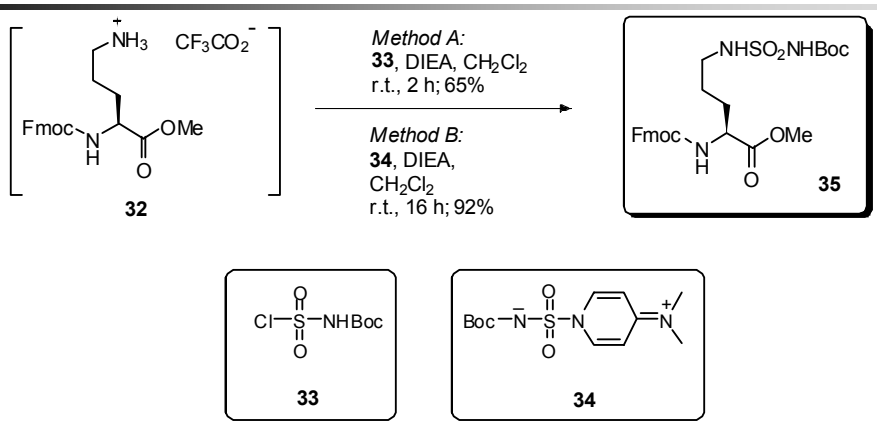


31 **32**

The α-amino function in the side-chain of **32** was finally functionalized using the sulfamoylating reagent **33** (Scheme 18, Method A), in the presence of DIEA. The reagent **33** was efficiently prepared from commercially available CSI and *tert*-butanol in dichloromethane, under the experimental conditions already described for conventional routes to this reagent.[89] Although **33** is rapidly formed in a quantitative yield by this approach, its use in the derivatization of the salt **32** proved to be troublesome. The chloride **33** is very unstable, and it cannot be stored for a long period. This aspect limits the possibility of an exact stoichiometric dosage of **33** imposing that the reagent must be prepared immediately before any sulfamoylation step. Moreover, under the experimental conditions adopted for the experiment in which **33** is used, the methyl ester **35** can be recovered as a pure product only after chromatography, and in yields not exceeding 65%. We found the zwitterionic azanide **34** to be the optimal reagent for the sulfamoylation of the trifluoroacetate **32**

(Scheme 18, Method B). This reagent can be obtained in very high yields and purity by a known procedure.[90] Since **34** is a stable crystalline solid, it can also be stored for prolonged periods and easily dosed for any purpose.

Scheme 18.

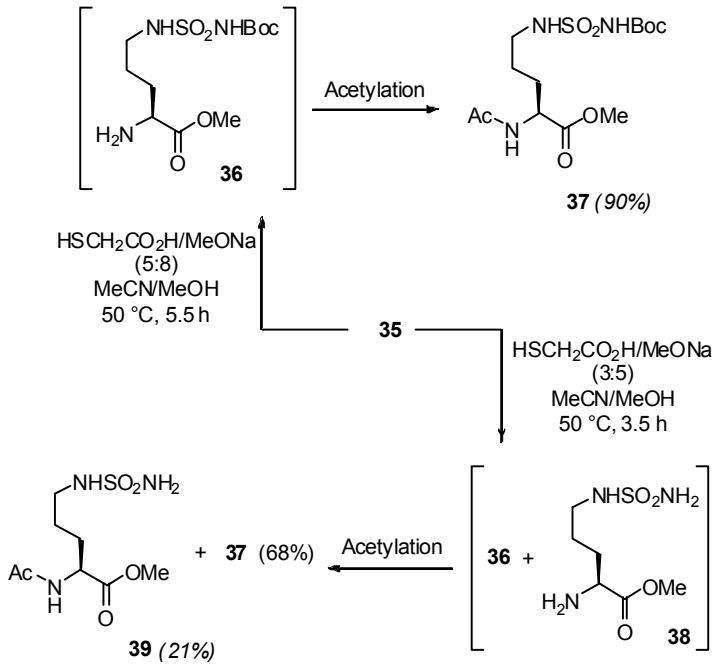


The reaction of trifluoroacetate **32** with **34** was performed in the presence of DIEA to generate the free δ-amino group; a simple work-up of the reaction mixture allowed the recovery of the methyl ester **35** in yields of 92%, with no need for chromatography. The use of a base, e.g. DIEA, is strongly recommended: without the base, compound **35** can be recovered pure only after column chromatography and in yields not higher than 65%. DMAP produced during the sulfamoylation did not provoke removal of the base-labile protecting group Fmoc. The modified methyl ester **35** was fully characterized by NMR analysis, and both 1D- and 2D-homonuclear techniques confirmed the structure proposed for the new arginine-like derivative.

The Fmoc group was removed from the α-amino function of **35** avoiding the use of nitrogenated species. This was done in order to facilitate the separation of the unprotected methyl ester from the crude

reaction mixture. As reported elsewhere,[91] the Fmoc group can alternatively be removed from the corresponding methyl esters by reagent systems composed of $AlCl_3$ and toluene or *N*,*N*-dimethylaminoaniline, but acid-labile protecting groups, e.g. Boc, are not compatible with the experimental conditions adopted for the Lewis acid-assisted treatment. Fmoc group was straightforwardly removed by treating **35** with the reagent system composed of mercaptoacetic acid and sodium methoxide, succesfully used to remove the Nosyl group (Scheme 19).[33,42]

Scheme 19.



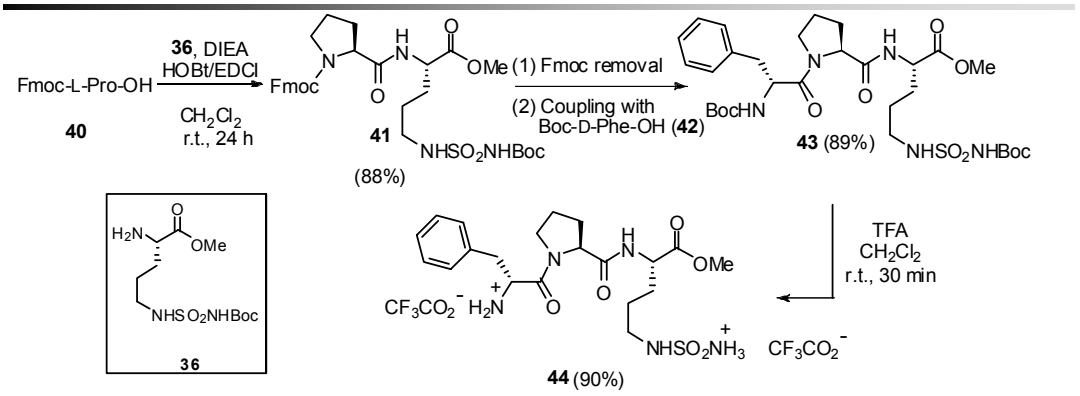
The appropriate stoichiometric ratio between the two components of the reagent system, referred to one equivalent of substrate, was found to be 5:8. Upon these conditions, the treatment of modified amino acid **35** at 50 °C for 5.5 h afforded **36** (Scheme 19), which was not separated and

immediately transformed into the corresponding acetyl derivative **37** by treating the crude material obtained from the unblocking step with an excess of acetic anhydride. Compound **37** was obtained in yields of 90%.

Since proline occupies a special place among the natural amino acids, **36** was coupled with *N*-Fmoc-L-proline (**40**). The sequence Pro-Arg, in fact, is often selected as an ideal chemical probe in conformational studies, and in determining the absolute specificity and bioavailability of peptide inhibitors of proteolytic enzymes.[92]

In order to evaluate the structural and conformational role of **35** in short peptide sequences, we prepared the dipeptide **44** by adopting a Fmoc-chemistry protocol usually used for the solution synthesis of peptides, upon conditions that can avoid the racemisation of the reaction partners.[93] *N*-Fmoc-L-proline (**40**; Scheme 20**)** was activated with the system HOBt/EDCI/DIEA and allowed to react with **36**. The only product obtained from the reaction was the dipeptide **41**, which was recovered in yields of 88% after flash column chromatography.

Scheme 20.



1D- and 2D- homonuclear NMR analysis of dipeptide showed resonances which were consistent with the proposed structure. The 1D ^1H spectrum, recorded in $CDCl_3$ at 298 K, showed the presence of two

sets of rotamers arising from the slow *trans*/*cis* isomerisation around the CO-N linkage between the Fmoc group and the proline ring. Based on the integration of non-overlapping signals, e.g. those appearing at 6.75 and 6.57 ppm, attributable to the α-NH proton of the ornithine skeleton included in the *trans* form and the same proton in the *cis* rotamer, respectively, we calculated a *trans/cis* ratio of 75:25 for the rotamers. The precise ratio was determined by the integration of the signals of both the α-NH protons which were assigned to the respective rotamer in analogy with the results reported in the literature for small dipeptides containing proline as the *N*-terminal residue.[94] 2D-homonuclear ^{1}H-NMR showed resonances clearly attributable to the protons belonging to the proline and the ornithine skeletons, respectively. In particular, the cross-signals appearing in the contour plot of the TOCSY analysis performed in $CDCl_3$ at 298 K confirmed the presence of the trans/cis rotamer mixture and showed correlation patterns typical of the peptide sequence Pro-Orn. No other signals relative to protons attributable to any possible diastereomer of **41** appeared in the 1D- and 2D- proton spectra. On the other hand, the 1D- and 2D homonuclear ^{1}H- NMR analysis of a sample of the crude dipeptide **41**, revealed resonances attributable only to the given structure, confirming that, limited to the sensitivity of the used NMR techniques, no racemisation of the α-carbon atom present in **35** occurred during the coupling; thus, the stereochemistry of the protected arginine-like derivative is retained both during its synthetic process and the Fmoc removal, as well as in the final coupling step.

In an effort to assess the biological role of the sulfamoyl group placed in the L-ornithine side-chain, **35** was finally used as a building block in the synthesis of some serine-protease inhibitors. In particular, **35** was coupled with the dipeptide Boc-D-Phe-Pro-OH to prepare tripeptide analogues of the *C*-terminal subsequence of fibrinogen, the natural

thrombin regulator. Thrombin, the blood-clotting enzyme, is a serine protease with trypsin-like specificity and is able to cleave Arg-Xaa peptide bonds but only in a very limited number of substrates.[95] This enzyme has a critical position in the blood coagulation cascade and thus a central role in the regulation of haemostasis.[96] Moreover, for the prevention and treatment of thrombosis the control of thrombin activity is a key target[97] and the discovery of new classes of inhibitors of this enzyme could lead to useful drugs for treating thrombotic disorders, which constitute a serious source of mortality and morbidity in patients worldwide.

The synthesis of tripeptides containing **36** as P1 residue is reported in Scheme 20. Dipeptide **41**, after removal of Fmoc protecting group, was then coupled with Boc-D-Phe-OH in the presence of the system HOBt/EDCI/DIEA. The fully protected tripeptide **43** was recovered pure by flash-column chromatography in yields of 89%, and ^{1}H and ^{13}C NMR analysis confirmed the expected structure. Acidolysis of **43** afforded **44** which was obtained by precipitation of the crude product from a 1:2 MTBE/*n*-pentane mixture and further lyophilisation in order to remove all the residues of *tert*-butanol and any trace of possible co-products generated during the unblocking step. The ^{1}H NMR spectrum of the trifluoroacetate salt **44** registered at 298 K in DMSO-d_6/D_2O showed exchange of all protons on the N atoms, except for those of the SO_2NH_2 moiety, but did not display well resolved resonances for the other protons.

Both tripeptides **43** and **44** were subjected to *in vitro* coagulation assays in order to assess their inhibitory potency against human thrombin. The TT (Thrombin Time), a measure of the thrombin-fibrinogen reaction *in vitro*,[98] and the APTT (Activated Partial Thromboplastin Time), the parameter used to evaluate the anticoagulant

effect on the thrombin produced by the intrinsic pathway of the coagulation cascade,[99] were determined using pooled human plasma treated with samples of **43** and **44** at different concentrations. Among the two tripeptides, only **44** exhibited a strong and dose dependent activity, as determined by the clotting time of human plasma in TT and APTT tests. Tripeptide **43** did not show activity.

In conlusion, we have synthesized the non-natural arginine-like α-amino acid derivative **35** which displays on the side-chain a masked sulfamoyl moiety. Compound **35**, previously converted in the substrate **36**, can be easily coupled with the dipeptide motif D-Phe-Pro to produce tripeptides of high biological interest. Since the neutral polar function in the side-chain of **35**, at physiological pH values, is certainly capable of H-bonding through its heteroatoms,[100] it could be regarded as an efficient anchoring moiety for a wide series of substrates and inhibitors in their binding with the recognition subsites of biological receptors. In particular, rational modification of the *C*-terminal tripeptide lead structure found in fibrinogen by inserting **35** in substitution of the natural arginine at P1 position generates compounds which are active as human thrombin inhibitors. Thus, the non-proteinogenic amino acid **35** can really regarded as an arginine mimic and could be strategic for studies on the importance of the physiological role of different kinds of proteases, especially in the discovery of promising candidates for future development of clinically useful agents.

7. Bergapten-free bergamot essential oils extracted from peeled fruits by vegetable matrix direct distillation

Bergamot essential oil is widely used in alimentary and cosmetic industries, and in preparing sanitary formulations.[101] Oils intended for these applications must possess extremely low contents of bergapten and coumarins, whose undesired and often grave side-effects for human health are well known.[102] For these reasons, many countries limit imports and uses of bergamot essential oils containing bergaptene and the other phototoxic and mutagenic coumarins.

Production of oils characterized by low contents of bergapten requires either a distillation process or alkaline treatments of cold-pressed bergamot oils. However, all these procedures remarkably modify the oil composition, rending it worthless from a commercial point of view. [103]

In this work it was proposed a procedure based on the direct distillation of the vegetable matrix obtainable from the peel of bergamot fruits.[104] Essential oil produced by this kind of distillation is completely bergapten-free, showing for all the other components relative percentage amounts quite similar to those observed for cold-pressed oils.

Bergapten is generally removed together with the other coumarin components of the oil by fractionated distillation under vacuum. This procedure causes loss of product and a considerable impoverishment of the composition of the thus obtained oil.[103] The same troubles are encountered when bergapten is removed by treatment with sodium hydroxide, another procedure that produces bergamot oil with altered compositions.

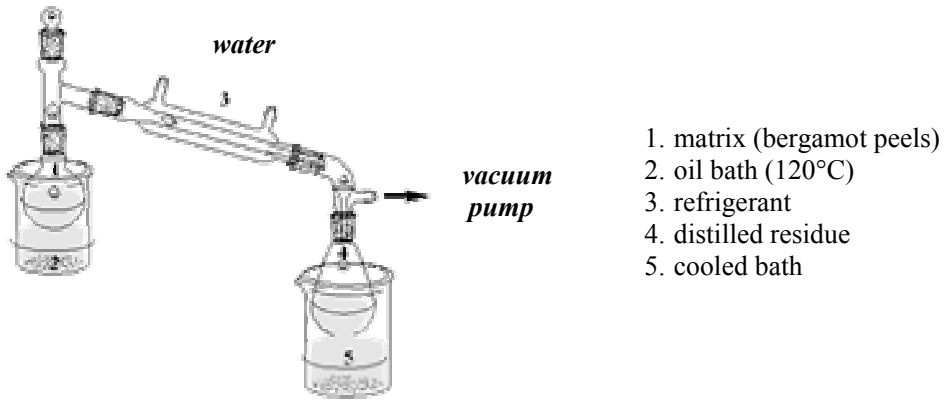
We thought that a simplified distillation procedure for extracting the bergamot essential oil should be planned starting from the following

hypotheses:

- direct use of the vegetable matrix, without any pre-treatment;
- vacuum conditions previously settled, and a rapid heating both to facilitate the kinetic of the process and to avoid a prolonged exposure of the matrix components to hard operative conditions;
- no addition of external water, since the only natural water content of the vegetable matrix could be enough to transform the process into a distillation of products realized by a steam distillation under reduced pressure.

In a typical experiment, a weighted amount of bergamot fruits was peeled, and the recovered peels were homogenized. The homogeneous mixture was subjected to vacuum distillation by immerging the reactor in a previously heated oil bath (120 °C). Distillate was collected in a cooled flask, and the essential oil separated from the aqueous phase produced during distillation (Figure 24).

Figure 24.



The obtained average amount of bergamot essential oil was 310.5 g for quintal of fruits. An aliquot of the final bergamot essential oil (0.01 mL) was diluted with diethyl ether (5 mL), and 1 μL of the ethereal solution was used for the GC-MS analysis.

The essential oil obtained by the procedure is characterized by the presence of cyclic and acyclic monoterpens, which can or not possess oxygen atoms in their structures. The most important components are the open-chain monoterpens β-myrcene, linalool, and linalyl acetate. (*Z*)-β-Ocimene, (*E*)-β-ocimene, nerol, neryl acetate, geranyl acetate, farnesene are also present.

The linalyl acetate and linalool are generally present in a 3.6 relative ratio: this value is a diagnostic index for the good quality of the obtained oils.

The volatile fraction of oils obtained from vegetable matrix direct distillation contains also oxygenated molecules, featuring aldehydic functions, such as neral, geranial, nonanal, and decanal.

The chromatographic profile shows cyclic monoterpens not containing oxygen atom, such as thujene, α-pinene, sabinene, β-pinene, α-terpinene, γ-terpinene, and terpinolene. Limonene represents the major component of the profile (average value 43.67%). Finally, it is important to underline that in the obtained essential oil are also present cyclic oxygenated monoterpens, e.g. terpinen-4-ol, and α-terpineol.

Low contents of alcoholic components, and the absence of oxydated monoterpenes are characteristic of the oils obtained by this way. In particular, cis- and trans-limonene oxide are not detected, unlike distilled oils. The components of the oil were identified by using standard samples of limonene, linalool, linalyl acetate, α-pinene, β-pinene, β-myrcene, α-terpinene, γ-terpinene, p-cymene, (*Z*)-β-ocimene, α-terpineol, terpinen-4-ol, neral, geranial, and geranyl acetate. Standard samples

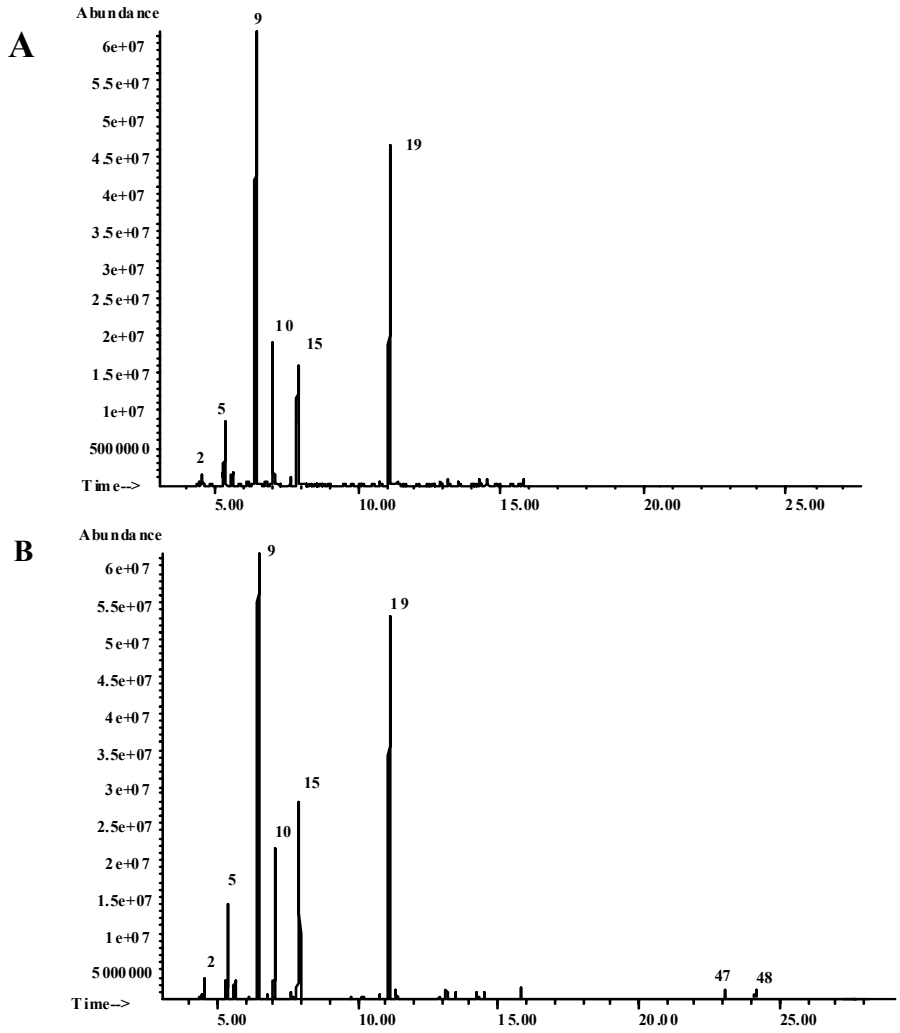
matched the retention time values and mass spectra of the corresponding components of the oil subjected to analysis. The other components, not identified by standard sample injections, were characterized comparing the respective experimental mass spectrum with that reported in the NIST98 data base. Data already reported in the literature were finally used to confirm the identification.[105] Limonene is the principal component of bergamot oils, and its content is strictly related to the fruit harvest period. Fruits harvested in different seasons[103] show also linalool percentage amounts that can vary between 5% and 11%. The presence of bergapten and other coumarins, responsible for undesired health effects has determined the complete prohibition or severe limitations in the cosmetic use of bergamot oils containing these harmful substances.[106] In order to take off bergapten, and to reduce the amounts of the other coumarins in cold-pressed bergamot oils, distillation is usually performed under vacuum or at atmospheric pressure. This procedure provokes a relevant variation of the oil composition. Distillation furnishes linalool enriched oils: the percentage of this compound rises up to 37%, a value higher than those generally observed for cold-pressed oils.[107] The relative content of linalool represents an important parameter for identifying bergamot oils produced by distillation.[103] In distilled bergamot oils, variations of the alcoholic components coming from hydration of monoterpens is generally observed; linalool, nerol, sabinene hydrate, and terpinen-4-ol can therefore show increased concentrations.

