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Doctoral Thesis

Modified amino acids as useful tool for peptide synthesis and their measurement in complex matrices

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Abstract

Negli ultimi anni sono stati scoperti e caratterizzati numerosi peptidi biologicamente attivi che, mediante interazioni con opportuni recettori, influenzano le cellule in una serie di funzioni vitali quali il metabolismo, le difese immunitarie, la proliferazione cellulare.

Questi peptidi, tuttavia, nella loro forma nativa non possono essere utilizzati come farmaci, data la loro scarsa stabilità metabolica e il difficile assorbimento dopo somministrazione orale; bisogna perciò disporre di pepdidomimetici, che siano in grado di mimare gli effetti farmacologici dei peptidi naturali, ma che presentino allo stesso tempo una maggiore stabilità e biodisponibilità.

Nella realizzazione della sintesi peptidica riveste un ruolo importante la scelta di gruppi protettori utili a mascherare la funzione amminica e carbossilica di amminoacidi e peptidi. La necessità di una sequenziale protezione e deprotezione delle varie funzionalità presenti fa si che, sia l'introduzione, che la rimozione di un gruppo protettore richiedano un'accurata progettazione sintetica, al fine di garantire il grado richiesto di ortogonalità tra i vari gruppi protettori utilizzati. Nella maggior parte dei casi per proteggere la funzione carbossilica di α-amminoacidi si utilizzano gruppi protettori semipermanenti che rimangono inalterati durante la costruzione della catena

peptidica e vengono rimossi alla fine della sintesi. La formazione di esteri è stata allo scopo ampiamente utilizzata. In particolare il gruppo carbossilico viene convertito in estere metilico, benzilico, tert-butilico e benzidrilico. Tuttavia, il ripristino della funzione carbossilica al termine della sintesi peptidica presenta delle difficoltà. Infatti, in questi casi la deprotezione della funzione carbossilica prevede l'idrolisi basica degli stessi esteri in presenza di solventi organici. Questa procedura, anche quando è eseguita in condizioni strettamente controllate, può causare epimerizzazione del peptide e altre reazioni collaterali.

Allo scopo è stata realizzata la deprotezione della funzione carbossilica di esteri metilici di alfa-amminoacidi utilizzando ilidi allo zolfo in particolare la dimetilsulfossonio metilide.

In un tipico esperimento l'estere metilico della *N*-nosil-L-alanina è stato trattato con la metilide di dimetilsolfossonio in THF nel rapporto molare 1:2 per trenta minuti. A seguito di idrolisi acida si recupera con resa quantitativa il corrispondente amminoacido deprotetto sulla funzione carbossilica. La reazione è stata applicata ad una serie di esteri metilici di alfa-amminoacidi protetti sulla funzione amminica con il gruppo terbutossicarbonile (Boc), nosile (Ns)e benzilossicarbonile (Cbz) e di esteri metilici di acidi carbossilici. In tutti gli esperimenti eseguiti il corrispondente acido carbossilico è stato recuperato con rese elevate e in forma pura. La deprotezione della funzione

carbossilica è stata realizzata con successo anche con substrati amminoacidici protetti in catena laterale con gruppi protettori acido-labili.

Inoltre, eseguendo la procedura su substrati enantiopuri, è stato dimostrato che la reazione avviene senza racemizzazione. Questa metodologia è generale e può essere considerata una valida alternativa all'idrolisi degli esteri quando un substrato è sensibile alle condizioni di idrolisi.

La dimetilsulfossonio metilide è stata usata, anche, come unico ed utile reagente per la deprotezione simultanea della funzione carbossilica protetta come estere e della funzione amminica protetta con il 9-fluorenilmetossicarbonile (Fmoc) di α-amino acidi e oligopeptidi. La nuova metodologia è stata applicata con successo nella sintesi peptidica, basata sulla "Fmoc-chemistry", sia in fase solida che in soluzione.

I corrispondenti amminoacidi e peptidi liberi sulla funzione carbossilica e sulla funzione amminica vengono recuperati in tempi brevi ed in ottime rese. La nuova procedura è stata applicata con ottimi risultati, anche, su peptidi contenenti amino acidi protetti in catena laterale con gruppi protettori acido-labili. Inoltre, è stato studiato l'andamento stereochimico della reazione di deprotezione mediante risonanza magnetica nucleare (^{1}H-NMR). I dati ^{1}H-NMR di miscele di prodotti diastereoisomerici tra di loro indicano chiaramente che le

condizioni adottate non causano epimerizzazione degli stereocentri presenti nei substrati di partenza.

Nel mio lavoro di ricerca mi sono occupata, inoltre, dello sviluppo di nuove metodologie per la sintesi di amminoacidi *N*-alchilati, utili building blocks per la sintesi di peptidi biologicamente attivi.

In questo campo è stata messa a punto un' efficiente metodologia "one-pot" per l' *N*-alchilazione di una serie di *N*-arilsolfonil-α-amminoacidi metil esteri recanti sostituenti diversi in posizione 4 dell'anello aromatico solfonammidico. In particolare, si è confrontata la reattività di queste specie con diazometano e trimetilossonio tetrafluoroborato in processi di *N*-metilazione e con trietilossonio tetrafluoroborato in processi di *N*-etilazione. La metilazione con diazometano ha avuto esito negativo per i derivati *N*-arilsolfon-ammidici contenenti gruppi elettron-donatori sull'anello aromatico. In questi casi il trimetilossonio tetrafluoroborato si è mostrato il reagente di scelta per la *N*-metilazione diretta e quantitativa. Il trietilossonio tetrafluoroborato ha dimostrato essere un reagente molto efficace per la preparazione di *N*-etil derivati di tutti gli *N*-arilsolfonil-α-ammino acidi metil esteri testati.

Nell'ultima parte del mio lavoro di ricerca mi sono occupata del riconoscimento e della misura di analiti contenuti in matrici di interesse alimentare. Nella carne il contenuto di amminoacidi

liberi ed il contenuto di acidi grassi liberi sono due parametri importanti utilizzati per determinare la sua qualità. Questi composti hanno un ruolo molto importante nella definizione delle caratteristiche sensoriali dei prodotti a base di carne. E' stata sviluppata, a tale proposito, una procedura innovativa per la misurazione del contenuto di amminoacidi e acidi grassi liberi nella carne e nei prodotti a base di carne. La procedura messa a punto prevede un singolo esperimento per la determinazione simultanea del profilo degli acidi grassi e degli amminoacidi. Gli analiti di interesse sono rapidamente estratti dalla matrice carnea e derivatizzati usando il metil cloroformiato. Questo reagente consente la trasformazione dei due gruppi di analiti nei corrispondenti *N*-metilossicarbonil aminoacidi metil esteri ed esteri metilici degli acidi grassi, che possono essere facilmente estratti per la loro successiva identificazione e quantificazione.

La misurazione dei derivati degli acidi grassi e degli amminoacidi ottenuti viene eseguita mediante GC/MS. Il principale vantaggio del protocollo messo a punto è la determinazione simultanea di due importanti classi di analiti che sono di grande importanza nell'analisi e caratterizzazione degli alimenti.

In un altro lavoro sono poi stati messi a confronto alcuni parametri importanti quali il contenuto di amminoacidi liberi e ammine biogeniche di prodotti a base di carne stagionati industriali e fatti in casa. A questo scopo, la "soppressata" e la

"salsiccia", due tipici salumi stagionati prodotti nel Sud Italia, sono stati analizzati. Le salsicce fatte in casa hanno mostrato un livello più alto di ammine biogeniche libere rispetto al contenuto dei prodotti industriali, molto probabilmente perché la formazione di ammine biogeniche nei prodotti industriali è limitata dall'uso di colture starter. Le salsicce industriali sono caratterizzate, invece, da un più alto contenuto di amminoacidi liberi totali.

Table of contents

Introduction

The discovery of the physiological role of a great number of peptides stimulated researchers all over the world towards the design and synthesis of peptidomimetics. Peptidomimetics are compounds whose essential elements (pharmacophores) mimic a natural peptide or protein in 3D space and retain the ability to interact with the biological target and produce the similar or better biological properties. Peptidomimetics are designed to circumvent some of the problems associated with a natural peptide, for example stability against proteolysis and poor bioavailability. Certain other properties, such as receptor selectivity or potency, often can be substantially improved. Hence mimics have great potential in drug discovery. The design process begins by developing structure–activity relationships that can define a minimal active sequence or major pharmacophore elements, and identify the key residues that are responsible for the biological effect. The demand for modified peptides with improved stability profiles and pharmacokinetic properties is driving extensive research effort in this field. Many structural modifications of peptides guided by rational design and molecular modeling have been established to develop novel synthetic approaches.

In this contest my research project was mainly based on the synthesis of modified amino acids and their introduction into peptide chains and on the development of new peptide synthetic strategies. The aim was to obtain useful building blocks to be used in the synthesis of peptide and peptidomimetics. In particular I worked on the development of new and efficient synthetic methodologies for the introduction and removal of protecting groups of the amino and carboxyl function of α-amino acid.

Furthermore my attention was focused on the synthesis of *N*-alkylated amino acids. *N*-Alkyl-α-amino acids are a representative class of amino acid derivatives. They find application as synthetic building blocks in medicinal chemistry as well as molecular probes in studies related to the structural properties and biological activities of *N*-alkylated peptides and peptidomimetics.

Finally part of my research activity was devoted to the food chemistry, in particular to the extraction and derivatization of amino acids and free fatty acids in meat products and to the comparison of free amino acids and biogenic amines content in industrial and homemade products.

Chapter 1

New methodologies for the protection and deprotection of amino acid functionalities in peptide synthesis

1. Carboxyl function deprotection of esters using sulphur ylides

1.1. Methodologies for esters cleavage

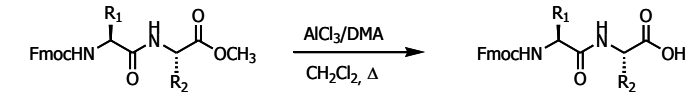
In peptide synthesis the semipermanent protection of the carboxyl terminal function is required. To this aim the formation of esters has been widely used, in particular the carboxylic group is converted into methyl, benzyl and *tert*-butyl ester. The protecting groups should be kept intact through the chain-building process and removed at its completion.

In solution phase peptide synthesis the protection of the carboxyl function of α-amino acids as a methyl ester is advantageous since it is stable during the entire synthetic process.[1] However, the regeneration of the free carboxyl function presents some difficulties. In peptide synthesis, the most used method for methyl ester cleavage is basic hydrolysis.[2] This procedure, even when it is carried out under strictly

controlled conditions, could cause racemization and other side reactions.[3]

In the light of the above-discussed limits, it is essential to identify milder methodologies for the deprotection of the carboxyl function of α-amino acids**.**

For this reason, alternative strategies have been developed that involve the use of nucleophiles that act directly on the carbon atom of the methyl ester function (*Scheme 1*).[4]



1 **2**

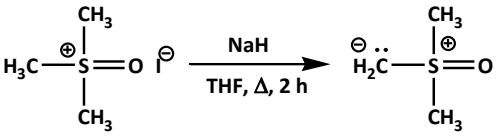
DMA = *N,N*-dimethylaniline $AlCl_3$/DMA = 9 :15

Scheme 1. Ester cleavage with the system $AlCl_3$/N,N dimethylaniline

1.2. Esters cleavage with dimethylsulfoxonium methylide

The deprotection strategy developed is based on the use of a nucleophilic reagent characterized also by the presence of an electrophilic site. Sulfur ylides were used as deprotecting reagents. The dimethylsulfoxonium methylide **4**, was prepared by the treatment of trimethylsulfoxonium iodide **3** with sodium

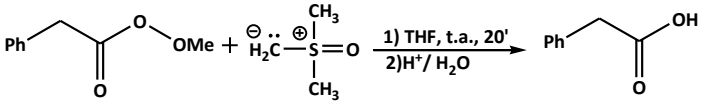
hydride in dry tetrahydrofuran under inert atmosphere. The mixture was heated under reflux for 2 hours (*Scheme 2*):



3 4

Scheme 2. Synthesis of dimethylsulfoxonium methylide (4)

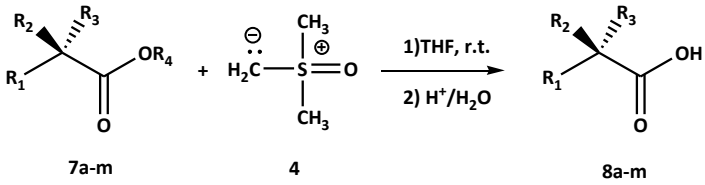
In a preliminary experiment the deprotection reaction was performed with methyl phenylacetate. The methyl phenylacetate **5** (1 mmol) was dissolved in tetrahydrofuran and treated with dimethylsulfoxonium methylide **4** (2 mmol). The resulting mixture was maintained under an inert atmosphere (N_2) and stirred at room temperature for 20 minutes (S*cheme 3*). The corresponding acid **6** was recovered, after a simple work up, with high yields (90%) without any further purification.



5 4 6

Scheme 3. Reaction of methyl phenylacetate (5) with dimethylsulfoxonium methylide.

The treatment of methyl esters with dimethylsulfoxonium methylide, leads to the efficient cleavage of the ester function and the obtainment of the corresponding carboxyl acids. The reaction can be considered a convenient alternative route to the ester hydrolysis. In the light of the interesting results and in order to validate this procedure, the reaction was applied to a series of *N*-protected-α-amino acid esters and to a set of carboxyl esters. (*Scheme 4*, *Table 1*)



7a-m **4** **8a-m**

Scheme 4. Reaction of esters 7a–m with dimethylsulfoxonium methylide.

Ester	R_1	R_2	R_3	R_4	Product (Yield %)
7a	NosNH	CH_3	H	CH_3	**8a (95)**
7b	NosNH	$CH(CH_3)CH_2CH_3$	H	CH_3	**8b (80)**
7c	NosNH	H	$CH(CH_3)CH_2CH_3$	CH_3	**8c (81)**
7d	NosNH	$(CH_2)_4NH(Boc)$	H	CH_3	**8d (75)**
7e	NosNH	$CH_2C_6H_4O(Bn)$	H	CH_3	**8e (97)**
7f	NosNH	$CH_2S(Trt)$	H	CH_3	**8f (70)**
7g	CbzNH	CH_3	H	CH_3	**8g (94)**
7h	BocNH	CH_2Ph	H	CH_3	**8h (99)**
7i	NosNH	CH_3	H	CH_2CH_3	**8a (70)**
7j	CbzalanylNH	CH_2Ph	H	CH_3	**8i (80)**
7k	H	CH_3 $(CH_2)_{10}$	H	CH_3	**8k (91)**
7l	H	Ph	H	C_6H_5	**6 (72)**
7m	H	Ph	H	CH_2Ph	**6 (70)**

Table 1. Results of the reaction of esters 7a–m with dimethylsulfoxonium methylide.

In each experiment the corresponding carboxyl acid was obtained in high yield and purity. The successful results make the reaction a valid procedure of general use for the ester cleavage. Figure 1 shows the ^1H NMR spectrum of *N*-nosyl-L-alanine (**8a**)

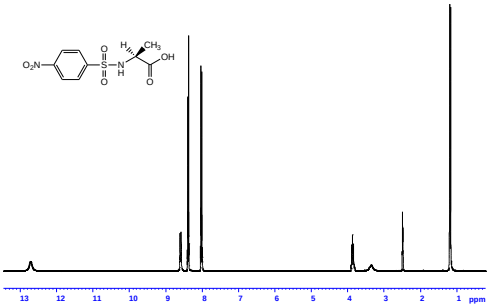


Figure 1. ^{1}H NMR spectrum of N-nosyl-L-alanine (8a).

We also verified that the configuration of the amino acid chiral center was retained by using *N*-nosyl-L-isoleucine methyl ester [(2*S*,3*S*)-*N*-nosylisoleucine, **7b**] as a starting substrate. Ester **7b** was treated with ylide **4** under the conditions adopted for this methodology. The reaction was complete in 30 min and provided *N*-nosyl-L-isoleucine (**8b**) as a unique product.

When *N*-nosyl-D-alloisoleucine methyl ester [(2*R*,3*S*)-*N*-nosylisoleucine,**7c**] was treated with methylide **4** under the same conditions, the corresponding *N*-nosyl-D-alloisoleucine (**8c**) was the only reaction product. The ^{1}H NMR spectroscopic data of **8b** and **8c** did not show any signals from possible epimers resulting from an inversion of the configuration at the α-carbon atom. Also the GC–MS analysis of each of the two crude products (**8b** and **8c**) revealed the presence of only one diastereomer. Moreover, a sample containing a mixture of crude

8b (20 mg) and **8c** (10 mg) was analyzed by GC–MS, and the corresponding chromatogram confirmed the complete separation of the two products (*Figure 2*).

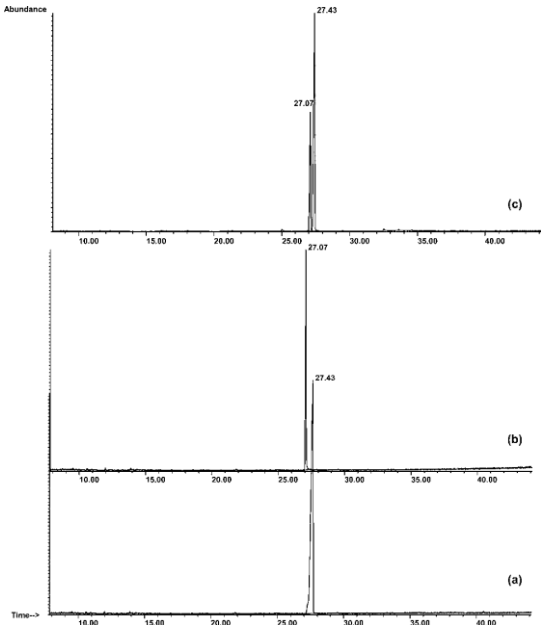


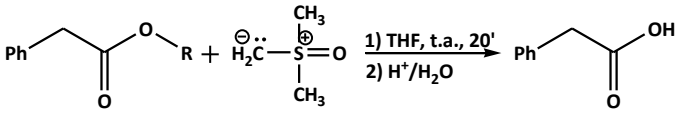
Figure 2. GC–MS analysis of (a) 8b, (b) 8c, and (c) mixture of 8b (20 mg) and 8c (10 mg).

The reaction has been utilized to convert the methyl esters of Nosyl-, Cbz- and Boc- protected amino acid into the corresponding amino acids. The deprotection of the carboxyl

function is fast and produces the corresponding acid in high yield also for the lysine N^{ε}-Boc-protected (**7d**), the *S*-trityl protected cysteine (**7f**) and the *O*-Bn-tyrosine (**7e**). The above result confirms the applicability of the procedure also in presence of acid-sensitive protecting groups in side chain.

With the same purpose, the procedure was attempted on the benzyl phenyl acetate and on the phenyl phenyl acetate (S*cheme 5*) (**7m** and **7l**). **7m**, treated with the ylide **4** in the usual conditions, provided as reaction product the phenyl acetic acid (**6**) in 70% yield. In the basic extract the benzyl alcohol was present. The reaction, monitored by TLC, showed the formation of the benzyl alcohol before the work-up treatment. These data suggest that the ester cleavage doesn't occur during the work-up step.

The reaction was also applied to the phenyl phenyl acetate (**7l**). In this case the phenyl acetic acid (**6**) was isolated in 72% yield and the phenol was detected in the slightly basic solutions.



7l-m **4** **6**

7l: R=Ph; 7m: R=CH_2Ph

Scheme 5. Results of the reaction of esters 7l-m with dimethyl-sulfoxonium methylide

The substrate **7l** lacks of a sp^3 hybridized carbon atom in the alcoholic moiety. As a consequence, the mechanism does not proceed through a nucleophilic attack of the methylide **4** on the carbon atom of the alcoholic moiety. In the light of the obtained results we decided to perform a series of experiments to exclude that the formation of carboxylic acid from the corresponding ester and the dimethylsulfoxonium methylide was due to a hydrolysis reaction related to the sodium hydroxide eventually present in the sodium hydride used in the procedure or formed during the reaction.

In previous experiments we have already observed that the addition of sodium hydroxide to the reaction mixture containing the phenyl acetic acid methyl ester and the dimethylsulfoxonium methylide decreases the reaction rate.

For this reason, an additional experiment was undertaken treating the methyl phenyl acetate with the dimethylsulfoxonium methylide in presence of a large excess of ^{18}O-labeled sodium hydroxide. The sodium hydroxide with a defined isotopic composition was used as a possible isotopic marker in the reaction of the ylide and phenyl acetic acid methyl ester. The labeled sodium hydroxide was added in large excess (5:1 respect to the methyl ester) to the reaction mixture. However the reaction gave as product only the carboxylic acid

containing oxygen 16 (*Figure 3*), confirming that the acid is not obtained by reaction of the hydroxide with the methyl ester.

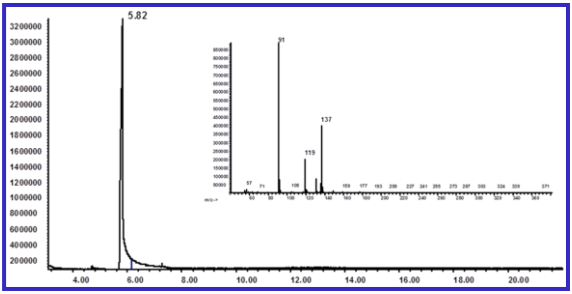


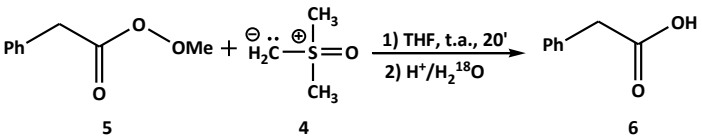
Figure 3. GC/MS analysis of phenyl acetic acid containing oxygen 16.

Therefore we assumed that the carboxyl acid doesn't result from an hydrolysis reaction and that the oxygen contained in the carboxyl acid doesn't belong to the used hydroxide.

Based on the obtained results and considering that the alcohol moiety is generated before the work-up treatment, we hypothesized that the conversion can proceed through an intermediate formed from the two reagents with subsequent loss of alcohol. This adduct can hydrolyze to carboxylic acid during the final work up. Another hypothesis is that during the reaction the intermediate evolves into carboxylic acid including the ylide's oxygen atom.

A last experiment that involved the use of ^{18}O-labeled water as an isotopic marker of the reaction products has provided important indications about the reaction mechanism. The

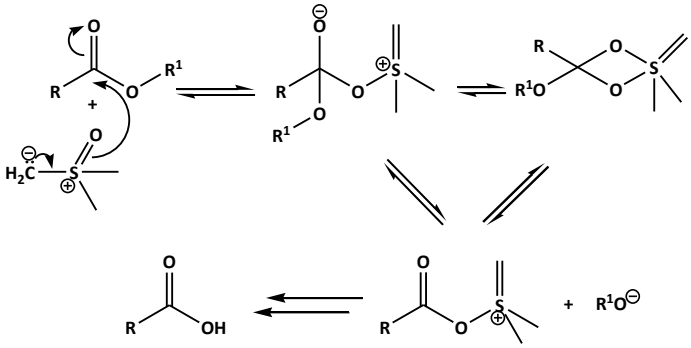
reaction of the methyl phenyl acetate with the methylide **4** was carried out under the usual conditions. However, the hydrolytic treatment in the final work up was performed using $H_2{}^{18}O$ (*Scheme 6*):



Scheme 6. Hydrolytic treatment with $H_2{}^{18}O$

The product analysis by mass spectrometry showed that the phenyl acetic acid contained in the acidic extracts had not incorporated the ^{18}O atom.

These experimental data are compatible with the formation of an intermediate (*Scheme 7*) consisting of a molecular adduct between the ylide and the ester. This intermediate is formed after nucleophile attack of the ylide on the carbon atom of the carbonyl group probably activated by the positively charged sulfur atom which coordinate the carbonyl oxygen. The intermediate first loss the alkoxy group and then probably forms the carboxylate incorporating the ylide's oxygen atom, after action of a base which could be the alkoxy group or another molecule of ylide.



Scheme 7. Hypothetical reaction mechanism between dimethyl-sulfoxonium methylide and a carboxylic ester.

None of the attempts to isolate the residue containing the sulfur atom was successful. Neither the GC/MS nor the ^{1}H-NMR analysis of the reaction crude products showed the presence of any by-products containing sulfur and different from the trimethylsulfoxonium ion.

The reaction of methylide **4** with esters highlights a new aspect in the reactivity of ylides. Resulting in the cleavage of the ester functionality and simulating a hydrolysis process, the reaction is general and proceeds with complex esters. Occurring rapidly under mild conditions, the reaction appears useful for solution-phase peptide synthesis when the final cleavage of the C-terminal amino acid ester function is required. Moreover, the configuration of the chiral center is maintained during the

reaction. All these considerations make this a valid and important procedure for ester cleavage in organic chemistry.

2. Simultaneous deprotection of amino and carboxyl function of N-Fmoc-α-amino acid and N-Fmoc-peptide esters with dimethylsulfoxonium methylide

2.1. Fmoc chemistry in peptide synthesis

One of the most widely used strategies in peptide synthesis is based on the masking of the amino function of α-amino acids by the 9-fluorenylmethoxycarbonyl (Fmoc) group.[5] This approach has found extended applications due to many advantages presented by the Fmoc protecting group. It is stable under various reaction conditions such as oxidation and reduction during the course of multi-step synthesis or total synthesis of natural products.[6] In addition it shows high stability towards trifluoroacetic acid (CF_3COOH), hydrogen bromide (HBr), which on the contrary, remove Boc (*tert*-butoxycarbonyl) and Cbz (benzyloxycarbonyl) groups, and tertiary amines. The Fmoc group is easily and typically removed by treatment of *N*-Fmoc-α-amino acids with a large excess of 20 % piperidine solution in DMF.[7]

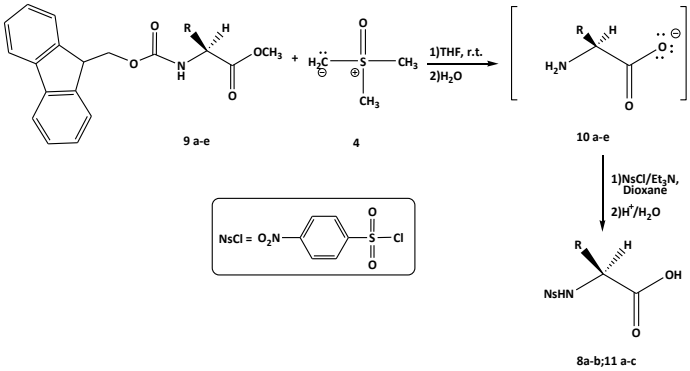
However, some limitations exist when this procedure is applied to solution phase peptide synthesis[8] due to the low volatility of

these solvents and the formation of the highly reactive dibenzofulvene (DBF) that induces various side reactions (polymerization, addition of amines and so on) causing difficulties in the purification step of the deprotected product. The sensitivity of the Fmoc group to Lewis acid is also reported.[9]

2.2 Deprotection of amino and carboxyl function of N-Fmoc-α-amino acid and N-Fmoc-peptide esters in solution phase peptide synthesis

We studied the reactivity of α-amino acid methyl esters protected on the amino function with 9-fluorenyl-methoxycarbonyl group (Fmoc) with dimethylsulfoxonium methylide. In a preliminary experiment *N*-Fmoc-L-alanine methyl ester (**9a**) (1 mmol) was treated at room temperature with dimethylsulfoxonium methylide (**4) (**2 mmol), freshly prepared from trimethylsulfoxonium iodide and sodium hydride. The reaction was monitored by thin-layer chromatography (TLC) and the conversion of the starting material was completed in 20 min. TLC analysis of the reaction mixture clearly revealed the formation of a new product with a low Rf value which gave a positive ninhydrin reaction. Our hypothesis was that the observed product it was the L-alanine (**10a**) (*Scheme 8*), deprotected on both amino and carboxyl function, suggested us

to recover the product after derivatization of amino function. To this aim, the crude reaction mixture, after hydrolysis, was treated with Nosyl chloride under basic conditions to obtain the corresponding *N*-Nosyl derivative **8a** (*Scheme 8*).



Scheme 8. Reaction of N-Fmoc protected α-amino acid methyl esters 9a-e with dimethylsulfoxonium methylide (4).

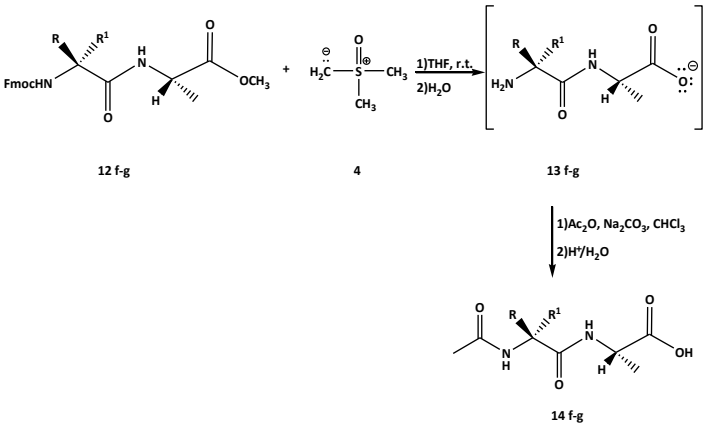
	R	Yield (%)
8a	CH_3	90
11a	$CH_2CH(CH_3)_2$	87
11b	$CH(CH_3)_2$	88
11c	CH_2Ph	88
8b	$CH(CH_3)CH_2\,CH_3$	87

Table 2. Results of the reaction of N-Fmoc-α-amino acid methyl esters 9a-e with dimethylsulfoxonium methylide (4).

^{1}H-NMR spectrum of the reaction product, recovered after acidic work up, clearly showed the presence of only *N*-Nosyl-L-Alanine (**8a**). This result demonstrated that the reaction of *N*-Fmoc-L-alanine methyl ester (**9a**) (*Scheme 8, Table 2*) with the ylide **4** provided the corresponding amino acid L-alanine **10a** free on both amino and carboxyl function. Encouraged by this result, the methodology was applied to other α-amino acid methyl esters *N*-Fmoc protected **9b-e** (*Scheme 8, Table 2*). The corresponding *N*-Nosyl-α-amino acids **8a-b** e **11b-c** were obtained in high yields (87-90%).