Bergamot oil obtained by our procedure is totally bergapten-free. The absence of this undesirable component was verified by parallel analysis of an authentic sample of bergapten. Under the experimental conditions applied for the GC-MS analysis of the bergamot oil, standard bergapten showed a retention time value of 24.10 min.

Chromatogram of the bergamot oil obtained by the exposed procedure

was characterized by the absence of any peak attributable to bergapten (Figure 25, chromatogram **A**). In the cold-pressed oil (Figure 25, chromatogram **B**) bergapten is present (peak 48; bergapten was confirmed by injecting of a standard sample).

Figure 25.



Another important compound, 5,7-dimethoxycoumarin, is detectable in the sample **B** at the retention time value of 23.05 min (peak 47), but is

absent in the chromatogram **A**.

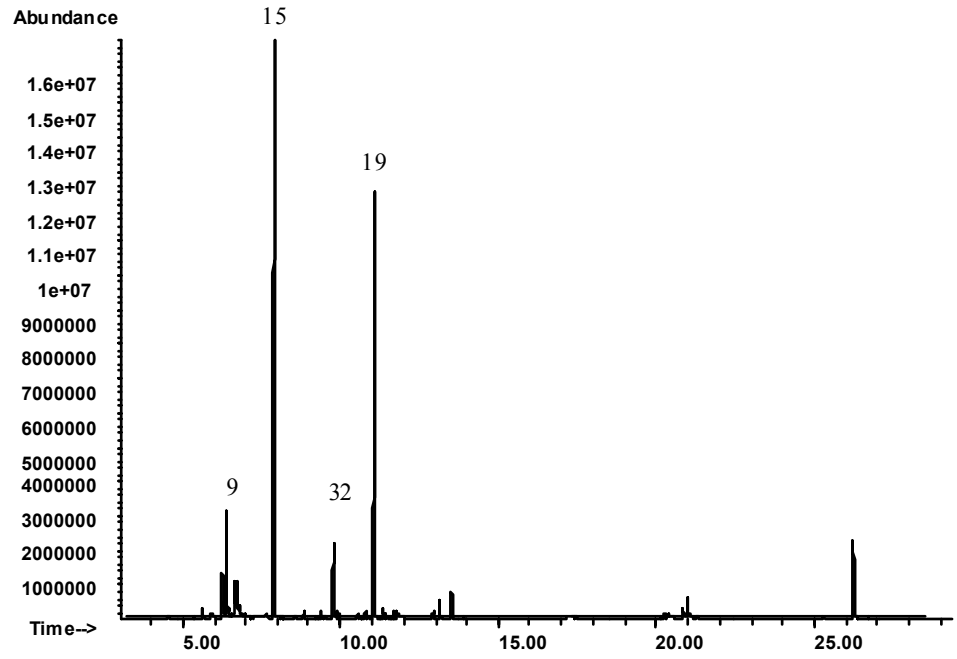
Also the solid residue obtained during storage at low temperature of the cold-pressed oil was analyzed by GC-MS: Bergaptene is the principal component of the residue, and a minimal amount of 5,7-dimethoxycoumarin was also detected.

Composition of bergamot essential oil obtained by vegetable matrix direct distillation of fruit peels, is similar to that of the cold pressed essential oil. Chromatographic profiles of the oils obtained by the two procedures slightly differ only in the relative abundances of some components. Furthermore, "vegetable matrix direct distillation procedure" successfully furnished oils containing all the other components in percentages quite equal to those featured by cold-pressed oils. Unlike distilled oils, no remarkable increments of the relative concentrations of alcoholic components are observed for oils directly obtained from the vegetable matrix. Comparison between chromatograms **A** and **B** highlights only an inversion of the relative ratio for γ-terpinene (peak 10) and linalool (peak 15) in the bergapten-free oil, with respect to the cold-pressed oil. Presumably, our procedure determines an evident increase of γ-terpinene. All the other components of the oil are present in relative percentages which perfectly fit those already reported[106b] for the composition of cold-pressed oils. Moreover, bergamot oil produced by direct distillation of the vegetable matrix shows high concentrations of limonene and low contents of linalool, peculiarities which are not featured by distilled oils.[103] In fact, oils obtained by direct distillation of cold-pressed oils, are characterized by high contents of alcoholic components, the most important of which is linalool, and by low amounts of limonene.

It is possible to identify commercial bergamot essential oils, probably distilled, by simply comparing them with a sample of bergamot oil

obtained by the exposed procedure. For example, high relative concentrations of linalool can be detected for essential bergamot oils used to soak commercial refreshing serviettes (Figure 26).

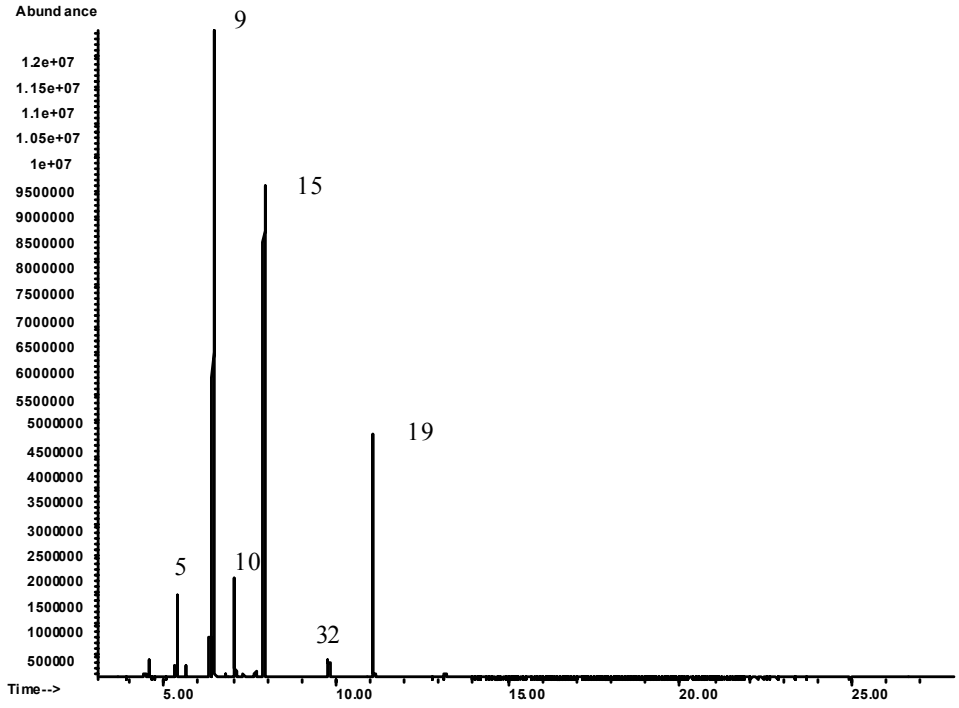
Figure 26.



Chromatogram depicted in Figure 26 shows a low content of limonene (peak 9) and a high relative amount of linalool (peak 15). The 0.62 linalyl acetate/linalool ratio (peaks 19 and 15, respectively) is indicative of a bergamot essential oil probably obtained by distillation. Peak appearing at the retention time value of 26.24 min was not attributed. It was not associable to any component of the oil recovered from the refreshing serviette. The GC-MS analysis was extended to a sample of an alcoholic solution of bergamot oil, a commercially available sanitary preparation (Figure 27). In this case also, the linalool content is very high (29.88%, peak 15), denoting that the bergamot oil used as an ingredient

of the alcoholic solution probably originates from distillation. In fact, the linalyl acetate/linalool ratio is 0.45, confirming that the analyzed bergamot oil is produced by further elaboration of a cold-pressed oil. Thus, distillation causes a sensible variation of the cold-pressed oil composition, lowering also the quality of the bergamot oil. Chromatograms reported in Figure 3 and 4 show also a consistent relative amount of α-terpineol (peak 32) in both the analyzed samples of commercial bergamot oils. A high α-terpineol content is another index useful for identifying distilled oils, as already well established.[103]

Figure 27.



In conclusion, bergamot oil produced by direct distillation of the vegetable matrix procedure shows remarkable similarities with a cold-pressed oil, and can easily be distinguished by oils obtained from further

distillation of cold-pressed oils.

The methodology here disclosed also allows the production of oils in which the linalyl acetate vs. linalool ratio results to be inverted with respect to the values observed for distilled oils.

GC-MS analysis of oils recovered by this wai reveals that bergaptene and the other coumarins are totally absent.

This essential oil featuring ideal characteristics which are invaluable in producing food (e.g., Earl Grey tea), and ideal for cosmetic and sanitary formulations. Oil composition is in fact in accordance with the law provisions of the European Community and Western Countries.

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CHAPTER 3

Experimental section

1. General Experimental Methods

All solvents were purified and dried by standard procedures and distilled prior to use. Melting points were recorded on a Kofler hot-stage apparatus and are uncorrected. The ^{1}H and ^{13}C NMR spectra were recorded at 298 K, at 300 and 75 MHz, respectively, and using $CDCl_3$, CD_3OD, CD_3COCD_3, or DMSO-d_6 as solvents. Chemical shifts (δ) are reported in ppm, and referred to the tetramethylsilane (TMS) used as internal standard. All coupling constants (J) are reported in Hz. GC/MS analyses were performed using a GC-MS instrument equipped with a PhMesiloxane 5 % (30 m × 0.25 mm) capillary column. The mass detector was operated in the electron impact ionization mode (EIMS) with an electron energy of 70 eV. The progress of all reactions was monitored by thin-layer chromatography using silica gel 60-F254 precoated glass plates, and UV light (254 nm) or 0.2% ninhydrin in ethanol and charring as visualizing agent. Kieselgel 60H without gypsum was used for flash column chromatography. When required, the reactions were performed using flame dried glassware and under an inert atmosphere (dry N_2). The methylene chloride solution of diazomethane was prepared from *N*-methyl-*N*-nitrosourea using a classical procedure. The concentration of the diazomethane solution (0.66 M) was obtained by back-titration performed with a standard benzoic acid solution. (CAUTION: diazomethane is highly toxic; hence, this reagent must be handled carefully). Methylene chloride solutions of diazomethane are stable for long periods if stored on KOH pellets at –20°C. All reagents were used as purchased and without further purification.

2. *N*-Fmoc-*N*-methyl and *N*-methyl-*N*-Nosyl α-amino acids

Preparation of Diphenyldiazomethane 2

A solution of benzophenone hydrazone (1.05 mmol) in dry dichloromethane (10 ml) was treated with Magtrieve^{TM} (15 mmol). The reaction mixture was stirred at room temperature and immediately took on a purple color characteristic of diphenyldiazomethane. The oxidation, monitored by TLC analysis (EtOAc/hexane 1:5 v/v), was completed within 15 min.

Synthesis of *N*-Nosyl-α-amino acid benzhydryl esters 3a-f. General Procedure.

A solution of *N*-Nosyl-α-amino acid **1a-f** (1 mmol) in dichloromethane was added to the purple solution of diphenyldiazomethane **2** (1.05 mmol). The resulting mixture was maintained under an inert atmosphere (N_2) and stirred at room temperature. TLC analysis (EtOAc/hexane, 1:5 v/v) showed complete conversion of the precursors **1a-f** after 40 min. After the reaction was finished, Magtrieve^{TM} was retrieved by filtration through celite and the solution was concentrated under reduced pressure to afford the corresponding benzhydryl esters **3a-f** in quantitative yields.

***N*-Nosyl-L-valine benzhydryl esters (3a):** Yellow solid, Mp 95-97 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.98-8.01 (m, 2 H, *o*-NO_2), 7.82-7.92 (m, 2 H, *m*-NO_2), 7.08-7.36 (m, 10 H, $RCO_2CH(C_6H_5)_2$), 6.62 (s, 1 H, RCO_2CHPh_2), 5.85 (d, J =9.2 Hz, 1 H, -N*H*), 4.00 (m, 1 H, α-C*H*), 2.24 (m, 1 H, $(CH_3)_2CH$-), 0.98-1.02 (m, 3 H, $(CH_3)_2$CH-), 0.82-0.88 (m, 3 H, $(CH_3)_2$CH-) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 170.1, 149.8, 145.3, 138.9, 128.6, 128.4, 128.2, 127.8, 127.5, 126.9, 126.8, 126.8, 126.6, 124.1, 78.4, 61.3, 31.6, 19.2, 17.0 ppm. GC/MS (EI) m/z (%) 257 (100), 186 (20), 167 (54), 122 (35). Anal. Calcd for $C_{24}H_{24}N_2O_6S$: C, 61.52; H, 5.16; N, 5.98; O, 20.49; S, 6.84. Found: C, 61.49; H, 5.17; N, 5.96.

***N*-Nosyl-D-valine benzhydryl ester (3b):** Yellow solid, Mp 96-98 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.92 (d, J = 9.1 Hz, 2 H, *o*-NO_2), 7.81 (d, J=9.1 Hz, 2 H, *m*-NO_2), 7.18-7.39 (m, 10 H, $RCO_2CH(C_6H_5)_2$), 6.65 (s, 1 H, RCO_2CHPh_2), 5.88 (d, J=9.2 Hz, 1 H, -N*H*), 4.02 (m, 1 H, α-C*H*), 2.22 (m, 1 H, $(CH_3)_2CH$-), 1.02 (m, J= 6.6 Hz, 3 H, $(CH_3)_2$CH-), 0.82 (d, J= 6.6 Hz, 3 H, $(CH_3)_2$CH-) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 170.1, 149.8, 145.3, 138.9, 128.6, 128.4, 128.2, 127.8, 127.5, 126.9, 126.8, 126.6, 126.8, 124.1, 78.4, 61.3, 31.6, 19.2, 17.0 ppm. GC/MS (EI) m/z (%) 257 (100), 186 (20), 167 (54), 122 (35). Anal. Calcd for $C_{24}H_{24}N_2O_6S$ C, 61.52; H, 5.16; N, 5.98; O, 20.49; S, 6.84. Found: C, 61.45; H, 5.15; N, 5.95.

***N*-Nosyl-L-leucine benzhydryl ester (3c):** Yellow solid, Mp 95-97 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.95 (d, J = 9.2 Hz, 2 H, *o*-NO_2), 7.82 (d, J=9.2 Hz, 2 H, *m*-NO_2), 7.12-7.38 (m, 10 H, $RCO_2CH(C_6H_5)_2$), 6.58 (s, 1H, RCO_2CHPh_2), 5.52 (d, J=9.3 Hz, 1 H, -N*H*), 4.15 (m, 1 H, α-C*H*), 1.80 (m, 1 H, $(CH_3)_2CHCH_2$-), 1.52-1.60 (m, 2 H, $(CH_3)_2CHCH_2$-), 0.82-1.03 (m, 6 H, $(CH_3)_2CHCH_2$-) ppm. GC/MS (EI) m/z (%) 271 (60), 215 (22), 186 (28), 167 (100), 122 (21). Anal. Calcd for $C_{25}H_{26}N_2O_6S$: C, 62.23; H, 5.43; N, 5.81; O, 19.89; S, 6.64. Found: C, 62.33; H, 5.41; N, 5.79.

***N*-Nosyl-S-benzyl-L-cysteine benzhydryl ester (3d):** Yellow solid, Mp 91-93 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.06 (d, J = 9.1 Hz, 2 H, *o*-NO_2), 7.88 (d, J=9.1 Hz, 2 H, *m*-NO_2), 7.08-7.42 (m, 15 H, $RCO_2CH(C_6H_5)_2$ and $–SCH_2C_6H_5$), 6.70 (s, J = 9.3 Hz, 1 H, RCO_2CHPh_2), 6.06 (d, J =9.1 Hz, 1 H, -N*H*), 4.35 (m, 1 H, α-C*H*), 3.60-3.70 (m, 2 H, -SCH_2Ph), 2.81-2.88 (m, 2 H, -CH_2S-Bzl) ppm. Anal. Calcd for $C_{29}H_{26}N_2O_6S_2$: C, 61.90; H, 4.66; N, 4.98; O, 17.06; S, 11.40. Found: C, 62.03; H, 4.65; N, 4.97.

***N*-Nosyl-L-isoleucine benzhydryl ester (3e):** Yellow solid, Mp 92-94 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.85 (d, J = 9.2 Hz, 2 H, *o*-NO_2), 7.75 (d, J=9.2 Hz, 2 H, *m*-NO_2), 7.21-7.40 (m, 10 H, $RCO_2CH(C_6H_5)_2$), 6.58 (s, 1 H, RCO_2CHPh_2), 5.60 (s, J=9.3 Hz, 1 H, -N*H*), 3.98 (d, J = 8.3 Hz, 1 H, α-C*H*), 1.90 (m, 1 H, -$CH(CH_3)CH_2CH_3$), 1.50 (m, 1 H, -$CH(CH_3)CH_2CH_3$), 1.22 (m, 1 H, -$CH(CH_3)CH_2CH_3$),

0.94 (d, J= 4.8 Hz, 3 H, -CH(C*H*$_3$)CH$_2$CH$_3$), 0.81 (t, J= 7.3 Hz, 3 H, -CH(CH$_3$)CH$_2$C*H*$_3$) ppm. GC/MS (EI) m/z (%) 271 (60), 186 (28), 167 (100), 122 (21). Anal. Calcd for $C_{25}H_{26}N_2O_6S$: C, 62.18; H, 5.43; N, 5.81; O, 19.89; S, 6.64. Found: C, 62.23; H, 5.41; N, 5.82.

***N*-Nosyl-L-alanine benzhydryl esters (3f):** Yellow solid, Mp 100-102 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.04 (d, J = 9.2 Hz, 2 H, *o*-NO$_2$), 7.88 (d, J=9.2 Hz, 2 H, *m*-NO$_2$), 7.19-7.38 (m, 10 H, RCO$_2$CH(C$_6$*H*$_5$)$_2$), 6.64 (s, 1 H, RCO$_2$C*H*Ph$_2$), 5.76 (d, J=9.1 Hz, 1 H, -N*H*), 4.23 (m, 1 H, α-C*H*), 1.48 (d, J= 6.0 Hz, 3 H, -C*H*$_3$) ppm. GC/MS (EI) m/z (%) 229 (44), 186 (18), 167 (100), 122 (15). Anal. Calcd for $C_{22}H_{20}N_2O_6S$: C, 59.99; H, 4.58; N, 6.36; O, 21.79; S, 7.28. Found C, 59.99; H, 4.59; N, 6.34.

Synthesis of *N*-Methyl-*N*-nosyl-α-amino acid benzhydryl esters 4a-f. General Procedure.

A 0.66 M solution of diazomethane in dry dichloromethane (8 mmol) was added cautiously dropwise to a magnetically stirred solution of the *N*-Nosyl-α-amino acid benzhydryl esters **3a-f** (1 mmol) in dry dichloromethane (10 ml). The resulting mixture was maintained at room temperature and under N_2. TLC analysis (EtOAc/hexane, 1:5 v/v) showed the complete conversion of the precursors **3a-f** after 1.5 h. Evaporation of the solvent under reduced pressure afforded the *N*-methyl-*N*-Nosyl- α-amino acid benzhydryl esters **4a-f** in quantitative yields.