It is worth marking that the removal of the Fmoc group from the *N*-protected-α-amino acid derivatives **9a-e** occurred without evidence of racemization at the chiral α-carbon atoms. This aspect was investigated applying the reaction to *N*-Fmoc-L-isoleucine methyl ester (**9e**). In fact, the ^{1}H-NMR analysis of the crude reaction product **9e** did not show any signals relative to a possible epimer resulting from an inversion of the configuration at the α carbon atom. Another interesting goal of our study was the extension of the above described deprotection procedure to *N*-Fmoc protected peptide methyl esters. The deprotection methodology was applied to *N*-Fmoc-L-alanyl-L-alanine methyl ester (**12f**) (*Scheme 9*). The treatment of dipeptide **12f** with the ylide **4** gave the corresponding dipeptide **13f** free on both amino

and carboxyl function after only 20 min. The product **13f** was recovered as *N*-acetyl derivative 1**4f** after treatment of the hydrolyzed reaction mixture with acetic anhydride (*Scheme 9*).



Scheme 9. Reaction of N-Fmoc dipeptide methyl esters 12f-g with dimethylsulfoxonium methylide (4).

	R	R^1	Yield (%)
14f	CH_3	H	90
14g	H	CH_3	88

Table 3. Results of the reaction of N-Fmoc dipeptide methyl esters 12f-g with dimethylsulfoxonium methylide (4).

The stereochemistry of the reaction was also investigated performing the deprotection reaction on the *N*-Fmoc-D-alanyl-L-alanine methyl ester (**12g**, *Scheme 9*) diastereoisomer of **12f** in order to obtain the *N*-Acetyl-D-alanyl-L-alanine (**14g**) diastereoisomer of **14f** (*Table 3*). The crude *N*-acetylated dipeptides (**14f** and **14g**) were analyzed by ^{1}H-NMR. The corresponding spectra (**a** and **b**, *Figure 4*) revealed the presence of signals relative to only one diastereoisomer in each spectrum. To confirm this result a mixture of **14d** (10 mg) and **14f** (10 mg) was also analyzed by ^{1}H-NMR. The ^{1}H-NMR spectrum of the mixture (**c**, *Figure 4*) showed the separation of the singlets at 1.81 and 1.82 ppm relative to the methyl protons of acetyl group of the two diastereoisomers and the separation of the doublets at 1.24 and 1.26 ppm relative to the protons of one of the side chain methyl group of the two diastereoisomers. On the contrary, in the ^{1}H-NMR spectrum of each diastereoisomer **14f** and **14g** (*Figure 4*) we observed only one peak at 1.26 and 1.81 ppm, in the case of **14f** and at 1.24 and 1.82 in the case of **14g**.

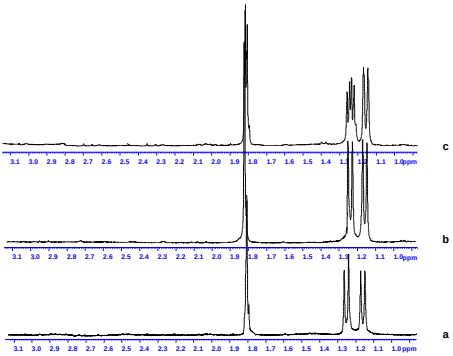
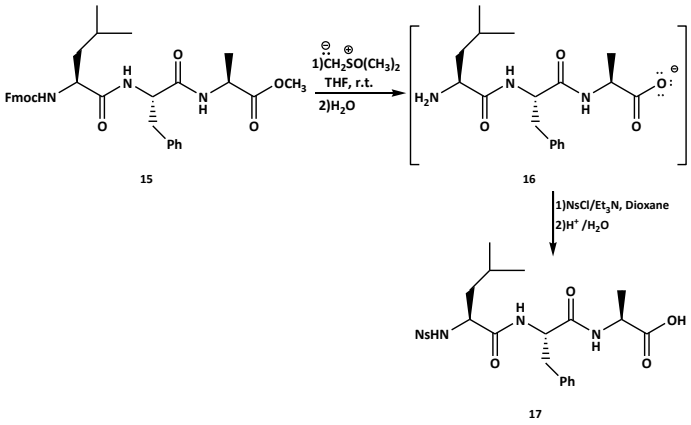


Figure 4. a) ^{1}H-NMR (DMSO-d_6) analysis of 14f; b) ^{1}H-NMR (DMSO-d_6) analysis of 14g; c) ^{1}H-NMR (DMSO-d_6) analysis of the mixture 14f and 14g.

These data clearly indicate that the deprotection reaction proceeds without inversion of configuration at the chiral centers present on the starting substrate. The simultaneous deprotection of both amino and carboxyl function was tested also on a tripeptide system, the *N*-Fmoc-L-leucyl-L-phenylalanyl-L-alanine methyl ester (**8**, *Scheme 10*), obtained by automated synthesizer and subsequently methylated on carboxyl function with a diethyl ether solution of diazomethane 0.66 M.



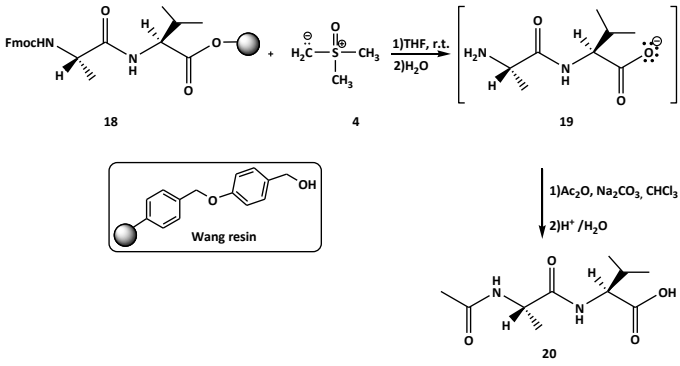
Scheme 10. Reaction of N-Fmoc tripeptide methyl ester 15 with dimethylsulfoxonium methylide (4).

The conversion of **15** into the total deprotected tripeptide was completed in 25 min. The deprotected product **16** was recovered as *N*-Nosyl derivative **17** (*Scheme 10*). All these successful results suggested us to apply the reaction also to resin anchored peptide systems. In particular, the methodology could represent a useful procedure for the cleavage of peptides from a solid support, when the peptide is anchored to the resin through an ester bond.

2.3 Deprotection of amino and carboxyl function of N-Fmoc-α-amino acid and N-Fmoc-peptide esters in solid phase peptide synthesis

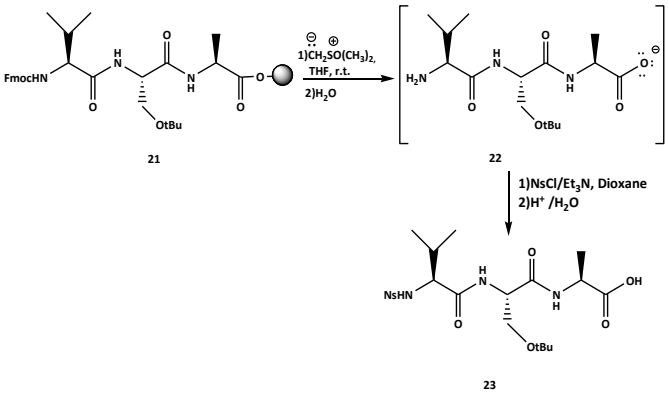
All these successful results suggested us to apply the reaction also to resin anchored peptide systems. In particular, the methodology could represent a useful procedure for cleavage of peptide from solid support, when the peptide is anchored to the resin through an ester bond.

In a first experiment a *N*-Fmoc protected dipeptide anchored by carboxyl function to the Wang resin, the *N*-Fmoc-L-alanyl-L-valine Wang resin (**18**), was treated with dimethylsulfoxonium methylide (**4**) in a molar ratio 1:2 with respect to the total active groups of the resin, assuming that the loading of the Wang resin with the first amino acid, L-alanine, was quantitative (*Scheme 11*).



Scheme 11. Reaction of N-Fmoc-L-Alanyl-L-Valine Wang resin (15) with dimethylsulfoxonium methylide (4).

After 30 min the reaction mixture was hydrolyzed and treated with acetic anhydride under basic conditions in order to convert the deprotected product **19** into the corresponding *N*-acetyl derivative **20**, that was recovered in 90% yield. To confirm this result, also a tripeptide system containing an acid-labile side chain protecting group, the *N*-Fmoc-L-valyl-L-*O*-*t*-butyl-serinyl-L-alanine Wang resin (**21**), was treated with the dimethylsulfoxonium methylide (**4**) in 1:2 molar ratio with respect to the total active groups of the resin (*Scheme 12*).



Scheme 12. Reaction of N-Fmoc-L-valyl-L-O-tert-butyl-serinyl-L-alanine Wang resin (21) with dimethylsulfoxonium methylide (4).

The conversion of **21** into the deprotected product **22** was complete in 30 min. **22** was recovered as Nosyl derivative **23** in 80% yield, keeping unchanged the acid-sensitive protecting group on the side chain.

All these experiments demonstrated that dimethylsulfoxonium methylide is an efficient reagent to deprotect in short time and in good yields the amino function of *N*-Fmoc protected peptides and to perform simultaneously their cleavage from Wang resin. We have developed a new methodology to deprotect simultaneously the amino and carboxyl function of *N*-Fmoc-α-amino acid and *N*-Fmoc-peptide methyl esters. The procedure

involves the use of dimethylsulfoxonium methylide and can be applied successfully to both solution and solid phase peptide synthesis. In solid phase peptide synthesis the dimethylsulfoxonium methylide deprotects, in short time and in good yields, the amino function of *N*-Fmoc protected peptides performing simultaneously their cleavage from Wang resin. Both the deprotection reactions occur rapidly with the same rate under mild conditions and do not cause any loss of the optical integrity at the chiral centers of the peptide. Furthermore in solution phase peptide synthesis, the use of methylide as deprotecting reagent of the amino function of *N*-Fmoc protected derivatives allows to separate easily under basic conditions the dibenzofulvene (DBF), an undesirable byproduct of the deprotection reaction. All these considerations make this methodology a valid and important procedure to obtain peptides free on both amino and carboxyl function at the end of a synthetic process using the Fmoc chemistry.

3. The N-acyl 4-nitrobenzenesulfonamides as "activatable protection" of carboxylic function in solution phase peptide synthesis

3.1. Activatable protecting groups of carboxyl function

Solution phase peptide synthesis is based on the appropriate combination of protecting groups together with an efficient method for the activation of the carboxyl group prior to reaction with the amino component. Formation of the desired amide bond can occur upon activation of the free carboxyl group. In general, transformation of a protecting group into an activating group towards a specific reaction at the preselected stage of a synthesis should be a useful tool in organic chemistry. Only a few examples of "activatable protecting groups" of carboxyl function are reported in the literature for peptide synthesis. Methylthioethyl esters[10] and 2-(p-nitrophenylthio)ethyl esters[11] are protecting groups of carboxyl function which result stable throughout the course of a peptide synthesis and are converted into the sulphonium salt or the sulphone at the final stage of liberation of the carboxyl group. The *p*-(methylthio)phenyl ester was described as an unactivated ester that is activated on oxidation to the sulfone, which then serves as an activated ester

in peptide synthesis.[12] Also the 2-(diphenylphosphino)ethyl ester is cleaved after quaternization with methyl iodide.[13] The 2-(benzyloxy)phenyl and the 2-(phenacyloxy)phenyl esters were described as protecting groups that are converted into the catechol monoester, an active ester easy removable at the end of the synthesis.[14] However, all these groups were described a long time ago and are rarely used in applied peptide synthesis due to the drastic conditions of activation which could affect the stability of the peptide or of potential protecting groups present in side chain.

3.2. N-acyl 4-nitrobenzenesulfonamides as "activatable protection" of carboxylic function in solution phase peptide synthesis

The development of new and efficient synthetic methodologies for the introduction and removal of amino acid protecting groups is an important target in peptide synthesis. Acylsulfonamide functionalities have been used in solid-phase peptide synthesis to connect the growing peptide chain to a sulfonamide anchor.[15] This linker is stable towards acidic and basic reaction conditions and nucleophiles, but is finally activated for cleavage by N-alkylation. The activation step

provides an activated C-terminus of the peptide which may react with diverse nucleophiles.[10]

We applied the sulfonamide-type Cα protection in solution-phase peptide synthesis (F*igure 5*).

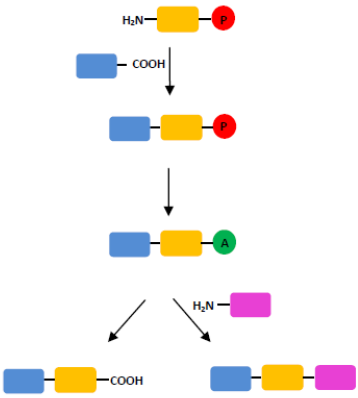
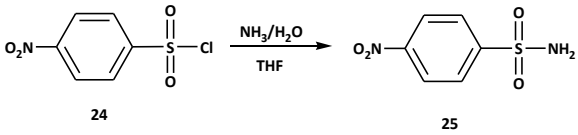


Figure 5. Graphical description of an "activatable protecting group" of carboxyl function.

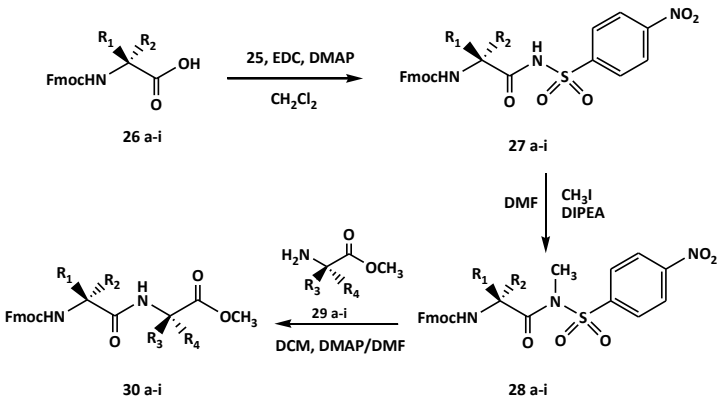
First, following a classical literature procedure,[16] we prepared the 4-nitrobenzenesulfonamide (nosylamide) by treatment of 4-nitrobenzenesulfonyl chloride (nosyl chloride) with an aqueous solution of ammonia (*Scheme 13*).



24 25

Scheme 13. Synthesis of 4-nitrobenzenesulfonamide (25)

The 4-nitrobenzenesulfonamide **25** was obtained in excellent yield (97%) and the spectroscopic data confirmed its purity. The 4-nitrobenzenesulfonamide, **25**, was used to synthesize *N*-acylsulfonamide derivatives. *N*-Fmoc-L-valine (**26a**) was chosen as model system and treated with 4-nitrobenzenesulfonamide **25** in presence of the condensing agent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and the dimethylaminopyridine (DMAP) as base (*Scheme 14*). The corresponding *N*-Fmoc-L-valine-4-nitrobenzenesulfonamide (**27a**) was recovered in high yield after 5 h stirring at room temperature (*Table 4*).



Scheme 14. Synthesis of N-Fmoc-α-aminoacyl-4-nitrobenzene-sulfonamides (27a-i), N-Fmoc-α-aminoacyl-N-methyl-4-nitrobenzene-sulfonamides (28a-i), and N-Fmoc-dipeptide methyl esters (30a-i).

Entry	R^1	R^2	R^3	R^4	Yield 27 (%)	Yield 28(%)	Yield 30 (%)
a	$CH(CH_3)_2$	H	H	CH_3	95	85	70
b	CH_2Ph	H	H	CH_3	94	86	68
c	CH_3	H	H	$CH_2CH(CH_3)_2$	80	92	75
d	$CH_2CH(CH_3)_2$	H	H	CH_3	85	90	76
e	$CH(CH_3)_2$	H	CH_3	H	95	80	72
f	H	CH_2Ph	H	CH_3	90	80	70
g	CH_2OtBu	H	H	CH_3	92	84	80
h	$CH_2C_6H_4OtBu$	H	H	CH_3	86	85	74
i	$CH_2CH_2COOtBu$	H	H	CH_3	80	87	75

Table 4. Results of the reactions of synthesis of N-Fmoc-α-aminoacyl-4-nitrobenzene-sulfonamides (27a-i), N-Fmoc-α-aminoacyl-N-methyl-4-nitrobenzene-sulfonamides (28a-i), and N-Fmoc-dipeptide methyl esters (30a-i).

In order to verify its activatability by *N*-alkylation in solution phase, we decided to methylate the sulfonamide nitrogen atom of **27a**. To this aim, we treated *N*-Fmoc-L-valinyl-4-nitrobenzenesulfonamide (**27a**) with methyl iodide and diisopropylethyl amine (DIPEA) for 3 h at room temperature. The corresponding *N*-methyl derivative **28a** was recovered, after a simple work-up, in very good yield (85%, *Table 4*). The subsequent coupling of the *C*-activated Fmoc-L-valine **28a** with the L-alanine methyl ester free on amino function (**29a**) required the use of a base (DMAP) and warming (*Scheme 14*). The completion of the reaction was monitored by TLC. After column chromatography, the corresponding dipeptide **30a** was recovered in good yield and high purity (*Table 4*). In the light of the successful result and to validate this carboxylic function activation procedure, the same reactions were applied to a series of *N*-Fmoc-α-amino acids (**26b-i**, *Table 4*).

In each experiment the corresponding dipeptide was obtained in good yield. These results make the 4-nitrobenzenesulfonamido group methylated on nitrogen atom a good activating agent of carboxyl function. The coupling, in fact, proceeds successfully also in the case of amino acids sterically hindered or functionalized in side chain (see *Table 4*). We also wanted to study that the configuration of the amino acid chiral centers was

retained during the coupling reaction by synthesizing dipeptides Fmoc-L-valinyl-L-alanine methyl ester (**30a**) and Fmoc-L-valinyl-D-alanine methyl ester (**30e**) and dipeptides Fmoc-L-phenylalanyl-L-alanine methyl ester **30b** and Fmoc-D-phenylalanyl-L-alanine methyl ester **30f**. The coupling of compounds **28** with compounds **29** was performed under the conditions usually adopted for this methodology. Each dipeptide was analyzed by ^{1}HMNR (*Figure 6a and 6b*). Furthermore, a sample containing a mixture of crude Fmoc-L-valinyl-L-alanine methyl ester (**30a**, 11 mg) and Fmoc-L-valinyl-D-alanine methyl ester (**30e**, 19 mg) was analyzed by ^{1}H-NMR and the corresponding spectrum showed the complete separation of the signal at 6.27 and 6.46 ppm relative to the amidic proton of the two dipeptides (*Figure 6c*). This confirmed the absence of any signal relative to possible epimers in the ^{1}H-NMR spectra of the single dipeptides **30a** and **30e** and, in this way, showed the total retention of configuration of the chiral centers during the coupling reaction. Also Fmoc-L-phenylalanyl-L-alanine methyl ester (**30b**) and Fmoc-d-phenylalanyl-L-alanine methyl ester (**30f**) were analyzed by ^{1}H NMR to compare the signals relative to the two diastereoisomers (*Figure 6*). Also in this case there was a good separation of some signals, in particular of the signal at 6.43 and 6.16 ppm relative to the amidic proton. This last result

confirmed the presence of only one distereoisomer in the spectrum of each crude dipeptide **30b** and **30f** and showed the retention of the chiral centers during the coupling reaction.

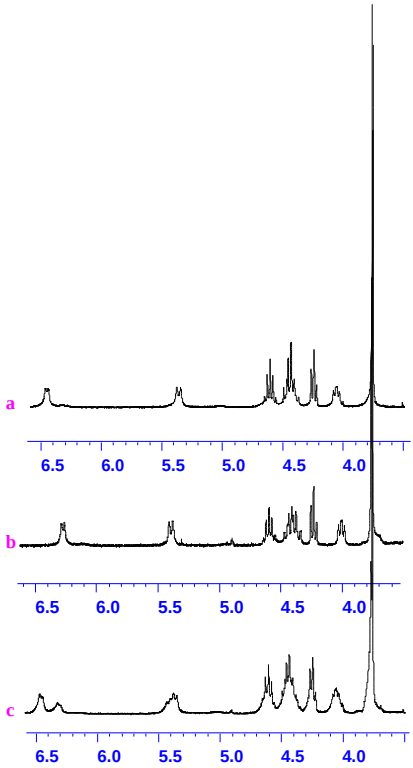


Figure 6. a) ^{1}H NMR spectrum of Fmoc-L-valinyl-D-alanine methyl ester (30e), b) ^{1}H NMR spectrum of Fmoc-L-valinyl-L-alanine methyl ester (30a), c) ^{1}H NMR spectrum of a mixture of 30e (19 mg) and 30a (11 mg).

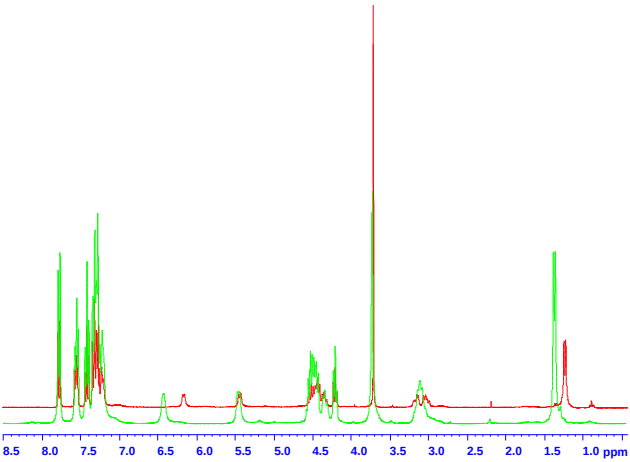
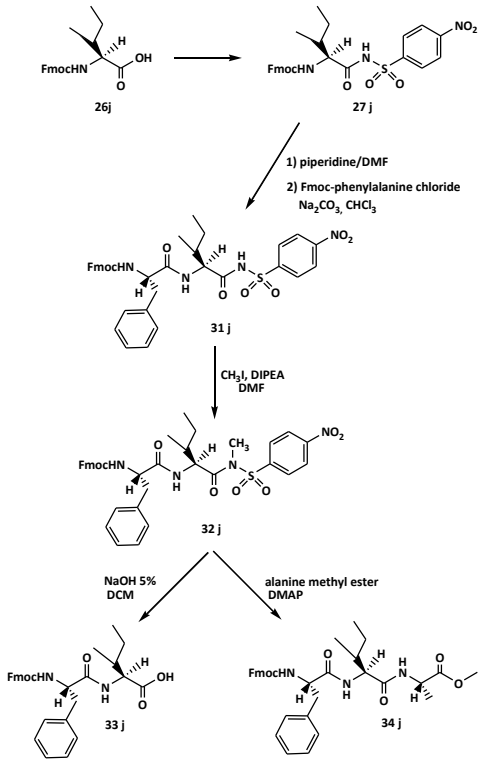


Figure 7. Overlapped ^{1}H NMR spectra of Fmoc-L-phenylalanyl-L-alanine methyl ester (30b, green) and Fmoc-D-phenylalanyl-L-alanine methyl ester (30f, red).

Once verified the possibility of activation of the *N*-acyl sulfonamide group towards acyl nucleophilic substitution through *N*-alkylation, we wanted to confirm the stability of the same group unmethylated under the conditions usually adopted in solution-phase peptide synthesis (Fmoc-chemistry). As consequence, the *N*-Fmoc-L-isoleucinyl-4-nitrobenzen-sulfonamide (**27j**) was synthesized as previously described (*Scheme 14*). **27j** was then deprotected on amino function using a solution of piperidine in DMF. The corresponding isoleucinyl-4-nitrobenzenesulfonamide free on amino function was immediately coupled with the *N*-Fmoc-L-phenylalanine activated

as chloride (*Scheme 15*). The dipeptide *N*-Fmoc-L-phenylalanyl-L-isoleucinyl-4-nitrobenzenesulfonamide **31j** was recovered in high yield (*Scheme 15*, 85%). Thus confirmed the stability of *N*-acyl sulfonamide towads Fmoc deprotection and coupling condition and, in this way, the suitability of 4-nitrobenzenesulfonamido group as protecting group of carboxyl function in peptide synthesis. The subsequent *N*-methylation of the sulfonamide nitrogen of **31j** gave the corresponding methylated *N*-Fmoc-L-phenylalanyl-L-isoleucinyl-4-nitrobenzenesulfonamide (**32j**), a dipeptide activated on carboxyl function (*Scheme 15*). In this form the peptide could result enabled for the coupling with another amino acid in N → C direction or for the basic hydrolysis to obtain the free carboxyl function. To confirm this trend, we performed two experiments in parallel. In a first experiment **32j** was treated with the L-alanine methyl ester (*Scheme 15*) adopting the coupling condition previously described. The tripeptide methyl ester **34j** was recovered in high yield (75%, *Scheme 15*) after a simple acidic treatment. Another reaction was performed to verify the activation of carboxyl function also towards basic hydrolysis condition. In this case, the same activated dipeptide **32j** was treated with a 5% aqueous solution of NaOH (*Scheme 15*). After 4 h reaction at room temperature, the dipeptide *N*-Fmoc-

phenylalanylisoleucine (**33j**) was recovered in very good yield (82 %).



Scheme 15. Synthesis of N-Fmoc-L-phenylalanyl-L-isoleucinyl-4-nitrobenzenesulfonamide (31j), its conversion into N-Fmoc-L-phenylalanyl-L-isoleucinyl-N-methyl-4-nitrobenzenesulfonamide (32j) and the subsequent basic hydrolysis to obtain the dipeptide 33j or coupling in N → C direction to obtain the tripeptide methyl ester 34j.

In the light of the obtained results it appears evident that this method of protection of carboxyl function of α-amino acids and its subsequent activation could be of great utility in peptide synthesis. The 4-nitrobenzenesulfonamido group could be simply used as protecting group of carboxyl function, resulting stable under the conditions adopted in solution phase peptide synthesis, or as activating group of carboxyl function after *N*-methylation, or even as "activatable protecting group" when the synthesis requires a change of direction C → N versus N → C such as in convergent peptide synthesis.

Chapter 2

Synthesis of modified amino acids

1. N-Alkylation of N-arylsulfonyl-α-amino acid methyl esters by trialkyloxonium tetrafluoroborates

1.1. N-Alkyl-α-amino acids

N-Alkyl-α-amino acids are a representative class of amino acid derivatives. They find application as synthetic building blocks in medicinal chemistry as well as molecular probes in studies related to the structural properties and biological activities of *N*-alkylated peptides and peptidomimetics.[17] These are species largely represented in nature especially in marine organisms.[18] *N*-Alkyl- α-amino acids are not only biologically active.

N-Methyl-α-amino acids, in particular, are found in nature as free compounds and as structural constituents of various peptides belonging to the cyclosporine,[19] dolastatin,[20] and didemnin families.[21] The incorporation of *N*-methyl and in general of different *N*-alkyl-α-amino acids into peptide chains often improves proteolytic stability, conformational rigidity, lipophilicity, and transport properties.[22]

One of the most widely employed processes of *N*-methylation of α-amino acids by alkylation under various conditions is to utilize

their *N*-arylsulfonyl protected derivatives. The sulfonamide protection in fact greatly enhances the acidity of the α-NH function,[23] allowing a rapid deprotonation under basic conditions. In the presence of the required alkylating reagent it furnishes the desired *N*-arylsulfonyl-*N*-methyl-α-amino acids.

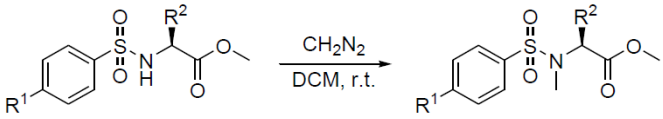
N-Arylsulfonyl groups are also used as pharmacophores, especially when substituted with alogens at the 4-position of the aryl portion.[24] *N*-Arylsulfonyl-α- amino acids are in fact constituents of metalloproteases and carbonic anhydrase inhibitors.

1.2. Synthesis of N-Alkyl-α-amino acids

We exploited the possible use of different methods for the N-alkylation of 4-substituted N-arylsulfonyl-α-amino acid methyl esters. Diazomethane and trimethyloxonium tetrafluoroborate showed a sensibly different behavior in the N-methylation.

We started our evaluation by subjecting the series of *N*-arylsulfonyl- α-amino acid methyl esters **35a-i** (*Table 5*) to the *N*-methylation with diazomethane (*Scheme 16*), according to the synthetic procedure previously used for the process performed on a number of *N*-nosyl protected derivatives. *N*-Arylsulfonyl-α-amino acid methyl esters **35a-i** have been prepared in excellent yields by reacting the corresponding α-amino acid methyl ester

hydrochloride with the required arylsulfonyl chloride in the presence of triethylamine and applying a general experimental protocol. [25]



35a-i **36a-i**

Scheme 16. Diazomethylation of 35a-i under neutral conditions

Entry	R^1	R^2	Product	Yield(40')	Yield(120h)
35a	NO_2	CH_3	**36a**	96	-
35b	NO_2	$CH(CH_3)_2$	**36b**	89	-
35c	NO_2	$CHCH_3CH_2CH_3$	**36c**	95	-
35d	NO_2	CH_2Ph	**36d**	99	-
35e	F	CH_3	**36e**	23	62
35f	Cl	CH_3	**36f**	19	59
35g	CH_3	CH_3	**36g**	12	50
35h	OCH_3	CH_3	**36h**	7	47
35i	H	CH_3	**36i**	15	55

Table 5. N-Methylation of N-arylsulfonyl-α-amino acid methyl esters 35a-i: use of diazomethane.

Our first aim was to compare the reactivity of a series of *N*-arylsulfonyl-α-amino acid methyl esters containing electron-withdrawing (F, Cl), and electron-releasing (CH_3, OCH_3) groups at the 4 position of the aromatic portion of the sulfonamide moiety, as opposed to their *N*-nosyl protected analogues.

Diazomethane presents optimal properties for quantitative *N*-methylation of the α-amino acid derivatives **35a-d**, without need of other reagent assistance in a "one-pot" process. However, it is worth to note that, in the case of the *N*-arylsulfonyl derivatives **35e-i** containing 4-substituents which are different from the NO_2 group (*Table 5*), this alkylating reagent determines variable overall yields in the corresponding final products **36e-i**. Results are referred to the yields in isolated products after a maximum reaction time of 40 min. It is important to note that conversion of the starting materials **35a-d** is complete after this time, while it is evident that *N*-arylsulfonyl derivatives **35e-i** show to be much less reactive than the 4-NO_2 substituted analogues. In the case of sulfonamides **35e-i** in fact very low yields in isolated products are obtained after 40 min. Moreover the amount of the respective *N*-methylated derivatives does not appreciably increase even after longer times of treatment.

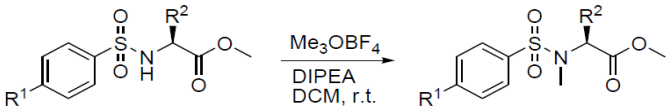
Conversions of the less acidic *N*-arylsulfonyl derivatives containing a halogen atom (F, Cl), or electron-releasing groups

(CH_3, OCH_3), or featuring no substitution at the 4-position of the aromatic ring, are not complete also after 120 hours.

The protocol based on the use of diazomethane fails when the acidity of the sulfonamide NH moiety is sensibly reduced. In the cases of N-arylsulfonamides not containing substituents at the 4-position of the aromatic ring, or bearing electron-releasing groups at the same carbon atom, the treatment with diazomethane is ineffective.

To this aim, we have shifted our attention to different and more efficient N-alkylating "one-pot" methodologies, we selected trialkyloxonium tetrafluoroborates species, because it is a method general in its applicability, convenient, mild, and which would not require the use of hazardous reagents. As recently proposed by our research group, triethyloxonium tetrafluoroborate can successfully be used for the chemospecific *N*-ethylation of *N*-nosyl protected α-amino acid methyl esters.

The N-methylation with trimethyloxonium tetrafluoroborate was performed in dichloromethane and in the presence of DIPEA, at room temperature (*Scheme 17*). The reaction resulted to be fast and chemospecific in all the analyzed cases in table 6.



35a-i **36a-i**

Scheme 17. Reaction of 35a-i with trimethyloxonium tetrafluoroborate.

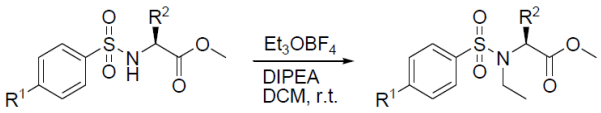
Entry	R^1	R^2	Product	Yield[(a)]	Time[(b)]
35a	NO_2	CH_3	36a	Quantitative	15
35b	NO_2	$CH(CH_3)_2$	36b	Quantitative	15
35c	NO_2	$CHCH_3CH_2CH_3$	36c	Quantitative	15
35d	NO_2	CH_2Ph	36d	Quantitative	15
35e	F	CH_3	36e	Quantitative	60
35f	Cl	CH_3	36f	Quantitative	75
35g	CH_3	CH_3	36g	Quantitative	90
35h	OCH_3	CH_3	36h	Quantitative	110
35i	H	CH_3	36i	Quantitative	90

[a]Isolated products. [b]Times expressed in min.

Table 6. N-Methylation of N-arylsulfonyl-α-amino acid methyl esters 35a-i: use of trimethyloxonium tetrafluoroborate.

Contrary to diazomethane, the reaction of **35a-i** with trimethyloxonium tetrafluoroborate (*Scheme 17*), resulted to be clean and rapid in all the analyzed cases. The desired products **36a-i** were isolated in quantitative yields. Our work continued

with the exploitation of the potentialities of trialkyloxonium salts in obtaining *N*-alkylated derivatives of *N*-arylsulfonyl-α-amino acid methyl esters in which the aromatic ring is substituted with groups other than NO_2. This further test was triggered by our interest in the biological implications that some *N*-ethyl derivatives of α-amino acids and peptides can have.[26] The *N*-arylsulfonyl-α-amino acid methyl esters **35a-d**, **35g-m** were subjected to treatment with trialkyloxonium tetrafluoroborate at room temperature, in dichloromethane and in the presence of the base DIPEA (*Scheme 18*).



35a-m **37a-m**

Scheme 18. Reaction with triethyloxonium tetrafluoroborate.

Entry	R^1	R^2	Product	Yield(%)[a]	Time[b]
35a	NO_2	CH_3	**37a**	96	10
35b	NO_2	CH $(CH_3)_2$	**37b**	89	10
35c	NO_2	$CH(CH_3)CH_2CH_3$	**37c**	95	10
35d	NO_2	CH_2Ph	**37d**	99	10
35g	CH_3	CH_3	**37g**	91	90
35h	OCH_3	CH_3	**37h**	90	120
35i	H	CH_3	**37i**	93	85
35l	F	CH $(CH_3)_2$	**37l**	94	65
35m	Cl	CH_2CH $(CH_3)_2$	**37m**	93	70

[a]Isolated products.[b]Times expressed in min.

Table 7. N-Ethylation of N-arylsulfonyl-α-amino acid methyl esters: use of triethyloxonium tetrafluoroborate.

The data collected about the *N*-ethylation with triethyloxonium tetrafluoroborate of the same systems further demonstrate the validity of the discussed methodology in providing N-ethylated derivatives of *N*-arylsulfonyl-α-amino acid methyl esters.

Trialkyloxonium tetrafluoroborates are powerful reagents for the mild, rapid, clean, and chemospecific *N*-alkylation of *N*-arylsulfonyl-α-amino acid methyl esters. These alkylating species can be safely handled and offers great advantages in terms of effectiveness and applicability. As disclosed here, trimethyloxonium tetrafluoroborate represents the reagent of reference especially in those cases in which diazomethane

results to be ineffective. Furthermore, the *N*-ethylation is easily realized using triethyloxonium tetrafluoroborate. In all cases, the alkylated products are obtained pure without need for chromatography.

We exploited the possible use of different methods for the *N*-alkylation of 4-substituted *N*-arylsulfonyl-a-amino acid methyl esters. Diazomethane and trimethyloxonium tetrafluoroborate showed a significantly different behavior in the *N*-methylation. For both methylating agents, the reaction seemed to be controlled by the acidity of the sulfonamide NH functionality. Systems characterized by a drastically enhanced acidity of the NH residue, e.g., *N*-nosyl derivatives, can efficiently be *N*-methylated either by trimethyloxonium tetrafluoroborate or diazomethane. The latter showed to be basic enough to generate the conjugated base of the sulfonamide derivatives, allowing diazomethylation under neutral conditions.

However, the protocol based on the use of diazomethane fails when the acidity of the sulfonamide NH moiety is sensibly reduced. In the cases of *N*-arylsulfonamides not containing substituents at the 4-position of the aromatic ring, or bearing electron releasing groups at the same carbon atom, the treatment with diazomethane is ineffective. In these circumstances, the base DIPEA, able to assists the *N*-methylation

when combined with trimethyloxonium tetrafluoroborate, allowed the rapid, clean, and quantitative reaction also in the presence of *N*-arylsulfonyl-α-amino acid methyl esters containing a less acidic NH functionality.

The effect of the different types of substituent placed at the 4-position of the *N*-arylsulfonamide moiety is crucial for the NH properties also when *N*-methylated derivatives have to be prepared by using trimethyloxonium tetrafluoroborate. This reagent can successfully replace diazomethane. In fact, trimethyloxonium tetrafluoroborate is more efficient than diazomethane in the *N*-methylation of less reactive substrates and it is much safer. The data collected about the *N*-ethylation with triethyloxonium tetrafluoroborate of the same systems further demonstrate the validity of the discussed methodology in providing *N*-ethylated derivatives of *N*-arylsulfonyl-α-amino acid methyl esters.

Chapter 3

New methodologies for the extraction and measurement of amino acids and free fatty acid in meat products.

1. Simultaneous extraction and derivatization of amino acids and free fatty acids in meat products

1.1. Important parameters used to establish the quality of meat products

In Italy, different types of dry fermented sausages are produced; these are often typical and characteristic of places and regions and can be associated with the Mediterranean people's history. In many cases these products are produced in consumers' homes (homemade) using traditional recipes handed down from generation to generation.

Today, traditional sausages are also produced in the factories by adapting modern technologies to the needs of industrial production and trying to maintain as much as possible the organoleptic characteristics of homemade products. Dry fermented sausages are the product of microbiological, biochemical, physical, and sensory changes, occurring in meat paste stuffed into natural or synthetic casings during its

maturation under certain conditions of humidity and temperature.[27] The typical flavor of a dry fermented sausage is a result of the bacterial and also muscle enzyme metabolism of carbohydrates, proteins, and lipids.

Lipolytic[28] and proteolytic processes[29] are catalyzed by endogenous and microbial proteases and lipases. Proteases of microbial origin, together with the endogenous enzymes, are responsible for the hydrolysis of proteins and the subsequent formation of smaller degradation products: low-molecular-weight peptides and free amino acids.[30] Peptides and free amino acids are major components of the non-protein nitrogen fraction in fermented meat products.[31] Free amino acids directly affect the basic taste of dry fermented sausages.[32] Moreover, acting as precursors of volatile compounds, free amino acids may be decarboxylated and deaminated to yield amines and organic acids, respectively, which may in turn lead to other changes, yielding volatile flavor compounds in the final product.[33] The evaluation of free amino acid composition serves as quality and typicality parameter of several salami varieties.

Moreover, during the ripening of meat products, with particular reference to fermented sausages, lipases are also active. These bacterial and/or endogenous enzymes affect the structure of glycerides, determining their partial hydrolysis. Consequently,

the formation of variable amounts of free fatty acids is observed.[34] The influence of the fermentation process on fatty acid level, especially in sausages, is of great interest in the recent literature.[35]

As consequence, it seems of primary importance to evaluate the effectiveness of simple and highly efficient procedures to determine the amino acid and fatty acid contents and their changes during processing, fermentation, ripening, and storage of meat products.

A series of extractive and chromatographic methods for the evaluation of the amino acid and free fatty acid profiles in meats are available.

Several protocols require the use of aqueous media for the extraction of the analytes from meat matrices. Precipitation of the protein content and ultracentrifugation of the resulting suspension are the two necessary steps for the sampling. The qualitative and quantitative analysis of amino acids is then performed by conventional instrumental methods.

The analysis of free fatty acids in meat products may be problematic, especially due to the high protein and peptide contents.[36] It is obvious that it is necessary to employ an effective deproteinization technique also in the case of free fatty acid sampling from natural matrices. The most widely used

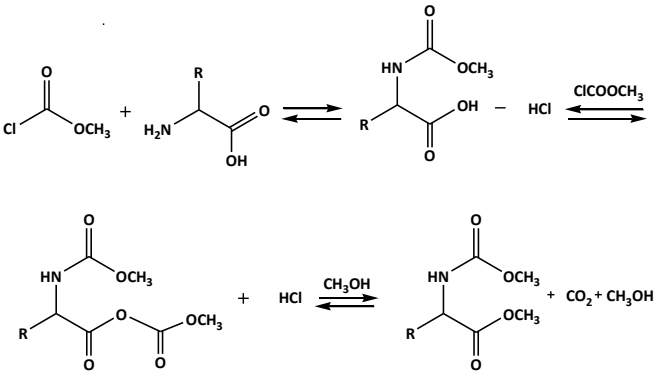
methods include precipitation with acids, ultrafiltration, and chemical and/or physical separation with organic solvents and membranes of different cut-off.

The aim of the present study is to propose a highly efficient treatment for the simultaneous derivatization and extraction of amino acids and free fatty acids in meat products and to further evaluate at once their profiles. "Salsiccia piccante", a traditional fermented pork sausage produced in Calabria, a region in Southern Italy, was selected as case of study.

1.2. Extraction and derivatization of amino acids and free fatty acids

We designed a highly efficient protocol for the easy and simultaneous extraction and derivatization of amino acids and free fatty acids contained in meat and its related products, followed by GC-MS analysis in order to evaluate their profiles. The procedure should be of general application for many kinds of meat matrices. It also should present some important characteristics: (i) the exclusive use of organic solvents, avoiding aqueous environments during the extraction and derivatization steps; (ii) the chemical derivatization and the extraction of analytes are performed simultaneously after homogenization of the meat matrix; (iii) formation of analytes with drastically

enhanced solubility in organic solvents; (iv) all the amino and carboxyl groups of the analytes can be chemically derivatized, with no exclusion of the reactive functions on the amino acid side-chains, by using only one reagent system. MCF[37] was selected as derivatizing agent. Amino acids are transformed into their *N*-methyloxycarbonyl derivatives. Moreover, carboxylic moiety of these intermediates can further interact with the same reagent to give the corresponding anhydrides which, in turn, generate methyl esters in the presence of methanol (*Scheme 19*). In analogy, free fatty acids are transformed into the corresponding methyl esters.



Scheme 19. Derivatization of amino acids and their transformation into their N-methyloxycarbonyl methyl esters.

In our procedure, methanol acts at the same time as a non aqueous solvent for the extraction of analytes from the meat matrices and as reagent for the methyl ester formation of free fatty acids and *N*-methyloxycarbonyl amino acids. For each class of analytes, all components were quantified by the corresponding peak area value according to those of both the internal standards. Figure 8 shows a typical GC–MS chromatogram obtained from the analysis of samples of cured sausages.

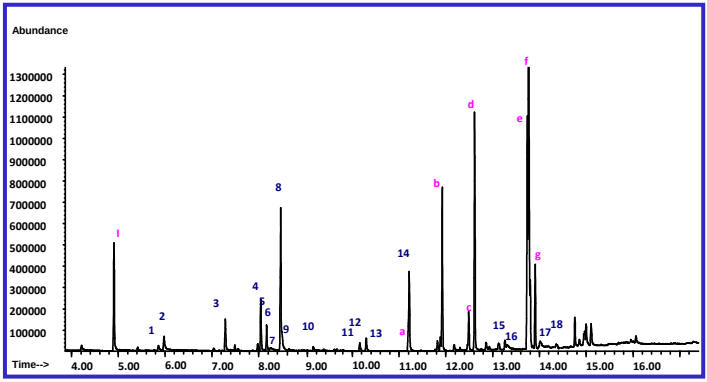


Figure 8. GC–MS (EI) analysis of a sample of "salsiccia"

The GC‑MS chromatogram clearly displays the peaks relative to the free amino acid derivatives that can be detected and identified (numbered peaks) and to the free fatty acid methyl esters (peaks indicated by letters). In the same chromatogram two additional peaks (**8** and **b**) are clearly visible: they are generated by *N*-methyloxycarbonyl norleucine methyl ester (peak **8**) and methyl pentadecanoate (peak **b**), used as internal standards. In the extract obtained from the sausages subjected to analysis 13 amino acid and 6 fatty acid derivatives were unequivocally recognized. The completeness of the reaction between MCF and amino acids was verified by further performing a set of three experiments at variable times (*Table 8*).

The results clearly indicated that only 10 min were required for the best performance of the treatment, and that more prolonged times are not necessary. To test the repeatability, the measurement of the concentration levels of each amino acid and free fatty acid, was repeated three times for each reaction time. The results are reported in Table 8 and show the good repeatability of the procedure.

Peak	Analyte	Amount[a] (t = 10 min)	Amount[a] (t = 20 min)	Amount[a] (t = 5h)
1	Gly	9.8 ± 0.3	9.3 ± 0.4	8.1 ± 0.3
2	Ala	74.5 ± 1.8	73.8 ± 2.6	54.6 ± 1.5
3	Val	29.9 ± 0.6	33.3 ± 1.2	44.3 ± 1.2
4	Leu	59.7 ± 1.2	60.3 ± 1.2	55.3 ± 1.5
6	Ile	43.9 ± 0.8	31.8 ± 1.5	34.2 ± 1.5
7	Thr	21.2 ± 0.7	19.4 ± 0.3	18.0 ± 0.9
9	Pro	9.4 ± 0.4	8.5 ± 0.3	11.8 ± 0.5
10	Asp	9.5 ± 0.4	8.8 ± 0.3	7.1 ± 0.3
11	Glu	31.6 ± 1.1	28.2 ± 0.7	24.8 ± 1.1
12	Met	19.2 ± 0.6	20.1 ± 0.6	15.9 ± 0.5
14	Phe	67.2 ± 1.5	64.6 ± 1.7	62.4 ± 1.6
15	Lys	81.5 ± 1.6	67.2 ± 1.4	65.4 ± 1.7
18	Tyr	6.8 ± 0.2	4.6 ± 0.2	4.3 ± 0.2
a	Miristic acid	11.4 ± 0.6	12.7 ± 0.3	10.0 ± 0.5
c	Palmitoleic acid	77.6 ± 2.0	62.8 ± 1.7	59.7 ± 2.1
d	Palmitic acid	197.0 ± 3.5	196.2 ± 2.4	175.5 ± 3.1
e	Linoleic acid	251.0 ± 6.7	207.3 ± 4.7	199.0 ± 3.6
f	Oleic acid	362.3 ± 6.0	310.4 ± 7.0	328.5 ± 5.9
g	Stearic acid	70.5 ± 2.7	71.3 ± 2.4	65.7 ± 2.1

[a]Amounts are expressed as mg of analytes/100 g of matrix and reported as mean value ± SD (n = 3)

Table 8. Determination of analytes at different reaction times.

We have also exploited the possible role that can be exerted by MCF in the extraction-derivatization process of the amino acid and free fatty acid contents present in the sausages case of study.

We was performed two experiments characterized, respectively, by extraction in presence of MCF and extraction and post-

derivatization with MCF, clearly indicated that MCF plays a crucial role in the procedure. In fact, the derivatizating reagent is able to react either with amino acids or free fatty acids, determining a shift of the equilibrium of solubility of the target analytes, and making their extraction from the meat matrix easy at the same time. In order to verify that during the extraction-derivatization with MCF no transesterification products are produced from triacylglycerols and/or phospholipids present in the meat matrix, a further experiment was performed, in which tristearine was exposed to the action of the system methanol/dichloromethane/pyridine/MCF. No traces of stearic acid methyl ester were detected by mono-dimensional high-resolution proton NMR analysis of the recovered reaction product. From all samples, analytes were recovered with percentage values ranging from 88% to 97% for amino acids, and from 92 to 98% for free fatty acids (*Table 9*).

Analyte	Recovery[a] %
Gly	96 ± 1
Ala	91 ± 1
Val	88 ± 3
Leu	93 ± 2
Ile	94 ± 1
Thr	94 ± 6
Pro	95 ± 1
Asp	89 ± 3
Glu	89 ± 1
Met	90 ± 4
Phe	97 ± 0
Lys	90 ± 2
Tyr	93 ± 5
Miristic Acid	94 ± 2
Palmitoleic Acid	92 ± 4
Palmitic Acid	98 ± 0
Linoleic Acid	95 ± 1
Oleic Acid	92 ± 1
Stearic Acid	94 ± 3

[a]Mean values ± SD (n = 3)

Table 9. Recovery test.

Finally, we compared the proposed method with an extraction procedure already reported in literature and widely used for the measurement of amino acids in natural matrices (*Table 10*).[38]

Peak	Analyte	Amount[a] (MCF-assisted extraction)	Amount[a] (Literature procedure [23])
1	Gly	9.8 ± 0.3	5.6 ± 0.2
2	Ala	70.9 ± 2.1	35.8 ± 0.8
3	Val	51.6 ± 2.3	20.7 ± 0.6
4	Leu	68.0 ± 2.4	38.5 ± 1.3
6	Ile	34.0 ± 1.5	19.0 ± 0.5
7	Thr	25.5 ± 0.9	20.5 ± 0.6
9	Pro	14.4 ± 0.6	24.2 ± 0.4
10	Asp	13.4 ± 0.5	13.8 ± 0.2
11	Glu	123.3 ± 3.0	125.0 ± 2.0
12	Met	23.3 ± 1.2	20.7 ± 0.8
14	Phe	69.5 ± 2.3	50.0 ± 1.0
15	Lys	134.6 ± 3.9	137.7 ± 1.5
18	Tyr	5.2 ± 0.4	3.7 ± 0.2
a	Miristic Acid	13.3 ± 0.7	18.4 ± 0.8
c	Palmitoleic Acid	70.1 ± 3.1	55.8 ± 1.4
d	Palmitic Acid	213.2 ± 5.8	168.3 ± 2.8
e	Linoleic Acid	264.4 ± 7.7	210.1 ± 0.9
f	Oleic Acid	436.4 ± 10.7	348.2 ± 3.9
g	Stearic Acid	82.8 ± 2.3	63.1 ± 1.7

[a]Amounts are expressed in mg of analyte/100 g of matrix and reported as mean value ± SD (n = 3)

Table 10. Comparison between the MCF-assisted extraction and a literature procedure.[17]

We developed a protocol for the accurate measurement of free amino acids and fatty acids in meat and meat derivatives. Free amino acids and fatty acids are extracted and derivatized simultaneously by a fast reaction with MCF under heterogeneous phase conditions, in a system not containing water. The method shows good analyte recovery, repeatability, and accuracy and can be considered an improvement over

conventional techniques usually employed for the analysis of meat derivatives.

2. *Comparison of Free Amino Acids and Biogenic Amines content between Industrial and Homemade Products*

Industrial fermented sausages were manufactured using a commercially available lyophilized starter formulation (SAGA T, Kerry Ingredients & Falvours Italia S.p.A.) including P. pentosaceus and S. carnosus. S. carnosus, either added as starter culture or present in the raw materials, ensures color development and stability by its nitrate reductase and catalase activities, respectively[39] and contributes to the typical aroma mainly by modulating the level and the nature of lipid oxidation products.[40] P. pentosaceus are categorized as "lactic acid bacteria" because the end product of its metabolism is lactic acid.[41] P. pentosaceus, like most lactic acid bacteria, are anaerobic and ferment sugars. The bacteriocin Pediocin p., produced by P. pentosaceus, inhibits several species of food pathogens such as Listeria monocytogenes, which can cause listeriosis.[42] Homemade sausages were prepared according to traditional recipes coming from the historical and cultural heritage of people of Southern Italy. In particular, no starter

cultures were added avoiding also the use of additives different from salt and pepper.

Homemade and industrially manufactured dry-fermented sausages were initially compared on the basis of the macronutrient composition and the free amino acid and biogenic amine contents after about 3 months of curing. The analytical data obtained after 80 days of curing showed some differences between the physical and chemical characteristics of industrial products (**1C**, **2C**, **3C** and **5C**) and those homemade (**4C** and **6C**) (Table 1).

2.1 Biogenic amines in industrial and homemade products

Biogenic amines are basic organic molecules with an aliphatic, aromatic, or heterocyclic structure, produced mainly by microbial decarboxylation of free amino acids that are formed during the maturation process.

Biogenic amine release in meat foods is due to the presence of free amino acids and to all the factors affecting the decarboxylase activity and bacterial growth.[43] During the ripening process of sausages, the formation of biogenic amines is favored by the growth of some lactic bacteria, which cause the formation of putresceine, cadaverine, hystamine, and tyramine.[44] The analytical procedure for determining biogenic

amines consisted of extraction with perchloric acid, pre-separation of crude extracts, and conversion of the amines into their trifluoroacetyl derivatives. The biogenic amines were identified by GC/MS analysis comparing their retention times and their mass spectra with those of derivatized authentic samples (*Figure 9*).

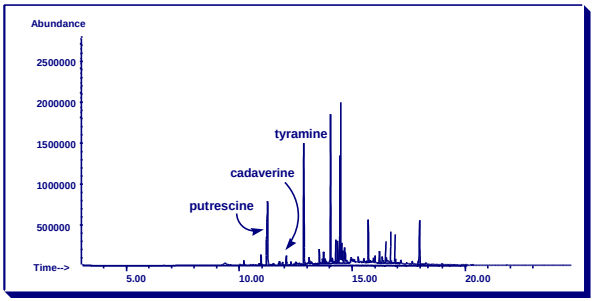


Figure 9. GC–MS chromatogram of biogenic amines of a sample of "salsiccia".

Tyramine and putrescine were the most abundantly detected in the examined samples. In all sausage samples examined the enzymatic decarboxylation of free amino acids was modest. In fact, in industrial samples of dry "salsiccia" and "soppressata" (**1C**-**3C**, **5C**), after 80 days of curing, putrescine, cadaverine, and tyramine were detected in only small concentrations that did not exceed 90 mg per kg of product (*Table 11*).

Sample[a]	1C[b]	2C[b]	3C[b]	5C[b]
Putrescine	9.70±0.51	54.12±0.93	37.22±1.83	62.90±2.13
Cadaverine	2.71±0.18	12.12±0.74	10.38±0.55	69.34±1.64
Tyramine	21.33±0.54	88.99±1.64	79.68±1.67	62.77±1.89

[a] Samples analyzed after 80 days of ripening.
[b]Results are expressed as means ± standard deviation (SD) of three replicates in mg/kg of fermented sausage

Table 11. Biogenic amine amounts measured in industrial "soppressata" and "salsiccia" after 80 days of curing.

Sample[a]	4C[b]	6C[b]
Putrescine	137.40±1.70	147.63±1.72
Cadaverine	72.73±1.19	49.09±1.94
Tyramine	165.13±3.32	179.15±2.82

[a] Samples analyzed after 80 days of ripening.
[b]Results are expressed as means ± standard deviation (SD) of three replicates in mg/kg of fermented sausage.

Table 12. Biogenic amine amounts measured in homemade "soppressata" and "salsiccia" after 80 days of curing.

In homemade samples, after 80 days of curing, we observed the same biogenic amines in higher concentrations (*Table 11, 12*) compared to those of industrial products. The values found, however, gave an indication of good-quality meats. In fact, in the literature the content of total biogenic amines up to 200 mg/kg is still considered an indicator of good hygiene in the production of sausages.[45]

2.2 Free amino acids in industrial and homemade products

The adopted procedure[46] for the determination of free amino acids was applied to each sample three lots of industrial "soppressata" (**1**, **2**, **3**), a homemade "soppressata" (**4C**), a batch of industrial "salsiccia" (**5**) and a homemade "salsiccia" (**6C**) were tested. The industrial batches were analyzed at the time of preparation (**1A**, **2A**, **3A**, **5A**), after 20 days (**1B**, **2B**, **3B**, **5B**), and after 80 days of ripening (**1C**, **2C**, **3C**, and **5C**). The homemade sausages (**4C** and **6C)** were analyzed after 80 days. The free amino acid level of the industrial batches of sausages was determined at the time of preparation, after 20 days, and after 80 days of ripening. Measurement of free amino acids in the meat products under evaluation required extraction and subsequent derivatization of amino acids by converting them into less polar and more volatile compounds to be analyzed by GC/MS. The individual amino acids were identified by GC/MS and comparing their retention times and mass spectra with those of derivatized authentic samples. From the obtained results it was observed that in all samples of "soppressata" and "salsiccia" the total free amino acid content tended to increase during aging. In particular, after 80 days, in the case of sample **1C** (*Table 15*), a total free amino acid content of 497.22 mg per 100 g of product was observed, while in the sample **1A** (*Table*

13) and in the sample **1B** (*Table 14*) the contents of total free amino acids were respectively 93.07 mg and 118.13 mg per 100 g . The trend of the total free amino acid contents is obviously in agreement with an increase in concentration of single amino acids over time (*Table 13, 14, 15 and 16*).

Sample[a]	1A[b]	2A[b]	3A[b]	4C[b]
Gly	0.40±0.02	0.90±0.04	0.51±0.02	12.35±0.55
Ala	6.00±0.17	15.05±0.27	8.44±0.33	41.24±0.78
Val	4.02±0.15	3.70±0.15	2.02±0.08	34.80±1.16
Leu	9.89±0.19	5.88±0.22	4.88±0.13	43.30±0.79
Ile	7.23±0.25	2.81±0.16	1.11±0.07	24.09±0.83
Thr	2.67±0.11	2.74±0.12	0.62±0.03	3.16±0.13
Pro	3.60±0.08	5.20±0.19	1.96±0.06	-
Asp	0.80±0.02	0.57±0.09	3.13±0.14	10.23±0.49
Glu	11.44±0.32	4.05±0.18	3.93±0.16	37.13±1.03
Met	1.54±0.06	0.49±0.02	0.31±0.02	12.47±0.61
Phe	34.30±0.37	21.43±0.31	11.40±0.27	65.34±1.79
Lys	11.18±0.29	9.30±0.27	9.19±0.24	37.62±1.92
Total	93.07	72.12	47.50	321.73

[a] Samples 1,2, and 3 are industrially manufactured, sample 4 is homemade. Samples were analyzed at the time of preparation (A), and after 80 days of ripening (C).[b] Results are expressed as means ± standard deviation (SD) of three replicates in mg/100 g of fermented sausage.

Table 13. Amino acid amounts measured in "soppressata" samples at the time of preparation.

Sample[a]	1B[b]	2B[b]	3B[b]	4C[b]
Gly	1.55±0.06	1.90±0.04	1.03±0.03	12.35±0.55
Ala	25.20±0.68	31.42±0.45	16.74±0.64	41.24±0.78
Val	9.57±0.26	24.14±0.40	9.56±0.37	34.80±1.16
Leu	16.44±0.30	38.34±0.52	27.61±0.43	43.30±0.79
Ile	10.64±0.26	20.68±0.90	11.41±0.43	24.09±0.83
Thr	2.19±0.09	-	4.32±0.19	3.16±0.13
Pro	-	-	5.99±0.09	-
Asp	1.78±0.10	-	6.00±0.08	10.23±0.49
Glu	4.68±0.14	16.72±0.31	31.38±0.79	37.13±1.03
Met	2.04±0.08	7.43±0.41	0.93±0.05	12.47±0.61
Phe	13.21±0.15	46.89±1.70	45.17±0.84	65.34±1.79
Lys	30.83±0.23	16.54±0.61	23.10±0.71	37.62±1.92
Total	118.13	204.06	183.24	321.73

[a] Samples 1,2, and 3 are industrially manufactured, sample 4 is homemade after 20 days of ripening (B), and after 80 days of ripening (C).

[b]Results are expressed as means ± standard deviation (SD) of three replicates in mg/100 g of fermented sausage.

Table 14. Amino acid amounts measured in "soppressata" samples after 20 days of ripening.