***N*-methyl-*N*-Nosyl-L-valine benzhydryl esters (4a):** Yellow solid, Mp 91-93 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.95 (d, J = 8.4 Hz, 2 H, *o*-NO$_2$), 7.78 (d, J=8.4 Hz, 2 H, *m*-NO$_2$), 7.16-7.38 (m, 10 H, RCO$_2$CH(C$_6$*H*$_5$)$_2$), 6.62 (s, 1 H, RCO$_2$C*H*Ph$_2$), 4.35 (d, J= 10.5 Hz, 1 H, α-C*H*), 2.92 (s, 3 H, -NC*H*$_3$), 2.18 (m, 1 H, (CH$_3$)$_2$C*H*-), 1.05 (d, J= 6.3 Hz, 3 H, (C*H*$_3$)$_2$CH-), 0.88 (d, J= 6.3 Hz, 3 H, (C*H*$_3$)$_2$CH-) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 168.9, 149.8, 145.3, 138.9, 128.8, 128.5, 128.4, 128.3, 128.2, 127.0, 126.9, 124.3, 77.9, 65.2, 30.3, 28.2, 19.3

ppm. GC/MS (EI) m/z (%) 271 (100), 186 (20), 167 (62), 122 (35). Anal. Calcd for $C_{25}H_{26}N_2O_6S$: C, 62.23; H, 5.43; N, 5.81; O, 19.89; S, 6.64. Found: C, 62.15; H, 5.42; N, 5.79.

***N*-methyl-*N*-Nosyl-D-valine benzhydryl ester (4b):**Yellow solid, Mp 95-97 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.92 (d, J= 8.4 Hz, 2 H, *o*-NO_2), 7.75 (d, J= 8.4 Hz, 2 H, *m*-NO_2), 7.14-7.35 (m, 10 H, $RCO_2CH(C_6H_5)_2$), 6.67 (s, 1 H, RCO_2CHPh_2), 4.39 (d, J= 10.5 Hz, 1 H, α-C*H*), 2.94 (s, 3 H, -NCH_3), 2.21 (m, 1 H, $(CH_3)_2CH$-), 1.03 (d, J= 6.3 Hz, 3 H, $(CH_3)_2CH$-), 0.85 (d, J= 6.3 Hz, 3 H, $(CH_3)_2CH$-) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 168.9, 149.8, 145.3, 138.9, 128.8, 128.5, 128.4, 128.3, 128.2, 127.0, 126.9, 124.3, 77.9, 65.2, 30.3, 28.2, 19.3 ppm. GC/MS (EI) m/z (%) 271 (100), 186 (20), 167 (62), 122 (35). Anal. Calcd for $C_{25}H_{26}N_2O_6S$: C, 62.23; H, 5.43; N, 5.81; O, 19.89; S, 6.64. Found: C, 62.41; H, 5.44; N, 5.79.

***N*-methyl-*N*-Nosyl-L-leucine benzhydryl ester (4c):** Yellow solid, Mp 93-95 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.95 (d, J= 9.0 Hz, 2 H, *o*-NO_2), 7.75 (d, J= 9.0 Hz, 2 H, *m*-NO_2), 7.18-7.38 (m, 10 H, $RCO_2CH(C_6H_5)_2$), 6.60 (s, 1 H, RCO_2CHPh_2), 4.84 (m, 1 H, α-C*H*), 2.90 (s, 3 H, -NCH_3), 1.69-1.78 (m, 3 H, $(CH_3)_2CHCH_2$- and $(CH_3)_2CHCH_2$-), 1.03 (d, J= 6.0 Hz, 3 H, $(CH_3)_2CHCH_2$), 0.99 (d, J= 6.0 Hz, 3 H, $(CH_3)_2CHCH_2$) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 169.8, 144.3, 139.1, 130.0, 128.6, 128.5, 126.7, 124.0, 78.0, 57.7, 38.2, 30.0, 24.5, 23.0, 21.0 ppm. GC/MS (EI) m/z (%) 285 (100), 186 (22), 167 (42), 122 (15). Anal. Calcd for $C_{26}H_{28}N_2O_6S$: C, 62.89; H, 5.68; N, 5.64; O, 19.33; S, 6.46. Found: C, 63.01; H, 5.69; N, 5.63.

***N*-methyl-*N*-Nosyl-S-benzyl-L-cysteine benzhydryl ester (4d):** Amorphous yellow solid. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.06 (d, J= 9.1 Hz, 2 H, *o*-NO_2), 7.88 (d, J= 9.1 Hz, 2 H, *m*-NO_2), 7.18-7.42 (m, 15 H, $RCO_2CH(C_6H_5)_2$ and –$SCH_2C_6H_5$), 6.70 (s, 1 H, RCO_2CHPh_2), 4.90 (m, 1 H, α-C*H*), 3.76-3.82 (m, 2 H, -SCH_2Ph), 2.98 (m, 1 H, -CH_2S-Bzl), 2.81 (s, 3 H, -NCH_3), 2.65 (m, 1 H, -CH_2S-Bzl) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 168.1, 149.8, 139.1, 138.9, 137.3,

128.5, 128.4, 128.3, 128.0, 126.7, 124.1, 78.6, 58.6, 35.7, 30.2, 30.1 ppm. Anal. Calcd for $C_{30}H_{28}N_2O_6S_2$: C, 62.48; H, 4.89; N, 4.86; O, 16.65; S, 11.12. Found: C, 62.22; H, 4.91; N, 4.84.

***N*-methyl-*N*-Nosyl-L-isoleucine benzhydryl ester (4e):** Yellow solid, Mp 100-102 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.88 (d, J= 9.0 Hz, 2 H, *o*-NO_2), 7.75 (d, J= 9.0 Hz, 2 H, *m*-NO_2), 7.20-7.42 (m, 10 H, $RCO_2CH(C_6H_5)_2$), 6.60 (s, 1 H, RCO_2CHPh_2), 4.47 (d, J= 12.3 Hz, 1 H, α-C*H*), 2.94 (s, 3 H, -NCH_3), 1.97 (m, 1 H, -C*H*$(CH_3)CH_2CH_3$), 1.62 (m, 1 H, -CH$(CH_3)CH_2$ CH_3), 1.26 (m, 1 H, -CH$(CH_3)CH_2CH_3$), 0.95 (t, J= 7.2 Hz, 3 H, -CH$(CH_3)CH_2CH_3$), 0.82 (d, J= 6.9 Hz, 3 H, -CH$(CH_3)CH_2CH_3$) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 169.0, 149.7, 144.3, 128.3, 128.2, 127.5, 127.0, 124.0, 77.8, 63.8, 34.2, 30.5, 25.3, 15.3, 10.4 ppm. GC/MS (EI) m/z (%) 285 (100), 229 (32), 186 (21), 167 (72). Anal. Calcd for $C_{26}H_{28}N_2O_6S$: C, 62.89; H, 5.68; N, 5.64; O, 19.33; S, 6.46. Found: C, 62.96; H, 5.64; N, 5.67.

***N*-methyl-*N*-Nosyl-L-alanine benzhydryl esters (4f):** Yellow solid, Mp 102-105 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.02 (d, J= 9.3 Hz, 2 H, *o*-NO_2), 7.80 (d, J= 9.3 Hz, 2 H, *m*-NO_2), 7.10-7.42 (m, 10 H, $RCO_2CH(C_6H_5)_2$), 6.68 (s, 1 H, RCO_2CHPh_2), 4.93 (m, 1 H, α-C*H*), 2.85 (s, 3 H, -NCH_3), 1.52 (d, J= 7.2 Hz, 3 H, -CH_3) ppm. GC/MS (EI) m/z (%) 243 (100), 186 (26), 167 (64), 122 (20). Anal. Calcd. for $C_{23}H_{22}N_2O_6S$: C, 60.78; H, 4.88; N, 6.16; O, 21.12; S, 7.06. Found: C, 60.95; H, 4.87; N, 6.17.

Synthesis of *N*-methyl-*N*-Nosyl-α-amino acid 5a-f. General Procedure.

Trifluoroacetic acid (5-7mL $mmol^{-1}$) and toluene (2 nnmol) were added to a solution of the *N*-methyl-*N*-Nosyl-α-amino acid benzhydryl esters **4a-f** in dichloromethane. The resulting mixture was stirred at room temperature and under N_2 for 1 h monitoring the conversion of **4a-f** by TLC (EtOAc/hexane, 1:5 v/v). After evaporation of the solvent under reduced pressure, saturated aqueous Na_2CO_3 was added and the aqueous solution was extracted with dichloromethane

(3 × 20 mL). Aqueous 2 N HCl was then added and the acidified solution was extracted with ethyl acetate (3 × 20 mL). The combined organic extracts were dried over Na_2SO_4 and evaporated under vacuum to afford the *N*-methyl-*N*-Nosyl- α-amino acid **5a-f** in 94-98 % overall yields.

***N*-methyl-*N*-Nosyl-L-valine (5a):** Yield 94%. Yellow solid. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.29-8.42 (m, 2 H), 7.92-8.05 (m, 2 H), 4.18 (d, J= 10.7 Hz, 1 H), 2.92 (s, 3 H), 2.13 (m, 1 H), 1.02 (d, J= 6.8 Hz, 3 H), 0.96 (d, J= 6.8 Hz, 3 H) ppm. Anal. Calcd for $C_{12}H_{16}N_2O_6S$: C, 45.56; H, 5.10; N, 8.86; O, 30.35; S, 10.14. Found: C, 45.46; H, 5.11; N, 8.85.

***N*-methyl-*N*-Nosyl-D-valine (5b):** Yield 94%. Yellow solid. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.29-8.42 (m, 2 H), 7.92-8.05 (m, 2 H), 4.18 (d, J= 10.7 Hz, 1 H), 2.92 (s, 3 H), 2.13 (m, 1 H), 1.02 (d, J= 6.8 Hz, 3 H), 0.96 (d, J= 6.8 Hz, 3 H) ppm. Anal. Calcd for $C_{12}H_{16}N_2O_6S$: C, 45.56; H, 5.10; N, 8.86; O, 30.35; S, 10.14. Found: C, 45.62; H, 5.09; N, 8.87.

***N*-methyl-*N*-Nosyl-L-leucine (5c):** Yield 94%. Yellow solid. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.35 (d, J= 9.2 Hz, 2 H), 8.00 (d, J= 9.2 Hz, 2 H), 4.71 (m, 1 H), 2.95 (s, 3 H), 1.61-1.72 (m, 3 H), 0.92-1.01 (m, 6 H, $(CH_3)_2CHCH_2$) ppm. Anal. Calcd for $C_{13}H_{18}N_2O_6S$: C, 47.26; H, 5.49; N, 8.48; O, 29.06; S, 9.71. Found: C, 47.34; H, 5.50; N, 8.46.

***N*-methyl-*N*-Nosyl-S-benzyl-L-cysteine (5d):** Yield 95%. Yellow solid. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.36 (d, J= 9.3 Hz, 2 H), 8.08 (d, J= 9.3 Hz, 2 H), 7.18-7.32 (m, 5 H), 4.62 (m, 1 H), 3.72 (m, 2 H), 2.88 (m, 1 H), 2.77 (s, 3 H), 2.72 (m, 1 H) ppm. ^{13}C-NMR (75 MHz, $CDCl_3$) δ 170.5, 150.1, 144.9, 138.4, 137.3, 129.5, 129.2, 128.8, 127.4, 124.8, 59.0, 30.7, 30.4 ppm. Anal. Calcd for $C_{17}H_{18}N_2O_6S_2$: C, 49.74; H, 4.42; N, 6.82; O, 23.39; S, 15.62. Found: C, 49.91; H, 4.41; N, 6.83.

***N*-methyl-*N*-Nosyl-L-isoleucine (5e):** Yield 98%. Yellow solid. ^{1}H-NMR (300

MHz, DMSO-d_6) δ 8.30 (d, J= 9.3 Hz, 2 H), 8.00 (d, J= 9.3 Hz, 2 H), 4.26 (d, J= 10.4 Hz, 1 H), 2.92 (s, 3 H), 1.88 (m, 1 H), 1.65 (m, 1 H), 1.54 (m, 1 H), 0.87 (m, 6 H) ppm. ^{13}C-NMR (75 MHz, DMSO-d_6) δ 171.1, 151.3, 144.2, 127.6, 129.1, 68.8, 33.3, 30.8, 28.2, 15.6, 11.4 ppm. Anal. Calcd for $C_{13}H_{18}N_2O_6S$: C, 47.26; H, 5.49; N, 8.48; O, 29.06; S, 9.71. Found: C, 47.32; H, 5.50; N, 8.47.

***N*-methyl-*N*-Nosyl-L-alanine (5f):** Yield 98%. Yellow solid. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.24-8.38 (d, J= 9.3 Hz, 2 H), 7.88-7.98 (d, J= 9.3 Hz, 2 H), 4.60 (q, J= 7.8 Hz, 1 H), 2.79 (s, 3 H), 1.22 (d, J= 6.8 Hz, 3 H) ppm. Anal. Calcd for $C_{10}H_{12}N_2O_6S$: C, 41.66; H, 4.20; N, 9.72; O, 33.30; S, 11.12. Found: C, 41.52; H, 4.20; N, 9.73.

***N*-Deprotection of *N*-methyl-*N*-Nosyl-α-amino acid benzhydryl esters 4a-f. General Procedure.**

Mercaptoacetic acid (3 mmol) was added to a solution of **4a-f** (1 mmol) in dry acetonitrile (10 mL) under N_2 at 50 °C. Sodium methoxide (7 mmol) was then gradually added to the solution with with a variable amount of methanol to facilitate the sodium methoxide solubilization. The resulting mixture was stirred for 40 min monitoring the conversion of the precursors **4a-f** by TLC (EtOAc/hexane, 1:5 v/v) and the formation of the *N*-deprotected products by ninhydrin test. Aqueous 1 N HCl was then added and the acidified solution (pH 2) was extracted with ethyl acetate (3 × 10 mL). The aqueous phase was basified with saturated aqueous Na_2CO_3 and then extracted with ethyl acetate (3 × 10 mL). The combined organic extracts were dried over Na_2SO_4 and evaporated under vacuum to afford the *N*-Methyl-α-amino acid benzhydryl esters **6a-f** in 90-96% overall yields.

***N*-methyl-L-valine benzhydryl esters (6a):** White oil (96%). GC/MS (EI) m/z (%)167 (46), 86 (100).

***N*-methyl-D-valine benzhydryl ester (6b):** White oil (96%). GC/MS (EI) m/z

(%)167 (46), 86 (100).

***N*-methyl-L-leucine benzhydryl ester (6c):** White oil (95%). GC/MS (EI) m/z (%) 167 (42), 100 (100).

***N*-methyl-S-benzyl-L-cysteine benzhydryl ester (6d):** Yellow oil (92%). MS (ESI-TOF) m/z Calcd for $C_{24}H_{26}NO_2S^+$ 392.1684; Found: 392.1697

***N*-methyl-L-isoleucine benzhydryl ester (6e):** White oil (95%). GC/MS (EI) m/z (%) 167 (46), 86 (100).

***N*-methyl-L-alanine benzhydryl ester (6f):** White oil (90%). GC/MS (EI) m/z (%) 167 (64), 58 (100).

Synthesis of *N*-Nosyl-dipeptides benzhydryl esters 8a-b. General Procedure.

The appropriate *N*-methyl-α-amino acid benzhydryl esters **6a-b** (1 mmol) was suspended in an aqueous solution of $NaHCO_3$ (6 mL, pH = 8). A solution of *N*-Nosyl-D-alanine chloride (1 mmol) in dichloromethane (6 mL) was added gradually and the resulting mixture was stirred at room temperature for 1 h monitoring the conversion of the precursors **6a-b** by TLC (EtOAc:hexane = 1:5) and GC/MS analysis. After the reaction was finished the organic layer was separated and the aqueous phase was extracted with three additional portions of dichloromethane (3 × 10 mL). The combined organic extracts were dried over Na_2SO_4 and evaporated under vacuum to afford **8a-b** in 89-91 % yields.

***N*-Nosyl-D-alanyl-*N*-methyl-L-valine benzhydryl ester (8a):** Amorphous yellow solid (89%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.28 (d, *J* = 9.2 Hz, 2 H, *o*-NO_2), 8.12 (d, J= 9.3 Hz, 1 H, -N*H*), 8.02 (d, J= 9.2 Hz, 2 H, *m*-NO_2), 7.22-7.39 (m, 10 H, $RCO_2CH(C_6H_5)_2$), 6.88 (s, 1 H, RCO_2CHPh_2), 4.85 (d, J= 10.8 Hz, 1 H, -C*H*$CH(CH_3)_2$), 4.32 (m, 1 H, -C*H*CH_3), 2.75 (s, 3 H, -NCH_3), 2.13 (m, 1 H, -

C*H*(CH_3)$_2$), 1.2 (d, J= 6.9 Hz, 3 H, -C*H*$_3$), 0.87 (d, J= 6.9 Hz, 3 H, -CH(C*H*$_3$)$_2$), 0.46 (d, J= 6.9 Hz, 3H, -CH(C*H*$_3$)$_2$) ppm. Anal. Calcd for $C_{28}H_{31}N_3O_7S$: C, 60.74; H, 5.64; N, 7.59; O, 20.23; S, 5.79. Found: C, 60.68; H, 5.65; N, 7.58.

***N*-Nosyl-D-alanyl-*N*-methyl-D-valine benzhydryl ester (8b):** Amorphous yellow solid (91%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.14 (d, J= 9.2 Hz, 2 H, *o*-NO_2), 8.02 (d, J= 9.2 Hz, 2 H, *m*-NO_2), 7.95 (d, J= 9.3 Hz, 1 H, -N*H*), 7.20-7.42 (m, 10 H, $RCO_2CH(C_6H_5)_2$), 6.85 (s, 1 H, RCO_2CHPh_2), 4.75 (d, *J* = 10.8 Hz, 1 H, -C*H*CH(CH_3)$_2$), 4.30-4.40 (m, 1 H, -C*H*CH_3), 2.82 (s, 3 H, -NC*H*$_3$), 2.20 (m, 1 H, -C*H*(CH_3)$_2$), 1.30 (d, J= 6.9 Hz, 3 H, -C*H*$_3$), 0.87 (d, J= 6.9 Hz, 3 H, -C*H*(CH_3)$_2$), 0.72 (d, J= 6.9 Hz, 3 H, -C*H*(CH_3)$_2$) ppm. Anal. Calcd for $C_{28}H_{31}N_3O_7S$: C, 60.74; H, 5.64; N, 7.59; O, 20.23; S, 5.79. Found: C, 60.93; H, 5.62; N, 7.61.

Synthesis of *N*-Nosyl-dipeptides 9a-b. General Procedure.

To a solution of the *N*-Nosyl-dipeptide benzhydryl esters **8a-b** in dichloromethane (2 mL) trifluoroacetic acid (5-7 mL) and toluene (2 mmol) were added. The resulting mixture was stirred at room temperature for 1 h monitoring the conversion of **8a-b** by TLC (EtOAc:hexane = 1:5). After evaporation of the solvent under reduced pressure, saturated aqueous Na_2CO_3 was added and the aqueous solution was extracted with dichloromethane (3 × 20 mL). Aqueous 2 N HCl was then added and the acidified solution was extracted with EtOAc (3 × 20 mL). The combined organic extracts were dried over Na_2SO_4 and evaporated under vacuum to afford **9a-b** in 98% overall yields.

***N*-Nosyl-D-alanyl-*N*-methyl-L-valine (9a):** Yield 89%. Yellow solid. Mp 107-111 °C. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.48 (d, J= 9.3 Hz, 1 H), 8.48 (d, J= 9.0 Hz, 2 H, *o*-NO_2), 8.05 (d, J= 9.0 Hz, 2 H, *m*-NO_2), 4.33-4.42 (m, 1 H, -C*H*CH(CH_3)$_2$), 3.88 (m, 1 H, -C*H*CH_3), 2.96 (s, 3 H, -NC*H*$_3$), 2.02 (m, 1 H, -C*H*(CH_3)$_2$), 1.14 (d, J= 7.4 Hz, 3 H, -C*H*$_3$), 0.88 (d, J= 7.0 Hz, 3 H, -C*H*(CH_3)$_2$), 0.52 (d, J= 7.0 Hz, 3 H, -C*H*(CH_3)$_2$) ppm. Anal. Calcd for $C_{15}H_{21}N_3O_7S$: C,

46.50; H, 5.46; N, 10.85; O, 28.91; S, 8.28. Found: C, 46.48; H, 5.45; N, 10.87.

***N*-Nosyl-D-alanyl-*N*-methyl-D-valine (9b):** Yield 98%. Yellow solid. Mp 105-108 °C. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.53 (d, J= 9.3 Hz, 1 H, *o*-NO_2), 8.48 (d, J= 9.0 Hz, 2 H, *m*-NO_2), 8.05 (d, J= 9.0 Hz, 2 H, -N*H*), 4.52 (d, J= 10.8 Hz, 1 H, -C*H*CH$(CH_3)_2$), 3.88 (m, 1 H, -C*H*CH_3), 2.96 (s, 3 H, -NC*H*$_3$), 2.02 (m, 1 H, -C*H*$(CH_3)_2$), 1.14 (d, J= 7.4 Hz, 3 H, -C*H*$_3$), 0.88 (d, J= 7.0 Hz, 3 H, -CH(C*H*$_3)_2$), 0.52 (d, J= 7.0 Hz, 3 H, -CH(C*H*$_3)_2$) ppm. Anal. Calcd for $C_{15}H_{21}N_3O_7S$: C, 46.50; H, 5.46; N, 10.85; O, 28.91; S, 8.28. Found: C, 46.49; H, 5.43; N, 10.89.