Sample[a]	1C[b]	2C[b]	3C[b]	4C[b]
Gly	1.33±0.06	2.73±0.11	4.21±0.19	12.35±0.55
Ala	21.57±0.18	45.42±0.43	70.14±1.92	41.24±0.78
Val	91.83±0.29	41.21±0.63	51.56±1.67	34.80±1.16
Leu	72.09±0.72	54.88±0.91	68.14±1.24	43.30±0.79
Ile	32.05±0.71	27.58±1.05	33.86±1.07	24.09±0.83
Thr	2.74±0.12	5.71±0.23	9.39±0.46	3.16±0.13
Pro	3.84±0.17	-	10.22±0.55	-
Asp	13.68±0.24	3.58±0.18	8.50±0.44	10.23±0.49
Glu	95.05±1.78	38.05±0.82	77.68±1.27	37.13±1.03
Met	21.24±0.48	14.83±0.51	20.80±0.93	12.47±0.61
Phe	93.67±1.61	66.23±0.83	71.80±1.32	65.34±1.79
Lys	48.13±0.56	57.71±0.65	82.29±1.04	37.62±1.92
Total	497.22	357.93	508.59	321.73

[a] Samples 1,2, and 3 are industrially manufactured, sample 4 is homemade and after 80 days of ripening (C).

[b]Results are expressed as means ± standard deviation (SD) of three replicates in mg/100 g of fermented sausage.

Table 15. Amino acid amounts measured in "soppressata" samples after 80 days of ripening.

	5A[b]	5B[b]	5C[b]	6C[b]
Gly	0.29±0.01	0.21±0.01	2.18±0.06	6.29±0.27
Ala	5.04±0.20	2.71±0.09	37.04±0.81	28.61±0.68
Val	2.24±0.09	23.16±0.80	20.04±0.82	18.04±0.82
Leu	4.73±0.21	7.13±0.32	30.05±0.86	26.41±1.33
Ile	3.54±0.15	10.11±0.29	14.98±0.47	12.84±0.50
Thr	7.90±0.28	-	-	-
Pro	1.71±0.06	2.97±0.11	14.97±0.11	7.39±0.39
Asp	2.62±0.13	0.59±0.14	18.04±0.38	18.19±0.40
Glu	2.82±0.14	11.51±0.22	18.05±0.28	5.69±0.25
Met	-	-	-	-
Phe	10.34±0.43	20.36±0.67	36.01±1.09	33.93±1.23
Lys	9.46±0.41	20.51±0.40	82.00±0.85	86.34±0.90
Total	50.69	99.26	273.36	243.73

[a] Samples 5 are industrially manufactured, sample 6 is homemade. Samples were analyzed respectively : at the time of preparation (A), after 20 days of ripening (B), and after 80 days of ripening (C).

[b] Results are expressed as means ± standard deviation (SD) of three replicates in mg/100 g of fermented sausage.

Table 16. Amino acid amounts measured in "salsiccia" sausage samples.

Some of these values have the same order of magnitude as those reported for similar products.[47] The amino acid values obtained for the industrial products were compared with those derived from samples of homemade "soppressata" (**4C**, *Table 13*) and "salsiccia" (**6C**, *Table 16*) after 80 days of maturation which, in the case of homemade products, took place at room temperature (10-15 °C). In particular, in the case of the sample

of homemade "soppressata" (**4C**) the total free amino acid level was of 321.73 mg per 100 g of product (*Table 15*), a value belonging to the lowest range of values observed for industrial products.

The highest content of total free amino acids in some industrial "soppressata" can be due to the proteolytic activity of the *Staphylococcus carnosus* used for the industrial preparation of the sausage.[48] In particular the main differences in amino acid content were observed for glutamic acid, lysine, valine and leucine. Moreover, for homemade "salsiccia" (**6C**), a relatively low total free amino acid content of 243.73 mg per 100 g of product was observed (*Table 16*), in agreement with the value obtained with the industrial "salsiccia" (**5C,** *Table 16*).

Moreover, a higher amount of free amino acid in the dried-out samples of "salsiccia" and "soppressata" was observed (*Table 17*).

	1C[b]	2C[b]	4C[b]	5C[b]	6C[b]
Gly	2.09±0.14	4.27±0.23	18.70±0.56	3.51±0.11	9.44±0.50
Ala	34.87±1.53	72.93±2.15	62.67±2.01	57.63±1.97	42.43±1.81
Val	146.77±3.10	65.87±3.33	52.88±2.49	32.02±1.98	26.99±1.68
Leu	135.42±3.28	87.76±1.87	65.76±3.10	47.99±2.15	39.56±1.75
Ile	71.64±1.07	44.21±2.66	32.14±1.72	23.24±0.65	19.34±0.64
Thr	4.32±0.24	9.13±0.40	4.93±0.11	10.83±0.61	-
Pro	6.08±0.15	-	-	24.04±0.78	11.14±0.63
Asp	21.86±0.90	5.81±0.26	11.61±0.53	27.87±0.34	28.41±0.85
Glu	171.51±2.42	60.82±3.97	56.55±1.66	27.89±0.34	8.53±0.68
Met	21.88±1.02	23.74±1.06	18.94±1.01	-	-
Phe	149.86±2.51	105.87±2.36	99.21±1.05	55.81±1.75	50.91±1.71
Lys	77.04±2.95	92.32±2.10	57.22±1.21	127.12±1.17	129.48±2.13
Total	843.34	572.73	480.61	437.95	366.23

[a] Samples analyzed after 80 days of curing.

[b] Results are expressed as means ± standard deviation (SD) of three replicates in mg/100 g of fermented sausage.

Table 17. Amino acid amounts measured in "salsiccia" and "soppressata" samples.

The lower total free amino acid level observed in both industrial and artisanal "salsiccia" than in "soppressata" can be attributed to the smaller size of the sausage. For this reason, in fact, a major water loss occurred in "salsiccia" during the maturation and, consequently, a lower degree of enzymatic hydrolysis took place.

The results showed that the total free amino acid content and the concentration of each amino acid increased with age, both in industrial and homemade products.

In particular, in the cured products, we observed an important increase in lysine, phenylalanine, valine, methionine, and glutamic acid in all tested samples. These compounds are taste-active and may also exert a strong influence on the final flavor. In particular, lysine, valine, and methionine have been related to an improvement in the aged taste of fermented meat derivatives, glutamic acid to saltiness,[49] and phenylalanine to the acid taste.

The high protein content observed in the traditional artisanal products (dry fermented "salsiccia" and "soppressata") can be justified by a more careful choice of meat and cuts used in their production. The low content of biogenic amines observed in industrial products is due to the use of the microbial starter culture and preservatives that reduce their production and also to the controlled maturing conditions. The higher content of biogenic amines in homemade products is a consequence of degradation of free amino acids in a product containing a higher amount of proteins and matured in uncontrolled conditions. The industrial sausages are characterized by a higher total free amino acid content than the homemade products. The proteolytic activity of Staphylococcus starters used for the industrial manufacture of sausages increases the formation of free amino acids during the ripening.

Experimental section

General

Solvents were purified and dried by standard procedures and distilled prior to use. Melting point were recorded on Kofler hot-stage apparatus and are uncorrected.

Proton nuclear magnetic resonance (^{1}H-NMR) spectra, and proton homodecoupling experiments were recorded at 300 MHz on a Bruker Avance 300 spectrometer. Carbon nuclear magnetic resonance (^{13}C-NMR) spectra. All NMR spectra were recorded at 25°C, using standard pulse sequence programs from the Bruker BioSpin firm. Samples were solubilized in DMSO-d_6 and $CDCl_3$. Chemical shift values (δ) are expressed in ppm relative to the residual proton of the solvent fixed at 2.50 ppm (central line of the quintet) for ^{1}H-NMR spectra, and relative to the DMSO-d_6 resonance fixed at 39.5 ppm (central line of the quintet) for ^{13}C-NMR spectra. All coupling constants (J) are reported in Hertz (Hz).

GC/MS analyses were performed with an HP-5MS (30 m x 0.25 mm, PhMesiloxane 5%) capillary column. The mass detector was operated in the electron impact ionization mode (EI-MS) with an electron energy of 70 eV. GC-MS analyses were carried out in split mode, using helium as the carrier gas (1 ml/min flow rate).

Elemental analysis was performed on a Perkin-Elmer Elemental Analyzer.

All reactions were monitored by thin-layer chromatography (TLC), using silica gel 60-F_{254} precoated glass plates. When required, the reactions were carried out under an inert atmosphere (N_2). The dichloromethane solution of diazomethane was prepared from *N*-methyl-*N*-nitrosourea with 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. Dichloromethane solution of diazomethane are stable for long periods if stored on KOH pellets at -20°C.

Preparation of Dimethylsulfoxonium Methylide (4)

Sodium hydride (1.1 mmol) in a 60% mineral oil dispersion was placed in a threenecked round-bottomed flask (100 mL) and washed with *n*-hexane (3 x 10 mL) by swirling to allow the hydride to settle and then decanting to remove the mineral oil. The flask was immediately fitted with a sealed mechanical stirrer and reflux condenser. Trimethylsulfoxonium iodide (220 mg, 1 mmol) and dry tetrahydrofuran (15 mL) were introduced, and the system was placed under nitrogen. The mixture was heated

to reflux and stirred for 2 h. During the reaction, the evolution of hydrogen and subsequent formation of a milky-white suspension was observed. The mixture was cooled to room temperature and used directly for the reaction with esters.

Reaction of esters of carboxylic acids (5, 7K, 7l, 7m) with Dimethylsufoxonium Methylide (4)

Esters of carboxylic acids (**5, 7K, 7l, 7m**, 1.13 mmol) was added to the solution of the dimethylsufoxonium methylide **4** (2.26 mmol), and the mixture was stirred under an inert atmosphere at room temperature. The reaction, monitored by TLC (chloroform/methanol, 90:10, v/v), was completed after 20 min. The mixture was evaporated to dryness under reduced pressure, and the residue was dissolved in water (10 mL). The aqueous solution was extracted with ethyl acetate (3 x 10 mL) and then acidified to pH = 2 using a solution of HCl (1 N). The resulting aqueous solution was extracted with ethyl acetate (3 x 10 mL). The combined organic layers were dried with Na_2SO_4, and the solvent was evaporated under reduced pressure to afford the corresponding carboxylic acid (70- 90%);

*Phenylacetic acid (**6**)*

m.p. 76–78 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.81 (br. s, 1 H, COOH), 7.29–7.41 (m, 5 H, Ar), 3.67 (s, 2 H, CH_2Ph) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 177.23, 133.450, 129.31, 128.12, 127.30, 41.04 ppm. MS (CI): *m/z* (%) = 137 (45) $[M - H]^+$, 119 (25), 91 (100). $C_8H_8O_2$ (136.15): elemental analysis calcd (%) calcd. C 70.57, H 5.92; found C 70.35, H 5.90.

*Lauric acid (**8k**)*

m.p. 42-45 °C; ^{1}H NMR ($CDCl_3$): δ = 2.35 (t, *J* = 7.2 Hz, 2H, CH_2CH_2COOH), 1.58-1.69 (m, 2H, $CH_2(CH_2)_8CH_3$), 1.19-1.42 (m, 16H, $CH_2(CH_2)_8CH_3$), 0.88 (t, *J*= 6.6 Hz, 3H, CH_2CH_3) ppm; ^{13}C NMR ($CDCl_3$): δ = 180.21, 34.07, 31.90, 29.58, 29.43, 29.32, 29.24, 29.06, 24.67, 22.68, 14.10 ppm; MS (EI): m/z (%): 200 (17) $[M^{\cdot+}]$, 171 (11), 157 (30), 143 (10), 129 (40), 115 (20), 101 (14), 85 (23), 73 (100), 60 (81), 43 (42), 41 (40); elemental analysis calcd (%) for $C_{12}H_{24}O_2$: C 71.95, H 12.08; found: C 72.15, H12.11.

Reaction of N-nosyl-α-amino acid esters (7a-c, 7i) with Dimethylsufoxonium Methylide (4).

N-nosyl-α-amino acid esters (**7a-c, 7i**, 1.13 mmol) was added to the solution of the dimethylsufoxonium methylide **4** (2.26 mmol), and the mixture was stirred under an inert atmosphere

at room temperature. The reaction, monitored by TLC (chloroform/methanol, 90:10, v/v), was completed after 20 min. The mixture was evaporated to dryness under reduced pressure, and the residue was dissolved in water (10 mL). The aqueous solution was extracted with ethyl acetate (3 x 10 mL) and then acidified to pH = 2 using a solution of HCl (1 N). The resulting aqueous solution was extracted with ethyl acetate (3 x 10 mL). The combined organic layers were dried with Na_2SO_4, and the solvent was evaporated under reduced pressure to afford the corresponding *N*-nosyl-α-amino acid (**8a-c, 8i**).

*N-nosyl-L-alanine (**8a**)*

m.p. 140-143 °C; ^{1}H NMR (DMSO-d_6): δ = 12.72 (s broad, 1H, COOH), 8.59 (d, J = 8.4 Hz, 1 H, NH,), 8.38 (d, J = 9.0 Hz, 2H, ArH,), 8.02 (d, J = 9.0 Hz, 2H, ArH,), 3.81-3.91 (m, 1H, CHCOOH), 1.18 (d, J = 7.2 Hz, 3H, CH_3,) ppm; ^{13}C NMR (DMSO-d_6): δ = 173.34, 149.89, 147.45, 128.50, 124.77, 51.75, 18.90 ppm; elemental analysis calcd (%) for $C_9H_{10}N_2O_6S$: C 39.42, H 3.68, N 10.21, S 11.69; found: C 39.54, H 3.69, N 10.24, S 11.67.

*N-Nosyl-L-isoleucine (**8b**)*

m.p. 126-129 °C; ^{1}H NMR (DMSO-d_6): δ = 12.70 (s broad, 1 H, COOH), 8.49 (d, J = 9.0 Hz, 1H, NH,), 8.38 (d, J = 8.7 Hz, 2 H, ArH,), 8.01 (d, J = 8.7 Hz, 2H, ArH,), 3.60-3.78 (m, 1 H, C*H*COOH), 1.63-1.85 (m, 1 H, C*H*CH_3), 1.27-1.42 (m, 1 H, C$H_2$$CH_3$), 1.01-1.19 (m, 1 H, C$H_2$$CH_3$), 0.71-0.89 (m, 6 H, $CH_2C H_3$ + CHCH_3) ppm; ^{13}C NMR (DMSO-d_6): δ = 172.28, 149.86, 147.17, 128.65, 124.71, 60.82, 37.25, 24.77, 15.89, 11.39 ppm; MS (EI): m/z (%): 271 (100) [$M^{\cdot+}$], 215 (41), 186 (27), 122 (26), 88 (48); elemental analysis calcd (%) for $C_{12}H_{16}N_2O_6S$: C 45.56, H 5.10, N 8.86, S 10.14; found: C 45.41, H 5.12, N 8.90, S 10.08.

*N-Nosyl-D-alloisoleucine (**8c**)*

^{1}H NMR (DMSO-d_6): δ = 12.71 (s broad, 1H, COOH), 8.39 (d, J= 8.4 Hz, 1H, NH), 8.37 (d, J = 8.7 Hz, 2 H, ArH), 8.02 (d, J = 8.7 Hz, 2H, ArH,), 3.79 (dd, J = 4.8 Hz, J = 8.4 Hz, 1 H, CHCOOH), 1.68-1.84 (m, 1 H, $CHCH_3$), 1.21-1.38 (m, 1 H, CH_2CH_3), 1.03-1.20 (m, 1 H, CH_2CH_3), 0.70-0.88 (m, 6 H, CH_2CH_3, $CHCH_3$) ppm; ^{13}C NMR (DMSO-d_6): δ = 172.59, 147.23, 149.81, 128.69, 124.66, 59.56, 37.31, 26.02, 15.11, 11.68 ppm; MS (EI): m/z (%): 271 (100) [$M^{\cdot+}$], 215 (43), 186 (26), 122 (24), 88 (45); elemental analysis calcd (%) for $C_{12}H_{16}N_2O_6S$: C 45.56, H 5.10, N 8.86, S 10.14; found: C 45.69, H 5.12, N 8.89, S 10.08.

Reaction of N-nosyl-α-amino acid esters with acid-sensitive protecting groups in the side chain (7d-f) with Dimethylsufoxonium Methylide (4).

N-nosyl-α-amino acid esters (**7d-f**) (1.13 mmol) was added to the solution of the dimethylsufoxonium methylide (**4**) (2.26 mmol) and the mixture allowed to react under stirring at room temperature. The conversion of (**7d-f**) into the corresponding carboxylic acid was monitored by TLC (chloroform/methanol 90:10 v/v). After 20 minutes the reaction mixture was evaporated to dryness under reduced pressure and redissolved in 10 ml of water. The aqueous solution was extracted with ethyl acetate (3 × 10 ml) and acidified to a pH 3 with a 5% potassium hydrogen sulphate solution. The acidic aqueous solution was extracted with ethyl acetate (3 × 10 ml) and the organic layers dried (Na_2SO_4) and evaporated to dryness under reduced pressure to afford the corresponding **8d-f**.

*N-nosyl-N^{ε}-Boc-protected lysine (**8d**)*

m.p. 153.4-154.8°C; ^{1}H NMR (DMSO-d_6): δ = 12.72 (s broad, 1H, COOH), 8.58 (s broad, 1H, $N^{\alpha}H$), 8.37 (d, J = 9.0 Hz, 2H, ArH,), 8.01 (d, J = 9.0 Hz, 2H, ArH,), 6.68-6.77 (m, 1H, $N^{\varepsilon}H$), 3.68-3.78 (m, 1H, CHCOOH), 2.72-2.83 (m, 2H, ε-CH_2), 1.41-1.62 (m, 2H, β-

CH_2), 1.34 (s, 9H, $C(CH_3)_3$), 1.09-1.29 (m, 4H, δ-CH_2 + γ-CH_2) ppm; ^{13}C NMR (DMSO-d_6): δ = 172.97, 155.97, 149.85, 147.29, 128.53, 124.75, 79.42, 77.79, 56.16, 32.04, 29.22, 28.71, 22.77 ppm; elemental analysis calcd (%) for $C_{17}H_{25}N_3O_8S$: C 47.32, H 5.84, N 9.74, S 7.43; found: C 47.17, H 5.86, N 9.78, S 7.46.

*N-Nosyl-O-Benzyl-L-tyrosine methyl ester (**8e**)*

^{1}H NMR (DMSO-d_6): δ = 12.83 (s broad, 1H, COOH), 8.69 (d, J = 9.0 Hz, 1H, NH,), 8.19 (d, J = 8.7 Hz, 2H, ArHNs,), 7.71 (d, J = 8.7 Hz, 2H, ArHNs,), 7.25-7.48 (m, 5H, ArH), 6.99 (d, J = 8.7 Hz, 2H, ArH,), 6.71 (d, J = 8.7 Hz, 2H, ArH,), 4.94 (s, 2H, CH_2Ph), 3.83-3.95 (m, 1H, α-CH), 2.92 (dd, J = 2.4 Hz, J = 13.8 Hz, 1H, α-$CHCH_2$,), 2.62 (dd, J = 10.5 Hz, J = 13.8 Hz, 1H, α-$CHCH_2$,) ppm;
^{13}C NMR (DMSO-d_6): δ = 172.75, 157.58, 149.39, 147.22, 137.49, 130.67, 129.17, 128.78, 128.24, 128.15, 128.07, 124.47, 114.67, 69.60, 58.39, 37.14 ppm; elemental analysis calcd (%) for $C_{22}H_{20}N_2O_7S$: C 57.89, H 4.42, N 6.14, S 7.02; found: C 58.05; H 4.43, N 6.16, S 7.05

*N-nosyl-S-trityl-protected cysteine (**8f**)*

m.p. 99-101°C; ^{1}H NMR (DMSO-d_6): δ = 8.35 (d, J = 8.4 Hz, 2H, ArHNs ,), 7.96 (d, J = 8.4 Hz, 2H, ArHNs,), 7.08-7.35 (m, 15H, ArH), 3.49-3.65 (m, 1H, CHCOOH), 2.22-2.38 (m, 2H, CH_2S) ppm;

^{13}C NMR (DMSO-d_6): δ = 172.55, 171.35, 155.59, 149.85, 146.98, 128.57, 127.65, 126.65, 124.81, 124.67, 65.36, 55.24, 21.40 ppm; elemental analysis calcd (%) for $C_{28}H_{24}N_2O_6S_2$: C 61.30, H 4.41, N 5.11, S 11.69; found: C 61.11, H 4.42, N 5.13, S 11.62.

Reaction of N-Cbz-L-alanine methyl ester (7g) with dimethylsufoxonium methylide (4).

N-Cbz-L-alanine methyl ester (**7g**) (267.8 mg, 1.13 mmol) was added to the solution of the dimethylsufoxonium methylide (**4**) (2.26 mmol) and the mixture allowed to react at room temperature and under nitrogen inert atmosphere. The conversion of **7g** into the corresponding carboxylic acid **8g** was monitored by TLC (ethyl ether/petroleum ether 70:30 v/v). After 20 minutes the reaction mixture was evaporated to dryness under reduced pressure and redissolved in 10 ml of water. The aqueous solution was extracted with ethyl acetate (3 × 10 ml) and acidified to pH 5 with a 5% potassium hydrogen sulphate solution. The acidic aqueous solution was extracted with ethyl acetate (3 × 10 ml) and the organic layers dried (Na_2SO_4) and evaporated to dryness under reduced pressure to afford the corresponding *N*-Cbz-L-alanine (**8g**) (236.9 mg) in 94% yield.

*N-Cbz-L-alanine(**8g**)*

m.p. 83-85°C; ^{1}H NMR ($CDCl_3$): δ = 8.22 (s broad, 1H, COOH), 7.31-7.39 (m, 5H, ArH), 5.48 (d, J= 7.2 Hz, 1H, NH,), 5.12 (d, J= 12.6 Hz, 1H, , CH_ACH_BPh), 5.10 (d, J = 12.6 Hz, 1H, CH_ACH_BPh), 4.32- 4.44 (m, 1H, $CHCH_3$), 1.44 (d, J = 7.2 Hz, 3H, CH_3) ppm; ^{13}C NMR ($CDCl_3$): δ = 176.81, 155.80, 136.20, 128.56, 128.23, 128.12, 67.05, 49.52, 18.47 ppm; elemental analysis calcd (%) for $C_{11}H_{13}NO_4$: C 59.19, H 5.87, N 6.27; found: C 59.36, H 5.89, N 6.29.

Reaction of N-Boc-L-phenylalanine methyl ester (7h) with dimethylsufoxonium methylide (4).

N-Boc-L-phenylalanine methyl ester (**7h**) (315.3 mg, 1.13 mmol) was added to the solution of the dimethylsufoxonium methylide (**4**) (2.26 mmol) and the mixture allowed to react at room temperature and under nitrogen inert atmosphere. The conversion of **7h** into the corresponding carboxylic acid **8h** was monitored by TLC (ethyl ether/petroleum ether 70:30 v/v). After 20 minutes the reaction mixture was evaporated to dryness under reduced pressure and redissolved in 10 ml of water. The aqueous solution was extracted with ethyl acetate (3 × 10 ml) and acidified to pH 3 with a 5% potassium hydrogen sulfate solution. The acidic aqueous solution was extracted with ethyl

acetate (3 × 10 ml) and the organic layers dried (Na_2SO_4) and evaporated to dryness under reduced pressure to afford the corresponding *N*-Boc-L-phenylalanine (**8h**) (296.4 mg) in 99% yield.

*N-Boc-L-phenylalanine (**8h**)*

m.p. 84-86°C; ^{1}H NMR ($CDCl_3$): δ = 8.89 (s broad, 1H, COOH), 7.15-7.33 (m, 5H, ArH), 5.02 (d, J = 8.1 Hz, 1H, NH,), 4.57-4.67 (m, 1H, $CHCH_2Ph$), 3.21 (dd, J = 5.4 Hz, J = 13.8 Hz, 1H, CH_2Ph,), 3.07 (dd, J = 6.0 Hz, J = 13.8 Hz, 1H, CH_2Ph,), 1.42 (s, 9H, $(CH_3)_3C$) ppm; ^{13}C NMR ($CDCl_3$): δ = 176.20, 155.44, 136.01, 129.44, 128.57, 127.02, 80.22, 54.37, 37.86, 28.31 ppm; elemental analysis calcd (%) for $C_{14}H_{19}NO_4$: C 63.38, H 7.22, N 5.28; found: C 63.17, H 7.25, N 5.29.

Reaction of N-Cbz-D-alanine-L-phenylalanine methyl ester (7j) with dimethylsufoxonium methylide (4).

N-Cbz-D-alanine-L-phenylalanine methyl ester (**7j**) (404.5 mg, 1.13 mmol) was added to the solution of the dimethylsufoxonium methylide (**4**) (2.26 mmol) and the mixture allowed to react at room temperature and under nitrogen inert atmosphere. The conversion of **7j** into the corresponding dipeptide deprotected on the carboxyl function (**8i**) was

monitored by TLC (chloroform/methanol 70:30 v/v). After 20 minutes the reaction mixture was evaporated to dryness under reduced pressure and redissolved in 10 ml of water. The aqueous solution was extracted with ethyl acetate (3 × 10 ml) and acidified to pH 3 with a 5% potassium hydrogen sulphate solution. The acidic aqueous solution was extracted with chloroform (3 × 10 ml) and the organic layers dried (Na_2SO_4) and evaporated to dryness under reduced pressure to afford the corresponding *N*-Cbz-D-alanine-L-phenylalanine (**8i**) (311.0 mg) in 80% yield. **8i** was then treated with diazomethane to afford the corresponding methyl ester **7j** in quantitative yield.

*N-Cbz-D-alanine-L-phenylalanine (**8i**)*

^{1}H NMR ($CDCl_3$): δ = 7.05-7.38 (m, 10H, Ar*H*), 6.89 (d, *J* = 7.5 Hz, 1H, CON*H*,), 5.79 (d, *J* = 7.8 Hz, 1H, OCON*H*,), 5.07 (d, *J* = 12.0 Hz, 1H, C*HA* H_BPh,), 5.01 (d, *J* = 12.0 Hz, 1H, CH_ACH_BPh,), 4.79-4.87 (m, *J* = 6.6 Hz, *J* = 13.8 Hz, 1H, C*H*COOH), 4.31-4.41 (m, 1H, C*H*CONH), 3.21 (dd, *J* = 5.1 Hz, *J* =13.8 Hz, 1H, CH_2Ph,), 3.04 (dd, 1H, CH_2Ph,), 1.23 (d, 3H, *J*= 6.9, CH_3CH) ppm; ^{13}C NMR ($CDCl_3$): δ = 173.88, 172.81, 156.43, 135.83, 129.47, 128.58, 128.50, 128.29, 128.07, 127.11, 67.23, 53.07, 50.24, 37.42, 18.94 ppm; elemental analysis calcd (%) for $C_{20}H_{22}N_2O_5$: C 64.85, H 5.99, N 7.56; found: C 64.65,H 6.00, N 7.59.

*N-Cbz-D-alanine-L-phenylalanine methyl ester (**7j**)*

^{1}H NMR ($CDCl_3$): δ = 7.05-7.40 (m, 10H, Ar*H*), 6.48 (d, *J* =6.9 Hz, 1H, CON*H*,), 5.27 (d, *J* = 6.9 Hz, 1H, OCON*H*CHO,), 5.12 (d, *J* = 12.3 Hz, 1H, CH_AH_BPhCbz,), 5.07 (d, *J* = 12.3 Hz, 1H, CH_AH_BPhCbz,), 4.85 (dd, J_{AX} = 6 Hz, J_{BX} = 13.5 Hz, 1H, $CH_xCH_ACH_B$Ph); 4.18-4.29 (m, 1H, C*H*CH_3), 3.73 (s, 3H, OCH_3), 3.14 (dd, *JAB* = 13.8 Hz, 1H, $CH_xCH_ACH_B$Ph,), 3.07 (dd, J_{AB} = 13.8 Hz, 1H, $CH_xCH_ACH_B$Ph), 1.34 (d, *J* = 6.9 Hz, 3H, CH_3CH) ppm; ^{13}C-NMR ($CDCl_3$): δ = 171.75, 171.67, 137.80,136.13, 135.62, 129.26, 128.60, 128.25, 128.21, 127.19, 125.01, 67.08, 53.16, 52.41, 50.41, 8.42 ppm; elemental analysis calcd (%) for $C_{20}H_{22}N_2O_5$: C 64.85, H 5.99, N 7.56; found: C 64.65, H 6.00, N 7.59.

Synthesis of ^{18}O-sodium hydroxide.

^{18}O-labeled water (0.4 mL) was added dropwise to a suspension of NaH (19.9 mmol, 60% mineral oil dispersion) in *n*-hexane. The formation of a insoluble white solid was observed (Na^{18}OH). The solvent was evaporated under reduced pressure and the Na^{18}OH was recovered in quantitative yield.

Reaction of methyl phenylacetate (5) with dimethylsufoxonium methylide (4) and ^{18}O-sodium hydroxide.

Methyl phenyl acetate (1.13 mmol) and ^{18}O-labeled sodium hydroxide (5.65 mmol) were added to the THF solution of the dimethylsufoxonium methylide (2.26 mmol) and the mixture allowed to stir at room temperature. The reaction, monitored by TLC, showed the conversion of the ester into the corresponding carboxyl acid after 6 hours. The reaction mixture was then acidified with gaseous HCl to pH = 2 and 10 mL (6.65 mmol) of a 0.66 M solution of diazomethane in DCM were added dropwise to the reaction mixture. The solvent was evaporated under reduced pressure and the reaction mixture redissolved in 10 ml of water. The aqueous solution was extracted with ethyl acetate (3 x 10 ml). The organic layers were dried (Na_2SO_4) and evaporated under reduced pressure. The corresponding phenyl acetic acid containing ^{16}O was recovered in quantitative yield.

*Phenyl acetic acid (**6**)*

^{1}H NMR (DMSO-d_6): δ = 7.21-7.38 (m, 5H, Ar*H*), 3.68 (s, 2H, CH_2Ph), 3.62 (s, 3H, OCH_3) ppm; ^{13}C-NMR (DMSO-d_6): δ = 172.13, 135.12, 129.91, 128.87, 127.11, 52.21, 41.10 ppm; MS (EI): m/z (%): 152 (1), 150 (34) [$M^{\cdot+}$], 119 (2), 91 (100), 77 (1), 65 (11);

elemental analysis calcd (%) for $C_9H_{10}O_2$: C 71.98, H 6.71; found: C 70.16, H 6.69.

Reaction of methyl phenylacetate (5) with dimethylsufoxonium methylide (4) (work up with $H_2{}^{18}O$).

Methyl phenylacetate (**5**) (169.50 mg, 1.13 mmol) was added to the solution of the dimethylsufoxonium methylide (**4**) (2.26 mmol) and the mixture allowed to stir under inert atmosphere at room temperature.

The conversion of the ester into the corresponding carboxylic acid was monitored by TLC (chloroform/methanol 90:10 v/v). After 40 minutes the reaction was completed and the mixture was evaporated to dryness under reduced pressure and 0.2 ml of $H_2{}^{18}O$ water were added. The resulting aqueous solution was extracted with ethyl acetate (3 × 10 ml) and acidified to pH 2 with a few drops of a 96% solution of H_2SO_4. The aqueous solution was extracted with ethyl acetate (3 × 10 ml). The acid organic layers were dried (Na_2SO_4) and the solvent evaporated to afford the corresponding phenyl acetic acid (**6)** in 70% yield.

*Phenyl acetic acid (**6**)*

^{1}H NMR (DMSO-d_6): δ = 7.21-7.38 (m, 5H, Ar*H*), 3.68 (s, 2H, CH_2Ph), 3.62 (s, 3H, OCH_3) ppm; ^{13}C-NMR (DMSO-d_6): δ = 172.13,

135.12, 129.91, 128.87, 127.11, 52.21, 41.10 ppm; MS (EI): m/z (%): 152 (1), 150 (34) [$M^{\cdot+}$], 119 (2), 91 (100), 77 (1), 65 (11); elemental analysis calcd (%) for $C_9H_{10}O_2$: C 71.98, H 6.71; found: C 70.16, H 6.69.

Reaction of N-Fmoc-α-amino acid methyl esters (9a-e) with dimetilsulfoxonium methylide (4).

The appropriate *N*-Fmoc-α-amino acid methyl esters (**9a-e**, 1 mmol) were added to a solution of the dimethylsulfoxonium methylide (**4**, 2 mmol) in THF and the mixture was stirred at room temperature under inert N_2 atmosphere. The reaction monitored by TLC (chloroform/methanol, 95:5 v/v) was completed after 20 min. The mixture was evaporated to dryness under reduced pressure and redissolved in 10 ml of water. The aqueous solution was extracted with diethyl ether (3 × 10ml). The corresponding deprotected products **10a-e** were recovered as *N*-Nosyl derivatives **8a-b** e **11a-c**. To this aim the aqueous solution containing the α-amino acids (**10a-e**, 1 mmol) was allowed to react with nosyl chloride (1 mmol) in dioxane at room temperature for 1h. The mixture was made basic with triethylamine (Et_3N) (3 mmol). The mixture was evaporated to dryness under reduced pressure. An aqueous solution of HCl 1N was then added and the acidified solution (pH 2) was extracted

with ethyl acetate (3 × 10ml). The organic layer was dried ($Na_2SO_{4)}$ and evaporated to dryness under reduced pressure to give the corresponding *N*-Nosyl derivatives **8a-b** e **11a-c**.

*N-Nosyl-L-alanine-OH (**8a**)*

Yield 90%. m.p. 140-143 °C; ^{1}H NMR (DMSO-d_6): δ = 12.72 (s broad, 1H, COOH), 8.59 (d, J = 8.4 Hz, 1 H, NH,), 8.38 (d, J = 9.0 Hz, 2H, ArH,), 8.02 (d, J = 9.0 Hz, 2H, ArH,), 3.81-3.91 (m, 1H, CHCOOH), 1.18 (d, J = 7.2 Hz, 3H, CH_3,) ppm; ^{13}C NMR (DMSO-d_6): δ = 173.34, 149.89, 147.45, 128.50, 124.77, 51.75, 18.90 ppm; elemental analysis calcd (%) for $C_9H_{10}N_2O_6S$: C 39.42, H 3.68, N 10.21, S 11.69; found: C 39.54, H 3.69, N 10.24, S 11.67.

*N-Nosyl-L-leucine-OH (**11a**)*

Yield 87%. ^{1}H-NMR (300 MHz, DMSO-d_6): 0.72 (d, 3H, J=6,5 Hz, $CH(CH_3)_2$); 0.81 (d, 3H, J=6,5 Hz, $CH(CH_3)_2$); 1.31-1.49 (m, 2H, $CHCH_2$); 1.58 (m, 1H, $CH(CH_3)_2$); 3.74 (m, 1H α-CH); 7.98-8.03 (d, 2H, J= 8.6 Hz, ArH); 8.32-8.40 (d, 2H, J= 8.6 Hz, ArH); 8.58 (d, 1H, J=9.0 Hz, NH); 12.67 (br s, 1H, COOH). ^{13}C NMR (75 MHz, DMSO-d_6): 21.37, 23.04, 24.39, 41.17, 54.65, 124.74, 128.58, 147.25, 149.87, 173.27. elemental analysis calcd (%) for $C_{12}H_{16}N_2O_6S$: C, 45.56; H, 5.10; N, 8.86; Found: C, 45.72; H, 5.11; N, 8.88.

*N-Nosyl-L-valine-OH (**11b**)*

Yield 88%. ^{1}H-NMR (300 MHz, DMSO-d_6): 0.80 (d, 3H, J=6,9 Hz, $CH(CH_3)_2$); 0.84 (d, 3H, J=6,9 Hz, $CH(CH_3)_2$); 1.98 (m, 1H, $CH(CH_3)_2$); 3.49-3.51 (m, 1H, α-CH); 8.03 (d, 2H, J=9.0 Hz, ArH); 8.38 (d, 2H, J=9.0 Hz, ArH); 8.45 (d, 1H, J=9.3 Hz, NH). ^{13}C NMR (75 MHz, DMSO-d_6): 18.21, 19.49, 30.74, 61.89, 124.69, 128.64, 147.23, 149.72, 172.29. Elemental analysis calcd (%) for $C_{11}H_{14}N_2O_6S$: C, 43.70; H, 4.67; N, 9.27; Found: C, 43.83; H, 4.69; N, 9.30.

*N-Nosyl-L-Phenylalanine-OH (**11c**)*

Yield 88%. ^{1}H-NMR (300 MHz, DMSO-d_6): 2.69 (dd, 1H, J=13.7 Hz, J=4.2 Hz, $PhCH_aH_bCH$); 2.97 (dd, 1H, J=13,7 Hz, J=10.2 Hz, $PhCH_aH_bCH$); 3.94 (m, 1H, α-CH); 7.04-7.15 (m, 5H, $C_6H_5CH_2$); 7.71 (d, 2H, J=8.7 Hz, ArH-Ns); 8.17 (d, 2H, J=8.7 Hz, ArH-Ns); 8.76 (d, 1H, J=9.0 Hz, NH).^{13}C NMR (75 MHz, DMSO-d_6): 38.04, 58.10, 124.57, 126.84, 128.10, 128.56, 129.65, 137.13, 147.11, 149.57, 172.58. elemental analysis calcd (%) for $C_{15}H_{14}N_2O_6S$: C, 51.42; H, 4.03; N 8.00; Found: C, 51.60; H, 4.01; N, 8.03.

*N-Nosyl-L-isoleucine (**8b**)*

m.p. 126-129 °C; ^{1}H NMR (DMSO-d_6): δ = 12.70 (s broad, 1 H, COOH), 8.49 (d, J = 9.0 Hz, 1H, NH,), 8.38 (d, J = 8.7 Hz, 2 H, ArH,), 8.01 (d, J = 8.7 Hz, 2H, ArH,), 3.60-3.78 (m, 1 H, CHCOOH), 1.63-1.85 (m, 1 H, $CHCH_3$), 1.27-1.42 (m, 1 H, CH_2CH_3), 1.01-1.19 (m, 1 H, CH_2CH_3), 0.71-0.89 (m, 6 H, CH_2CH_3 + $CHCH_3$) ppm; ^{13}C NMR (DMSO-d_6): δ = 172.28, 149.86, 147.17, 128.65, 124.71, 60.82, 37.25, 24.77, 15.89, 11.39 ppm; MS (EI): m/z (%): 271 (100) [$M^{\cdot+}$], 215 (41), 186 (27), 122 (26), 88 (48); elemental analysis calcd (%) for $C_{12}H_{16}N_2O_6S$: C 45.56, H 5.10, N 8.86, S 10.14; found: C 45.41, H 5.12, N 8.90, S 10.08.

Synthesis of N-Fmoc-L-alanyl-L-alanine methyl ester and N-Fmoc-D-alanyl-L-alanine methyl ester (12f-g).

To a magnetically stirred solution of the L-alanine methyl ester hydrochloride (1 mmol) in 5% aqueous Na_2CO_3 (7 mL) was added dropwise the appropriate *N*-Fmoc amino acid chloride (0.8 mmol) in dry chloroform (10 mL).[12] The resulting mixture was stirred for 2-3 h, monitoring the conversion of *N*-Fmoc-L-amino acid chloride by TLC (chloroform/methanol, 95:5). The organic phase was separated and washed with 1 N HCl (3 × 10 mL). The organic extract was washed with brine, then dried (Na_2SO_4) and

evaporated to dryness to give compounds **12f-g**, in 84–92% overall yields.

*N-Fmoc-L-alanyl-L-alanine methyl ester (**12f**)*

Yield 95%. ^{1}H-NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 1.33-1.49 (m, 6H, $CHCH_3$); 3.76 (s, 3H, OCH_3); 4.17-4.36 (m, 2H, CH-Fmoc, CHCONH); 4.39 (d, 2H, J=6.9 Hz, CH_2-Fmoc); 4.58 (m, 1H,CHCOOMe); 5.51 (br s, 1H, NH-Fmoc); 6.62 (br s, 1H, CONH); 7.29-7.43 (m, 4H, ArH); 7.58-7.64 (m, 2H, ArH); 7.77 (d, 2H, J=7.5 Hz, ArH). ^{13}C NMR (75 MHz, $CDCl_3$): 18.34, 18.82, 47.08, 48.13, 50.40, 52.61, 67.09, 120.03, 125.09, 127.10, 127.61, 141.30, 143.74, 155.92, 171.83, 173.17. elemental analysis calcd (%) for $C_{22}H_{24}N_2O_5$: C, 66.65; H, 6.10; N, 7.07. Found: C, 66.87; H, 6.08; N, 7.08.

Reaction of N-Fmoc-dipeptides methyl esters 12f-g with dimethylsulfoxonium methylide (4).

The appropriate *N*-Fmoc-dipeptide methyl ester **12f-g** (1 mmol) was added to a solution of the dimethylsulfoxonium methylide (**4**, 2 mmol) in THF and the mixture was stirred at room temperature under inert N_2 atmosphere. The reaction monitored by TLC (chloroform/methanol, 90:10 v/v) was completed after 20 min. The mixture was evaporated to dryness

under reduced pressure and redissolved in 10 ml of water. The aqueous solution was extracted with diethyl ether (3 × 10ml). The products **13f-g** were recovered as *N*-Acetyl derivatives. The aqueous solution containing the dipeptide **13f-g** was treated with acetic anhydride (6 mmol) in chloroform (10ml) at room temperature for 30 min. The basic conditions were maintained by a saturated Na_2CO_3 aqueous solution, then the mixture was extracted with chloroform (3 × 10 ml).The organic layer was washed with distilled water (2 × 10 ml), then dried (Na_2SO_4) and evaporated to dryness to afford the *N*-Acetyl derivatives **14f** and **14g** in high yields.

*N-Acetyl-L-alanyl-L-alanine (**14f**)*

Yield 90%, ^{1}H NMR (300 MHz, DMSO-d_6) δ (p.p.m.): 1.16 (d, 3H, J=6.9 Hz, $CHCH_3$); 1.26 (d, 3H, J=6.9 Hz, $CHCH_3$); 1.81 (s, 3H, CH_3CO); 4.18 (m, 1H, α-CH); 4.31 (m, 1H, α-CH); 8.09 (d, 1H, J=7.5 Hz, NH); 8.20 (d, 1H, J=6.9 Hz, NH); 12.50 (br s, 1H, COOH). ^{13}C NMR (75 MHz, DMSO-d_6): 17.56, 18.72, 22.97, 47.87, 48.22, 171.73, 172.79, 174.39. elemental analysis calcd (%) for $C_8H_{14}N_2O_4$: C, 47.52; H, 6.98; N, 13.85. Found: C, 47.68; H, 7.01; N, 13.83.

*N-Acetyl-D-alanyl-L-alanine (**14g**)*

Yield 88%.[1]H-NMR (300 MHz, DMSO-d_6) δ (p.p.m.): 1.16 (d, 3H, J=6.9 Hz, $CHCH_3$); 1.24 (d, 3H, J=6.9 Hz, $CHCH_3$); 1.82 (s, 3H, CH_3CO); 4.16 (m, 1H, α-CH); 4.32 (m, 1H, α-CH); 8.01 (d, 1H, J=8.1 Hz, NH); 8.14 (d, 1H, J=7.2 Hz, NH). ^{13}C NMR (75 MHz, DMSO-d_6):17.77, 19.06, 22.98, 47.82, 48.17, 143.84, 169.06, 172.35. elemental analysis calcd (%) for $C_8H_{14}N_2O_4$: C, 47.52; H, 6.98; N, 13.85. Found C, 47.67; H, 6.96; N, 13.88.

Synthesis of methyl ester of N-Fmoc-L-leucyl-L-phenylalanyl-L-alanine (15).

A 0.66 M solution of diazomethane[2] in dichloromethane (8 mmol) was added cautiously dropwise to a stirred solution of the *N*-Fmoc-L-leucyl-L-phenylalanyl-L-alanine (1 mmol) synthesized by automated synthesizer in dry dichloromethane (10 mL). The resulting mixture was maintained under an inert atmosphere (N_2) and stirred at room temperature. TLC analysis (chloroform/methanol, 80:20 v/v) showed complete conversion of the precursor after 10 min. Evaporation of the solvent under reduced pressure afforded the *N*-Fmoc-L-leucyl-L-phenylalanyl-L-alanine methyl ester **15** in 90% yields.

*N-Fmoc-L-leucyl-L-phenylalanyl-L-alanine methyl ester (**15**)*

Yield 90%.^{1}H-NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 0.81-0.91 (m, 6H, $CH(CH_3)_2$); 1.05 (m, 1H, $CH(CH_3)_2$); 1.17-1.49 (m, 5H, $CHCH_3$, $CH_2CH(CH_3)_2$); 3.01-3.09 (m, 2H, $CH_2C_6H_5$); 3.60 (s, 3H, OCH_3); 4.12-4.25 (m, 2H, CH-Fmoc, α-CH_{leu}); 4.31 (m, 1H, α-CH_{phe}); 4.40-4.55 (m, 2H, CH_2-Fmoc); 4.81 (m, 1H, α-CH_{ala}); 5.30 (d, 1H, J=7.8 Hz, NH-Fmoc); 6.66 (d, 1H, J=7.8 Hz, NH); 6.82 (d, 1H, J=7.8 Hz, NH); 7.12-7.20 (m, 5H, $C_6H_5CH_2$); 7.25-7.36 (m, 2H, ArH-Fmoc); 7.37-7.48 (m, 2H, ArH-Fmoc); 7.58 (d, 2H, J= 7.2 Hz, ArH-Fmoc); 7.71-7.82 (m, 2H, ArH-Fmoc). elemental analysis calcd (%) for $C_{34}H_{39}N_3O_6$: C, 69.72; H, 6.71; N, 7.17. Found: C, 70.02; H, 6.73; N, 7.20.

Reaction of N-Fmoc-L-leucyl-L-phenylalanyl-L-alanine methyl ester (15) with dimethylsulfoxonium methylide (4).

The *N*-Fmoc-tripeptide methyl ester **15** (1 mmol) was added to a solution of the dimethylsulfoxonium methylide (**4**, 2 mmol) in THF and the mixture was stirred at room temperature under inert N_2 atmosphere. The reaction monitored by TLC (chloroform/methanol 90:10 v/v) was completed after 20 min. The mixture was evaporated to dryness under reduced pressure and redissolved in 10 ml of water. The aqueous solution was

extracted with diethyl ether (3 × 10ml). The deprotected product **16** was recovered as *N*-nosyl derivative. The aqueous solution containing the tripeptide **16** (1 mmol) was allowed to react with nosyl chloride (1 mmol) in dioxane at room temperature for 1h. The mixture was made basic with triethylamine (Et_3N) (3 mmol). The mixture was evaporated to dryness under reduced pressure. An aqueous solution of HCl 1N was then added and the acidified solution (pH 2) was extracted with ethyl acetate (3 × 10ml). The organic layer was dried (Na_2SO_4) and evaporated to dryness under reduced pressure to give *N*-Nosyl derivative **17**.

*N-Nosyl-L-leucyl-L-phenylalanyl-L-alanine (**17**)*

Yield 80%. ^{1}H-NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 0.61 (d, 3H, J=6.5 Hz, $CH(CH_3)_2$); 0.79 (d, 3H, J=6.5 Hz, $CH(CH_3)_2$); 0.81-0.98 (m, 2H, $CH_2CH(CH_3)_2$); 1.23-1.49 (m, 4H, $CH(CH_3)_2$, $CHCH_3$); 2.95 (m, 1H, $CH_aH_bC_6H_5$); 3.18 (m, 1H, $CH_aH_bC_6H_5$); 3.74 (m, 1H, α-CH_{leu}); 4.40 (m, 1H, α-CH_{ala}); 4.72 (m, 1H, α-CH_{phe}); 6.31 (br s, 1H, NH); 7.11 (d, 1H, J=9.0 Hz, NH); 7.15-7.38 (m, 5H, $C_6H_5CH_2$); 7.44 (d, 1H, J=9.0 Hz, NH); 8.05 (d, 2H, J=9.3 Hz, ArH-Ns); 8.28 (d, 2H, J=9.3 Hz, ArH-Ns). ^{13}C NMR (75 MHz, $CDCl_3$): 17.50, 21.47, 23.34, 24.23, 37.80, 40.84, 47.97, 53.92, 55.51, 124.43, 126.74, 128.40, 129.78, 138.16, 146.94, 149.56, 170.90, 171.01, 174.35.

elemental analysis calcd (%) for $C_{24}H_{30}N_4O_8S$: C, 53.92; H, 5.66; N, 10.48. Found: C, 54.13; H, 5.68; N, 10.44.

Reaction of N-Fmoc-L-Alanyl-L-Valine Wang resin (18) with dimethylsulfoxonium methylide (4).

N-Fmoc-L-Alanyl-L-Valine Wang resin (**18**) (1 mmol) was added to the solution of the dimethylsulfoxonium methylide in THF (**4**, 2 mmol) and the mixture was stirred at room temperature under inert N_2 atmosphere. The reaction monitored by TLC (chloroform/methanol, 90:10 v/v) was completed after 20 min. The mixture was hydrolyzed and filtered. After evaporation of THF under reduced pressure, the filtrate was extracted with diethyl ether (3 × 10ml). The aqueous solution containing the deprotected dipeptide **19** was treated with acetic anhydride (6 mmol) in chloroform (10 ml) at room temperature for 30 min. The basic conditions were maintained by adding of a saturated $NaHCO_3$ aqueous solution. The mixture was acidified with HCl 1N and extracted with chloroform (3 × 10 ml).The organic layer was washed with distilled water (2 × 10 ml), then dried (Na_2SO_4) and evaporated to dryness to afford *N*-Acetyl-L-Alanyl-L-Valine (**20**) in 90% yield.

*N-Acetyl-L-Alanyl-L-Valine (**20**)*

Yield 90%.^{1}H-NMR (300 MHz, DMSO- d_6) δ (p.p.m.): 0.86 (d, 6H, J=6.8 Hz, $CH(CH_3)_2$); 1.17 (d, 3H, J=6.8 Hz, $CHCH_3$); 1.91 (s, 3H, CH_3CONH); 2.02 (m, 1H, $CH(CH_3)_2$); 3.97-4.20 (m, 1H, α-CH_{val}); 4.30-4.41 (m, 1H, α-CH_{ala}), 7.86 (d, 1H, J=10.6 Hz, NH), 8.11 (d, 1H, J=10.6 Hz, NH). elemental analysis calcd (%) for $C_{10}H_{18}N_2O_4$: C, 52.16; H, 7.88; N, 12.17. Found: C, 52.08; H, 7.91; N, 12.20.

Reaction of N-Fmoc-L-valyl-O-tert-butyl-L-serinyl-L-alanine Wang resin (21) with dimethylsulfoxonium methylide (4).

N-Fmoc-L-valyl-*O-tert*-butyl-L-serinyl-L-alanine Wang resin (**21**, 1 mmol) was added to the solution of the dimethylsulfoxonium methylide (**4**, 2 mmol) in THF and the mixture was stirred at room temperature under inert N_2 atmosphere. The reaction monitored by TLC (chloroform/methanol, 90:10 v/v) was completed after 20 min. The mixture was hydrolyzed and filtered. After evaporation of THF under reduced pressure, the filtrate was extracted with diethyl ether (3 × 10ml). The aqueous solution containing the tripeptide L-valyl-*O-tert*-butyl-L-serinyl-L-alanine (**21**) was allowed to react with Nosyl chloride (1 mmol) in dioxane at room temperature for 1h. The mixture was made

basic with triethylamine (Et_3N) (3 mmol). The mixture was then evaporated to dryness under reduced pressure, acidified with a 5% $NaHSO_4$ aqueous solution (pH 5) and extracted with ethyl acetate (3 × 10ml). The organic layer was dried (Na_2SO_4) and evaporated to dryness under reduced pressure to give pure *N*-Acetyl-L-valyl-*O-tert*-butyl-L-serinyl-L-alanine **23** in 80% yield.

*N-Nosyl- L-valyl- O-tert-butyl-L-serinyl-L-alanine (**23**)*

Yield 80%.^{1}H-NMR (300 MHz, DMSO-d_6) δ (p.p.m.): 0.82 (d, 6H, J=7.0 Hz, $CH(CH_3)_2$); 1.22 (d, 3H, J=7.2 Hz, $CHCH_3$); 1.32 (s, 9H, $(CH_3)_3C$); 1.85 (m, 1H, $CH(CH_3)_2$); 3.34 (d, 2H, J=6.3 Hz, CH_2O-*t*Bu); 3.70 (dd, 1H, J=7.2 Hz, J=9.3 Hz, α-CH_{val}); 4.01 (m, 1H, α-CH_{ser}); 4.18 (m, 1H, α-CH_{ala}); 7.92 (d, 1H, J=7.2 Hz , NH); 8.01 (d, 2H, J=8.4 Hz, ArH); 8.04 (d, 1H, J=7.8 Hz, NH); 8.23 (d, 1H, J=9.3 Hz, NH); 8.34 (d, 2H, J=8.4 Hz, ArH); 12.50 (br s, 1H, COOH). ^{13}C NMR (75 MHz, DMSO- d_6): 17.71, 18.73, 19.45, 29.05, 31.39, 47.99, 55.08, 61.89, 62.33, 124.57, 128.63, 147.19, 149.73, 169.73, 170.11, 174.41. elemental analysis calcd (%) for $C_{21}H_{32}N_4O_9S$: C, 48.83; H, 6.24; N, 10.85. Found: C, 49.02; H, 6.26; N, 10.82.

Reaction of N-Fmoc-L-alanine methyl esters (9a) with dimetilsulfoxonium methylide (4) in ratio molar 1:1.

N-Fmoc-L-alanine methyl esters (**9a**, 1 mmol) was added to a solution of the dimethylsulfoxonium methylide (**4**, 1 mmol) and the mixture was stirred at room temperature under inert N_2 atmosphere in THF. Then 30 min the reaction, analyzed by TLC (chloroform/methanol, 95:5 v/v), does not go to completion. However, the mixture was evaporated to dryness under reduced pressure, redissolved in 10 ml of water and extract with diethyl ether. The organic layer, dried (Na_2SO_4), was evaporated to dryness under reduced pressure to give the *N*-Fmoc-l-alanine methyl ester in 45% yield. The aqueous solution was derivatized with Nosyl chloride (1 mmol) and triethylamine (Et_3N) (3 mmol) in dioxane at room temperature for 1h. The mixture after evaporation to dryness under reduced pressure was acidified with aqueous solution of HCl 1N (pH 2) and extracted with ethyl acetate (3 × 10ml). The organic layer was dried (Na_2SO_4) and evaporated to dryness under reduced pressure to give the corresponding *N*-Nosyl-L-alanine (**8a**) in 55% yield. The product **8a** was analyzed by GC/MS.

*N-Nosyl-L-alanine (**8a**)*

GC-MS (E.I.) m/z: 274 [$M^{\cdot+}$] (1%), 229 (100), 186 (33), 122 (19).

Synthesis of 4-nitrobenzenesulfonamide (25).

4-nitrobenzenesulfonyl chloride (**24**, 1.35 mmol) dissolved in 15 mL of dry THF was added dropwise to a 37% aqueous solution of ammonia (5.4 mmol) in a two-necked round-bottomed flask (100 mL) immersed in an ice bath. The reaction, monitored by TLC (chloroform/methanol, 95:5, v/v), was completed after 1 h. The mixture was evaporated to dryness under reduced pressure and the residue was dissolved in a 1N aqueous solution of HCl (10 mL). The aqueous solution was extracted with ethyl acetate (3 × 10 mL). The organic layers were collected, washed with brine solution (1 × 10 mL), dried with Na_2SO_4, and filtered. Evaporation of the solvent afforded the 4-nitrobenzenesulfonamide (**25**) in 97% yield.

*4-nitrobenzenesulfonamide (**25**)*

^{1}H NMR (300 MHz, DMSO-d_6) δ (p.p.m.): 8.41 (d, J = 8.7 Hz, Ar-H, 2 H), 8.06 (d, J = 8.7 Hz, Ar-H, 2 H), 7.75 (s, NH_2, 2 H). ^{13}C NMR (75 MHz, DMSO-d_6): 149.90, 149.67, 128.21, 124.89. Elemental

analysis calcd (%) for $C_6H_6N_2O_4S$: C, 35.64; H, 2.99; N, 13.86; found C, 35.72; H, 3.00; N, 13.90.

Synthesis of N-Fmoc-α-aminoacyl-4-nitrobenzene sulfonamides (26a-j).

The *N*-Fmoc amino acid (**26a-j**, 1 mmol) was dissolved in dry DCM (10 mL) in a 100 mL round-bottomed flask and the 4-nitrobenzenesulfonamide (**25**, 1 mmol) together with the EDC (1.1 mmol) and the DMAP (1.1 mmol) was added. The mixture was stirred under an inert atmosphere at room temperature. The reaction, monitored by TLC (chloroform/methanol, 85:15, v/v), was completed after 2 h. The reaction mixture was washed with a 5% $NaHSO_4$ aqueous solution (3 × 10 mL), then with a 5% $NaHCO_3$ aqueous solution (3 × 10 mL) and with a brine solution (1 × 10 mL). The organic solution was dried with Na_2SO_4, filtered and evaporated to dryness under reduced pressure to afford the *N*-Fmoc-α-aminoacyl-4-nitrobenzenesulfonamides (**27a-j**) in very good yield (80-95 %).

*N-Fmoc-L-valinyl-4-nitrobenzenesulfonamide (**27a,e**)*

Yield: 95%. ^{1}H NMR (300 MHz, DMSO-d_6) δ (p.p.m.): 8.23 (d, J = 8.7 Hz, Ar-H_{Ns}, 2H), 8.16 (d, J = 8.7 Hz, Ar-H_{Ns}, 2H), 7.87 (d, J = 7.8 Hz, Ar-H_{Fmoc}, 2H), 7.73 (dd, J = 3.0 Hz, J = 7.2 Hz, Ar-H_{Fmoc}, 2H),

7.44-7.35 (m, Ar-H_{Fmoc}, $NHSO_2Ar$, 3H), 7.33-7.26 (m, Ar-H_{Fmoc}, 2H), 6.61 (d, J = 9.0 Hz, NH, 1H), 4.26-4.14 (m, CH_{2Fmoc}, CH_{Fmoc}, 3H), 3.68 (dd, J = 5.1 Hz, J = 7.2 Hz, α-CH, 1H), 2.09-2.02 (m, β-CH, 1H), 0.75 (d, J = 6.9 Hz , $CH(CH_3)_2$, 3H), 0.66 (d, J = 6.9 Hz , $CH(CH_3)_2$, 3H).^{13}C NMR (75 MHz, DMSO-d_6): 176.15, 156.45, 152.72, 148.43, 144.30, 141.12, 128.73, 128.03, 127.53, 125.87, 123.41, 120.50, 66.04, 62.08, 47.17, 31.54, 20.07, 17.89. . Elemental analysis calcd (%) for $C_{26}H_{25}N_3O_7S$: C, 59.65; H, 4.81; N, 8.03; found C, 59.91; H, 4.80; N, 8.05.

*N-Fmoc-L-phenylalanyl-4-nitrobenzenesulfonamide (**27b**)*

Yield: 94%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 8.23 (d, J = 8.9 Hz, Ar-H_{Ns}, 2H), 8.01 (d, J = 8.9 Hz, Ar-H_{Ns}, 2H), 7.80 (d, J = 7.2 Hz, Ar-H_{Fmoc}, 2H), 7.57-7.39 (m, Ar-H_{Fmoc}, 4H), 7.35-7.27 (m, Ar-H_{Fmoc}, $NHSO_2Ar$, 2H), 7.06-6.95 (m, Ar-H_{Ph}, 3H), 6.91-6.89 (m, Ar-H_{Ph}, 2H), 5.44 (d, J = 8.4 Hz, NH, 1H), 4.59-4.48 (m, α-CH, 1H), 4.45-4.38 (m, CH_{Fmoc}, 1H), 4.26-4.12 (m, CH_{2Fmoc}, 2H), 2.93 (app t, J = 6.9 Hz, CH_2Ph, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): 169.92, 162.44, 143.90, 143.56, 141.36, 141.30, 134.65, 129.73, 129.08, 128.75, 128.02, 127.44, 127.25, 125.06, 124.08, 120.15, 67.96, 56.20, 46.74, 37.91. . Elemental analysis calcd (%) for $C_{30}H_{25}N_3O_7S$: C, 63.04; H, 4.41; N, 7.35; found C, 63.28; H, 4.43; N, 7.33.