Synthesis of *N*-Nosyl-*N*-methyl-dipeptides 10a-b. General Procedure.

A 0.66 M solution of diazomethane in dry dichloromethane (8 mmol) was added cautiously dropwise to a suspension of the dipeptides **9a-b** (1 mmol) in dry dichloromethane (10 ml). The resulting mixture was magnetically stirred at room temperature and under N_2. Evaporation of the solvent under reduced pressure afforded the N-methylated dipeptides **10a-b** in quantitative yields.

***N*-methyl-*N*-Nosyl-D-alanyl-*N*-methyl-L-valine methyl ester (10a):** Yield 98%. Yellow oil. GC/MS (EI) m/z (%) 243 (100), 229 (20), 186 (18), 122 (28), 56 (24). Anal. Calcd for $C_{17}H_{25}N_3O_7S$: C, 49.15; H, 6.07; N, 10.11; O, 26.96; S, 7.72. Found: C, 48.98; H, 6.08; N, 10.09.

***N*-methyl-*N*-Nosyl-D-alanyl-*N*-methyl-D-valine methyl ester (10b):** Yield 98%. Yellow oil. GC/MS (EI) m/z (%) 243 (100), 229 (20), 186 (15), 122 (24), 56 (20). Anal. Calcd for $C_{17}H_{25}N_3O_7S$: C, 49.15; H, 6.07; N, 10.11; O, 26.96; S, 7.72. Found: C, 49.23; H, 6.06; N, 10.12.

Synthesis of *N*-Fmoc-α-amino acid benzhydryl esters 11a-f. General Procedure.

The *N*-methyl-α-amino acid benzhydryl esters **6a-f** (1 mmol) were suspended

in aqueous $NaHCO_3$ (6 mL, pH = 8). A solution of FmocCl (1 mmol) in dichloromethane (6 mL) was added gradually and the resulting mixture was stirred at room temperature for 1 h monitoring the conversion of the precursors **6a-b** by TLC (EtOAc:hexane = 1:5) and GC/MS analysis. After the reaction was finished the dichloromethane layer was separated and the aqueous phase was extracted with three additional portions of dichloromethane (3 ×10 mL). The combined organic extracts were dried over Na_2SO_4 and evaporated under vacuum to afford the *N*-Fmoc-α-amino acid benzhydryl esters **11a-f** in 85-94 % overall yields.

***N*-Fmoc-*N*-methyl-L-valine benzhydryl esters (11a):** Yield 92%. Amorphous white solid. ^{1}H-NMR (300 MHz, $CDCl_3$) 55:45 mixture of two rotamers A and B δ 7.65-7.90 (m, 8 H), 7.25-7.48 (m, 10 H, $RCO_2CH(C_6H_5)_2$), 6.99 (s, 1 H, A, RCO_2CHPh_2), 6.94 (s, 1 H, B, RCO_2CHPh_2), 4.76 (d, J= 10.3 Hz, 1 H, α-C*H*), 4.50-4.64 (m, 2 H), 4.29 (m, 1 H), 2.87 (s, 3 H, -NCH_3), 2.30 (m, 1 H, A, $(CH_3)_2CH$-), 2.18 (m, 1 H, B, $(CH_3)_2CH$-), 1.02 (d, J= 6.5 Hz, 3 H, A, $(CH_3)_2$CH-), 0.96 (d, J= 6.5 Hz, 3 H, B, $(CH_3)_2$CH-), 0.90 (d, J= 6.5 Hz, 3 H, B, $(CH_3)_2$CH-), 0.77 (d, J= 6.5 Hz, 3 H, A, $(CH_3)_2$CH-) ppm. Anal. Calcd for $C_{34}H_{33}NO_4$: C, 78.59; H, 6.40; N, 2.70; O, 12.32. Found: C, 78.70; H, 6.41; N, 2.69.

***N*-Fmoc-*N*-methyl-D-valine benzhydryl ester (11b):** Yield 92%. Amorphous white solid. ^{1}H-NMR (300 MHz, $CDCl_3$), 55:45 mixture of two rotamers A and B δ 7.62-7.88 (m, 8 H), 7.21-7.42 (m, 10 H, $RCO_2CH(C_6H_5)_2$), 7.00 (s, 1 H, A, RCO_2CHPh_2), 6.94 (s, 1 H, B, RCO_2CHPh_2), 4.80 (d, J= 10.3 Hz, 1 H, α-C*H*), 4.52-4.62 (m, 2 H), 4.30 (m, 1 H), 2.88 (s, 3 H, -NCH_3), 2.28 (m, 1 H, A, $(CH_3)_2CH$-), 2.12 (m, 1 H, B, $(CH_3)_2CH$-), 0.99 (d, J= 6.5 Hz, 3 H, A, $(CH_3)_2$CH-), 0.95 (d, J= 6.5 Hz, 3 H, B, $(CH_3)_2$CH-), 0.89 (d, J= 6.5 Hz, 3 H, A, $(CH_3)_2$CH-), 0.75 (d, J= 6.5 Hz, 3 H, B, $(CH_3)_2$CH-) ppm. Anal. Calcd for $C_{34}H_{33}NO_4$: C, 78.50; H, 6.40; N, 2.70; O, 12.32. Found: C, 78.59; H, 6.41; N, 2.79.

***N*-Fmoc-*N*-methyl-L-leucine benzhydryl ester (11c):** Yield 90%. Amorphous white solid. ^{1}H-NMR (300 MHz, $CDCl_3$) 55:45 mixture of two rotamers A and B

δ 8.20 (d, J= 9.3 Hz, 2 H), 7.82-7.90 (m, 2 H), 7.59-7.68 (m, 4 H), 7.12-7.43 (m, 10 H, $RCO_2CH(C_6H_5)_2$), 6.76 (s, 1 H, A, RCO_2CHPh_2), 6.61 (s, 1 H, B, RCO_2CHPh_2), 4.78-4.86 (m, 2 H, A+B, α-C*H*), 4.22-4.65 (m, 3 H, Fmoc-C*H* and Fmoc-CH_2), 2.98 (s, 3 H, -NCH_3), 1.71-1.75 (m, 3 H, $(CH_3)_2CHCH_2$- and $(CH_3)_2CHCH_2$-), 0.98-1.20 (m, 6 H, A+B, $(CH_3)_2CHCH_2$-) ppm. Anal. Calcd for $C_{35}H_{35}NO_4$: C, 78.77; H, 6.61; N, 2.62.

***N*-Fmoc-*N*-methyl-S-benzyl-L-cysteine benzhydryl ester (11d):** Yield 95%. Amorphous yellow solid. ^{1}H-NMR (300 MHz, $CDCl_3$) 55:45 mixture of two rotamers A and B δ 7.92-8.02 (m, 4 H, Ar_{Fmoc}), 7.50-7.60 (m, 2 H, Ar_{Fmoc}), 7.22-7.45 (m, 17 H, $RCO_2CH(C_6H_5)_2$ and $-SCH_2C_6H_5$ and Ar_{Fmoc}), 6.82 (s, 1 H, A, RCO_2CHPh_2), 6.72 (s, 1 H, B, RCO_2CHPh_2), 4.98 (m, 1 H, A, α-C*H*), 4.85 (m, 1 H, B, α-C*H*), 4.10-4.52 (m, 3 H, Fmoc-C*H* and Fmoc-CH_2), 3.64-3.72 (m, 2 H, -CH_2SBzl), 3.08 (m, 1 H, B, -CH_2SBzl), 3.02 (m, 1 H, A, -CH_2SBzl), 2.86 (s, 3 H, A, -NCH_3), 2.81 (s, 3 H, B, -NCH_3), 2.45-2.72 (m, 2 H, A+B, -CH_2S-Bzl) ppm. Anal. Calcd for $C_{39}H_{35}NO_4S$: C, 76.32; H, 5.75; N, 2.28; O, 10.43; S, 5.22. Found: C, 76.48; H, 5.73; N, 2.29.

***N*-Fmoc-*N*-methyl-L-isoleucine benzhydryl ester (11e):** Yield 94%. Amorphous white solid. ^{1}H-NMR (300 MHz, $CDCl_3$) 55:45 mixture of two rotamers A and B δ 8.08 (d, J= 9.3 Hz, 2 H), 7.70-7.78 (m, 2 H), 7.52-7.60 (m, 4 H), 7.20-7.48 (m, 10 H, $RCO_2CH(C_6H_5)_2$), 6.84 (s, 1 H, A, RCO_2CHPh_2), 6.82 (s, 1 H, B, RCO_2CHPh_2), 4.75 (d, J= 12.3 Hz, 1 H, α-C*H*), 4.42-4.52 (m, 4 H, A+B, Fmoc-CH_2), 4.15-4.30 (m, 1 H, Fmoc-C*H*), 2.78 (s, 3 H, -NCH_3), 1.99 (m, 1 H, A, -$CH(CH_3)CH_2CH_3$), 1.92 (m, 1 H, B, -$CH(CH_3)CH_2CH_3$), 1.38 (m, 1 H, A -$CH(CH_3)CH_2CH_3$), 1.24 (m, 1 H, B, -$CH(CH_3)CH_2CH_3$), 1.06 (m, 1H, -$CH(CH_3)CH_2CH_3$), 0.85-0.90 (m, 3 H, -$CH(CH_3)CH_2CH_3$), 0.73-0.79 (m, 3 H, -$CH(CH_3)CH_2CH_3$) ppm. Anal. Calcd for $C_{35}H_{35}NO_4$: C, 78.77; H, 6.61; N, 2.62; O, 11.99. Found: C, 78.57; H, 6.62; N, 2.61.

***N*-Fmoc-*N*-methyl-L-alanine benzhydryl ester (11f):** Yield 88%. Amorphous white solid. ^{1}H-NMR (300 MHz, $CDCl_3$), 60:40 mixture of two rotamers A and

B δ 7.96 (d, J= 9.3 Hz, 2 H), 7.64-7.71 (m, 2 H), 7.12-7.35 (m, 14 H, $RCO_2CH(C_6H_5)_2$ and Ar_{Fmoc}), 6.90 (s, 1 H, A, RCO_2CHPh_2), 6.88 (s, 1 H, B, RCO_2CHPh_2), 4.95 (m, 1 H, A, α-C*H*), 4.85 (m, 1 H, B, □-C*H*), 4.35-4.48 (m, 3 H, Fmoc-C*H* and Fmoc-CH_2), 2.88 (s, 3 H, B, -NCH_3), 2.82 (s, 3 H, A, -NCH_3), 1.38-1.41 (m, 3 H, -CH_3) ppm. Anal. Calcd for $C_{33}H_{31}NO_3$: C, 80.95; H, 6.38; N, 2.86; O, 9.80. Found: C, 81.13; H, 6.37; N, 2.84.

Synthesis of *N*-methyl-*N*-Fmoc-α-amino acid 12a-f. General Procedure.

To a solution of the appropriate *N*-methyl-*N*-Fmoc-α-amino acid benzhydryl esters **11a-f** in dichloromethane (2 mL) trifluoroacetic acid (5-7 mL) and toluene (2 mmol) were added. The resulting mixture was stirred at room temperature for 1 h monitoring the conversion of **4a-f** by TLC (EtOAc:hexane = 1:5). After evaporation of the solvent under reduced pressure, saturated aqueous Na_2CO_3 was added and the aqueous solution was extracted with dichloromethane (3 × 20 mL). Aqueous 2 N HCl was then added and the acidified solution was extracted with EtOAc (3 × 20 mL). The combined organic extracts were dried over Na_2SO_4 and evaporated under vacuum to afford the *N*-methyl-*N*-Fmoc-α-amino acid **12a-f** in 94-98 % overall yields.

***N*-Fmoc-*N*-methyl-L-valine (12a):** Yield 96%. White solid. Mp 187-189 °C. ^{1}H-NMR (300 MHz, DMSO-d_6) 55:45 mixture of two rotamers A and B δ 7.28-7.89 (m, 8 H), 4.25-4.45 (m, 3 H), 4.00 (d, J= 10.8 Hz, 1 H, A), 3.86 (d, J= 10.8 Hz, 1 H, B), 2.72 (s, 3 H, A), 2.70 (s, 3 H, B), 2.15 (m, 1 H, A), 1.99 (m, 1 H, B), 0.91 (d, J= 6.6 Hz, 3 H, A), 0.80 (d, J= 6.6 Hz, 3 H, B), 0.72 (d, J= 6.6 Hz, 3 H, A), 0.55 (d, J= 6.6 Hz, 3 H, B) ppm. ^{13}C-NMR (75 MHz, DMSO-d_6) mixture of the two rotamers A and B δ 173.7, 173.6, 153.2, 153.1, 144.3, 144.24, 134.1, 128.1, 128.0, 127.5, 127.4, 125.4, 120.6, 67.1, 64.3, 64.3, 47.2, 47.1, 30.6, 27.4, 27.3, 20.2, 20.1, 19.3, 19.1 ppm. Anal. Calcd for $C_{21}H_{23}NO_4$: C, 71.37; H, 6.56; N, 3.96; O, 18.11. Found: C, 71.40, H, 6.58, N, 3.98.

***N*-Fmoc-*N*-methyl-D-valine (12b):** Yield 96%. Amorphous white solid. ^{1}H-

NMR (300 MHz, DMSO-d_6) 55:45 mixture of two rotamers A and B δ 7.28-7.89 (m, 8 H), 4.25-4.45 (m, 3 H), 4.00 (d, J= 10.8 Hz, 1 H, A), 3.86 (d, J= 10.8 Hz, 1 H, B), 2.72 (s, 3 H, A), 2.70 (s, 3 H, B), 2.15 (m, 1 H, A), 1.99 (m, 1 H, B), 0.91 (d, J= 6.6 Hz, 3 H, A), 0.80 (2, J= 6.6 Hz, 3 H, B), 0.72 (d, J= 6.6 Hz, 3 H, A), 0.55 (d, J= 6.6 Hz, 3 H, B) ppm. ^{13}C-NMR (75 MHz, DMSO-d_6) mixture of the two rotamers A and B δ 173.7, 173.6, 153.2, 153.1, 144.3, 144.2, 134.1, 128.1, 128.0, 127.5, 127.4, 125.4, 120.6, 67.1, 64.3, 64.1, 47.2, 47.1, 30.6, 27.4, 27.3, 20.2, 20.1, 19.3, 19.1 ppm. Anal. Calcd for $C_{21}H_{23}NO_4$: C, 71.37; H, 6.56; N, 3.96; O, 18.11. Found: C, 71.12; H, 6.57; N, 3.97.

***N*-Fmoc-*N*-methyl-L-leucine (12c):** Yield 98%. White solid. Mp 113-116 °C. ^{1}H-NMR (300 MHz, DMSO-d_6) 67:33 mixture of two rotamers A and B δ 8.40 (s br, 1 H), 7.23-7.80 (m, 8 H), 4.40-4.65 (m, 3 H), 0.83-1.00 (m, 6 H), 4.22-4.33 (m, 1 H), 2.90 (s, 3 H, A), 2.85 (s, 3 H, B), 1.15-1.65 (m, 3 H) ppm. ^{13}C-NMR (75 MHz, DMSO-d_6) mixture of the two rotamers A and B δ 173.5, 173.4, 156.5, 156.2, 144.2, 144.1, 139.6, 128.2, 128.1, 127.6, 127.5, 125.3, 125.2, 120.6, 67.2, 56.6, 56.4, 47.2, 37.5, 37.2, 30.6, 30.4, 24.9, 23.6, 23.4, 21.4, 21.3 ppm. Anal. Calcd for $C_{22}H_{25}NO_4$: C, 71.91; H, 6.86; N, 3.81; O, 17.42. Found: C, 71.72; H, 6.87; N, 3.79.

***N*-Fmoc-*N*-methyl-S-benzyl-L-cysteine (12d):** Yield 94%. Yellow solid. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 7.31-7.75 (m, 8 H), 7.10-7.25 (m, 5 H), 4.15-4.70 (m, 4 H), 3.54-3.90 (m, 2 H), 2.90-3.10 (m, 4 H), 2.70 (m, 1 H) ppm. Anal. Calcd for $C_{26}H_{25}NO_4S$: C, 69.78; H, 5.63; N, 3.13; O, 14.30; S, 7.16. Found: C, 69.59; H, 5.64; N, 3.11.

***N*-Fmoc-*N*-methyl-L-isoleucine (12e):** Yield 98%. White solid. Mp 181-183 °C. ^{1}H-NMR (300 MHz, DMSO-d_6) 67:33 mixture of two rotamers A and B δ 7.25-7.90 (m, 8 H), 4.3-4.5 (m, 3 H), 4.25 (d, J= 9.9 Hz, 1 H, A), 3.95 (2, J = 9.9 Hz, 1 H, B), 2.70 (s, 3 H), 1.70-1.90 (m, 1 H), 1.05-1.25 (m, 2 H), 0.70-0.90 (m, 6 H) ppm. ^{13}C-NMR (75 MHz, DMSO- d_6) mixture of the two rotamers A and B δ 172.5, 156.4, 144.3, 144.1, 141.2, 128.1, 127.5, 125.4, 120.5, 67.1, 62.9, 62.6,

47.2, 47.1, 33.1, 30.4, 25.0, 24.7, 11.1, 10.9 ppm. Anal. Calcd for $C_{22}H_{25}NO_4$: C, 71.91; H, 6.86; N, 3.81; O, 17.42. Found: C, 71.66; H, 6.88; N, 3.80.

***N*-Fmoc-*N*-methyl-L-alanine (12f):** Yield 96%. White solid. Mp 140-142 °C. ^{1}H-NMR (300 MHz, DMSO-d_6) 61:39 mixture of two rotamers A and B δ 12.85 (s br, 1 H), 7.25-7.90 (m, 8 H), 4.64 (m, 1 H, A), 4.52 (m, 1 H, B), 4.18-4.45 (m, 3 H), 2.82 (s, 3 H, A), 2.79 (s, 3 H, B), 1.28 (d, J= 7.2 Hz, 3 H, A), 1.22 (d, J= 7.2 Hz, 3 H, B) ppm. ^{13}C-NMR (75 MHz, DMSO-d_6) mixture of the two rotamers A and B δ 170.8, 170.7, 162.3, 161.8, 144.3, 144.2, 141.2, 128.1, 128.0, 127.6, 127.5, 125.5, 120.6, 67.2, 54.6, 54.5, 47.1, 47.0, 30.7, 30.6, 15.0, 15.1 ppm. Anal. Calcd for $C_{19}H_{19}NO_4$: C, 70.14; H, 5.89; N, 4.31; O, 19.67. Found: C, 70.39; H, 5.86; N, 4.30.

3. Methylation of α-amino acids and derivatives using trimethylsilyldiazomethane

Treatment of *N*-nosyl-L-alanine (1f) with 1 equiv. of diazomethane.

A 0.66 M ethereal solution of diazomethane (1.94 ml, 1 mmol) was added cautiously to a stirred solution of *N*-nosyl-L-alanine (**1f**) (350 mg, 1 mmol) in dry dichloromethane (10 ml). The resulting mixture was stirred at room temperature for about 1 h, monitoring the reaction by TLC (Ethyl ether/Petroleum ether 70:30 v/v). After evaporation of the solvent under reduced pressure, the residue was dissolved in saturated aqueous Na_2CO_3 and the resulting solution was extracted with ethyl acetate (3 × 20 mL). The combined organic extracts were dried over Na_2SO_4 and evaporated under vacuum. The residue was purified by column chromatography (Ethyl ether/Petroleum ether 70:30 v/v) to afford 218 mg of the *N*-Nosyl-L-alanine methyl ester (60% yield) and 38 mg of the *N*-methyl-*N*-Nosyl-L-alanine methyl ester (10% yield).

Synthesis of *N*-Nosyl-α-amino acid methyl esters 14a-g. General Procedure.

A 2.0 M ethereal solution of trimethylsilyldiazomethane (**13**) (5 mmol) was added cautiously to a stirred solution of the appropriate *N*-Nosyl-α-amino acid **1a-g** (1 mmol) in dry dichloromethane (10 ml) and dry methanol (7 mmol). The resulting mixture was stirred at room temperature for about 2-5 min., until TLC analysis (chloroform/methanol 90:10 v/v) of the reaction mixture showed complete conversion of the precursor into the corresponding methyl ester. Evaporation of the solvent under reduced pressure, afforded the respective *N*-Nosyl-α-amino acid methyl ester **14a-g**, in quantitative yield.

***N*-Nosyl-L-valine methyl ester (14a):** yellow solid, p.f. 97-100 °C. ^{1}H-NMR: (300 MHz, $CDCl_3$), δ (ppm): 0.88 (d, J= 6.8 Hz, 3H), 0.96 (d, J= 6.8 Hz, 3H),

2.11 (m,1H), 3.52 (s, 3H), 3.85 (dd, J_1= 4.9 Hz, J_2= 9.8 Hz, 1H), 5.56 (d, J= 9.8 Hz, 1H), 8.02-8.09 (m, 2H), 8.32-8.39 (m, 2H). GC/MS (E.I.), *m/z*: 257 (100%), 122 (48), 88 (35), 186 (30). Anal. Calcd. for $C_{12}H_{16}N_2O_6S$; C, 45.56; H, 5.10; N, 8.86; O, 30.35; S, 10.14. Found: C, 45.70; H, 5.11; N, 8.81; O, 30.42; S, 10.12.

***N*-Nosyl-L-leucine methyl ester (14c):** yellow solid, p.f. 97-100 °C. ^{1}H-NMR: (300 MHz, $CDCl_3$), δ (ppm): 0.88 (d, J= 6.8 Hz, 3H), 0.96 (d, J= 6.8 Hz, 3H), 2.11 (m, 1H), 3.52 (s, 3H), 3.85 (dd, J_1= 4.9 Hz, J_2= 9.8 Hz, 1H), 5.56 (d, J= 9.8 Hz, 1H), 8.02-8.09 (m, 2H), 8.32-8.39 (m, 2H). GC/MS (E.I.), *m/z*: 271 (100%), 186 (58), 122 (52), 215 (40), 229 (26). Anal. Calcd. for $C_{13}H_{18}N_2O_6S$: C, 47.26; H, 5.49; N, 8.48; O, 29.06; S, 9.71. Found: C, 47.42; H, 5.48; N, 8.50; O, 28.95; S, 9.73.

***N*-Nosyl-L-isoleucine methyl ester (14e):** yellow solid, p.f. 94-96 °C. ^{1}H-NMR: (300 MHz, $CDCl_3$), δ (ppm): 0.83-0.95 (m, 6H), 1.15 (m, 1H), 1.37 (m, 1H), 1.83 (m, 1H), 3.50 (s, 3H), 3.89 (m,1H), 5.62 (d, J= 9.8 Hz, 1H), 8.03-8.08 (m, 2H), 8.33-8.39 (m, 2H). GC/MS (E.I.), *m/z*: 88 (100%), 271 (90), 215 (55), 186 (42), 122 (40). Anal. Calcd. for $C_{13}H_{18}N_2O_6S$: C, 47.26; H, 5.49; N, 8.48; O, 29.06; S, 9.71. Found: C, 47.18; H, 5.47; N, 8.49; O, 29.20; S, 9.68.

***N*-Nosyl-L-alanine methyl ester (14f):** yellow solid, p.f. 111-113 °C. ^{1}H-NMR: (300 MHz, $CDCl_3$), δ (ppm): 1.23 (d, J= 7.1 Hz, 3H), 3.47 (s, 3H), 3.97-4.03 (m, 1H), 5.60 (d, J= 9.8 Hz, 1H), 8.01-8.08 (m, 2H), 8.40-8.46 (m, 2H). GC/MS (E.I.), *m/z*: 229 (100%), 122 (54), 186 (36). Anal. Calcd. for $C_{10}H_{12}N_2O_6S$: C, 41.66; H, 4.20; N, 9.72; O, 33.30; S, 11.12. Found: C, 41.56; H, 4.21; N, 9.69; O, 33.36; S, 11.09.

***N*-Nosyl-L-phenylalanine methyl ester (14g):** yellow solid, p.f. 151-155 °C. ^{1}H-NMR: (300 MHz, $CDCl_3$), δ (ppm): 2.77 (dd, J_1= 9.6 Hz, J_2= 13.5 Hz, 2H), 2.99 (dd, J_1 = 5.2 Hz, J_2= 13.5 Hz, 2H), 3.45 (s, 3H), 4.09 (m,1H), 5.58 (d, J = 9.8 Hz, 1H), 7.09-7.19 (m, 5H), 7.75-7.81 (m, 2H), 8.21-8.28 (m, 2H). GC/MS (E.I.), *m/z*: 91 (100%), 162 (90), 186 (58), 273 (50), 122 (42), 305 (25). Anal. Calcd. for

$C_{16}H_{16}N_2O_6S$: C, 52.74; H, 4.43; N, 7.69; O, 26.35; S, 8.80. Found: C, 52.56; H, 4.44; N, 7.68; O, 26.41; S, 8.81.

Synthesis of *N*-methyl-*N*-Nosyl-α-amino acid methyl esters 15a-g. General Procedure.

A 2.0 M ethereal solution of trimethylsilyldiazomethane (**13**) (9 mmol) was added cautiously to a stirred solution of the appropriate *N*-Nosyl-α-amino acid **1a-g** (1 mmol) in dry dichloromethane (10 ml) and dry methanol (13 mmol). The resulting mixture was stirred at room temperature for about 35-120 min., and disappearance of starting material was monitored by TLC analysis (Ethyl ether/Petroleum ether 70:30 v/v). After complete conversion of the precursor, evaporation of the solvent under reduced pressure, afforded the respective *N*-methyl-*N*-Nosyl-α-amino acid methyl ester **15a-g**, in quantitative yield.

***N*-methyl-*N*-Nosyl-L-valine methyl ester (15a):** yellow solid, p.f. 53-56°C. ^{1}H-NMR: (300 MHz, $CDCl_3$), δ (ppm): 0.96 (d, J= 6.8 Hz, 3H), 1.02 (d, J= 6.8 Hz, 3H), 2.13 (m, 1H), 2.92 (s, 3H) 3.46 (s, 3H), 4.18 (d, J= 10.7 Hz, 1H), 7.92-8.05 (m, 2H), 8.29-8.42 (m, 2H). GC/MS (E.I.), *m/z*: 271 (100%), 84 (60), 122 (40), 287 (36), 186 (30). Anal. Calcd. for $C_{13}H_{18}N_2O_6S$: C, 47.26; H, 5.49; N, 8.48; O, 29.06; S, 9.71. Found: C, 47.13; H, 5.50; N, 8.50; O, 29.28; S, 9.70.

***N*-methyl-*N*-Nosyl-L-leucine methyl ester (15c):** yellow solid, p.f. 89-91 °C. ^{1}H-NMR: (300 MHz, $CDCl_3$), δ (ppm): 0.92-1.01 (m, 6H), 1.61-1.72 (m, 3H), 2.85 (s, 3H) 3.47 (s, 3H), 4.71 (m, 1H), 7.90-7.99 (m, 2H), 8.27-8.39 (m, 2H). GC/MS (E.I.), *m/z*: 285 (100%), 186 (25), 122 (24), 229 (18). Anal. Calcd. for $C_{14}H_{20}N_2O_6S$: C, 48.83; H, 5.85; N, 8.13; O, 27.87; S, 9.31. Found: C, 48.95; H, 5.84; N, 8.11; O, 27.95; S, 9.30.

***N*-methyl-*N*-Nosyl-L-isoleucine methyl ester (15e**): yellow solid, p.f. 95-92 °C. ^{1}H-NMR: (300 MHz, $CDCl_3$), δ (ppm): 0.87-0.98 (m, 6H), 1.10-1.25 (m, 1H), 1.54-1.65 (m, 1H), 1.86-1.99 (m, 1H), 2.92 (s, 3H), 3.43 (s, 3H), 4.26 (d, J= 10.7

Hz, 1H), 7.95-8.03 (m, 2H), 8.31-8.38 (m, 2H). GC/MS (E.I.), *m/z*: 271 (100%), 186 (62), 122 (46), 229 (38). Anal. Calcd. for $C_{14}H_{20}N_2O_6S$: C, 48.83; H, 5.85; N, 8.13; O, 27.87; S, 9.31. Found: C, 48.77; H, 5.84; N, 8.14; O, 27.79; S, 9.32.

***N*-methyl-*N*-Nosyl-L-alanine methyl ester (15f):** yellow solid, p.f. 74-76°C. ^{1}H-NMR: (300 MHz, $CDCl_3$), δ (ppm): 1.35 (d, J= 6.8 Hz, 3H), 2.81 (s, 3H), 3.47 (s, 3H), 4,76 (q, J= 7.8 Hz, 1H), 7.88-7.98 (m, 2H), 8.24-8.38 (m, 2H). GC/MS (E.I.), *m/z*: 243 (100%), 122 (25), 186 (20). Anal. Calcd. for $C_{11}H_{14}N_2O_6S$: C, 43.70; H, 4.67; N, 9.27; O, 31.75; S, 10.61. Found: C, 43.65; H, 4.68; N, 9.26; O, 31.87; S, 10.64.

Synthesis of *N*-methyl-*N*-Nosyl-α-amino acid methyl esters 15a-g by methylation of 1a-g. General Procedure.

A 2.0 M solution of trimethylsilyldiazomethane in diethyl ether (**13**) (14 mmol) was added cautiously to a stirred solution of the appropriate *N*-Nosyl-α-amino acid **1a-g** (1 mmol) in dry dichloromethane (10 ml) and dry methanol (17 mmol). The resulting mixture was stirred at room temperature for 1-2 h, until TLC analysis (chloroform/methanol 90:10 v/v) of the reaction mixture showed complete conversion of the precursor. Evaporation of the solvent under vacuum afforded the respective *N*-methyl-*N*-Nosyl-α-amino acid methyl ester **15a-g** in quantitative yields.

Synthesis of *N*-methyl-*N*-Nosyl-α-amino acid benzhydryl esters 4a-f. General Procedure.

A 2.0 M solution of trimethylsilyldiazomethane in diethyl ether (**13**) (12 mmol) was added cautiously to a stirred solution of the appropriate *N*-Nosyl-α-amino acid benzhydryl ester **3a-f** (1 mmol) in dry dichloromethane (10 ml) and dry methanol (17 mmol). The resulting mixture was stirred at room temperature for about 3-3.5 h, until TLC analysis (ethyl acetate/hexane 1:5 v/v) of the reaction mixture showed complete conversion of the precursor. Evaporation of the solvent

under vacuum afforded the respective *N*-methyl-*N*-Nosyl-α-amino acid benzhydryl ester 4**a-f** in quantitative yield.

***N*-methyl-*N*-Nosyl-L-valine benzhydryl ester (4a):** yellow solid, m.p.: 94-96 °C. ^{1}H-NMR (300 MHz, $CDCl_3$), δ (ppm): 0.85 (d, 3H, J= 6.3 Hz), 1.03 (d, 3H, J= 6.3Hz), 2.21 (m, 1H), 2.94 (s, 3H), 4.39 (d, 1H, J=10.5 Hz), 6.67 (s, 1H), 7.14-7.35 (m, 10H), 7.75 (d, 2H, J= 8.4 Hz), 7.92 (d, 2H, J=8.4Hz). ^{13}C-NMR (75 MHz, $CDCl_3$), δ (ppm): 168.9, 149.8, 145.3, 138.9, 128.8, 128.5, 128.4, 128.3, 128.2, 127.0, 126.9, 124.3, 77.9, 65.2, 30.3, 28.2, 19.3. GC/MS (E.I.), *m/z*: 271 (100%), 167 (62), 186 (20), 122 (35). Anal. Calcd. for $C_{25}H_{26}N_2O_6S$: C, 62.23; H, 5.43; N, 5.81; O, 19.89; S, 6.64. Found: C, 62.41; H, 5.44; N, 5.79; O, 19.87; S, 6.65.

***N*-methyl-*N*-Nosyl-L-leucine benzhydryl ester (4c):** yellow solid, m.p.: 93-95 °C.^{1}H-NMR (300 MHz, $CDCl_3$), δ (ppm): 0.99 (d, 3H, J=6Hz), 1.03 (d, 3H, J=6Hz), 1.69-1.78 (m, 3H), 2.90 (s, 3H), 4.84 (m, 1H), 6.60 (s, 1H), 7.18-7.38 (m, 10H), 7.75 (d, 2H, J= 9 Hz), 7.95 (d, 2H, J= 9Hz). ^{13}C-NMR (75 MHz, $CDCl_3$), δ (ppm): 21.0, 23.0, 24.5, 30.0, 38.2, 57.7, 78.0, 124.0, 126.7, 128.5, 128.6, 130.0, 139.1, 144.3, 169.8. GC/MS (E.I.), *m/z*: 285 (100 %), 167 (42), 186 (22), 122 (15). $C_{26}H_{28}N_2O_6S$: C, 62.89; H, 5.68; N, 5.64; O, 19.33; S, 6.46. Found: C, 63.01; H, 5.69; N, 5.63; O, 19.27; S, 6.44.

***N*-methyl-*N*-Nosyl-L-isoleucine benzhydryl ester (4e):** yellow solid, m.p.: 100-102°C. ^{1}H-NMR: (300 MHz, $CDCl_3$), δ (ppm): 0.82 (d, 3H, J= 6.9 Hz), 0.95 (t, 3H, J=7.2Hz), 1.26 (m, 1H), 1.62 (m, 1H), 1.97 (m, 1H), 2.94 (s, 3H), 4.47 (d, 1H, J= 12.3 Hz), 6.60 (s, 1H), 7.20-7.42 (m, 10H), 7.75 (d, 2H, J=9 Hz), 7.88 (d, 2H, J=9 Hz). ^{13}C-NMR (75 MHz, $CDCl_3$), δ (ppm): 10.4, 15.3, 25.3, 30.5, 34.2, 63.8, 77.8, 124.0, 127.0, 127.5, 128.2, 128.3, 144.3, 149.7, 169.0. GC/MS (E.I.), *m/z*: 285 (100 %), 167 (72), 229 (32), 186 (21). Anal. Calcd. for $C_{26}H_{28}N_2O_6S$: C, 62.89; H, 5.68; N, 5.64; O, 19.33; S, 6.46. Found: C, 62.96; H, 5.64; N, 5.67; O, 19.28; S, 6.47.

***N*-methyl-*N*-Nosyl-L-alanine benzhydryl ester (4f):** yellow solid, m.p.: 102-

105°C. ^{1}H-NMR: (300 MHz, $CDCl_3$), δ (ppm): 1.52 (d, J= 7.2 Hz, 3H), 2.85 (s, 3H), 4.93 (m, 1H), 6.68 (s, 1H), 7.10-7.42 (m, 10H), 7.80 (d, 2H, J= 9.3 Hz), 8.02 (d, 2H, J= 9 Hz). GC/MS (E.I.), *m/z*: 243 (100%), 167 (64), 186 (26), 122 (20). Anal. Calcd. for $C_{23}H_{22}N_2O_6S$: C, 60.78; H, 4.88; N, 6.16; O, 21.12; S, 7.06. Found: C, 60.95; H, 4.87; N, 6.17; O, 21.18; S, 7.04.

Synthesis of *N*-methyl-*N*-Nosyl-α-amino acids 5a-f. General Procedure.

A solution of the appropriate *N*-methyl-*N*-Nosyl-α-amino acid benzhydryl ester **4a-f** (1 mmol) in dry dichloromethane (10 ml) was treated with trifluoroacetic acid (5-7 mL mmol^{-1}) containing toluene (2 mmol). The resulting mixture was stirred at room temperature for about 1 h monitoring the conversion of the precursor by TLC (ethyl acetate/hexane 1:5 v/v). After evaporation of the solvent under reduced pressure, the residue was dissolved in saturated aqueous Na_2CO_3 and the resulting solution was extracted with dichloromethane (3 × 20 mL). Aqueous 2 N HCl was then added and the acidified solution was extracted with ethyl acetate (3 × 20 mL). The combined organic extracts were dried over Na_2SO_4 and evaporated under vacuum to afford the respective *N*-methyl-*N*-Nosyl-α-amino acid **5a-f** in 94-98 % yields.

***N*-methyl-*N*-Nosyl-L-valine (5a):** yellow solid (94%). ^{1}H-NMR (300 MHz, DMSO-d_6), δ (ppm): 0.96 (d, 3H, J= 6.8 Hz), 1.02 (d, 3H, J= 6.8 Hz), 2.13 (m, 1H, 2.92 (s, 3H), 4.18 (d, 1H, J= 10.7 Hz), 7.92-8.05 (m, 2H), 8.29-8.42 (m, 2H). Anal. Calcd. for $C_{12}H_{16}N_2O_6S$: C, 45.56; H, 5.10; N, 8.86; O, 30.35; S, 10.14. Found: C, 45.46; H, 5.11; N, 8.85; O, 30.41; S, 10.15.

***N*-methyl-*N*-Nosyl-L-leucine (5c):** yellow solid (94%). ^{1}H-NMR (300 MHz, DMSO-d_6), δ (ppm): 0.92-1.01 (m, 6H), 1.61-1.72 (m, 3H), 2.95 (s, 3H), 4.71 (m, 1H), 8.00 (d, 2H, J=9.2 Hz), 8.35 (d, 2H, J=9.2 Hz). Anal. Calcd. for $C_{13}H_{18}N_2O_6S$: C, 47.26; H, 5.49; N, 8.48; O, 29.06; S, 9.71. Found: C, 47.34; H, 5.50; N, 8.46; O, 28.96; S, 9.70.

***N*-methyl-*N*-Nosyl-L-isoleucine (5e):** yellow solid (98%). ^{1}H-NMR (300 MHz, DMSO-d_6), δ (ppm): 0.87 (m, 6H), 1.54 (m, 1H), 1.65 (m, 1H), 1.88 (m, 1H), 2.92 (s, 3H), 4.26 (d, 1H, J= 10.4 Hz), 8.00 (d, 2H, J=9.3 Hz), 8.30 (d, 2H, J=9.3 Hz). ^{13}C-NMR (75 MHz, DMSO-d_6), δ (ppm): 11.4, 15.6, 28.2, 30.8, 33.3, 68.8, 127.6, 129.1, 144.2, 151.3, 171.1. Anal. Calcd. for $C_{13}H_{18}N_2O_6S$: C, 47.26; H, 5.49; N, 8.48; O, 29.06; S, 9.71. Found: C, 47.32; H, 5.50; N, 8.47; O, 29.13; S, 9.69.

***N*-methyl-*N*-Nosyl-L-alanine (5f):** yellow solid (98%). ^{1}H-NMR (300 MHz, DMSO-d_6), δ (ppm): 1.22 (d, 3H, J= 6.8 Hz), 2.79 (s, 3H), 4,60 (q, 1H, J=7.8 Hz), 7.88-7.98 (d, 2H, J=9.3 Hz), 8.24-8.38 (d, 2H, J=9.3 Hz). Anal. Calcd. for $C_{10}H_{12}N_2O_6S$: C, 41.66; H, 4.20; N, 9.72; O, 33.30; S, 11.12. Found: C, 41.52; H, 4.20; N, 9.73; O, 33.38; S, 11.10.

4. *N*-Methyl-*N*-Nosyl-β^3-amino acids

Synthesis of *N*-Nosyl-α-amino acid chlorides 16a-i. General procedure.

Thionyl chloride (12 mmol) was added to a solution of the appropriate *N*-Nosyl-α-amino acid **1a-i** (1 mmol) dissolved in dry ethanol free methylene chloride (15 mL). The resulting mixture was stirred under reflux for 20-30 min, until TLC analysis (chloroform/methanol 90:10 v/v) showed complete conversion of the precursor. Evaporation of the solvent under vacuum afforded the respective amino acid chlorides **16a-i** in quantitative yield, each one as a yellowish amorphous solid (Complete caracterization was effected by GC/MS and NMR spettroscopy, after conversion of substrates in the corresponding methyl esters, by treatment with dry methanol.)

Synthesis of *N*-methyl-*N*-Nosyl-α-aminoacyldiazomethanes 17a-i and 20. General procedure.

A solution of the appropriate *N*-Nosyl-α-aminoacyl chloride **16a-i** (1 mmol) in dry methylene chloride was added dropwise to a stirred 0.66 M methylene chloride solution of diazomethane (10 mmol) at 0 °C. The mixture was maintained under stirring for about 50-60 min, until TLC analysis (chloroform/diethyl ether 90:10 v/v) of the reaction mixture showed complete conversion of the precursor into the corresponding *N*-methyl diazoketone. The organic solvent was removed under vacuum and the oily residue was purified by column chromatography to afford the respective *N*-methyl-*N*-Nosyl-α-aminoacyldiazomethane **17a-i** in 69%-89% yields.