*N-Fmoc-L-alanyl-4-nitrobenzenesulfonamide (**27c**)*

Yield: 80%. ^{1}H NMR (300 MHz, DMSO-d_6) δ (p.p.m.): 8.42 (d, J = 8.9 Hz, Ar-H_{Ns}, 2H), 8.31 (s, $NHSO_2$ Ar, 1H), 8.15 (d, J = 8.9 Hz, Ar-H_{Ns}, 2H), 7.88 (d, J = 7.7 Hz, Ar-H_{Fmoc}, 2H), 7.74 (d, J = 7.0 Hz, NH, 1H), 7.69-7.63 (m, Ar-H_{Fmoc}, 2H), 7.43-7.35 (m, Ar-H_{Fmoc}, 2H), 7.34-7.24 (m, Ar-H_{Fmoc}, 2H), 4.26- 4.12 (m, CH_{2Fmoc}, CH_{Fmoc}, 3H), 4.09-3.92 (m, α-CH, 1H), 1.18 (d, J = 7.0 Hz , CHCH_3, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): 176.05, 156.11, 152.70, 148.13, 144.25, 141.10, 128.71, 128.02, 127.51, 125.85, 123.39, 120.52, 65.95, 47.20, 46.51, 17.86. Elemental analysis calcd (%) for $C_{24}H_{21}N_3O_7S$: C, 58.17; H, 4.27; N, 8.48; found C, 58.28; H, 4.28; N, 8.51.

*N-Fmoc-L-leucinyl-4-nitrobenzenesulfonamide (**27d**)*

Yield: 85%. ^{1}H NMR (300 MHz, DMSO-d_6) δ (p.p.m.): 8.40 (d, J = 8.1 Hz, Ar-H_{Ns}, 2H), 8.15 (d, J = 8.1 Hz, Ar-H_{Ns}, 2H), 7.86 (d, J = 7.2 Hz, Ar-H_{Fmoc}, 2H), 7.70 (d, J = 7.5 Hz, NH, 1H),7.64 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.44-7.33 (m, Ar-H_{Fmoc}, $NHSO_2$Ar, 3H), 7.32-7.22 (m, Ar-H_{Fmoc}, 2H), 4.28-4.13 (m, CH_{2Fmoc}, α-C*H*, 3H), 4.10-3.98 (m, CH_{Fmoc}, 1H), 1.65-1.49 (m, γ-CH, 1H), 1.47-1.26 (m, CH_2, 2H), 0.84 (d, J = 6.6 Hz, CH(CH_3)$_2$, 3H), 0.81 (d, J = 6.6 Hz, CH(CH_3)$_2$, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): 173.01, 156.45,

150.67, 144.81, 144.08, 141.13, 129.63, 128.10, 127.49, 125.66, 124.92, 120.58, 79.63, 66.12, 53.74, 47.01, 24.67, 23.42, 21.32. Elemental analysis calcd (%) for $C_{27}H_{27}N_3O_7S$: C, 60.32; H, 5.06; N, 7.82; found C, 60.21; H, 5.08; N, 7.84.

N-Fmoc-D-phenylalanyl-4-nitrobenzenesulfonamide (27f)

Yield: 90%. ^{1}H NMR (300 MHz, DMSO-d_6) δ (p.p.m.): 8.22 (d, J = 8.7 Hz, Ar-H_{Ns}, 2H), 7.97 (d, J = 8.7 Hz, Ar-H_{Ns}, 2H), 7.86 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.66-7.59 (m, Ar-H_{Fmoc}, 2H), 7.42-7.23 (m, Ar-H_{Fmoc}, $NHSO_2$ Ar, 5H), 7.17-7.04 (m, Ar-H_{Ph}, 5H), 6.86 (d, J = 8.9 Hz, NH, 1H), 4.17-4.04 (m, CH_{Fmoc}, CH_{2Fmoc}, 3H), 3.98-3.89 (m, α-CH, 1H), 3.03 (dd, J = 5.1 Hz, J = 13.8 Hz, CH_2Ph, 1H), 2.72 (dd, J = 9.3 Hz, J = 13.8 Hz, CH_2Ph, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): 175.81, 155.87, 155.20, 148.72, 144.33, 141.09, 139.06, 129.39, 128.84, 128.26, 128.01, 127.74, 126.33, 125.82, 123.62, 120.49, 65.91, 58.96, 47.09, 38.20. Elemental analysis calcd (%) for $C_{30}H_{25}N_3O_7S$: C, 63.04; H, 4.41; N, 7.35; found C, 63.25; H, 4.40; N, 7.32.

*N-Fmoc-L-isoleucinyl-4-nitrobenzenesulfonamide (**27j**)*

Yield: 90%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 8.23 (d, J = 8.8 Hz, Ar-H_{Ns}, 2H), 8.11 (d, J = 8.8 Hz, Ar-H_{Ns}, 2H), 7.75 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.52 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.41-7.33 (m,

Ar-H_{Fmoc}, 2H), 7.29-7.18 (m, Ar-H_{Fmoc}, $NHSO_2$ Ar, 3H), 5.65 (d, J = 9.2 Hz, NH, 1H), 4.40 (d, J = 7.2 Hz, CH_{2Fmoc}, 2H), 4.28-4.12 (m, CH_{Fmoc}, α-C*H*, 2H), 1.76-1.64 (m, C*H*CH_3, 1H), 1.44-1.26 (m, C*H*$_2CH_3$, 1H), 1.09-0.95 (m, C*H*$_2CH_3$, 1H), 0.83-0.71 (m CH_2CH_3, $CHCH_3$, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): 171.00, 156.85, 150.89, 143.93, 143.08, 141.15, 129.59, 127.83, 127.09, 124.89, 123.88, 120.28, 67.71, 59.40, 46.76, 37.49, 24.46, 15.05, 10.90. Elemental analysis calcd (%) for $C_{27}H_{27}N_3O_7S$: C, 60.32; H, 5.06; N, 7.82; found C, 60.23; H, 5.07; N, 7.81.

*N-Fmoc-O-tBu-L-serinyl-4-nitrobenzenesulfonamide (**27g**)*

Yield: 92%. ^{1}H NMR (300 MHz, DMSO-d_6) δ (p.p.m.): 8.19 (d, J = 8.7 Hz, Ar-H_{Ns}, 2H), 7.96 (d, J = 8.7 Hz, Ar-H_{Ns}, 2H), 7.86 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.69 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.44-7.25 (m, Ar-H_{Fmoc} , $NHSO_2$Ar, 5H), 6.50 (d, J = 8.4 Hz, NH, 1H), 4.23-4.16 (m, CH_{Fmoc}, CH_{2Fmoc}, 3H), 3.84-3.80 (m, α-CH, 1H), 3.55 (dd, J = 3.3 Hz, J = 8.7 Hz, CH_2OtBu, 1H), 3.46 (dd, J = 6.3 Hz, J = 8.7 Hz, CH_2O*t*Bu, 1H), 1.02 (s, C(C*H*$_3$)$_3$, 9H). ^{13}C NMR (75 MHz, DMSO-d_6): 174.77, 148.12, 144.41, 141.13, 128.57, 128.02, 127.53, 125.79, 123.41, 120.50, 72.61, 66.01, 63.65, 50.98, 47.14, 27.76. . Elemental analysis calcd (%) for $C_{28}H_{29}N_3O_8S$: C, 59.25; H, 5.15; N, 7.40; found C, 59.48; H, 5.17; N, 7.43.

*N-Fmoc-O-tBu-L-tyrosinyl-4-nitrobenzenesulfonamide(**27h**)*

Yield: 86%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 7.89-7.70 (m, Ar-H_{Ns}, 4H), 7.67- 7.56 (m, Ar-H_{Fmoc}, 2H), 7.29-7.18 (m, Ar-H_{Fmoc}, 2H), 7.09-6.92 (m, Ar-H_{Fmoc}, $NHSO_2Ar$, 5H), 6.77 (d, J = 8.7 Hz, Ar-H_{Tyr}, 2H), 6.62 (d, J = 8.7 Hz, Ar-H_{Tyr}, 2H), 5.50 (br s, NH, 1H), 4.40-4.23 (m, α-CH, 1H), 4.20-3.79 (m, CH_{Fmoc}, CH_{2Fmoc}, 3H), 2.98-2.65 (m, CH_{2Tyr}, 2H), 1.25 (s, $C(CH_3)_3$, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): 176.43, 156.95, 154.10, 149.15, 148.31, 143.53, 141.08, 129.56, 127.65, 126.99, 124.85, 123.90, 123.53, 119.98, 78.34, 67.26, 60.42, 46.77, 36.71, 28.73. Elemental analysis calcd (%) for $C_{34}H_{33}N_3O_8S$: C, 63.44; H, 5.17; N, 6.53; found C, 63.29; H, 5.19; N, 6.56.

*N-Fmoc-O-tBu-L-glutamyl-4-nitrobenzenesulfonamide (**4i**)*

Yield: 80%. ^{1}H NMR (300 MHz, DMSO-d_6) δ (p.p.m.): 8.21 (d, J = 8.4 Hz, Ar-H_{Ns}, 2H), 7.96 (d, J = 8.4 Hz, Ar-H_{Ns}, 2H), 7.90-7.81 (m, Ar-H_{Fmoc}, 2H), 7.72-7.63 (m, Ar-H_{Fmoc}, $NHSO_2Ar$, 3H), 7.44-7.35 (m, Ar-H_{Fmoc}, 2H), 7.34-7.25 (m, Ar-H_{Fmoc}, 2H), 6.74 (d, J = 9.0 Hz, NH, 1H), 4.32-4.13 (m, CH_{2Fmoc}, 2H), 3.82-3.71 (m, CH_{Fmoc}, 1H), 3.69-3.60 (m, α-CH, 1H), 2.20-1.83 (m, CH_2CH_2, 4H), 1.37 (s, $C(CH_3)_3$, 9H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): 173.01, 172.56, 157.60, 152.02, 144.72, 144.10, 141.12, 128.65, 128.02, 127.51,

125.85, 123.44, 120.49, 79.81, 62.12, 56.05, 47.17, 28.79, 28.21, 27.05 ppm. Elemental analysis calcd (%) for $C_{30}H_{31}N_3O_9S$: C, 59.10; H, 5.13; N, 6.89; found C, 59.30; H, 5.14; N, 6.91.

Synthesis of N-Fmoc-α-aminoacyl-N-methyl-4-nitrobenzenesulfonamides (28 a-i).

The *N*-Fmoc-α-aminoacyl 4-nitrobenzenesulfonamide (**27a-i**, 1 mmol) was dissolved in 10 mL of dry DMF in a 50 mL round-bottomed flask . Methyl iodide (5 mmol) and DIPEA (5 mmol) were added and the mixture stirred at room temperature under an inert atmosphere and monitored by TLC (chloroform/methanol, 90:10, v/v). The reaction was completed after 2 h. To the reaction mixture 10 mL of a 5% $NaHSO_4$ aqueous solution were added and the resulting solution was extracted with ethyl acetate (3 × 10 mL). The organic layers were collected and washed with a 5% $NaHCO_3$ aqueous solution (3 × 10 mL), with a brine solution (1 × 10 mL) and then dried (Na_2SO_4), filtered and evaporated to dryness. The corresponding *N*-Fmoc-aminoacyl-4-nitrobenzenesulfonamides *N*-methylated (**28 a-i**) were recovered in very good yield (80-92%).

*N-Fmoc-L-valinyl-N-methyl-4-nitrobenzenesulfonamide (**28a,e**)*

Yield: 85%. ^{1}H NMR (300 MHz, DMSO-d_6) δ (p.p.m.): 8.35 (d, J = 8.7 Hz, Ar-H_{Ns}, 2H), 8.20 (d, J = 8.7 Hz, Ar-H_{Ns}, 2H), 7.90-7.81 (m, Ar-H_{Fmoc}, NH, 3H), 7.53-7.51 (m, Ar-H_{Fmoc}, 2H), 7.44-7.23 (m, Ar-H_{Fmoc}, 4H), 4.45 (app t, J = 7.8 Hz, α-CH, 1H), 4.32-4.10 (m, CH_{Fmoc}, CH_{2Fmoc}, 3H), 3.46 (s, NCH_3, 3H), 2.06-1.93 (m, C*H*$(CH_3)_2$, 1H), 0.85 (d, J = 6.6 Hz, CH(C*H*$_3)_2$, 6H).^{13}C NMR (75 MHz, DMSO-d_6): 174.01, 150.73, 144.20, 144.12, 141.18, 129.89, 128.08, 127.48, 124.76, 120.56, 66.36, 58.94, 47.02, 34.02, 30.61, 19.13, 18.35. Elemental analysis calcd (%) for $C_{27}H_{27}N_3O_7S$: C, 60.32; H, 5.06; N, 7.82; found C, 60.56; H, 5.06; N, 7.80.

*N-Fmoc-L-phenylalanyl-N-methyl-4-nitrobenzene sulfonamide (**28b**)*

Yield: 86%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 8.36 (d, J = 8.9 Hz, Ar-H_{Ns}, 2H), 8.17 (d, J = 8.9 Hz, Ar-H_{Ns}, 2H), 7.86 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.48 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.45-7.10 (m, Ar-H_{Fmoc}, Ar-H_{Phe}, 9H), 5.48 (br s, NH, 1H), 4.40-4.20 (m, α-CH, CH_2Fmoc, 3H), 4.16-4.05 (m, CH_{Fmoc}, 1H), 3.20 (s, NCH_3, 3H), 2.98-2.60 (m, CH_2Ph, 2H).^{13}C NMR (75 MHz, $CDCl_3$): 173.41, 156.98, 150.81, 143.60, 143.53, 141.28, 135.09, 129.36, 129.32, 128.82, 127.80, 127.75, 127.08, 124.98, 124.41, 120.07, 67.30,

55.35, 46.93, 39.15, 33.23. Elemental analysis calcd (%) for $C_{31}H_{27}N_3O_7S$: C, 63.88; H, 5.19; N, 6.98; found C, 64.12; H, 5.21; N, 6.95.

*N-Fmoc-L-alanyl-N-methyl-4-nitrobenzenesulfonamide (**28c**)*

Yield: 92%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 8.38 (d, J = 9.0 Hz, Ar-H_{Ns}, 2H), 8.26 (d, J = 9.0 Hz, Ar-H_{Ns}, 2H), 7.81 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.65 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.46-7.25 (m, Ar-H_{Fmoc}, 4H), 5.36 (d, J = 9.0 Hz, NH, 1H), 5.23-5.10 (m, α-CH, 1H), 4.34 (d, J = 7.2 Hz, CH_{2Fmoc}, 2H), 4.27-4.09 (m, CH_{Fmoc} , 1H), 3.32 (s, NCH_3, 3H), 1.48 (d, J = 6.9 Hz, CH(CH_3), 3H).^{13}C NMR (75 MHz, $CDCl_3$): 174.60, 155.87, 143.86, 143.02, 141.30, 129.42, 127.81, 127.07, 125.00, 124.47, 120.06, 67.24, 50.29, 47.01, 33.27, 18.88. Elemental analysis calcd (%) for $C_{25}H_{23}N_3O_7S$: C, 58.93; H, 4.55; N, 8.25; found C, 58.72; H, 4.57; N, 8.22.

*N-Fmoc-L-leucinyl-N-methyl-4-nitrobenzenesulfonamide (**28d**)*

Yield: 90%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 8.36 (d, J = 8.7 Hz, Ar-H_{Ns}, 2H), 8.25 (d, J = 8.7 Hz, Ar-H_{Ns}, 2H), 7.77 (d, J = 7.2 Hz, Ar-H_{Fmoc}, 2H), 7.56 (d, J = 7.2 Hz, Ar-H_{Fmoc}, 2H), 7.42-7.37 (m, Ar-H_{Fmoc}, 2H), 7.34-7.27 (m, Ar-H_{Fmoc}, 2H), 5.30-5.10 (m, NH, α-CH, 2 H), 4.43-4.28 (m, CH_{2Fmoc}, 2H), 4.21-4.13 (m, CH_{Fmoc}, 1H), 3.32 (s, NCH_3, 3H), 1.85-1.47 (m, CH_2CH(CH_3)$_2$, 3H), 1.07-0.94 (m,

$CH(CH_3)_2$, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): 174.73, 156.27, 150.73, 143.60, 143.55, 141.30, 129.43, 127.82, 127.06, 124.98, 124.45, 120.05, 67.18, 53.04, 47.04, 41.72, 33.26, 24.92, 23.37, 21.06. Elemental analysis calcd (%) for $C_{28}H_{29}N_3O_7S$: C, 60.97; H, 5.30; N, 7.62; found C, 61.17; H, 5.32; N, 7.64.

*N-Fmoc-D-phenylalanyl-N-methyl-4-nitrobenzene sulfonamide (**28f**)*

Yield: 80%. ^1H NMR (300 MHz, DMSO-d_6) δ (p.p.m.): 8.37 (d, J = 8.7 Hz, Ar-H_{Ns}, 2H), 8.17 (d, J = 8.7 Hz, Ar-H_{Ns}, 2H), 8.01(m, NH, 1H), 7.82 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.66-7.54 (m, Ar-H_{Fmoc}, 2H), 7.43-7.06 (m, Ar-H_{Fmoc}, Ar-H_{Phe}, 9H), 4.86-4.77 (m, α-CH, 1H), 4.32-3.98 (m, CH_{Fmoc}, CH_{2Fmoc}, 3H), 3.30 (s, NCH_3, 3H), 2.98 (dd, J = 5.1 Hz, J = 13.8 Hz, CH_2Ph, 1H), 2.77 (dd, J = 9.3 Hz, J = 13.8 Hz, CH_2Ph, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): 173.71, 156.88, 143.98, 143.84, 141.15, 137.76, 129.71, 128.64, 128.08, 127.48, 124.84, 120.54, 79.63, 60.20, 55.56, 21.21, 14.54. Elemental analysis calcd (%) for $C_{31}H_{27}N_3O_7S$: C, 63.88; H, 5.19; N, 6.98; found C, 64.14; H, 5.20; N, 6.96.

N-Fmoc-O-tBu-serinyl-N-methyl-4-nitrobenzene sulfonamide ***(28g)***

Yield: 84%. ^{1}H NMR (300 MHz, DMSO-d_6) δ (p.p.m.): 8.37 (d, J = 8.9 Hz, Ar-H_{Ns}, 2H), 8.18 (d, J = 8.9 Hz, Ar-H_{Ns}, 2H), 7.90 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.71-7.58 (m, Ar-H_{Fmoc}, NH, 3H), 7.45-7.36 (m, Ar-H_{Fmoc}, 2H), 7.34-7.27 (m, Ar-H_{Fmoc}, 2H), 4.76-4.67 (m, α-CH, 1H), 4.12-4.32 (m, CH_{Fmoc}, CH_{2Fmoc}, 3H), 4.39-4.49 (m, CH_2OtBu, 2H), 3.34 (s, NCH_3, 3H), 1.05 (s, C(CH_3)$_3$, 9H).^{13}C NMR (75 MHz, DMSO-d_6): 182.11, 156.67, 150.96, 144.12, 141.18, 130.01, 128.10, 127.51, 124.71, 120.57, 73.59, 66.88, 60.21, 53.90, 46.96, 34.01, 27.93. Elemental analysis calcd (%) for $C_{29}H_{31}N_3O_8S$: C, 59.88; H, 5.37; N, 7.22; found C, 60.11; H, 5.35; N, 7.24.

N-Fmoc-(OtBu)-tyrosinyl-N-methyl-4-nitrobenzene sulfonamide ***(28h)***

Yield: 85%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 8.35 (d, J = 8.9 Hz, Ar-H_{Ns}, 2H), 8.19 (d, J = 8.9 Hz, Ar-H_{Ns}, 2H), 7.80 (d, J = 7.2 Hz, Ar-H_{Fmoc}, 2H), 7.53 (d, J = 7.2 Hz, Ar-H_{Fmoc}, 2H), 7.43-7.22 (m, Ar-H_{Fmoc} , 4H), 7.09 (d, J = 8.7 Hz, Ar-H_{Tyr}, 2H), 6.91 (d, J = 8.7 Hz, Ar-H_{Tyr}, 2H), 5.40 (d, J = 8.7 Hz, NH, 1H), 4.68-4.61 (m, α-CH, 1H), 4.50-4.08 (m, CH_{Fmoc}, CH_{2Fmoc}, 3H), 3.18 (s, NCH_3, 3H), 3.11-3.04

(m, CH_{2Tyr}, 1H), 2.95-2.84 (m, CH_{2Tyr}, 1H), 1.35 (s, $CH(CH_3)_3$, 9H).^{13}C NMR (75 MHz, $CDCl_3$): 173.52, 155.86, 154.81, 150.66, 143.76, 143.54, 141.28, 129.81, 129.53, 127.80, 127.08, 125.00, 124.21, 120.06, 67.31, 60.42, 55.36, 46.94, 38.65, 33.18, 29.01. . Elemental analysis calcd (%) for $C_{35}H_{35}N_3O_8S$: C, 64.17; H, 5.83; N, 6.24; found C, 64.37; H, 5.85; N, 6.26.

*N-Fmoc-(OtBu)-glutamyl-N-methyl-4-nitrobenzene sulfonamide (**28i**)*

Yield: 87%. ^{1}H NMR (300 MHz, DMSO-d_6) δ (p.p.m.): 8.38 (d, J = 8.7 Hz, Ar-H_{Ns}, 2H), 8.21 (d, J = 8.7 Hz, Ar-H_{Ns}, 2H), 7.96 (d, J = 8.9 Hz, NH 1H), 7.95-7.80 (m, Ar-H_{Fmoc}, 2H), 7.73 (d, J = 7.5 Hz , Ar-H_{Fmoc}, 2H), 7.44-7.25 (m, Ar-H_{Fmoc}, 4H), 4.33-4.02 (m, α-CH , CH_{Fmoc}, CH_{2Fmoc}, 4H), 3.33 (s, NCH_3, 3H), 2.31-2.19 (m, CH_{2Glu}, 2H), 2.02-1.74 (m, CH_{2Glu}, 2H), 1.38 (s, $CH(CH_3)_3$, 9H). ^{13}C NMR (75 MHz, DMSO-d_6): 178.16, 176.21, 157.81, 154.89, 144.56, 141.21, 128.09, 127.51, 126.05, 120.58, 80.05, 66.20, 53.01, 47.11, 30.61, 28.20, 26.76. Elemental analysis calcd (%) for $C_{31}H_{33}N_3O_9S$ C, 59.70; H, 5.33; N, 6.74; found C, 59.76; H, 5.34; N, 6.72.

Synthesis of N-Fmoc-dipeptide methyl esters (30 a-i).

The *N*-Fmoc-α-aminoacyl-*N*-methyl-4-nitrobenzenesulfonamide (1 mmol, **30a-i**) was dissolved in 10 mL of dry dichloromethane

in a 100 mL two-necked round-bottomed flask under a nitrogen inert atmosphere. The α-amino acid methyl ester (1 mmol, **29**), the DMAP (2 mmol) and 1 mL of DMF were added. The reaction mixture was stirred at reflux and the reaction monitored by TLC (Et_2O/petroleum ether 70:30 v/v). After about 2 h the reaction was completed and the reaction mixture was washed with a 9% aqueous solution of Na_2CO_3 (2 × 10 mL), then with a 5% $NaHSO_4$ aqueous solution (2 × 10 mL) and finally with a brine solution (1 × 10 mL). The organic layer was dried (Na_2SO_4), paper-filtered and evaporated under reduced pressure. The crude reaction product was purified by column chromatography (Et_2O/ petroleum ether 70:30 v/v) and the *N*-Fmoc-dipeptide methyl ester was recovered in good yield (68-80%).

*N-Fmoc-L-valinyl-L-alanine methyl ester (**30a**)*

Yield: 70%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 7.78 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.61 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.48-7.36 (m, Ar-H_{Fmoc}, 2H), 7.32-7.22 (m, Ar-H_{Fmoc}, 42H), 6.27 (d, J = 7.5 Hz , NH, 1H), 5.40 (d, J = 9.0 Hz , $NH_{urethane}$, 1H), 4.60 (app quin, J = 7.2 Hz, CH_{ala}, 1H), 4.48-4.33 (m, CH_{2Fmoc}, 2H), 4.27-4.19 (m, CH_{Fmoc}, 1H), 4.02 (dd, J = 6.6 Hz, J = 9.0 Hz, CH_{val}, 1H), 3.75 (s, OCH_3, 3H), 2.19-2.07 (m, C*H*$(CH_3)_2$, 1H), 1.43 (d, J = 7.2 Hz, CHC*H*$_3$, 3H), 0.98 (m, CH(C*H*$_3$)$_2$, 6H). ^{13}C NMR (75 MHz, $CDCl_3$):

173.46, 173.07, 143.98, 141.32, 127.73, 127.09, 125.08, 119.98, 67.07, 60.27, 52.53, 48.15, 47.20, 31.05, 19.00, 18.25. Elemental analysis calcd (%) for $C_{24}H_{28}N_2O_5$: C, 67.91; H, 6.65; N, 6.60; found C, 67.67; H, 6.68; N, 6.62.

*N-Fmoc-L-phenylalanyl-L-alanine methyl ester (**30b**)*

Yield: 68%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 7.78 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.59-7.51 (m, Ar-H_{Fmoc}, 2H), 7.46-7.38 (m, Ar-H_{Fmoc}, 2H), 7.37-7.16 (m, Ar-H_{Fmoc}, Ar-H_{Phe}, 7H), 6.43 (br s, NH, 1H), 5.44 (br s, $NH_{urethane}$, 1H), 4.58-4.41 (m, CH_{ala}, CH_{2Fmoc}, 3H), 4.39-4.28 (m, CH_{Phe}, 1H), 4.25-4.16 (m, CH_{Fmoc}, 1H), 3.72 (s, OCH_3, 3H), 3.19-3.02 (m, CH_{2Phe}, 2H), 1.35 (d, J = 7.2 Hz, CHCH_3, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): 172.76, 170.32, 156.01, 143.71, 141.30, 136.24, 128.98, 128.56, 128.01, 127.75, 125.02, 120.00, 67.11, 56.00, 52.48, 48.19, 47.12, 38.62, 18.31. Elemental analysis calcd (%) for $C_{28}H_{28}N_2O_5$: C, 71.17; H, 5.97; N, 5.93; found C, 71.35; H, 5.95; N, 5.95.

*N-Fmoc-L-alanyl-L-leucine methyl ester (**30c**)*

Yield: 75%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 7.77 (d, J = 7.4Hz, Ar-H_{Fmoc}, 2H), 7.55 (d, J = 7.4 Hz, Ar-H_{Fmoc}, 2H), 7.45-7.37 (m, Ar-H_{Fmoc}, 2H), 7.34-7.25 (m, Ar-H_{Fmoc}, 2H), 5.32 (d, J = 9.5 Hz, $NH_{urethane}$, 1H), 5.16 (m, CH_{ala}, 1H), 4.45-4.31 (m, CH_{2Fmoc}, CH_{Fmoc},

3H), 4.26-4.12 (m, CH_{leu}, 1H), 3.31 (s, OCH_3, 3H), 1.68-1.55 (m, CH_2, 4H), 1.47 (d, J = 6.9 Hz, $CHCH_3$, 3H), 1.45-1.32 (m, $CHCH_3$, 1H), 0.94-0.86 (m, $CH(CH_3)_2$, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): 173.10, 171.99, 153.55, 141.48, 134.66, 129.56, 127.70, 127.10, 120.00, 67.15, 53.44, 52.40, 48.10, 47.20, 41.60, 24.70, 22.99,18.21, 18.19. Elemental analysis calcd (%) for $C_{25}H_{30}N_2O_5$: C, 68.47; H, 6.90; N, 6.39; found C, 68.62; H, 6.92; N, 6.37.

*N-Fmoc-L-leucinyl-L-alanine methyl ester (**30d**)*

Yield: 76%. ^1H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 7.78 (d, J = 7.2 Hz, Ar-H_{Fmoc}, 2H), 7.61 (d, J = 7.2 Hz, Ar-H_{Fmoc}, 2H), 7.46-7.38 (m, Ar-H_{Fmoc}, 2H), 7.37-7.24 (m, Ar-H_{Fmoc}, 2H), 6.53 (d, J = 6.9 Hz, NH, 1H), 5.29 (d, J = 7.8 Hz, $NH_{urethane}$, 1H), 4.60 (app quin, J = 7.2 Hz, CH_{ala}, 1H), 4.48-4.35 (m, CH_{2Fmoc}, 2H), 4.29-4.18 (m, CH_{Fmoc}, CH_{leu}, 2H), 3.71 (s, OCH_3, 3H), 1.76-1.51 (m, $CH_2CH(CH_3)_2$, 3H), 1.43 (d, J = 7.2 Hz, $CHCH_3$, 3H), 1.05-0.70 (m, $CH(CH_3)_2$, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): 173.46, 171.90, 153.97, 141.31, 134.70, 129.09, 127.73, 127.08, 119.98, 67.05, 53.41, 52.50, 48.07, 47.17, 41.63, 24.63, 22.91,18.31, 18.29. Elemental analysis calcd (%) for $C_{25}H_{30}N_2O_5$: C, 68.47; H, 6.90; N, 6.39; found C, 68.14; H, 6.91; N, 6.42.

*N-Fmoc-L-valinyl-D-alanine methyl ester (**30e**)*

Yield: 72%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 7.78 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.61 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.47-7.30 (m, Ar-H_{Fmoc}, 4H), 6.46 (d, J = 7.0 Hz , NH, 1H), 5.36 (d, J = 8.9 Hz, $NH_{urethane}$, 1H), 4.61 (app quin, J = 7.2 Hz, CH_{ala}, 1H), 4.50-4.36 (m, CH_{2Fmoc}, 2H), 4.27-4.22 (m, CH_{Fmoc}, 1H), 4.10-4.01 (m, CH_{val}, 1H), 3.76 (s, OCH_3, 3H), 2.24-2.12 (m, C*H*$(CH_3)_2$, 1H), 1.43 (d, J = 7.2 Hz, $CHCH_3$, 3H), 1.02-0.99 (m, CH(C*H*$_3)_2$, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): 170.91, 170.37, 143.88, 141.33, 127.74, 127.09, 125.05, 120.01, 67.07, 60.27, 52.57, 48.15, 47.20, 29.98, 19.14, 18.39. Elemental analysis calcd (%) for $C_{24}H_{28}N_2O_5$: C, 67.91; H, 6.65; N, 6.60; found C, 68.17; H, 6.62; N, 6.63.

*N-Fmoc-D-phenylalanyl-L-alanine methyl ester (**30f**)*

Yield: 70%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 7.78 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.59-7.52 (m, Ar-H_{Fmoc}, 2H), 7.45-7.37 (m, Ar-H_{Fmoc}, 2H), 7.36-7.17 (m, Ar-H_{Fmoc} , Ar-H_{Phe}, 7H), 6.16 (d, J = 7.5 Hz, NH, 1H), 5.44 (d, J = 8.1 Hz, $NH_{urethane}$, 1H), 4.57-4.29 (m, CH_{ala}, CH_{2Fmoc}, CH_{Phe}, 4H), 4.20 (t, J = 6.9 Hz, CH_{Fmoc}, 1H), 3.71 (s, OCH_3, 3H), 3.19-3.11 (m, CH_2Phe, 1H), 3.07-2.98 (m, CH_2Phe, 1H), 1.23 (d, J = 7.2 Hz, CHC*H*$_3$, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): 172.91, 170.03, 143.70, 141.31, 136.32, 129.36, 128.79, 127.76,

127.18, 127.10, 125.06, 120.02, 67.10, 56.10, 52.56, 47.96, 47.11, 38.60, 18.13. Elemental analysis calcd (%) for $C_{28}H_{28}N_2O_5$: C, 71.17; H, 5.97; N, 5.93; found C, 71.43; H, 5.95; N, 5.96.

*N-Fmoc-OtBu-L-serinyl-L-alanine methyl ester (**30g**)*

Yield: 80%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 7.77 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.61 (d, J = 7.2 Hz, Ar-H_{Fmoc}, 2H), 7.51 (d, J = 6.9 Hz, NH, 1H), 7.45-7.26 (m, Ar-H_{Fmoc}, 4H), 5.82 (d, J = 6.0 Hz , $NH_{urethane}$, 1H), 4.65-4.52 (m, CH_{ala}, 1H), 4.40 (d, J = 7.2 Hz, CH_{2Fmoc}, 2H), 4.30-4.20 (m, CH_{Fmoc}, CH_{ser}, 2H), 3.86-3.80 (m, CH_{2ser}, 1H), 3.76 (s, OCH_3, 3H), 3.43-3.34 (m, CH_{2ser}, 1H), 1.44 (d, J = 7.2 Hz, CHC*H*$_3$, 3H), 1.25 (s, $C(CH_3)_3$, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): 173.07, 169.98, 156.04, 143.73, 141.29, 127.75, 127.10, 125.18, 120.03, 74.48, 67.12, 61.73, 54.01, 52.53, 48.32, 47.10, 27.38, 18.46. Elemental analysis calcd (%) for $C_{26}H_{32}N_2O_6$: C, 66.65; H, 6.88; N, 5.98; found C, 66.87; H, 6.89; N, 6.00.

*N-Fmoc-OtBu-L-tyrosinyl-L-alanine methyl ester (**30h**)*

Yield: 74%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 7.78 (d, J = 7.2 Hz, Ar-H_{Fmoc}, 2H), 7.60-7.54 (m, Ar-H_{Fmoc}, 2H), 7.44-7.25 (m, Ar-H_{Fmoc}, 4H), 7.10 (d, J = 8.1 Hz, Ar-H_{Tyr}, 2H), 6.92 (d, J = 8.1 Hz, Ar-H_{Tyr}, 2H), 6.33 (d, J = 6.7 Hz , NH, 1H), 5.43 (d, J = 7.9 Hz , $NH_{urethane}$, 1H), 4.53-4.31 (m, CH_{ala} , CH_{2Fmoc}, CH_{tyr}, 4H), 4.24-4.15

(m, CH_{Fmoc}, 1H), 3.72 (s, OCH_3, 3H), 3.17-3.06 (m, CH_{2tyr}, 1H), 3.05-2.94 (m, CH_{2tyr}, 1H), 1.39-1.21 (m, CHCH_3 , $C(CH_3)_3$, 12H). ^{13}C NMR (75 MHz, $CDCl_3$): 172.75, 170.33, 156.04, 154.47, 143.70, 141.29, 129.85, 127.76, 127.11, 125.05, 120.01, 78.47, 67.10, 56.10, 52.53, 48.20, 47.11, 38.02, 29.73, 18.33. Elemental analysis calcd (%) for $C_{32}H_{36}N_2O_6$: C, 70.57; H, 6.66; N, 5.14; found C, 70.71; H, 6.64; N, 5.12.

*N-Fmoc-OtBu-L-glutamyl-L-alanine methyl ester (**30i**)*

Yield: 75%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 7.78 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.61 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.48-7.26 (m, Ar-H_{Fmoc}, 4H), 6.89 (d, J = 7.2 Hz , NH, 1H), 5.75 (d, J = 7.5 Hz , $NH_{urethane}$, 1H), 4.68-4.52 (m, CH_{ala},1H), 4.48-4.36 (m, CH_{2Fmoc}, 2H), 4.31-4.20 (m, CH_{glu}, CH_{Fmoc}, 2H), 3.78 (s, OCH_3, 3H), 2.54-2.41 (m, CH_{2glu}, 1H), 2.19-1.90 (m, CH_{2glu}, 1H), 1.39-1.21 (m, CHCH_3 , $C(CH_3)_3$, 12H). ^{13}C NMR (75 MHz, $CDCl_3$): 173.12, 173.06, 172.71, 156.05, 143.96, 141.30, 127.73, 127.09, 125.14, 120.00, 81.18, 67.01, 53.99, 52.50, 48.19, 47.13, 31.60, 29.70, 28.52, 18.14. Elemental analysis calcd (%) for $C_{28}H_{34}N_2O_7$: C, 65.87; H, 6.71; N, 5.49; found C, 66.11; H, 6.73; N, 5.50.

Deprotection of N-Fmoc-L-isoleucinyl-4-nitrobenzenesulfonamide (27j).

N-Fmoc-L-isoleucinyl-4-nitrobenzenesulfonamide (**27j**) (1 mmol) was dissolved in 10 mL of dry DMF in a 100 mL round-bottomed flask. The piperidine (5 mmol) was added and the mixture left to stir at room temperature. The reaction, monitored by TLC (Et_2O/CH_3OH 90:10, v/v), was completed after 1 h. 10 mL of a 0.2 N aqueous solution of HCl were added to the reaction mixture and the resulting solution extracted with dichloromethane (3 × 10 mL). The aqueous phase was basified to pH 9 with a 0.2 N solution of NaOH and extracted with DCM (3 × 10 mL). The obtained L-isoleucinyl 4-nitrobenzenesulfonamide free on amino function was directly coupled with the *N*-Fmoc-L-phenylalanine.

Synthesis of N-Fmoc-L-phenylalanyl-L-isoleucinyl-4-nitrobenzenesulfonamide (31j).

To a magnetically stirred solution of L-isoleucinyl-4-nitrobenzenesulfonamide (1 mmol) in dry dichlomethane (5 mL) and DIPEA (0.8 mmol) was added dropwise a solution of *N*-Fmoc-L-phenylalanine chloride (0.8 mmol) in dry dichloromethane (5 mL). The resulting mixture was stirred at

room temperature under inert atmosphere for 1 h, monitoring the conversion of *N*-Fmoc-L-phenylalanine chloride by TLC (Et_2O/ CH_3OH, 90:10, v/v). The reaction was washed with 1 N HCl (3 × 10 mL), with a 5% aqueous $NaHCO_3$ solution (2× 10 mL) and with brine (1 × 10 mL), dried (Na_2SO_4) and evaporated to dryness to give the *N*-Fmoc-L-phenylalanyl-L-isoleucinyl-4-nitrobenzene-sulfonamide (**31j**) in 72% yields.

*N-Fmoc-L-phenylalanyl-L-isoleucinyl-4 nitrobenzenesulfonamide (**31j**)*

Yield: 72%. ^{1}H NMR (300 MHz, DMSO-d_6) δ (p.p.m.): 8.19 (d, J = 8.7 Hz, Ar-H_{Ns}, 2H), 7.94 (d, J = 8.7 Hz, Ar-H_{Ns}, 2H), 7.91-7.84 (m, Ar-H_{Fmoc}, 2H), 7.71-7.57 (m, Ar-H_{Fmoc}, 2H), 7.44-7.14 (m, Ar-H_{Fmoc} , Ar-H_{Phe} , NH, 10H), 4.06-4.26 (m, α-CH_{Ile} , α-CH_{Phe} , CH_{Fmoc}, CH_{2Fmoc}, 5H), 3.13-2.79 (m, CH_{2Phe}, 2H), 1.81-1.68 (m, C*H*CH_3, 1H), 1.20-0.91 (m, CH_{2Ile}, 1H), 0.90-0.78 (m, CH_{2Ile}, 1H), 0.77-0.55 (m, CHC*H*$_3$, CH_2C*H*$_3$, 6H).^{13}C NMR (75 MHz, DMSO-d_6): 180.81, 172.46, 160.12, 158.15, 148.47, 147.37, 143.58, 142.70, 129.68, 128.75, 128.09, 127.62, 127.48, 127.40, 125.78, 123.12, 120.21, 65.98, 56.21, 48.31, 38.85, 37.88, 24.85, 15.98, 12.32. Elemental analysis calcd (%) for $C_{36}H_{36}N_4O_8S$: C, 63.14; H, 5.30; N, 8.18; found C, 63.33; H, 5.32; N, 8.15.

Synthesis of N-Fmoc-L-phenylalanyl-L-isoleucinyl-N-methyl-4-nitrobenzenesulfonamide (32j).

N-Fmoc-L-phenylalanyl-L-isoleucinyl-4-nitrobenzenesulfonamide (**31j**, 1 mmol) was dissolved in 10 mL of dry DMF in a 50 mL round-bottomed flask. Methyl iodide (5 mmol) and DIPEA (5 mmol) were added and the mixture stirred at room temperature under an inert atmosphere and monitored by TLC (Et_2O/ CH_3OH, 90:10, v/v). The reaction was completed after 2 h. To the reaction mixture 10 mL of a 5% $NaHSO_4$ aqueous solution were added and the resulting solution extracted with ethyl acetate (3 × 10 mL). The organic layers were collected and washed with a 5% $NaHCO_3$ aqueous solution (3 × 10 mL), with a brine solution (1 × 10 mL) and then dried (Na_2SO_4), filtered and evaporated to dryness. The corresponding *N*-Fmoc-L-phenylalanyl-L-isoleucine-*N*-methyl-4-nitrobenzenesulfonamide (**32j**) was recovered in very good yield (78%).

*N-Fmoc-L-phenylalanyl-L-isoleucine-N-methyl-4-nitrobenzene-sulfonamide (**32j**)*

Yield: 78%. ^{1}H NMR (300 MHz, DMSO-d_6) δ (p.p.m.): 8.42 (d, J = 8.8 Hz, Ar-H_{Ns}, 2H), 8.22 (d, J = 8.8 Hz, Ar-H_{Ns}, 2H), 7.93-7.81 (m, Ar-H_{Fmoc}, 2H), 7.72-7.54 (m, Ar-H_{Fmoc}, 2H), 7.48-7.11 (m, Ar-H_{Fmoc},

Ar-H_{Phe} , NH, 10H), 4.31-4.08 (m, α-CH_{Ile} , α-CH_{Phe} , CH_{Fmoc}, CH_{2Fmoc}, 5H), 3.62 (s, NCH_3, 3H), 3.11-3.01 (m, CH_{2Phe}, 1H), 2.81-2.68 (m, CH_{2Phe}, 1H), 1.82-1.68 (m, $CHCH_3$, 1H), 1.32-1.17 (m, CH_{2Ile}, 2H), 0.89-0.64 (m, $CHCH_3$, CH_2CH_3, 6H). Elemental analysis calcd (%) for $C_{37}H_{38}N_4O_8S$: C, 63.60; H, 5.48; N, 8.02; found C, 63.84; H, 5.45; N, 8.05.

Synthesis of N-Fmoc-L-phenylalanyl-L-isoleucine (33j).

The *N*-Fmoc-l-phenylalanyl-l-isoleucinyl-*N*-methyl-4-nitro-benzenesulfonamide (**32j**) was dissolved in dry DCM (10 mL) in a 100 mL flask. A 5% aqueous solution of NaOH was added (10 mL) and the reaction mixture left to stir at room temperature. The reaction, monitored by TLC (Et_2O/ CH_3OH, 90:10, v/v) was completed after 2h. The organic solvent was evaporated and the reaction mixture acidified with a 1N HCl solution and extracted with ethyl acetate (3 × 10 mL). The organic layer were collected, dried (Na_2SO_4), filtered and evaporated to dryness. The *N*-Fmoc-L-phenylalanyl-L-isoleucine (**33j**) was recovered in very good yield (82%).

*N-Fmoc-L-phenylalanyl-L-isoleucine (**33j**)*

Yield: 82%. ^{1}H NMR (300 MHz, DMSO-d_6) δ (p.p.m.): 12.65 (br s, COOH, 1H), 8.07 (d, J = 8.7 Hz, NH, 1H), 7.88 (d, J = 7.5 Hz, Ar-

H_{Fmoc}, 2H), 7.70-7.57 (m, Ar-H_{Fmoc}, 4H), 7.48-7.20 (m, Ar-H_{Fmoc} , Ar-H_{Phe}, 7H), 7.19 (d, J = 7.5 Hz, $NH_{urethane}$, 1H), 4.42-4.31 (m, α-CH_{Phe}, 1H), 4.25-4.05 (m, α-CH_{Ile}, CH_{2Fmoc} , CH_{Fmoc}, 4H), 3.07-2.92 (m, CH_{2Phe}, 1H), 2.83-2.70 (m, CH_{2Phe}, 1H), 1.88-1.72 (m, CH_{Ile}, 1H), 1.51-1.34 (m, CH_{2Ile}, 1H), 1.28-1.11 (m, CH_{2Ile}, 1H), 0.95-0.74 (m, CHCH_3 , $CH_2C$$H_3$, 6H) . Elemental analysis calcd (%) for $C_{30}H_{32}N_2O_5$: C, 71.98; H, 6.44; N, 5.60; found C, 71.69; H, 6.46; N, 5.62.

Synthesis of N-Fmoc-L-phenylalanyl-L-isoleucinyl-L-alanine methyl ester (34j)

The *N*-Fmoc-L-phenylalanyl-L-isoleucinyl-*N*-methyl-4-nitro-benzenesulfonamide (1 mmol, **32j**) was dissolved in 10 mL of dry dichloromethane in a 100 mL two-necked round-bottomed flask under a nitrogen inert atmosphere. The L-alanine methyl ester (1 mmol) and the DMAP (2 mmol) and 1 mL of DMF were added. The reaction mixture was stirred at reflux and the reaction monitored by TLC (Et_2O/ CH_3OH, 90:10, v/v). After about 2 h the reaction was completed and the organic phase was washed with a 9% aqueous solution of Na_2CO_3 (2 × 10 mL), then with a 1N HCl solution (2 × 10 mL) and finally with a brine solution (1 × 10 mL). The organic layer was dried (Na_2SO_4), paper- filtered and evaporated under reduced pressure. The crude product was

purified by column chromatography (Et_2O/ CH_3OH, 90:10, v/v) and the *N*-Fmoc-L-phenylalanyl-L-isoleucinyl-L-alanine methyl ester (**34j**) was recovered in good yield (75%).

*N-Fmoc-L-phenylalanyl-L-isoleucinyl-L-alanine methyl ester (**34j**)*

Yield: 75%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 7.78 (d, J = 7.5 Hz, Ar-H_{Fmoc}, 2H), 7.51-7.57 (m, Ar-H_{Fmoc}, 2H), 7.15-7.45 (m, Ar-H_{Fmoc} , Ar-H_{Phe}, 9H), 6.51 (d, J = 7.5 Hz, NH, 1H), 6.42 (d, J = 7.5 Hz, NH, 1H), 5.40 (d, J = 7.5 Hz, $NH_{urethane}$, 1H), 4.41-4.57 (m, α-CH_{Phe}, α-CH_{ala}, α-CH_{ile}, 3H), 4.16-4.37 (m, CH_{2Fmoc} , CH_{Fmoc}, 3H), 3.75 (s, OCH_3, 3H), 3.03-3.15 (m, CH_{2Phe}, 2H), 1.40 (d, J = 6.0 Hz, CH_{3ala}, 3H), 1.24-1.30 (m, CH_{Ile}, 1H), 1.01-1.11 (m, CH_{2Ile}, 2H), 0.80- 0.95 (m, $CHCH_3$, CH_2CH_3, 6H). ^{13}C NMR (75 MHz, $CDCl_3$): 198.06, 170.98, 170.04, 154.97, 143.62, 141.02, 139.98, 129.31, 128.97, 127.76, 127.09, 124.99, 120.00, 66.95, 65.78, 57.88, 52.43, 48.05, 45.34, 41.45, 37.03, 25.01, 18.20, 15.23, 11.29. Elemental analysis calcd (%) for $C_{34}H_{39}N_3O_6$: C, 69.72; H, 6.71; N, 7.17; found C, 69.79; H, 6.68; N, 7.15.

General procedure for the reaction of N-arylsulfonyl-α-aminoacid methyl esters 36a-i with trimethyloxonium tetrafluoroborate.

To a solution of (**35a-i**, 1 mmol), in DCM (20 mL) were added DIPEA (3.5 mmol) and solid trimethyloxonium tetrafluoroborate (2.5 mmol). The reaction mixture was stirred for 15-110 min at room temperature and under an inert atmosphere. The mixture was then quenched with 1 N aqueous HCl until pH 2, and extracted with DCM (3x10 mL). The organic layer was washed with 1 N aqueous NaOH (3x10 mL) and then brine (10 mL). The combined organic layers were dried with Na_2SO_4, and evaporated to dryness under reduced pressure conditions to give the respective N-methylated derivatives **36a-i** as colorless oils in quantitative yields.

Synthesis N-Methyl-N-4-nitrophenylsulfonyl-L-alanine methyl ester (36a).

Treatment of a solution of *N*-4-nitrophenylsulfonyl-L-alanine methyl ester (**35a**) (100 mg, 0.347 mmol) in dry DCM (20 mL) with DIPEA (0.212 mL, 1.21 mmol) and trimethyloxonium tetrafluoroborate (128 mg, 0.868 mmol) for 15 min afforded the title compound **36a** (105 mg, quantitative yield).

Synthesis N-Methyl-N-4-nitrophenylsulfonyl-L-valine methyl ester (36b).

Treatment of a solution of *N*-4-nitrophenylsulfonyl-L-valine methyl ester (**35b**, 100 mg, 0.316 mmol) in dry DCM (20 mL) with DIPEA (0.193 mL, 1.11 mmol) and trimethyloxonium tetrafluoroborate (117 mg, 0.790 mmol) for 15 min afforded the title compound (**36b**, 104 mg, quantitative yield).

Synthesis N-Methyl-N-4-nitrophenylsulfonyl-L-isoleucine methyl ester (36 c).

Treatment of a solution of N-4-nitrophenylsulfonyl-L-isoleucine methyl ester (**35c**) (100 mg, 0.303 mmol) in dry DCM (20 mL) with DIPEA (0.185 mL, 1.10 mmol) and trimethyloxonium tetrafluoroborate (111 mg, 0.75 mmol) for 15 min afforded the title compound **36c** (104 mg, quantitative yield).

Synthesis N-Methyl-N-4-nitrophenylsulfonyl-L-phenylalanine methyl ester (36d).

Treatment of a solution of *N*-4-nitrophenylsulfonyl-L-phenylalanine methyl ester (**35d**) (100 mg, 0.274 mmol) in dry DCM (20 mL) with DIPEA (0.167 mL, 0.959 mmol) and trimethyloxonium tetrafluoroborate (99 mg, 0.67 mmol) for 15

min afforded the title compound **36d** (103 mg, quantitative yield).

Synthesis of N-Methyl-N-4-fluorophenylsulfonyl-L-alanine methyl ester (36e).

Treatment of a solution of *N*-4-fluorophenylsulfonyl-L-alanine methyl ester **35e** (100 mg, 0.383 mmol) in dry DCM (20 mL) with DIPEA (0.233 mL, 1.34 mmol) and trimethyloxonium tetrafluoroborate (142 mg, 0.958 mmol) for 60 min afforded the title compound **36e** (105 mg, quantitative yield) as a colorless oil.

*N-Methyl-N-4-fluorophenylsulfonyl-L-alanine methyl ester (**36e**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 1.38 (3H, d, J 7.3 Hz, CH_3), 2.84 (3H, s, N-CH_3), 3.56 (3H, s, OCH_3), 4.77 (1H, q, J 7.3 Hz, a-CH), 7.22-7.14 (2H, m, ArH o-F), 7.86-7.80 (2H, m, ArH m-F) ppm; ^{13}C NMR (75 MHz, CDCl3): d 15.6, 29.9, 52.1, 54.6, 116.1 (d, $J_{C,F}$=22.5 Hz), 129.9 (d, $J_{C,F}$=9.0 Hz), 135.3 (d, $J_{C,F}$=3.0 Hz), 165.1 (d, $J_{C,F}$=252.7 Hz), 171.3. MS (CI) (rel int.): m/z 304 (2, $MC_2H_5^+$), 276 (28, MH^+), 256 (2), 244 (3), 216 (100), 202 (2), 152 (5. Elemental analysis calcd (%) for $C_{11}H_{14}FNO_4S$: C, 47.99; H, 5.13; N, 5.09. Found: C, 47.79; H, 5.14; N, 5.11.

Synthesis of N-Methyl-N-4-chlorophenylsulfonyl-L-alanine methyl ester (36f).

Treatment of a solution of *N*-4-chlorophenylsulfonyl-L-alanine methyl ester **35f** (100 mg, 0.360 mmol) in dry DCM (20 mL) with DIPEA (0.219 mL, 1.26 mmol) and trimethyloxonium tetrafluoroborate (133 mg, 0.900 mmol) for 75 min afforded the title compound **36f** (105 mg, quantitative yield) as a colorless oil.

*N-Methyl-N-4-chlorophenylsulfonyl-L-alanine methyl ester (**36f**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 1.38 (3H, d, J 7.3 Hz, CH_3), 2.84 (3H, s, NCH_3), 3.56 (3H, s, OCH_3), 4.76 (1H, q, J 7.3 Hz, a-CH), 7.48 (2H, d, J 8.8 Hz, ArH o-Cl), 7.75 (2H, d, J 8.8 Hz, ArH m-Cl) ppm. ^{13}C NMR (75 MHz, CDCl3): d 15.4, 29.8, 52.2, 54.6, 128.6, 129.0, 137.6, 138.9, 171.1 ppm. MS (EI) (rel int.): m/z 234 (39), 232 (100), 177 (18), 175 (50), 113 (14), 111 (45), 75 (12. Elemental analysis calcd (%) for $C_{11}H_{14}ClNO_4S$: C, 45.28; H, 4.84; N, 4.80Found: C, 45.09; H, 4.85; N, 4.79.

Synthesis of N-Methyl-N-4-methylphenylsulfonyl-L-alanine methyl ester (36g).

Treatment of a solution of *N*-4-methylphenylsulfonyl-L-alanine methyl ester **35g** (100 mg, 0.389 mmol) in dry DCM (20 mL) with

DIPEA (0.237 mL, 1.36 mmol) and trimethyloxonium tetrafluoroborate (144 mg, 0.973 mmol) for 90 min afforded the title compound **36g** (106 mg, quantitative yield) as a colorless oil.

*N-Methyl-N-4-methylphenylsulfonyl-L-alanine methyl ester (**36g**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 1.32 (3H, d, J 7.3 Hz, CH_3), 2.42 (3H, s, $ArCH_3$), 2.83 (3H, s, NCH_3), 3.55 (3H, s, OCH_3), 4.76 (1H, q, J 7.3 Hz, a-CH), 7.31 (2H, d, J 8.3 Hz, ArH o-CH_3), 7.68 (2H, d, J 8.3 Hz, ArH m-CH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): 15.2, 21.3, 29.7, 51.9, 54.4, 127.1, 129.4, 135.9, 143.2, 171.4 ppm. MS (EI) (rel int): m/z 271 (1), 212 (100), 155 (60), 139 (2), 116 (3), 91 (75), 65(10%). Elemental analysis calcd (%) for $C_{12}H_{17}NO_4S$: C, 53.12; H, 6.32; N, 5.16.Found C, 52.90; H, 6.33; N, 5.14.

Synthesis of N-Methyl-N-4-methoxyphenylsulfonyl-L-alanine methyl ester (36h).

Treatment of a solution of *N*-4-methoxyphenylsulfonyl-L-alanine methyl ester **35h** (100 mg, 0.366 mmol) in dry DCM (20 mL) with DIPEA (0.223 mL, 1.28 mmol) and trimethyloxonium tetrafluoroborate (135 mg, 0.915 mmol) for 110 min afforded the title compound **36h** (105 mg, quantitative yield) as a colorless oil.

N-Methyl-N-4-methoxyphenylsulfonyl-L-alanine methyl ester ***(36h)***

^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 1.33 (3H, d, J 7.3 Hz, CH_3), 2.82 (3H, s, NCH_3), 3.57 (3H, s, OCH_3), 3.86 (3H, s, $ArOCH_3$), 4.76 (1H, q, J=7.3 Hz, a-CH), 6.97 (2H, d, J= 9.0 Hz, ArH o-OCH_3), 7.74 (2H, d, J= 9.0 Hz, ArH m-OCH_3) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): 15.3, 29.7, 51.3, 54.4, 55.3, 114.0, 129.2, 162.4, 171.5, ppm. MS (EI) (rel int.): m/z=287 (1), 228 (81), 214 (14), 171 (100), 155 (3), 123 (13), 107 (27), 92 (13), 77 (17%). Elemental analysis calcd (%) for $C_{12}H_{17}NO_5S$: C, 50.16; H, 5.96; N, 4.87. Found C, 49.97; H, 5.98; N, 4.85.

Synthesis of N-Methyl-N-phenylsulfonyl-L-alanine methyl ester (36i).

Treatment of a solution of *N*-phenylsulfonyl-L-alanine methyl ester **35i** (100 mg, 0.411 mmol) in dry DCM (20 mL) with DIPEA (0.251 mL, 1.44 mmol) and trimethyloxonium tetrafluoroborate (152 mg, 1.03 mmol) for 90 min afforded the title compound **36i** (106 mg, quantitative yield) as a colorless oil.

*N-Methyl-N-phenylsulfonyl-L-alanine methyl ester (**36i**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 1.28 (3H, d, J 7.3 Hz, CH_3), 2.79 (3H, s, NCH_3), 3.46 (3H, s, OCH_3), 4.71 (1H, q, J 7.2 Hz, a-CH), 7.43-7.57 (3H, m, ArH), 7.77-7.72 (2H, m, ArH) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): 15.1, 51.8, 54.4, 126.9, 128.7, 132.3, 138.8, 171.1 ppm; MS (EI) (rel int.): m/z=198 (80), 184 (4), 156 (28), 141 (52), 118 (33), 91 (28), 77 (100), 65 (3), 41 (37%). Elemental analysis calcd (%) for $C_{11}H_{15}NO_4$: C, 51.35; H, 5.88; N, 5.44. Found C, 51.53; H, 5.85; N, 5.43.

General procedure for the synthesis of N-ethyl-N-arylsulfonyl amino acid methyl esters 37g-i, 37l-m.

To a solution of **37g-i**, and **37l-m** (1 mmol), in DCM (20 mL) were added DIPEA (3.5 mmol) and solid triethyloxonium tetrafluoroborate (2.5 mmol). The reaction mixture was stirred for 15-120 min at room temperature and under an inert atmosphere. The mixture was then quenched with 1 N aqueous HCl until pH 2, and extracted with DCM (3x10 mL). The organic layer was washed with 1 N aqueous NaOH (3x10 mL) and then once with brine (10 mL). Finally the combined organic layers were dried with Na_2SO_4. Evaporation of the solvent gave the *N*-ethyl derivatives **37g-i** and **37l-m** in 90-94% overall yields.

Synthesis of N-Ethyl-N-4-methylphenylsulfonyl-L-alanine methyl ester (37g).

Treatment of a solution of *N*-4-methylphenylsulfonyl-L-alanine methyl ester **35g** (100 mg, 0.389 mmol) in dry DCM (20 mL) with DIPEA (0.237 mL, 1.36 mmol) and triethyloxonium tetrafluoroborate (185 mg, 0.973 mmol) for 90 min afforded the title compound **37g** (101 mg, 91% yield) as a colorless oil.

*N-4-methylphenylsulfonyl-L-alanine methyl ester (**35g**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 1.21 (3H, t, J=6.9 Hz, NCH_2CH_3), 1.41 (3H, d, J=7.5 Hz, CH_3), 2.41 (3H, s, $ArCH_3$), 3.21 (1H, m, NCH_2CH_3), 3.34 (1H, m, NCH_2CH_3), 3.53 (3H, s, OCH_3), 4.65 (1H, q, J =7.3 Hz, a-CH), 7.27 (2H, d, J 8.4 Hz, ArH *o*-CH_3), 7.69 (2H, d, J 8.4 Hz, ArH m-CH_3) ppm; ^{13}C NMR (75 MHz, CDCl3): 16.5, 16.6, 21.5, 40.4, 52.0, 54.9, 127.2, 129.4, 137.3, 143.1, 172.1 ppm; MS (CI) (rel int.): m/z=314 (7, $MC_2H_5^+$), 286 (45, MH^+), 226 (100), 155 (3%). Elemental analysis calcd (%) for $C_{13}H_{19}NO_4S$: C, 54.72; H, 6.71; N, 4.91. Found C, 54.93; H, 6.68; N, 4.90.

Synthesis of N-Ethyl-N-4-methoxyphenylsulfonyl-L-alanine methyl ester (37h).

Treatment of a solution of *N*-4-methoxyphenylsulfonyl-L-alanine methyl ester **35h** (100 mg, 0.366 mmol) in dry DCM (20 mL) with DIPEA (0.223 mL, 1.28 mmol) and triethyloxonium tetrafluoroborate (174 mg, 0.915 mmol) for 120 min afforded the title compound **37h** (100 mg, 90% yield) as a colorless oil.