***N*-methyl-*N*-Nosyl-L-valyldiazomethane (17a):** Pale yellow solid. 73% yield after flash column chromatography (petroleum ether/ethyl acetate 70:30 v/v). Mp: 88.6-90.3 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.41-8.34 (m, 2H), 8.04-7.97 (m, 2H), 5.57 (s, 1H), 3.92 (d, J= 10.86 Hz, 1H), 2.90 (s, 3H), 2.29-2.15 (m, 1H), 0.93 (d, J= 6.50 Hz, 3H), 0.66 (d, J= 6.50 Hz, 3H), ^{13}C-NMR (75

MHz, $CDCl_3$) δ: 190.2, 149.8, 145.0, 128.1, 124.1, 67.3, 55.9, 29.9, 26.3, 19.2. Anal. Calcd for $C_{13}H_{16}N_4O_5S$: C, 45.88; H, 4.74; N, 16.46. Found: C, 46.03; H, 4.73; N, 16.40.

***N*-methyl-*N*-Nosyl-L-leucyldiazomethane (17c):** Yellow oil. 77% yield after flash column chromatography (chloroform/ethyl acetate 98:2 v/v). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.44-8.32 (m, 2H), 8.08-7.95 (m, 2H), 5.60 (s, 1H), 4.59-4.48 (m, 1H), 2.85 (s, 3H), 1.73-1.62 (m, 1H), 1.50-1.35 (m, 1H), 1.30-1.19 (m, 1H), 0.89 (d, J= 6.6 Hz, 3H), 0.87 (d, *J*= 6.6 Hz, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 191.2, 150.0, 144.9, 128.4, 124.3, 60.9, 54.8, 36.6, 30.2, 24.7, 22.8, 21.9. Anal. Calcd for $C_{14}H_{18}N_4O_5S$: C, 47.45; H, 5.12; N, 15.81. Found: C, 47.50; H, 5.10; N, 15.78.

***N*-methyl-*N*-Nosyl-L-isoleucyldiazomethane (17e):** Yellow oil. 81% yield after flash column chromatography (chloroform/ethyl acetate 95:5 v/v). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.40-8.34 (m, 2H), 8.02-7.95 (m, 2H), 5.50 (s, 1H), 4.03 (d, J= 10.80 Hz, 1H), 2.88 (s, 3H), 2.05-1.92 (m, 1H),1.67-1.53 (m, 1H), 1.26-1.13 (m, 1H), 0.90 (d, J= 6.3 Hz, 3H), 0.86-0.79 (m, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 194.9, 159.5, 152.9, 128.4, 124.3, 66.1, 56.1, 32.4, 30.2, 25.1, 15.5, 10.6. Anal. Calcd for $C_{14}H_{18}N_4O_5S$: C, 47.45; H, 5.12; N, 15.81. Found: C, 47.60; H, 5.14; N, 15.75.

***N*-methyl-*N*-Nosyl-L-alanyldiazomethane (17f):** Pale yellow solid. 78% yield after flash column chromatography (chloroform/diethyl ether 90:10 v/v). Mp: 81.8-83.3 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.44-8.38 (m, 2H), 8.05-7.99 (m, 2H), 5.79 (s, 1H), 4.61 (q, J= 7.35, 1H), 2.81 (s, 3H), 1.09 (d, J= 7.35, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ 191.7, 149.4, 144.6, 128.4, 124.6, 58.9, 54.4, 30.2, 12.3. Anal. Calcd for $C_{11}H_{12}N_4O_5S$: C, 42.30; H, 3.87; N, 17.94. Found: C, 42.17; H, 3.86; N, 18.00.

***N*-methyl-*N*-nosyl-L-phenylalanyldiazomethane (17g):** Yellow oil. 77% yield after flash column chromatography (chloroform/diethyl ether 95:5 v/v). ^{1}H-NMR

(300 MHz, $CDCl_3$) δ (ppm): 8.09 (d, J= 8.70 Hz, 2H), 7.44 (d, J= 8.70 Hz, 2H), 7.26-7.10 (m, 3H), 7.06-6.99 (m, 2H), 5.83 (s, 1H), 4.85 (dd, J= 10.20, 5.25 Hz, 1H), 3.31 (dd, J= 14.70, 5.40 Hz, 1H), 2.92 (s, 3H), 2.69 (dd, J= 14.70, 10.05 Hz, 1H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 190.1 149.0, 144.0, 137.0, 129.0, 128.9, 128.0, 127.0, 124.1, 65.1, 55.2, 33.3, 30.1. Anal. Calcd for $C_{17}H_{16}N_4O_5S$: C, 52.57; H, 4.15; N, 14.43. Found: C, 52.70; H, 4.13; N, 14.38.

***N*-methyl-*N*-Nosyl-D-alanyldiazomethane (17h):** Pale yellow amorphous solid. 81% yield after flash column chromatography (chloroform/diethyl ether 90:10 v/v). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.44-8.38 (m, 2H), 8.05-7.99 (m, 2H), 5.79 (s, 1H), 4.61 (q, J= 7.35, 1H), 2.81 (s, 3H), 1.09 (d, J= 7.35, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ 191.7, 149.4, 144.6, 128.4, 124.6, 58.9, 54.4, 30.2, 12.3. Anal. Calcd for $C_{11}H_{12}N_4O_5S$: C, 42.30; H, 3.87; N, 17.94. Found: C, 42.39; H, 3.86; N, 17.87.

***N*-methyl-*N*-Nosyl-D-*allo*-isoleucyldiazomethane (17i):** Yellow oil. 89% yield after flash column chromatography (chloroform/ethyl acetate 98:2 v/v). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.40-8.31 (m, 2H), 8.09-7.95 (m, 2H), 5.55 (s, 1H), 4.01 (d, *J* = 11.10 Hz, 1H), 2.87 (s, 3H), 2.10-1.94 (m, 1H), 1.50-1.36 (m, 1H), 1.13-0.95 (m, 1H), 0.94-0.85 (m, 3H) δ 0.62 (d, *J* = 6.60 Hz, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 194.9, 159.5, 152.9, 128.3, 124.3, 66.1, 56.1, 32.7, 30.1, 25.9, 15.4, 11.2. Anal. Calcd for $C_{14}H_{18}N_4O_5S$: C, 47.45; H, 5.12; N, 15.81. Found: C, 47.31; H, 5.13; N, 15.85.

***N*$^{\alpha}$-methyl-*N*$^{\alpha}$-Nosyl-*N*$^{\varepsilon}$-Fmoc-L-lysinyldiazomethane (21):** Pale yellow oil. 69% yield after flash column chromatography (chloroform/ethyl acetate 60:40 v/v). ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.38-8.33 (m, 2H), 8.01-7.96 (m, 2H), 7.81-7.25 (m, 8H), 5.60 (s, 1H), 4.86-4.78 (m, 1H), 4.51-4.38 (m, 3H), 4.29-4.18 (m, 1H), 3.18-3.06 (m, 2H), 2.82 (s, 3H, NCH_3), 1.55-1.13 (m, 6H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 177.0, 157.0, 150.3, 145.1, 143.9, 142.0, 128.4, 127.7, 127.1, 125.0, 124.4, 120.0, 66.5, 63.0, 54.2, 47.3, 40.3, 30.1, 29.3, 27.1, 23.3.

Anal. Calcd for $C_{29}H_{29}N_5O_7S$: C, 58.87; H, 4.94; N, 11.84. Found: C, 59.08; H, 4.92; N, 11.80.

Synthesis of *N*-methyl-*N*-Nosyl-β^3-homoamino acids 18a-i. General procedure.

A solution of silver benzoate (0.13 mol equiv) dissolved in freshly distilled triethylamine (the volume of triethylamine was adjusted to one-eighth with respect to that of 1,4-dioxane/water solution) was added dropwise to a solution (0.1 M) of the appropriate *N*-methyl-*N*-Nosyl-α-aminoacyldiazomethane **17a-i** in 1,4-dioxane/water (4:1 v/v). The resulting mixture was stirred at room temperature for 20-30 min, until TLC analysis (chloroform/methanol 90:10 v/v) of the reaction mixture showed complete conversion of the precursor into the corresponding *N*-methyl-β^3-homoamino acid. The reaction mixture was filtered and the solvent was removed under vacuum. The residue was dissolved in saturated aqueous sodium hydrogen carbonate (20 mL) and washed with diethyl ether (3×10 mL). The aqueous layer was acidified to pH 2 by adding 1 N aqueous hydrochloric acid (10 mL) and extracted with ethyl acetate (3×20 mL). The combined organic layers were washed once with brine (10 mL) and dried over Na_2SO_4. Evaporation of the solvent under vacuum afforded the respective *N*-methylated β^3-homoamino acid **18a-i** in 62%-83% yields, without need of chromatography. The *N*-methyl-*N*-Nosyl-β^3-homoamino acids **18a-i** were analysed by GC-MS after their conversion into the corresponding methyl esters by treatment with a methylene chloride solution of diazomethane.

***N*-methyl-*N*-Nosyl-β^3-homo-L-valine (18a):** Pale yellow solid. 60 % yield. Mp: 111.8-114.6 °C. ^{1}H-NMR (300 MHz, CD_3OD) δ (ppm): 8.30-8.21 (m, 2H) 7.98-7.90 (m, 2H), 4.05-3.92 (m, 1H), 2.68 (s, 3H), 2.37 (dd, J= 15.64, 5.84 Hz, 1H), 2.06 (dd, J= 15.64, 7.19 Hz, 1H), 1.79-1.65 (m, 1H), 0.86-0.82 (m, 6H) ^{13}C-NMR (75 MHz, CD_3OD): δ 174.8, 151.3, 146.8, 129.8, 125.3, 62.7, 36.9, 32.6, 32.0, 20.6, 19.8. Anal. Calcd for $C_{13}H_{18}N_2O_6S$: C, 47.26; H, 5.49; N, 8.48. Found: C, 47.12; H, 5.50; N, 8.50.

***N*-methyl-*N*-Nosyl-β^3-homo-L-valine methyl ester:** GC/MS (EI): 301 (100%), 285 (1), 271 (11), 259 (15), 186 (12), 158 (6), 122 (33).

***N*-methyl-*N*-Nosyl-β^3-homo-L-leucine (18c):** Pale yellow solid. 74% yield. Mp: 128.2-129.1°C. ^{1}H-NMR (300 MHz, CD_3OD) δ (ppm): 8.46 -8.25 (m, 2H), 8.11-8.01 (m, 2H), 4.54-4.38 (m, 1H), 2.74 (s, 3H), 2.32 (dd, J= 15.30, 7.65 Hz, 1H), 2.11 (dd, J= 15.30, 6.30 Hz, 1H), 1.54-1.40 (m, 1H), 1.29-1.10 (m, 2H), 0.95-0.75 (m, 6H). ^{13}C-NMR (CD_3OD, 75 MHz) δ: 174.3, 151.5, 146.8, 129.7, 125.4, 54.5, 42.6, 38.5, 28.7, 25.6, 23.3, 22.1. Anal. Calcd for $C_{14}H_{20}N_2O_6S$: C, 48.83; H, 5.85; N, 8.13. Found: C, 48.98; H, 5.86; N, 8.12.
***N*-methyl-*N*-Nosyl-β^3-homo-L-leucine methyl ester:** GC/MS (EI): 301 (100%), 285 (50), 259 (17), 243 (10), 229 (7), 207 (16), 186 (27), 172 (39), 156 (11), 122 (41).

***N*-methyl-*N*-Nosyl-β^3-homo-L-isoleucine (18e):** Pale yellow solid. 65% yield. Mp: 137.9-139.9 °C. ^{1}H-NMR (300 MHz, CD_3OD) δ (ppm): 8.41-8.35 (m, 2H), 8.08-8.03 (m, 2H), 4.24-4.16 (m, 1H), 2.78 (s, 3H), 2.46 (dd, J= 15.60, 6.00 Hz, 1H), 2.13 (dd, J= 15.90, 6.90 Hz, 1H), 1.70-1.50 (m, 2H), 1.20-1.01 (m, 1H), 0.97-0.83 (m, 6H).^{13}C-NMR (75 MHz, CD_3OD) δ: 174.9, 151.4, 146.7, 129.8, 125.3, 61.1, 39.1, 36.7, 29.8, 27.0, 15.7, 10.4. Anal. Calcd for $C_{14}H_{20}N_2O_6S$: C, 48.83; H, 5.85; N, 8.13. Found: C, 49.01; H, 5.88; N, 8.10.
***N*-methyl-*N*-Nosyl-β^3-homo-L-isoleucine:** GC/MS (EI): 301 (100%), 285 (8), 259 (13), 186 (8), 172 (2), 156 (8), 122 (17).

***N*-methyl-*N*-Nosyl-β^3-homo-L-alanine (18f):** Pale yellow solid. 83% yield. Mp: 102.7-106.1 °C. ^{1}H-NMR (300 MHz, CD_3OD) δ (ppm): 8.46-8.36 (m, 2H) 8.10-8.04 (m, 2H), 4.56-4.43 (m, 1H), 2.81 (s, 3H), 2.48-2.32 (m, 2H), 1.06 (d, J= 6.60 Hz, 3H). Anal. Calcd for $C_{11}H_{14}N_2O_6S$: C, 43.70; H, 4.67; N, 9.27. Found: C, 43.81; H, 4.65; N, 9.25.
***N*-methyl-*N*-nosyl-β^3-homo-L-alanine methyl ester:** GC/MS (EI): 301 (4%), 243 (84), 186 (30), 130 (100), 122 (55), 98 (17), 88 (3), 76 (21).

***N*-methyl-*N*-nosyl-β^3-homo-L-phenylalanine (18g):** Yellow solid. 70 % yield. Mp: 142.8-149.4 °C. ^{1}H-NMR (300 MHz, CD_3OD) δ (ppm): 8.23-8.16 (m, 2H). 7.78-7.69 (m, 2H), 7.28-7.00 (m, 5H), 4.68-4.56 (m, 1H), 2.90 (s, 3H), 2.89-2.61 (m, 2H), 2.60-2.44 (m, 2H). ^{13}C-NMR (75 MHz, CD_3OD) δ: 174.3, 151.0, 147.2, 139.1, 130.2, 129.6, 129.3, 127.7, 125.2, 58.4, 39.5, 38.5, 29.2. Anal. Calcd for $C_{17}H_{18}N_2O_6S$: C, 53.96; H, 4.79; N, 7.40. Found: C, 53.99; H, 4.77; N, 7.38.
***N*-methyl-*N*-Nosyl-β^3-homo-L-phenylalanine methyl ester:** GC/MS (EI): 319 (5%), 301(100), 259 (10) 186 (5), 156 (4), 122 (11), 91 (12).

***N*-methyl-*N*-Nosyl-β^3-homo-D-alanine (18h):** Pale yellow amorphous solid. 82% yield. ^{1}H-NMR (300 MHz, CD_3OD) δ (ppm): 8.46-8.36 (m, 2H) 8.10-8.04 (m, 2H), 4.56-4.43 (m, 1H), 2.81 (s, 3H), 2.48-2.32 (m, 2H), 1.06 (d, J= 6.60 Hz, 3H). Anal. Calcd for $C_{11}H_{14}N_2O_6S$: C, 43.70; H, 4.67; N, 9.27.. Found: C, 43.61; H, 4.66; N, 9.23.
***N*-methyl-*N*-Nosyl-β^3-homo-D-alanine methyl ester:** GC/MS (EI): 301 (4%), 243 (84), 186 (30), 130 (100), 122 (55), 98 (17), 88 (3), 76 (21).

***N*-methyl-*N*-Nosyl-β^3-homo-D-*allo*-isoleucine (18i):** Yellow solid. 68% yield. Mp: 128.15-129.05 °C. ^{1}H-NMR (300 MHz, CD_3OD) δ (ppm): 8.39-8.33 (m, 2H) 8.07-8.01 (m, 2H), 4.25-4.15 (m, 1H), 2.79 (s, 3H), 2.48 (dd, J= 15.90, 5.55 Hz, 1H), 2.15 (dd, J= 15.60, 7.35 Hz, 1H), 1.67-1.45 (m, 2H), 1.21-1.08 (m, 1H), 0.95-0.87 (m, 6H). ^{13}C-NMR (75 MHz, CD_3OD) δ: 173.5, 154.9, 145.5, 128.3, 123.9, 59.5, 37.5, 35.5, 28.5, 25.3, 15.0, 10.6. Anal. Calcd for $C_{14}H_{20}N_2O_6S$: C, 48.83; H, 5.85; N, 8.13. Found: C, 48.65; H, 5.83; N, 8.15.
***N*-methyl-*N*-Nosyl-β^3-homo-D-*allo*-isoleucine methyl ester:** GC/MS (EI): 301 (100%), 285 (8), 259 (13), 186 (8), 172 (2), 156 (8), 122 (17).

***N*$^\alpha$-methyl-*N*$^\alpha$-Nosyl-*N*$^\zeta$-Fmoc-β^3-homo-L-lysine (22):** Yellow oil. 62% yield. ^{1}H-NMR (300 MHz, DMSO-d_6) δ (ppm): 8.37 (d, J= 8.40 Hz, 2H) 8.01 (d, J= 8.40 Hz, 2H), 7.91-7.28 (m, 9H), 4.33-4.11 (m, 4H), 2.99-2.86 (m, H), 2.69 (s, 3H), 2.35 (dd, J= 15.00, 7.80 Hz, 1H), 2.08 (dd, J= 15.30, 6.30 Hz, 1H), 1.55-1.00 (m, 6H). Anal. Calcd for $C_{29}H_{31}N_3O_8S$: C, 59.88; H, 5.37; N, 7.22. Found:

C, 59.68; H, 5.34; N, 7.24.

Synthesis of *N*-methyl-*N*-Nosyl-β^3-homoamino acid chlorides 23a-b. General procedure.

Thionyl chloride (12 mmol) was added to a solution of the appropriate *N*-methyl-*N*-Nosyl-β^3-homoamino acid **18a-b** (1 mmol) dissolved in dry ethanol free methylene chloride (15 mL). The resulting mixture was stirred under reflux for 20-30 min, until TLC analysis (chloroform/methanol 90:10 v/v) showed complete conversion of the precursor. Evaporation of the solvent under vacuum afforded the respective *N*-methyl-*N*-Nosyl-β^3-homoamino acid chlorides **23a-b** in quantitative yield, each one as a yellowish amorphous solid.

Synthesis of *N*-methyl-*N*-Nosyl-dipeptides 24a-b. General procedure.

The appropriate *N*-methyl-*N*-Nosyl-β^3-homoamino acid chloride **23a-b** (1 mmol) was dissolved in ethanol-free methylene chloride (10 mL) and treated with a solution of L-alanine methyl ester hydrochloride (1 mmol) in 5% aqueous sodium hydrogen carbonate (10 mL). The mixture was stirred at room temperature for 25-30 min, until TLC analysis (chloroform/methanol 95:5 v/v) showed complete conversion of the precursor. The organic layer was separated and the aqueous phase was extracted with methylene chloride (3×10 mL). The combined organic extracts were washed once with 1 N aqueous hydrochloric acid (10 mL), once with brine (10 mL), and then dried over Na_2SO_4. The solvent was evaporated under vacuum to afford the corresponding *N*-Nosyl-dipeptides **24a-b** in 64%-75% yields.

***N*-methyl-*N*-Nosyl-β^3-homo-L-alanyl-L-alanine methyl ester (24a):** 75% yield. Pale yellow oil. ^{1}H-NMR (300 MHz, DMSO-d_6) δ (ppm): 8.42-8.37 (m, 2H) 8.30 (d, J= 7.20, 1H), 8.07-8.00 (m, 2H), 4.42-4.33 (m, 1H), 4.24-4.12 (m, 1H), 3.59 (s, 3H), 2.73 (s, 3H), 2.32 (dd, J= 14.20, 8.10 Hz, 1H), 2.14 (dd, J= 14.16, 6.60 Hz, 1H), 1.22 (d, J= 7.28, 3H), 0.94 (d, J= 6.73 Hz, 3H). ^{13}C-NMR

(75 MHz, DMSO-d_6) δ: 173.4, 168.9, 149.9, 145.4, 128.3, 124.1, 52.5, 51.0, 48.1, 41.4, 28.6, 18.2, 17.7. GC/MS (EI): 328 (3%), 243 (51), 201 (100), 186 (16), 122 (24). Anal. Calcd for $C_{15}H_{21}N_3O_7S$: C, 46.50; H, 5.46; N, 10.85. Found: C, 46.31; H, 5.44; N, 10.88.

***N*-methyl-*N*-Nosyl-β^3-homo-D-alanyl-L-alanine methyl ester (24b):** Pale yellow oil. 64% yield. ^{1}H-NMR (300 MHz, DMSO-d_6) δ (ppm): 8.44-8.37 (m, 2H), 8.30 (d, J= 6.94, 1H), 8.08-8.00 (m, 2H), 4.39-4.30 (m, 1H), 4.19-4.08 (m, 1H), 3.60 (s, 3H), 2.72 (s, 3H), 2.29 (dd, J= 13.94, 8.40 Hz, 1H), 2.12 (dd, J= 13.90, 6.10 Hz, 1H), 1.19 (d, J= 7.30, 3H), 0.96 (d, J= 6.74 Hz, 3H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 173.3, 169.1, 149.9, 145.4, 128.4, 124.0, 52.5, 51.0, 48.0, 41.2, 28.5, 18.0, 17.6. GC/MS (E.I.): 328 (3%), 243 (51), 201 (100), 186 (16), 122 (24). Anal. Calcd for $C_{15}H_{21}N_3O_7S$: C, 46.50; H, 5.46; N, 10.85. Found: C, 46.60; H, 5.47; N, 10.82.

5. Reduction of *N*-methoxy-*N*-methylamides to the corresponding amines with $AlCl_3/LiAlH_4$

Synthesis of *N*-methoxy-*N*-methylamides (26a-g). General Procedure.

Hydrochloride of *N*,*O*-dimethylhydroxylamine (1.1 mmol) was dissolved in anhydrous chloroform (15 ml) then dry pyridine was added (2, 2 mmol), and after, the appropriate carboxylic acid chloride **25a-g** (1mmol). The reaction mixture proceeds under magnetic stirring, at room temperature for 1-2 hours, checking the disappearance of carboxylic acid chloride by TLC (CHCl3/CH3OH 95:5, v / v). After complete conversion of substrates, the mixture was extracted with an aqueous solution of 1 N HCl (2 X 7 ml), then with an aqueous solution of saturated sodium carbonate (2 X 7 ml). After anhydrification on sodium sulfate; and evaporation of the solvent under reduced pressure **26a-g** were recovered in the form of oil, with yields of between 84% and 99%.

***N*-methoxy-*N*-methylamide (26a):** 99% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.63-7.60 (m, 2 H, Ar*H*), 7.43-7.29 (m, 3 H, Ar*H*), 3.51 (s, 3 H, OCH_3), 3.32 (s, 3 H, NCH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ: 169.90, 134.08, 130.56, 128.09, 128.01, 61.02, 33.76. GC-MS (EI) *m/z*: 165 ($M^{\cdot+}$, 3%), 105 (100), 77 (41), 51 (10).

***N*-methoxy-*N*-methylamide (26b):** 93% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.57-7.54 (m, 2 H, Ar*H*), 7.24-7.19 (m, 2 H, Ar*H*), 3.54 (s, 3 H, OCH_3), 3.36 (s, 3 H, NCH_3), 2.39 (s, 3 H, Ar-CH_3). GC-MS (EI) *m/z*: 179 ($M^{\cdot+}$, 5%), 119 (100), 91 (100), 65 (39).

***N*-methoxy-*N*-methylamide (26c):** 91% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 7.74-7.68 (m, 2 H, Ar*H*), 6.92-6.84 (m, 2 H, Ar*H*), 3.80 (s, 3 H, ArOCH_3), 3.53 (s, 3 H, NOCH_3), 3.33 (s, 3 H, NCH_3). GC-MS (EI) *m/z*: 195 ($M^{\cdot+}$, 1%), 149 (1), 135 (100), 107 (7), 92 (9), 77 (14).

***N*-methoxy-*N*-methylamine (27d):** 86% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ(ppm): 7.68-7.59 (m, 2 H, Ar*H*), 7.38-7.30 (m, 2 H, Ar*H*), 3.50 (s, 3 H, OCH_3), 3.32 (s, 3 H, NCH_3). GC-MS (EI) *m/z*: 199 ($M^{\cdot+}$, 3%), 141 (32), 139 (100), 111 (30), 75 (14).

***N*-methoxy-*N*-methylamine (27e):** 84% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ(ppm): 8.27 (d, 2 H, Ar*H*, *J* = 9.0 Hz), 7.84 (d, 2 H, Ar*H*, *J* = 9.0 Hz), 3.54 (s, 3 H, OCH_3), 3.39 (s, 3 H, NCH_3). GC-MS (EI) *m/z*: 210 ($M^{\cdot+}$, 3 %), 179 (1), 150 (100), 120 (9), 104 (34), 92 (13), 76 (18).

***N*-methoxy-*N*-methylamide (26f):** 89% yield. ^{1}H-NMR (300 MHz, $CDCl_3$) δ(ppm): 7.45-7.20 (m, 5 H, Ar*H*), 3.80 (s, 2 H, CH_2), 3.61 (s, 3 H, OCH_3), 3.20 (s, 3 H, NCH_3). GC-MS (EI) *m/z*: 179 ($M^{\cdot+}$, 11%), 148 (3), 119 (14), 118 (64), 91 (100), 61 (9).

***N*-methoxy-*N*-methylamide (26g):** 90% yield. ^{1}H-NMR (300 MHz, $CDCl_3$) δ(ppm): 3.70 (s, 3 H, OCH_3), 3.19 (s, 3 H, NCH_3), 2.40 (t, 2 H, CH_2CO, *J* = 7.6 Hz), 1.68-1.56 (m, 2 H, H-3), 1.37-1.23 (m, 4 H, H-4, H-5), 0.89 (t, 3 H, CH_3, *J* = 6.9 Hz). GC-MS (EI) *m/z*: 159 ($M^{\cdot+}$, 2%), 144 (1), 130 (4), 99 (99), 71 (68), 61 (80), 55 (18), 43 (100).

Synthesis of *N*-methoxy-*N*-methylamides 28. General Procedure.

Hydrochloride of *N*,*O*-dimethylhydroxylamine (1.1 mmol) was dissolved in anhydrous chloroform (15 ml) then dry pyridine was added (2, 2 mmol), and after, *N*-Nosyl-alanyl chloride **16f** (1mmol). The reaction mixture proceeds under magnetic stirring, at room temperature for 1-2 hours, checking the disappearance of carboxylic acid chloride by TLC (CHCl3/CH3OH 9:1, v / v). After complete conversion of substrate, the mixture was extracted with an aqueous solution of 1 N HCl (2 X 10 ml), then with an aqueous solution of saturated sodium carbonate (2 X 10 ml). After anhydrification on sodium sulfate; and evaporation of the solvent under reduced pressure **28** were recovered in the form of oil, with yields of between 80%.

***N*-methoxy-*N*-methylamides 28:** ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.45-8.30 (m, 2 H, Ar*H*), 8.15-8.00 (m, 2 H, Ar*H*), 5.90 (d, 1 H, N*H*, *J*= 9.3 Hz), 4.56-4.40 (m, 1 H, C*H*), 3.69 (s, 3 H, OCH_3), 3.04 (s, 3 H, NCH_3), 1.35 (d, 3 H, CHCH_3, *J* = 6.9 Hz). GC-MS (EI) *m/z*: 317 ($M^{+\cdot}$, 1%), 302 (1), 229 (100), 186 (50), 122 (50), 106 (5), 92 (10), 76 (18), 61 (60).

Synthesis of *N*-methoxy-*N*-methylamines (27a-g). General Procedure.

$AlCl_3$ (2 mmol) was added to a solution of the appropriate *N*-methoxy-*N*-methylamide **26a-g** (1 mmol) in dry THF (10 mL) and $LiAlH_4$ (1 mmol) was added. The mixture was stirred for 10 min, until TLC analysis of reaction mixture showed complete conversion of the precursor to the corresponding amine. The reaction mixture was hydrolyzed with aq sat. Na_2CO_3 solution (5 mL) and the aqueous phase was extracted with ether (3 x 5 mL). The combined organic phase was washed with brine and dried with Na_2SO_4. The solvent was evaporated to give the *N*-methoxy-*N*-methylamines **27a-g** in 84-100% overall yields.

Preparation of *N*-methoxy-*N*-methylamines hydrochloride 27g.

Hydrogen chloride gas, prepared by adding dropwise concentrated sulphuric acid to sodium chloride, was bubbled through the ethereal solution of the amine 3g. The solvent was evaporated and the residue was dried in a vacuum desiccator with P_2O_5 to afford the hydrochloride salt 4 (0.15 g, 84%) as a yellow solid.

***N*-methoxy-*N*-methylamines (27a)**: Oil (0.13 g, 85%); ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm): 2.62 (s, 3H, NCH_3), 3.40 (s, 3H, OCH_3), 3.79 (s, 2H, CH_2), 7.25-7.48 (m, 3H, Ar-H), 7.59-7.71 (m, 2H, Ar-H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 44.9, 59.9, 62.7, 127.3, 128.2, 128.6, 129.5; GC-MS (EI, 70 eV): *m/z* (%) 51 (3), 65 (7), 77 (3), 91 (100), 136 (1), 151 (22) [$M^{+\cdot}$]; Anal. Calcd for $C_9H_{13}NO$: C, 71.49; H, 8.67; N, 9.26; Found: C, 71.71; H, 8.70; N, 9.24.

***N*-methoxy-*N*-methylamines (27b)**: Oil (0.14 g, 84%); ^{1}H NMR (300 MHz,

$CDCl_3$): δ (ppm): 2.35 (s, 3H, Ar-CH_3), 2.60 (s, 3H, NCH_3), 3.40 (s, 3H, OCH_3), 3.75 (s, 2H, CH_2), 7.10-7.30 (m, 4H, Ar-H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ: 21.2, 44.8, 59.9, 64.4, 128.9, 129.4, 134.4, 136.8; GC-MS (EI, 70 eV): *m/z* (%) 77 (9), 91 (7), 105 (100), 132 (16), 165 (12) [$M^{+\cdot}$]; Anal. Calcd for $C_{10}H_{15}NO$: C, 72.69; H, 9.15; N, 8.48; Found: C, 72.93; H, 9.12; N, 8.46.

***N*-methoxy-*N*-methylamines (27c)**: Oil (0.18 g, 100%); ^1H NMR (300 MHz, $CDCl_3$): δ (ppm): 2.60 (s, 3H, NCH_3), 3.40 (s, 3H, $NOCH_3$), 3.70 (s, 2H, CH_2), 3.80 (s, 3H, $ArOCH_3$), 6.80-6,90 (m, 2H, Ar-H), 7.20-7.30 (m, 2H, Ar-H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ: 45.0, 55.2, 60.0, 64.0, 113.8, 128.6, 130.7, 158.9; GC-MS (EI, 70 eV): *m/z* (%): 91 (3), 121 (100), 181 (3) [$M^{+\cdot}$]; Anal. Calcd for $C_{10}H_{15}NO_2$: C, 66.27; H, 8.34; N, 7.73; Found: C, 66.50; H, 8.31; N, 7.72.

***N*-methoxy-*N*-methylamines (27d)**: Oil (0.18 g, 97%); ^1H NMR (300 MHz, $CDCl_3$): δ(ppm): 2.60 (s, 3H, NCH_3), 3.35 (s, 3H, OCH_3), 3.71 (s, 2H, CH_2), 7.20-7.35 (m, 4H, Ar-H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ: 44.9, 60.0, 63.9, 128.3, 130.8, 133.0, 136.1; GC-MS (EI, 70 eV): *m/z* (%) 89 (9), 99 (3), 125 (100), 127 (32), 185 (15) [$M^{+\cdot}$]; Anal. Calcd for $C_9H_{12}ClNO$: C, 58.23; H, 6.52; N, 7.54; Found: C, 58.35; H, 6.50; N, 7.52.

***N*-methoxy-*N*-methylamines (27e)**: Oil (0.19 g, 95%); ^1H NMR (300 MHz, $CDCl_3$): δ (ppm): 2.65 (s, 3H, NCH_3), 3.31 (s, 3H, OCH_3), 3.84 (s, 2H, CH_2), 7.50-7.60 (m, 2H, Ar-H), 8.11-8.22 (m, 2H, Ar-H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ: 45.1, 60.0, 63.9, 123.4, 130.0, 145.5, 147.1; GC-MS (EI, 70 eV): *m/z* (%): 78 (38), 89 (42), 90 (39), 106 (49), 136 (100), 181 (32), 196 (92) [$M^{+\cdot}$]; Anal. Calcd for $C_9H_{12}N_2O_3$: C, 55.09; H, 6.16; N, 14.28; Found: C, 55.27; H, 6.17; N, 14.32.

***N*-methoxy-*N*-methylamines (27f):** Oil (0.14 g, 84%); ^1H NMR (300 MHz, $CDCl_3$): δ (ppm) 2.61 (s, 3H, NCH_3), 2.80-2.95 (m, 4H, CH_2CH_2), 3.58 (s, 3H, OCH_3), 7.15-7.40 (m, 5H, Ar-H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 33.9, 45.3, 60.2, 62.5, 126.1, 128.5, 128.6, 140.1; GC-MS (EI, 70 eV): *m/z* (%): 65 (6), 74

(100), 91 (15), 105 (2), 165 (1) [$M^{+\cdot}$]; Anal. Calcd for $C_{10}H_{15}NO$: C, 72.69; H, 9.15; N, 8.48; Found: C, 72.90; H, 9.12; N, 8.46.

***N*-methoxy-*N*-methylamines (27g):** (0.15 g, 84%) as a yellow solid. ^{1}H NMR (300 MHz, D_2O): δ (ppm) 0.71 (t, *J* = 7.0 Hz, 3H, CH_3), 1.11-1.30 (m, 6H, H-3, H-4, H-5), 1.51-1.69 (m, 2H, H-2), 3.05 (s, 3H, NCH_3), 3.24-3.40 (m, 2H, H-1), 3.78 (s, 3H, OCH_3); ^{13}C NMR (75.5 MHz, D_2O): δ: 13.1, 21.5, 22.5, 25.0, 30.2, 41.3, 58.0, 59.4; Anal. Calcd for $C_8H_{20}ClNO$: C, 52.88; H, 11.09; N, 7.71; Found: C, 53.08; H, 11.05; N, 7.73.

Synthesis of *N*-methoxy-*N*-methylamines 29. General Procedure.

$AlCl_3$ (4 mmol) was added to a solution of the appropriate *N*-methoxy-*N*-methylamide **28** (1 mmol) in dry THF (15 mL) and $LiAlH_4$ (2 mmol) was added. The mixture was stirred for 20 min, until TLC analysis of reaction mixture showed complete conversion of the precursor to the corresponding amine. The reaction mixture was hydrolyzed with aq sat. Na_2CO_3 solution (5 mL) and the aqueous phase was extracted with ether (3 x 5 mL). The combined organic phase was washed with brine and dried with Na_2SO_4. The solvent was evaporated to give the *N*-methoxy-*N*-methylamine **29** in 98% yield.

***N*-methoxy-*N*-methylamines 29:** ^{1}H-NMR (300 MHz, $CDCl_3$) δ (ppm): 8.42-8.31 (m, 2 H, Ar*H*), 8.19-8.06 (m, 2 H, Ar*H*), 5.75 (d, 1 H, N*H*, *J*= 4.5 Hz), 3.63-3.41 (m, 2 H, CH_2), 3.30 (s, 3 H, OCH_3), 2.57-2.52 (m, 1 H, $CHCH_3$), 2.49 (s, 3 H, NCH_3), 1.16 (d, 3 H, $CHCH_3$, *J*= 6.6 Hz). GC-MS (EI) *m/z*: 229 (1), 186 (2), 122 (4), 92 (1), 74 (100).

6. A new non-natural arginine-like amino acid derivative with a sulfamoyl group in the side-chain

Synthesis of N^{α}-Fmoc-N^{δ}-Boc-L-ornithine methyl ester (31).

A 0.66 M solution of diazomethane in CH_2Cl_2 (8.5 mL, 5.6 mmol) was added dropwise to a suspension of **30** (0.73 g, 1.6 mmol) in CH_2Cl_2 (5 mL). The resulting mixture was stirred at r.t. for 15 min, and the conversion of the precursor was monitored by TLC ($CHCl_3/CH_3OH$ 95:5, v/v). The solvent was removed under reduced pressure condition and the residue was solubilised in AcOEt (5 mL), and washed with a 5% aqueous solution of $NaHCO_3$ (2 × 5 mL) and once with brine (5 mL). The organic extracts were dried over Na_2SO_4, filtered and evaporated to dryness under vacuum. The solid residue was co-evaporated with a 1:1 Et_2O/*n*-hexane mixture and compound **31** was recovered as a white crystalline solid (0.72 g, 96%). Mp = 149-151 °C. R_f = 0.69. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.75 (d, J = 7.4 Hz, 2 H), 7.58 (d, J = 7.4 Hz; 2 H), 7.38 (t, J = 7.4 Hz; 2 H), 7.30 (t, J = 7.4 Hz; 2 H), 5.43 (d, J = 8.0; 1 H), 4.55 (t, J = 6.9; 1 H), 4.31-4-42 (m, 3 H), 3.74 (s, 3 H), 3.12 (m, 2 H), 1.79-1.94 (m, 1 H), 1.46-1.74 (m; 3 H), 1.42 (s, 9 H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ 172.8, 156.0, 143.8, 141.3, 128.9, 127.7, 125.1, 119.9, 79.3, 77.5, 67.0, 53.6, 52.5, 47.1, 40.0, 29.8, 28.4, 26.1. Anal. Calcd for $C_{26}H_{32}N_2O_6$: C, 66.65; H, 6.88; N, 5.98. Found: C, 66.86; H, 6.89; N, 5.96.

Synthesis N^{α}-Fmoc-L-ornithine methyl ester trifluoroacetate (32).

A solution of **31** (0.72 g; 1.54 mmol) in CH_2Cl_2 (3 mL) was treated with a solution of TFA in CH_2Cl_2 (9:1, v/v; 5 mL). The resulting mixture was stirred at 0 °C for 15 min. After this time the consumption of **32** was complete (TLC: $CHCl_3/CH_3OH$ 95:5, v/v; **32** furnished a yellow spot to the ninhydrin test), and the volatile components of the mixture were removed under vacuum. The oily residue was co-evaporated with toluene (3 × 5 mL) and finally with a 1:1 MTBE/*n*-hexane mixture. The obtained glassy solid was dried under vacuum and immediately subjected to the next reaction step. R_f = 0.11.

Synthesis of N^{α}-Fmoc-N^{δ}-Sulfamoyl(Boc)-L-ornithine methyl ester (35).

Method A: A solution of the sulfamoyl chloride **33** (0.95 g, 4.62 mmol), freshly prepared, in dry ethanol-free CH_2Cl_2 (3 mL) was added dropwise to a solution of **32** (0.57 g, 1.54 mmol; amount estimated on the basis of a quantitative transformation of **31** into **32** by acidolysis) in dry ethanol-free CH_2Cl_2 (3 mL) containing DIEA (1.18 mL, 6.78 mmol). The resulting mixture was stirred at r.t. for 2 h (TLC: $CHCl_3$/CH_3OH 95:5, v/v). After this time, the solvent was removed under vaccum and the oily residue was solubilised in AcOEt (5 mL). The organic solution was washed with a 5% aqueous solution of $KHSO_4$ (3 × 5 mL), a 5% aqueous $NaHCO_3$ (3 × 5 mL) and once with brine (5 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness under vacuum. The pale yellow solid residue was co-evaporated three times with a 1:1 Et_2O/*n*-hexane mixture to give **35** as a pale yellow glassy solid. Flash column chromatography afforded pure **35** as a pale yellow powder.

Method B:

(0.55 g, 65%). Mp = 119-121 °C. R_f = 0.56. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.01 (s broad, 1 H), 7.74 (d, *J* = 7.4 Hz; 2 H), 7.58 (d, J = 7.4 Hz; 2 H), 7.38 (t, *J* = 7.4; 2 H), 7.30 (t, *J* = 7.4 Hz; 2 H), 5.44 (d, *J* = 8.3; 1 H), 5.35 (t, *J* = 6.16; 1 H), 4.20-4.43 (m, 3 H), 4.19 (t, *J* = 6.7, 1 H), 3.73 (s, 3 H), 3.08 (m, 2 H), 1.83-2.00 (m, 1 H), 1.54-1.78 (m, 3 H), 1.46 (s, 9 H). ^{13}C-NMR DEPT (75 MHz, $CDCl_3$) δ 172.8 (C=O), 156.1 (C=O), 155.9 (C=O), 143.8 (C), 141.2 (C), 128.0 (CH), 127.6 (CH), 125.1 (CH), 119.9 (CH), 79.3 (C) , 67.0 (CH_2), 53.6 (CH), 52.5 (CH_3), 47.1 (CH), 40.0 (CH_2), 29.7 (CH_2), 28.4 (CH_3), 26.1 (CH_2). Anal. Calcd for $C_{26}H_{33}N_3O_8S$: C, 57.02; H, 6.07; N, 7.67. Found: C, 57.21; H, 6.09; N, 7.68.