*N-Ethyl-N-4-methoxyphenylsulfonyl-L-alanine methyl ester (**37h**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): d 1.22 (3H, t, J=7.1 Hz, NCH_2CH_3), 1.43 (3H, d, J=7.3 Hz, CH_3), 3.21 (1H, m, NCH_2CH_3), 3.34 (1H, m, NCH_2CH_3), 3.56 (3H, s, OCH_3), 3.86 (3H, s, $ArOCH_3$), 4.65 (1H, q, J=7.3 Hz, a-CH), 6.96 (2H, d, J =8.9 Hz, ArH o-OCH_3), 7.76 (2H, d, J=8.9 Hz, ArH m-OCH_3), ppm; ^{13}C NMR (75 MHz, $CDCl_3$): 16.5, 16.6, 40.3, 52.1, 54.7, 55.5, 113.9, 129.3, 162.7, 172.1 ppm. MS (EI) (rel int.): m/z=301 (1), 242 (100), 171 (95), 155 (2), 123 (9), 107 (18), 92(7), 77 (9%). Elemental analysis calcd (%) for $C_{13}H_{19}NO_5S$: C, 51.81; H, 6.35; N, 4.65. Found C, 52.01; H, 6.33; N, 4.63.

Synthesis of N-Ethyl-N-phenylsulfonyl-L-alanine methyl ester (37i).

Treatment of a solution of *N*-phenylsulfonyl-L-alanine methyl ester **35i** (100 mg, 0.411 mmol) in dry DCM (20 mL) with DIPEA (0.251 mL, 1.44 mmol) and triethyloxonium tetrafluoroborate (196 mg, 1.03 mmol) for 85 min afforded the title compound **37i** (104 mg, 93% yield) as a colorless oil.

*N-Ethyl-N-phenylsulfonyl-L-alanine methyl ester (**37i**)*

^{1}H NMR (300 MHz, $CDCl_3$) δ (p.p.m.): 1.28 (3H, t, J=7.1 Hz, NCH_2CH_3), 1.47 (3H, d, J=7.4 Hz, CH_3), 3.25 (1H, m, NCH_2CH_3), 3.40 (1H, m, NCH_2CH_3), 3.54 (3H, s, OCH_3), 4.70 (1H, q, J =7.3 Hz, a-CH), 7.50-7.64 (3H, m, ArH), 7.88-7.83 (2H, m, ArH); ^{13}C NMR (75 MHz, $CDCl_3$): 16.5, 16.6, 40.4, 51.9, 54.9, 127.1, 128.7, 132.4, 140.0, 171.8 ppm; MS (CI) (rel int.): m/z=300 (3,$MC_2H_5^+$), 272 (28, MH^+), 212 (100), 166 (2%). Elemental analysis calcd (%) for $C_{12}H_{17}NO_4S$: C, 53.12; H, 6.32; N, 5.16. Found C, 53.33; H, 6.29; N, 5.17.

Synthesis of N-Ethyl-N-4-fluorophenylsulfonyl-L-valine methyl ester (37l).

Treatment of a solution of *N*-4-fluorophenylsulfonyl-L-valine methyl ester **35l** (100 mg, 0.346 mmol) in dry DCM (20 mL) with DIPEA (0.211 mL,1.21 mmol) and triethyloxonium tetrafluoroborate (164 mg, 0.865 mmol) for 65 min afforded the title compound **37l** (105 mg, 94% yield) as a colorless oil.

*N-Ethyl-N-4-fluorophenylsulfonyl-L-valine methyl ester (**37l**)*

^{1}H NMR (300 MHz, $CDCl_3$): d 0.93 [3H, d, J=6.6 Hz, $CH(CH_3)_2$], 1.05 [3H, d, J=6.6 Hz, $CH(CH_3)_2$], 1.22 (3H, t, J =7.3 Hz, NCH_2CH_3), 2.09 [1H, m, $CH(CH_3)_2$], 3.45 (3H, s, OCH3), 3.54-3.40 (2H, m, NCH_2CH_3), 4.05 (1H, d, J 10.5 Hz, a-CH), 7.19-7.10 (2H, m, ArH o-F), 7.88-7.80 (2H, m, ArHm-F), ppm; ^{13}C NMR (75 MHz, $CDCl_3$): 16.3, 19.4, 19.6, 28.7, 40.3, 51.4, 65.7, 115.8 (d, $J_{C,F}$=22.5 Hz), 130.1 (d, $J_{C,F}$=9.0 Hz), 136.2 (d, $J_{C,F}$=3.0 Hz), 164.9 (d, $J_{C,F}$=252.8 Hz), 170.9 ppm. MS (CI) (rel int.): m/z=346 (9, $MC_2H_5^+$), 318 (19, MH^+), 258 (100), 239 (3), 194 (7), 158 (3%). Elemental analysis calcd (%) for $C_{14}H_{20}FNO_4S$: C, 52.98; H, 6.35; N, 4.41. Found C, 52.77; H, 6.37; N, 4.41.

Synthesis of N-Ethyl-N-4-chlorophenylsulfonyl-L-leucine methyl ester (37m).

Treatment of a solution of *N*-4-chlorophenylsulfonyl-L-leucine methyl ester **35m** (100 mg, 0.313 mmol) in dry DCM (20 mL) with DIPEA (0.192 mL, 1.10 mmol) and triethyloxonium tetrafluoroborate (149 mg, 0.783 mmol) for 70 min afforded the title compound **37m** (102 mg, 94% yield) as a colorless oil.

*N-Ethyl-N-4-chlorophenylsulfonyl-L-leucine methyl ester (**37m**)*

^{1}H NMR (300 MHz, $CDCl_3$): d 0.95 [3H, d, J=6.9 Hz, $CH(CH_3)_2$], 0.97 [3H, d, J=6.9 Hz, $CH(CH_3)_2$], 1.20 (3H, t, J=7.1 Hz, NCH_2CH_3), 1.70-1.62 (2H, m, $CHCH_2$), 1.75 [1H, m, $CH(CH_3)_2$], 3.40-3.10 (2H, m, NCH_2CH_3), 3.45 (3H, s, OCH_3), 4.55 (1H, dd, J= 8.8, 5.3 Hz, a-CH), 7.48 (2H, d, J= 8.1 Hz, ArH o-Cl), 7.75 (2H, d, J=8.1 Hz, ArHm-Cl); ^{13}C NMR (75 MHz, $CDCl_3$): 12.3, 21.7, 21.8, 23.4, 38.4, 39.8, 50.9, 57.2, 128.3, 127.8, 137.6, 137.9, 170.8 ppm. MS (CI) (rel int.): m/z=376 (7, $MC_2H_5^+$), 348 (21, MH^+), 288 (100), 208 (5%). Elemental analysis calcd (%) for $C_{15}H_{22}ClNO_4S$: C, 51.79; H, 6.37; N, 4.03. Found C, 51.59; H, 6.39; N, 4.04.

Simultaneous extraction and derivatization of amino acids and free fatty acids in meat products

Samples

The industrial batches of cured "salsiccia piccante" were supplied by the industry "San Vincenzo Salumi" SRL (Spezzano Piccolo, province of Cosenza, Calabria, Italy). Meat matrices were obtained from three different industrial batches (I–III, 3 months of ripening) of cured "salsiccia piccante" and were used for the analyses directly after delivery by the manufacturer. Sausages were chopped after gut peeling and finely grinded. Weighted aliquots of 1 g of matrix were used for the analysis.

Preparation of stock solutions 1-6

A solution of norleucine in 0.01 N aqueous HCl at concentration of 5.1 mg/mL (Stock 1) and a solution of pentadecanoic acid in dichloromethane at concentration of 205 mg/mL (Stock 2) were prepared. Stocks 1 and 2 were used as internal standards.

Stock 3 was prepared by dissolving 17 natural amino acids in 0.01 N aqueous HCl (250 mL). The resulting concentration of each amino acid was 0.4 mg/mL. An appropriate volume of Stock 3 was added to 0.4 mL of Stock 1 in a screw capped vial, and the resulting mixture was diluted to 5 mL with 0.01 N

aqueous HCl. Thus, 5 solutions at different concentration levels of amino acids were prepared:

solutions A–E contained 0.4 mg/mL of internal standard (Stock 1) and 0.34, 0.18, 0.08, 0.02 and 0.0068 mg/mL of amino acids,respectively.

Stocks 4 and 5 were prepared by dissolving the appropriate amounts of myristic, palmitic, palmitoleic, oleic, linoleic and stearic acid in dichloromethane. The resulting concentrations of each fatty acid were 2.5 mg/mL (Stock 4) and 25 mg/mL (Stock 5).

Stock 6 was prepared using 100 mg of each amino acid (see Table 3) in 0.01 N aqueous HCl (250 mL). The final concentration of each amino acid was 0.4 mg/mL.

An appropriate volume of Stock 4 was added to 0.1 mL of Stock 2 in a screw capped vial, and the resulting mixture was diluted to 5 mL with dichloromethane. Thus, three solutions at different concentration levels of free fatty acids were prepared and used for the derivatization: solutions F–H contained 4.1 mg/mL of internal standard (Stock 2) and 1, 0.5 and 0.25 mg/mL of free fatty acids, respectively.

Solutions for calibration curves

Solutions A–E, after concentration under vacuum, were treated with a 2:1 (v/v) solution of methanol and pyridine (18, 9, 4, 1.5 and 0.5 mL, respectively), and MCF (4, 2, 1, 0.2 and 0.07 mL, respectively) was added. The resulting mixtures were magnetically stirred or shaken at room temperature until complete development of carbon dioxide and then diluted with dichloromethane (10, 5, 2, 0.5 and 0.2 mL, respectively). The organic solutions were washed two times with distilled water (10, 5, 2, 0.5 and 0.2 mL, respectively), separated, dried over Na2SO4, filtered and evaporated to dryness under reduced pressure conditions. The collected residue recovered from each treatment was dissolved in dichloromethane (5 mL) and a 1 µL aliquot of each final solution was used for the GC–MS analysis to plot the calibration curve.

Solutions F, G, and H were treated with an excess of a 0.66 M dichloromethane solution of diazomethane for 10 min at room temperature, according to the literature.[50] All the reaction mixtures were evaporated to dryness under reduced pressure conditions and the respective residue was dissolved in dichloromethane (5 mL). A 1 µL aliquot of each final solution was analyzed by GC/MS to plot the calibration curve.

Extraction–derivatization with MCF. General procedure

A weighted aliquot (1 g) of meat matrix obtained from sausages was suspended in a 2:1 (v/v) solution of methanol and dichloromethane (60 mL). A 0.4 mL aliquot of Stock 1 and a 0.1 mL aliquot of Stock 2 were added. Stock 1 and Stock 2 were used as internal standards. The suspension was maintained under magnetic stirring for 5 min at room temperature, then pyridine (20 mL) and MCF (14 mL) were added. After 10 min, the suspension was filtered through a glass filter and the meat solid residue was washed with dichloromethane (2x10 mL). The collected organic filtrates were diluted with further dichloromethane (20 mL), washed once with distilled water (20 mL), dried on Na_2SO_4, paper-filtered, and evaporated to dryness under reduced pressure conditions.

The residue obtained was treated with a 1:1 (v/v) mixture of toluene and ethanol (3 x 10 mL) and co-evaporated to dryness under reduced pressure conditions, in order to remove all traces of the residual pyridine. The final residue (156 mg) was dissolved in dichloromethane (5 mL), and a 1 µL aliquot of this solution was subjected to the GC–MS analysis. This general procedure was repeated two times, by simply varying the stirring period (30 min and 5 h), in order to establish the minimal time necessary for the obtainment of the best yield in recovered final

residue. Starting from an equal aliquot of finely grinded meat matrix, the collected amounts of residue containing the analytes were 127 mg and 83 mg, after 30 min and 5 h of stirring, respectively. The procedure was performed in triplicate for each batch.

Extraction and post-derivatization with MCF

A weighted aliquot (1 g) of meat matrix obtained from sausages was suspended in a 2:1 (v/v) solution of methanol and dichloromethane (60 mL). A 0.4 mL aliquot of Stock 1 and a 0.1 mL aliquot of Stock 2 were added. Stock 1 and Stock 2 were used as internal standards. Pyridine (20 mL) was added. The suspension was maintained under magnetic stirring for 10 min at room temperature. The suspension was then filtered through a glass filter and the obtained extract was treated with MCF (14 mL) under stirring for 10 min. The final residue was recovered and analyzed as described in Section 2.5.

Extraction with water/chloroform/methanol

The extraction of analytes was performed by a conventional protocol already suggested for the determination of amino acids in natural matrices [23]. Derivatization of the recovered analytes was then performed with MCF as described before.

A 1 g aliquot of meat matrix was suspended in a 3:5:12 (v/v) mixture of water, chloroform and methanol (4 mL). The suspension was vigorously stirred at room temperature for 4 min and then centrifuged at 13,000 rpm for 4 min. The supernatant was removed and the obtained residue was treated again with a 3:5:12 (v/v) mixture of water, chloroform and methanol (4 mL). Centrifugation was repeated as for the previous step and the supernatant was removed and combined with the previous one. Chloroform (2 mL) and water (3 mL) were added to the collected supernatants. After centrifugation at full speed for 4 min the upper water/methanol phase was collected and evaporated to dryness under reduced pressure conditions.

The final residue was treated with a 2:1 (v/v) solution of methanol and pyridine (60 mL), and MCF (14 mL) was added. The final residue was recovered and analyzed as previously described.

Comparison of Free Amino Acids and Biogenic Amines content between Industrial and Homemade Products.

Samples

Three lots of industrial "soppressata" (**1**, **2**, **3**), a homemade "soppressata" (**4C**), a batch of industrial "salsiccia" (**5**) and a homemade "salsiccia" (**6C**) were tested. The industrial batches were analyzed at the time of preparation (**1A**, **2A**, **3A**, **5A**), after 20 d (**1B**, **2B**, **3B**, **5B**), and after 80 d of ripening (**1C**, **2C**, **3C**, and **5C**). The homemade sausages (**4C** and **6C**) were analyzed after 80 d of maturation.

All industrial sausages were furnished by a factory near Cosenza ("San Vincenzo Salumi", Spezzano Piccolo, Cosenza, Italy). All homemade sausages were appropriately prepared for this research by country people who usually make these products for personal consumption.

Industrial "soppressata" and "salsiccia".

The soppressata paste was obtained by mixing large ground pork ham and sirloin (95.5%), salt (2.40%), and other ingredients such as black pepper and other spices (0.6%), aromas (1%), dextrose and sucrose (0.45%), preservatives (sodium nitrite [E250] 0.01%), potassium nitrate [E252] 0.01%), antioxidants

(ascorbic acid [E300] 0.02%), and a microbial starter formulation (0.01%; SAGA T, 2 × 1010 ufc/g *Pediococcus pentosaceus* and *Staphylococcus carnosus*, Kerry Ingredients & Flavours Italia S.p.A.,Mozzo, Bergamo, Italy).

The salsiccia paste was obtained by mixing medium ground pork shoulder, bacon, and ham (95.5%) with salt (2.4%), spices (0.55%), aromas (1.04%), dextrose and sucrose (0.46%), preservatives (E250 0.01%, E252 0.01%), antioxidants (E300 0.02%), and a microbial starter formulation (0.01%; SAGA T, 2 × 1010 ufc/g *P. pentosaceus* and *S. carnosus*, Kerry Ingredients & Flavours Italia S.p.A.).

The meat paste, after being in a cold room to rest, was stuffed into casings. After the stuffing, it was first subjected to appropriate drying and then cured in a temperature- and humidity-controlled environment for 20 d. During the drying processes the temperature was maintained for 3 d at 24 to 26 ◦C with a relative humidity (RH) of 75% to 80% and then gradually decreased to 12 to 14 ◦C with 60% to 70% RH in the last 5 d of curing. The pH value of both “soppressata” and “salsiccia” sausage was 5.9 after 5 d stuffing.

Homemade "soppressata" and "salsiccia"

Homemade "soppressata" and "salsiccia" were made from pork meat during winter months (from December to March). After slaughter, pork meat was left to hang for 24 h. The "soppressata" matrix was made with cuts of ham (700 g/kg) and sirloin (300 g/kg). These were cut by hand into small pieces. For each kilogram of paste, chopped pork neck (70 g), salt (30 g), and black peppercorns (20 g) were added. The "salsiccia" matrix was made with cuts of shoulder (600 g/kg), ham (100 g/kg), and bacon (300 g/kg). These were cut by hand into small pieces. For each kilogram of paste, salt (30 g), red pepper (100 g), and fennel seeds (20 g) were added.

The meat paste was stuffed into casings. The drying and the ripening processes were carried out at room temperature (approximately 10 to 15 ◦C) for about 60 d (40 d in the case of "salsiccia") to assume the characteristics of the final product. The pH value of both "soppressata" and "salsiccia" sausage was 6.1 after 5 d stuffing.

Analytical determination of biogenic amines

The analytical procedure for determining biogenic amines consisted of extraction with perchloric acid, pre-separation of

crude extracts, and conversion of the amines into their trifluoroacetyl derivatives. The biogenic amines were quantified by GC/FID (Slemr and Beyermann 1984; Slemr and Beyermann 1985).

The biogenic amines were identified by GC/MS analysis comparing their retention times and their mass spectra with those of derivatized authentic samples.

Extraction

Dry meat (5 g) was treated with 30 mL of a 0.4 M solution of $HClO_4$ (Carlo Erba Group, Arese, Milano, Italy) with stirring for 30 min at room temperature. The mixture was filtered and basified with a 5% solution of NaHCO3, extracted with dichloromethane (3 x 10 mL) and the organic layers were dried (Na_2SO_4) and evaporated under reduced pressure.

Derivatization

The extract was dissolved in 5 mL of dry dichloromethane (DCM) and 5 mL trifluoroacetic anhydride, and 5 mL triethylamine was added to the solution. After 2 h, the solution was treated with a 0.1 M solution of HCl and extracted with DCM (3x10 mL). The organic layers were basified with a 5% solution of $NaHCO_3$, extracted with DCM (3 x 10 ml) and dried

(Na_2SO_4). The solvent was evaporated to recover 111 mg of extract; that was analyzed by GC/MS and GC/FID.

Single biogenic amine derivatization procedure

Biogenic amines were purchased from Fluka. Derivatized putrescine, cadaverine, and tyramine were prepared separately. Each biogenic amine (50 mg) was dissolved in 5 mL dry dichloromethane and 5 mL trifluoroacetic anhydride, and 5 mL of triethylamine was added to the solution. After 2 h, the solution was treated with a 0.1M solution of HCl and extracted with DCM (3 x 10 mL). The organic layers were basified with a 5% solution of $NaHCO_3$, extracted with DCM (3 $\times$ 10 mL), and dried (Na_2SO_4). The solvent was evaporated to recover the derivatized amine which was then analyzed by GC/MS and GC/FID.

References

1. Di Gioia, M. L.; Leggio, A.; Le Pera, A.; Liguori, A.; Napoli, A.; Siciliano, C.; Sindona, G. *J. Org. Chem.* **2003**, 68, 7416

2. (a) Answer, K. L.; Audhya, T. K.; Goldstein, G. *Tetrahedron Lett.* **1991**, 32, 327. (b) Salomon, C. J.; Mata, E. G.; Mascaretti, O. A. *Tetrahedron* **1993**, 49, 3691.

3. (a) McDermott, J. R.; Benoiton, N. L. *Can. J. Chem.* **1973**, 51, 2555. (b) Benoiton, N. L.; Cheung, S. T. *Can. J. Chem.* **1977**, 55, 916. (c) Schroder, E.; Lubke, K. In The Peptides. Methods of Peptide Synthesis; Academic Press: New York, **1965**; Vol. 1.

4. M.L. Di Gioia; A. Leggio; A. Le Pera; C. Siciliano; G. Sindona and A. Liguori *J.Peptide Res.* **2004,** 63, 383-387.

5. Wuts PGM, Greene TW, in *Greene's protective groups in organic synthesis*, Wiley, New York, 4rd edn, **2007**.

6. Nicolau KC et al, *J.Americ. Chem. Soc.*, **1990**, 112, 8193-8195.

7. a) Bayermann M., Biernet M., Niedrich H., Carpino L. A., Sadat-Aalaee D., *J.Org. Chem*, **1990**, 55, 721-728. b) Carpino L. A., Sadat-Aalaee D., Bayermann M., *J. Org. Chem*, **1990**, 55, 1673-1675.

8. L. A. Carpino et al.; *J. Org. Chem*. **1986**, 51, 3734-3736.

9. a) Leggio A., Liguori A., Napoli A., Siciliano C., Sindona G., *Eur.J. Org. Chem.,* **2000**, 573-575. b) M.L: Di Gioia, A. Leggio, A. Le Pera, A. Liguori, F. Perri, C. Siciliano *Eur.J. Org. Chem.,* **2004**, 4437-4441. c) M.L. Di Gioia, A. Leggio, A. Le Pera, C. Siciliano and A. Liguori, *J. Peptide Res*. **2004** 63, 383–387.

10. Amaral, M. J. S. A.; Barrett, G.C.; Rydon, H.N.; Willet, J.E. *J. Chem. Soc. C* **1966**, 807.

11. Amaral, M. J. S. A. *J. Chem. Soc. C* **1969**, 2495.

12. Johnson, B. J.; Ruettinger, T. A. *J. Org. Chem.* **1970**, 35, 255.

13. Chantreux, D.; Gamet, J.P.; Jacquier, R.; Verducci, J. *Tetrahedron* **1984**, 40, 3087.

14. Jones, J.H.; Young, G.T. *J. Chem. Soc. C* **1968**, 437.

15. Heidler, P.; Link, A. *Bioorg. Med. Chem.* 2005, 13, 585.

16. a) Di Gioia, M. L.; Leggio, A.; Le Pera, A.; Liguori, A.; Napoli, A.; Siciliano, C.; Sindona, G. *J. Org. Chem.* **2003**, 68, 7416–7421; b) Di Gioia, M. L.; Leggio, A.; Liguori, A.; Perri, F. *J. Org. Chem*. **2007**, 72, 3723–3728; c) Belsito, E. ; Di Gioia, M. L.; Greco, A.; Leggio, A.; Liguori, A.; Perri, F.; Siciliano, C.; Viscomi, M. C. *J. Org. Chem.* **2007**, 72, 4798–4802; d) Di Gioia, M. L.; Leggio, A.; Liguori, A.; Perri, F. ; Siciliano, C.; Viscomi, M. C. *Amino Acids* **2010**, 38, 133–143.

17. Gilon, C.; Dechantsreiter, M.A. ; Burkhart, F.; Friedler, A.; Kessler, H. in *Synthesis of Peptides and Peptidomimetics*, Houben-Weyl Methods of Organic Chemistry; Goodman, M.; Felix, A.; Moroder, L.; Toniolo, C. Eds; Thieme: Stuttgart, Germany, **2003**, vol. E 22c, pp. 215–271.

18. See for example: Bewley, C. A.; Faulkner, D. J. Angew. Chem. **1998**, 110, 2280–2297; Angew. Chem. Int. Ed. 1998, 37, 2162–2178.

19. a) Wenger, R. M. Helv. Chim. Acta 1983, 66, 2308–2321; b) Wenger, R. M. Helv. Chim. Acta **1983**, 66, 2672–2702; c) Wenger, R. M. Helv. *Chim. Acta* **1984**, 67, 502–525.

20. a) Jouin, P.; Poncet, J.; Dufour, M.-N.; Pantaloni, A.; Castro, B. *J. Org. Chem.* **1989**, 54, 617–627; b) Pettit, G. R.; Kamano, Y.; Herald, C. L.; Dufresne, C.; Bates, R. B.; Schmidt, J. M.; Cerny, R. L.; Kizu, H. *J. Org. Chem.* **1990**, 55, 2989–2990; c) Pettit, G. R.; Kamano, Y.; Herald, C. L.; Fujii, Y.; Kizu, H.; Boyd, M. R.; Boettner, F. E.; Doubek, D. L.; Schmidt, J. M. ; Chapuis, J.-C.; Michael, C. *Tetrahedron* **1993**, 49, 9151–9170.

21. Ramanjulu, J. M.; Ding, X.; Jouillié, M. M. *J. Org. Chem.* **1997**, 62, 4961–4969.

22. a) Witt, K. A. ; Gillespie, T. J.; Huber, J. D.; Egleton, R. D.; Davis, T. P. *Peptides* **2001**, 22, 2329–2343; b) Bodles, A. M.; El- Agnaf, O. M. A.; Greer, B.; Guthrie, D. J. S.; Brent Irvine, G. *Neuroscience* **2004**, 359, 89–93.

23. a) Remko, M. *J. Phys. Chem. A* **2003**, *107*, 720–725; b) Chataigner, I.; Panel, C.; Gérard, H.; Piettre, S. R. *Chem. Commun.* **2007**, 3288–3290; c) Şanli, S.; Altun, Y.; Şanli, N.; Alsancak, G.; Beltran, J. L. *J. Chem. Eng. Data* **2009**, *54,* 3014– 3021

24. a) Clare, B. W.; Scozzafava, A.; Supuran, C. T. *J. Med. Chem.* **2001**, 44, 2253–2258; b) Marques, S. M.; Nuti, E.; Rossello, A.; Supuran, C. T.; Tuccinardi, T.; Martinelli, A.; Santos, M. A. *J. Med. Chem.* **2008**, 51, 7968–7979.

25. Belsito, E. L.; De Marco, R.; Di Gioia, M. L.; Liguori, A.; Perri, F.; Viscomi, M. C. *Eur. J. Org. Chem*. **2010**, 4245–4252.

26. Hubler, F.; Ruckle, T.; Patiny, L.; Muamba, T.; Guichou, J. F.; Mutter, M.; Wenger, R. *Tetrahedron Lett.* **2000**, 41, 7193–7196.

27. Toldrà F, Reig M. **2007**. Sausages. In: Hui YH, editor. Handbook of food product manufacturing.Hoboken, N.J.: John Wiley and Sons. p 251–64.

28. Hierro E, De la Hoz L, Ordoñez JA. **1997**. Contribution of microbial and meat endogenous enzymes to the lipolysis of dry fermented sausages. *J Agric Food Chem* 45: 2989–95.

29. García de Fernando G, Fox PF. **1991**. Study of proteolysis during the processing of a dry fermented pork sausage. *Meat Sci* 30: 367–83.

30. a) Ordóñez JA, Hierro E, Bruna EM, de la Hoz L. **1999**. Changes in the components of dry fermented sausages during ripening. CRC Crit Rev *Food Sci Nutr* 39:329–67. b) Zapalena MJ, Astiasarán I, Bello J. **1999**. Dry fermented sausages made with a protease from *Aspergillus oryzae* and/or a starter culture. *Meat Sci* 52:403–9.

31. Candoğan K, Acton JC. **2001**. Proteolysis of sausage fermentation. GIDA 26:247–53.

32. Demeyer D, Blom H, Hinrichsen L, Johansson G, Molly K, Montel C, Perez-Martinez G, Sandtorv BF, Talon R, Verplaetse A, VanCleemputt I. **1995**; Cork, Ireland.

33. Aro Aro JM, Nyam-Osor P, Tsuji K, Shimada K, Fukushima M, Sekikawa M. **2010**, *Food Chem.* 119:279–85.

34. a)B. Herranz, L. de la Hoz, E. Hierro, M. Fernandez, J.A. Ordoñez, **2005**, *Food Chem.* 91, 673. b) M.C. Montel, F. Masson, R. Talon, **1998**, *Meat Sci.* 49, S111.

35. A. Marco, J.L. Navarro, M. Flores, *Meat Sci.* **2006**, 73, 660.

36. M.C. Aristoy, F. Toldra, *J. Agric. Food Chem.* 39 (1991) 1792.

37. P. Hušek, *J. Chromatogr. B*, **1998,** 717, 57.

38. C.T. Young, R.S. Matlock, M.E Mason, G.R. Waller, **1974**, *J. Am. Oil Chem. Soc.*, 5, 269.

39. Lücke FK, Hechelmann H. **1987**. Starter culture for dry sausages and raw ham. Composition and effect. *Fleischwirtschaft* 67:307–14.

40. Montel MC, Reitz J, Talon R, Berdagu´e JL, Rousset-Akrim S. **1996**. Biochemical activities of *Micrococcaceae* and their effects on the aromatic profiles and odours of a dry sausage model. *Food Microbiol* 13:489–99.

41. Pritchard G, Coolbear T. **1993**. The physiology and biochemistry of the proteolytic system in lactic acid bacteria. *FEMS Microbiol Rev* 12:179–206.

42. Osmanagaoglu O, Beyatli Y, Gunduz U. **2001**. Isolation and characterization of pediocin producing Pediococcus pentosaceus pep1 from vacuum-packed sausages. *Turk J Biol* 25:133–43.

43. Anastasio A, Draisci R, Pepe T, Mercogliano R, Delli Quadri F, Luppi G, Cortesi ML. **2010**. Development of biogenic amines during the ripening of Italian dry sausages. *J Food Prot* 73:114–8.

44. Brink B, Damink C, Joosten HM, Huis in't Veld JH. **1990**. Occurence and formation of biologically active amines in foods. *Intl J Food Microbiol* 1:73–84.

45. Eaerola S, Roig-Sagués AX, Salminen M, Hirvi TK. **1998**. Biogenic amines in Finnish dry sausages. J Food Safety 18:127-38, b) Suzzi G, Gardini F. **2003**. Biogenic amine in dry fermented sausages: a review. *Int J Food Microbiol* 88:41-54.

46. Antonella Leggio, Emilia Lucia Belsito, Rosaria De Marco, Angelo Liguori, Carlo Siciliano and Mariagiovanna Spinella "Simultaneous extraction and derivatization of amino acids and free fatty acids in meat products" *Journal of Chromatography A*, **2012**,1241, 96-102.

47. Aro Aro JM, Nyam-Osor P, Tsuji K, Shimada K, Fukushima M, Sekikawa M. **2010**. The effect of starter cultures on proteolytic changes and amino acid content in fermented sausages. *Food Chem* 119:279-85.

48. De Masi TW, Wardlaw FB, Dick RL, Acton JC. **1990**. Nonprotein nitrogen (NPN) and free amino acid content of dry, fermented and nonfermented sausages. *Meat Sci* 27:1-12.

49. Careri M, Mangia A, Barbieri G, Bouoni L, Virgili R, Parolari G. **1993**. Sensory Property Relationships to Chemical Data of Italian-type Dry-cured Ham. *J Food Sci* 58:968-72.

50. A. Leggio, A. Liguori, A. Napoli, C. Siciliano, G. Sindona, **2001**, *J. Org. Chem.* ,66, 2246.