Synthesis of N^{α}-Acetyl-N^{δ}-Sulfamoyl-L-ornithine methyl ester (37). *Removal of Fmoc group from **35** by the mercaptoacetic acid/sodium methoxide reagent system (molar ratio **35**/$HSCH_2CO_2H$/MeONa 1:5:8)*. Mercaptoacetic acid (0.09 mL, 1.26 mmol) was added to a solution of sodium methoxide (0.11 g, 2.1 mmol) in CH_3OH (1 mL) at 0 °C. A solution of **35** (0.23 g, 0.42 mmol) in CH_3CN (10 mL) was then added and the resulting mixture was maintained under

magnetic stirring at 50 °C. After 3.5 h, TLC (gradient: $CHCl_3/CH_3OH$ 95:5, v/v) showed the complete conversion of **35**. The mixture was made acidic (pH 5) by adding a 0.1 N aqueous solution of HCl, and extracted with Et_2O (3 × 10 mL). The ethereal extracts were discarded off and the aqueous phase was made basic (pH 8) with a 10% aqueous solution of $NaHCO_3$, and then extracted with AcOEt (3 × 10 mL). The collected organic layers were dried over Na_2SO_4, filtered and evaporated to dryness under vacuum to give an oily residue which was immediately treated with an excess of freshly distilled acetic anhydride, in a 1:1 mixture of CH_2Cl_2 (5 mL) and a 10% aqueous solution of $NaHCO_3$ (5 mL). The biphasic system was vigorously stirred at r.t., and the reaction was monitored by TLC (gradient: $CHCl_3/CH_3OH$ 95:5, v/v). After 1 h, the mixture was concentrated under vacuum and the aqueous residue was extracted with AcOEt (3 × 5 mL). The organic phase was washed with a 5 % aqueous solution of $NaHCO_3$ (3 × 5 mL) and once with brine (5 mL), then dried over Na_2SO_4, filtered, and evaporated to dryness under vacuum. The solid residue was purified by FCC to give the acetylated compounds **39** (0.03 g, 21%) and **37** (0.11 g, 68%).

39. Pale yellow foam. R_f = 0.34. ^{1}H-NMR (300 MHz, DMSO-d_6) δ 8.19 (d, J = 8.0; 1 H), 6.44 (m, 3 H), 4.19 (m, 1 H), 3.62 (s, 3 H), 2.85 (m, 2 H), 1.84 (s, 3 H), 1.37-1.81 (m, 4 H). ^{13}C-NMR (75 MHz, DMSO-d_6) δ 172.7, 169.4, 51.7, 41.9, 39.8, 28.3, 25.4, 22.1. Anal. Calcd for $C_8H_{17}N_3O_5S$: C, 35.95; H, 6.41; N, 15.72. Found: C, 36.05; H, 6.40; N, 15.72.

37. Pale yellow foam. R_f = 0.65. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.28 (s broad, 1 H), 6.54 (d, *J* = 7.9 Hz; 1 H), 5.74 (t, *J* = 6.8; 1 H), 4.56 (m, 1 H), 3.71 (s, 3 H), 3.05 (m, 2 H), 2.0 (s, 3 H), 1.52-1.96 (m, 4 H), 1.43 (s, 9 H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ 172.9, 170.6, 150.5, 83.5, 52.5, 51.6, 42.9, 29.5, 28.0, 25.1, 22.9. Anal. Calcd for $C_{13}H_{25}N_3O_7S$: C, 42.50; H, 6.86; N, 11.44. Found: C, 42.59; H, 6.88; N, 11.46.

Synthesis of dipeptide 41. A solution of Fmoc-Pro-OH (**40**) (0.14 g; 0.42 mmol) in dry ethanol-free CH_2Cl_2 (2 mL) was treated with HOBt monohydrate (0.07 g, 0.46 mmol), EDCI (0.1 g, 0.52 mmol), DIEA (0.18 mL, 1.03 mmol), and the resulting mixture was stirred at r.t. for 12 h. Compound **36** (0.14 g, 0.42

mmol; amount estimated on the basis of a complete conversion of **35** in the unblocking step performed as described for the preparation of **37**) solubilised in dry ethanol-free CH_2Cl_2 (2 mL) was then added, and the stirring was maintained for further 12 h. After this time, TLC (gradient: $CHCl_3/CH_3OH$ 95:5, v/v) showed the complete consumption of substrate. The mixture was evaporated to dryness under vacuum and the residue was solubilised with AcOEt (5 mL), washed with a 5% aqueous solution of $KHSO_4$ (3 × 5 mL), 5% aqueous solution of $NaHCO_3$ (3 × 5 mL), and once with brine (5 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness under vacuum to give a solid residue which was triturated with a 1:4 MTBE/*n*-hexane mixture. The precipitated was collected and dried under vacuum. Dipeptide **43** was recovered pure as a yellowish powder. Yield: 0.24 g, 88%. ^{1}H-NMR (300 MHz, $CDCl_3$) 75:25 mixture of rotamers δ 8.43 (s broad, 0.75 H), 7.74 (d, J = 7.4 Hz, 2 H), 7.60 (m, 2 H), 7.52 (s broad, 0.25 H), 7.38 (t, J = 7.4 Hz; 2 H), 7.29 (t, J = 7.4 Hz; 2 H), 6.75 (d, J = 8.1 Hz; 0.75 H), 6.57 (s broad, 0.25 H), 5.46 (t, J = 6.9 Hz; 0.75 H), 5.33 (s broad, 0.25 H), 4.58 (m, 1 H), 4.41 (m, 3 H), 4.13-4.30 (m, 2 H), 3.71 (s, 3 H), 3.31-3.65 (m, 2 H), 2.90-3.29 (m, 2 H), 2.11 (m, 2 H), 1.91 (m, 2 H), 1.56-1.78 (m, 4 H), 1.43 (s, 2.25 H), 1.39 (s, 6.75 H). ^{13}C-NMR (75 MHz, $CDCl_3$) 75:25 mixture of rotamers δ 172.3 and 171.9, 155.7, 150.7, 144.0 and 143.7, 141.3, 127.7, 127.1, 125.2, 120.0, 83.5, 67.9, 60.5, 52.5, 51.7, 47.1, 43.1, 31.6, 29.7 and 29.5, 28.9, 27.9, 24.8 and 24.7. Anal. Calcd for $C_{31}H_{40}N_4O_9S$: C, 57.75; H, 6.25; N, 8.69. Found: C, 57.94; H, 6.27; N, 8.67.

Synthesis of tripeptide 43. *(a) Unblocking of **41**.* A solution of **41** (0.32 g, 0.5 mmol) in CH_3CN (10 mL) was reacted with mercaptoacetic acid (0.18 mL, 2.5 mmol) and sodium methoxide (0.21 g, 4.0 mmol) in CH_3OH (2 mL). TLC (gradient: $CHCl_3/CH_3OH$ 95:5, v/v) showed complete conversion of substrate after 5.5 h at 50 °C. The mixture was made acidic (pH 5) by adding a 0.1 N aqueous solution of HCl, and extracted with Et_2O (3 × 10 mL). The ethereal extracts were discarded off and the aqueous phase was made basic (pH 8) with a 10% aqueous solution of $NaHCO_3$, and then extracted with AcOEt (3 × 10 mL). The collected organic layers were dried over Na_2SO_4, filtered and evaporated to

dryness under vacuum to give a glassy pale yellow solid which was used in the next step of coupling without further purification. *(b) Coupling.* A solution of Boc-D-Phe-OH (0.13 g; 0.5 mmol) in freshly distilled DMF (2 mL) was treated with EDCI (0.31 g, 0.52 mmol), HOBt monohydrate (0.08 g, 0.55 mmol), DIEA (0.27 mL, 1.6 mmol), and the resulting mixture was stirred at r.t. for 2 h. The glassy solid obtained from the unblocking of **41**, (0.21 g, 0.5 mmol; amount estimated on the basis of a complete conversion of **41** in the unblocking step performed as described) solubilised in freshly distilled DMF (2 mL) was then added dropwise to the mixture, and the stirring was maintained for further 24 h. After this time, TLC (gradient: $CHCl_3/CH_3OH$ 95:5, v/v) showed the complete consumption of Boc-D-Phe-OH. The mixture was evaporated to dryness under vacuum and the solid residue was solubilised with AcOEt (5 mL), washed with a 5% aqueous solution of $KHSO_4$ (3 × 5 mL), 5% aqueous solution of $NaHCO_3$ (3 × 5 mL), and once with brine (5 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated to dryness under vacuum to give a solid residue which was triturated with a 1:4 MTBE/*n*-pentane mixture. The precipitated was collected and dried under vacuum. Dipeptide **43** was recovered pure as a yellowish powder. Yield: 0.30 g, 89%. ^{1}H-NMR (300 MHz, $CDCl_3$) 70:30 mixture of rotamers δ 7.18-7.41 (m, 6 H), 7.06 (d, J = 7.1 Hz; 1 H), 6.24 (m, 0.3 H), 6.02 (m, 1 H), 5.89 (d, J = 6.9 Hz; 0.7 H), 4.52-4.74 (m, 0.3 H), 4.50 (m, 0.7 H), 4.30-4.48 (m, 0.3 H), 4.28 (m, 0.7 H), 4.17 (m, 1 H), 3.70-3.90 (m, 1 H), 3.76 (s, 3 H), 3.52 (m, 0.3 H), 3.49 (m, 1 H), 3.28 (m, 0.7 H), 2.90-3.16 (m, 0.3 H), 2.67 (m, 0.7 H), 1.10-2.15 (m, 8 H), 1.52 (s, 3 H), 1.41 (s, 3 H). ^{13}C-NMR (75 MHz, $CDCl_3$) 70:30 mixture of rotamers δ 172.2 and 172.0, 171.7, 170.9, 157.03, 155.3, 136.22, 134.7, 129.8 and 129.6, 129.4 and 129.1, 128.5 and 128.0, 127.1 and 126.8, 124.3, 120.4, 60.2, 55.7, 53.8, 52.44, 51.9, 51.4, 49.1 and 47.2, 43.0, 41.7, 40.4, 39.4, 33.9, 33.3 and 33.1, 32.7, 29.6, 28.3 and 28.1, 25.6, 25.3, 25.0 and 24.7. Anal. Calcd for $C_{30}H_{47}N_5O_{10}S$: C, 53.80; H, 7.07; N, 10.46. Found: C, 52.7; H, 7.33; N, 10.04.

Synthesis of tripeptide trifluoroacetate 44. A solution of **43** (0.30 g; 0.45 mmol) in CH_2Cl_2 (3 mL) was treated with a solution of TFA in CH_2Cl_2 (9:1, v/v;

5 mL). The resulting mixture was stirred at 0 °C for 30 min. After this time the consumption of **43** was complete (TLC: $CHCl_3/CH_3OH$ 95:5, v/v; **44** furnished a yellow spot to the ninhydrin test), and the volatile components of the mixture were removed under vacuum. The oily residue was co-evaporated with toluene (3 × 5 mL), absolute EtOH (3 × 5 mL), and triturated with a 1:2 MTBE/*n*-pentane mixture. Precipitate was collected, dried under vacuum and finally lyophilized to give **44** as a pale yellow glassy solid. Yield: 0.25 g, 90 %. R_f = 0.10. ^{1}H-NMR (300 MHz, DMSO-d_6/D_2O) δ 7.11-7.40 (m, 5 H), 6.50 (s broad, 2 H), 4.02-4-41 (m, 3 H), 3.61 (s, 3 H), 3.4-3.6 (m, 2 H, partially overlapped by the water signal), 2.68-3.14 (m, 4 H), 1.34-1.90 (m, 8 H). ^{13}C-NMR (75 MHz, DMSO-d_6/D_2O) δ 172.9, 171.8, 166.8, 134, 94, 130.0, 129.0, 127.9, 59.7, 52.3, 52.1, 49.2, 47.2, 42.5, 37.2, 29.6, 28.6, 26.0, 24.2. MALDI MS Calcd for $C_{20}H_{32}N_5O_6S^+$: *m/z* 470.2070. Found: m/z 470.2014 (Δm = -12 ppm). Anal. Calcd for $C_{22}H_{31}N_5O_5S \cdot CF_3CO_2H$: C, 45.28; H, 5.53; N, 12.00. Found: C, 44.02; H, 5.68; N, 11.65.

Clotting assays for tripeptides 43 and 44.

Materials. For in vitro clotting assays an automatic coagulometer was used with a maximum coagulation time limit of 500 sec. TT and APTT determinations were performed with the kits Dade BC Thrombin Reagent and Dade Actin FSL, respectively.

Preparation of stock solution. Tripeptides **43** and **44** to be tested were dissolved in DMSO just prior to use and diluted with PBS buffer (pH 8) to yield a final 152 mM stock solution for tripeptide **43**, and a 337 mM stock solution for tripeptide trifluoroacetate **44**.

Clotting assays. In vitro coagulation assays were performed with pooled human plasma. Clotting time was measured using an automatic coagulometer according to the manufacturer's instructions. In order to exclude any influence of DMSO in clotting assays, a blank prepared by mixing the pooled human plasma (800 μL) and DMSO (200 μL) was subjected to TT and APTT tests. Control values of both the parameters were not modified with respect to those registered for a blank prepared by mixing the pooled human plasma (800 μL) and PBS (200 μL).

Determination of Thrombin Time (TT). To each of the tubes containing plasma (800 μL), increasing amounts (25, 50, 75, 100, 125, and 150 μL; corresponding to 8.4, 16.8, 25.3, 33.7, 42.1, and 50.5 μmol, respectively) of the stock solutions of tripeptide **44** were added. The same protocol was applied to prepare samples of tripeptide **43**. The resulting samples were diluted to a total volume of 1 mL by adding PBS, vortexed and incubated at 37 °C for 1 minute, and then placed on the instrument sample wheel. The coagulometer automatically dispensed thrombin time reagent in the sample of each tube. The time for the appearance of a fibrin clot was measured and each value was obtained from a set of three separate experiments. Control value of clotting time for TT tests was determined for three experiments using a blank prepared by mixing the pooled human plasma (800 μL) and PBS (200 μL), and was 15.5 sec (mean value; SD = ± 5%). Clotting time values exceeding the coagulometer time limit were not determined.

Determination of Activated Partial Thromboplastin Time (APTT). A second series of samples prepared as described for TT determinations was treated with the activated partial thromboplastin time reagent. The coagulometer automatically dispensed the appropriate reagent in the sample of each tube. The time for the appearance of a fibrin clot was measured and each value was obtained from a set of three separate experiments. The same blank used for the control value of clotting time for TT tests was used to determine a control value of 31.2 sec (mean value; SD = ± 5%) for clotting time in APTT tests. Clotting time values exceeding the coagulometer limit were not determined.

Results. Tripeptide **43** did not show appreciable inhibitory potency in all the performed experiments. Trifloroacetate **44** (sample containing 8.4 μmol) prolonged the clotting time of pooled human plasma to three times the control value fibrin clot time in TT determination (217.2 sec; mean value, SD = ± 5%), and to twice in APTT measurements (65.4 sec; mean value, SD = ± 5%).

Tripeptide **44** (µmol in the sample)	**TT** (mean value in sec; SD = ± 5%)	APTT **(mean value in sec; SD = ± 5%)**
8.4	217.2	**65.4**
16.8	344.4	**87.9**
25.3	493.7	**123.8**
33.7	ND	**196.8**
42.1	ND	**ND**
50.5	**ND**	**ND**

7. Bergapten-free bergamot essential oils extracted from peeled fruits by vegetable matrix direct distillation

Bergamot (C. bergamia, Risso et Poiteau) was supplied by the "Consorzio del Bergamotto di Reggio Calabria" (Southern Italy). A sample of cold-pressed bergamot essential oil was provided by the Capua Co. (Campo Calabro, Reggio Calabria, Southern Italy). The Bergatrade Co. (Reggio Calabria, Southern Italy) supplied serviettes moisturized with bergamot oil. A commercial solution of distilled bergamot oil (Bergarital Co., Reggio Calabria, Southern Italy) was also analyzed. Tetradecane and standard samples of the bergamot essential oil components for the gas chromatography-mass spectrometry (GC-MS) analysis were purchased from Sigma-Aldrich (Milano, Italy) and from TCI Europe N.V. (Zwijndrecht, Belgium).

Extraction of essential oils. Vacuum distillation.

In a typical experiment, a weighted amount of bergamot fruits was peeled, and the recovered peels were chopped. The pulp was collected in a flask and subjected to vacuum distillation by immersion in a previously heated oil bath (120 °C). The essential oil was separated from the aqueous phase produced during distillation and dried over Na2SO4. The procedure was then applied to five bergamot fruit samples (weights: 297, 208, 206, 246, and 283 g). The recovered amounts of bergamot oil were 0.69, 0.69, 0.83, 0.74, and 0.90 g, respectively. The obtained average amount of bergamot essential oil was 311 g for quintal of fruits. An aliquot of the final bergamot essential oil (0.01 mL) was diluted with diethyl ether (5 mL), and 1 μL of the ethereal solution was used for the qualitative GC-MS analysis.

Analysis of Cold-pressed bergamot essential oil.

An aliquot of a commercial sample of cold-pressed bergamot essential oil (0.01 mL) was diluted with diethyl ether (5 mL). After filtration, 1 μL of the resulting solution was subjected to the qualitative GC-MS analysis. During storage at low

temperature, a sample of cold-pressed oil (120 mL) furnished a solid residue (0.13 g), which was collected, dried, solubilized in chloroform, and then subjected to the qualitative GC-MS analysis.

Analysis of bergamot essential oil from moisturized serviette.

A commercial refreshing serviette soak with bergamot essential oil was immerged in diethyl ether (10 mL) and kept in contact with the solvent for 2 h. The ethereal layer was separated and dried over Na_2SO_4. After filtration, an aliquot (1 μL) of the organic solution was subjected to the qualitative GC-MS analysis.

Analysis of bergamot essential oil conteined in commercial solutions.

A commercial solution of bergamot essential oil (0.01 mL) was diluted with diethyl ether (5 mL) and dried over Na_2SO_4. After filtration, an aliquot (1 μL) of the resulting solution was subjected to the qualitative GC-MS analysis.

GC/MS analysis condition.

GC-MS analyses were performed using a 6890N Network GC System (Agilent Technologies Inc., Palo Alto, CA) equipped with a HP-5MS (30 m × 0.25 mm i.d., 5% dimethylsiloxane; film thickness, 0.25 μm) capillary column and with a 5973 Network MSD mass spectrometer, operated in electron impact ionization mode (70 eV). GC-MS analyses were carried out in split mode (split ratio, 1:50), using helium as the carrier gas (1 mL/min flow rate). The injection port was heated at 250 °C. The column was maintained at an initial temperature of 60 °C for 2 min and then programmed at 7 °C/min to a final temperature of 280 °C, where it was maintained for 10 min. Quantitative GC-MS analysis was carried out in splitless mode (splitless time, 1 min), by using tetradecane as the internal standard.

Qualitative GC/MS analysis.

All analytes were characterized by injecting samples of the oils enriched with an authentic sample of each standard compound and by observing the enhancement of the corresponding peak area.

Quantitative GC/MS analysis.

For the quantitative analysis, two stock solutions were prepared. Stock 1 was prepared using 50 μL of each liquid oil component and 50 mg of each solid oil component (camphene, *cis*-sabinene hydrate, dodecanal, 5,7-dimethoxycoumarin, and bergaptene), by diluting the resulting mixture to 100 mL with chloroform. Aliquots of the stock 1 were then used to prepare three solutions containing, respectively, 0.5 (solution A), 5 (solution B), and 50 μL (solution C) of each analyte per liter. Tetradecane was added to solutions A–C before dilution to obtain a final concentration of the standard of 500 μL per liter. Stock 2 was prepared using 50 μL of each oil component and 50 mg of each solid oil component by diluting the resulting mixture to 10 mL with chloroform. Aliquots of the stock 2 were then used to prepare three solutions containing, respectively, 0.5 (solution D), 0.83 (solution E), and 2.5 mL (solution F) of each analyte per liter. Tetradecane was added to solutions D–F as the internal standard before dilution to obtain a final concentration of the standard of 500 μL per liter. Stocks 1 and 2, containing tetradecane (500 μL per liter), were also used for the quantitative analysis. Three aliquots of the oil isolated by vacuum distillation of the fruit peels (73.9, 123.2, and 172.5 mg) and three aliquots of cold-pressed oil (74.2, 125.7, and 174 mg), containing tetradecane (5 μL), were diluted to 10 mL with chloroform and then subjected to the quantitative analysis. Quantitative data were obtained by comparing the analyte/tetradecane area ratios in the standard solutions with the corresponding ratios in the oil samples solutions.

GC-flame ionization detection (FID) analysis.

Linear retention indices of all analytes were determined using a standard mixture of homologue hydrocarbons (C_8–C_{20}). Analyses were carried out by means of a GC-2010 system (Shimadzu, Japan), equipped with an Equity-5MS

column (Supelco, United States) having the following dimensions: 30 m × 0.25 mm i.d.; film thickness, 0.25 μm. The oven temperature program was as follows: 50 °C at 3°/min to 250 °C, held 10 min. Sample injection was in split mode (split ratio, 1:50). Injector and FID temperatures were set at 280 °C. The carrier gas was helium (1 mL/min flow rate).