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Reduction of methionine sulfoxide with NH_4I/TFA: Compatibility with peptides containing cysteine and aromatic amino acids

Marta Vilaseca, Ernesto Nicolás*, Fina Capdevila and Ernest Giralt*

Department of Organic Chemistry, University of Barcelona; 08028 Barcelona (Spain)

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Abstract

The reduction of methionine sulfoxide with ammonium iodide in trifluoroacetic acid has been studied in peptides containing cysteine, histidine, tyrosine or tryptophan residues. While histidine and tyrosine have proved to be stable under the experimental conditions, cysteine is oxidized to cystine and tryptophan dimerizes to form 2-indolylindolenine derivatives. The use of methyl sulfide to increase the reduction rate minimizes the problem and protection of indole ring with the formyl group avoids the side reaction for this amino acid.

Keywords: Solid-phase synthesis; Peptides; protecting groups; sulfoxides.

Solid-phase methodology has become a powerful tool for peptide synthesis. Despite the improvements since the first example was reported in 1963 [1], tremendous efforts are still being made in order to increase the scope of the application of this technique. Among the different research subjects related to this field, special attention has been paid to side-chain protection of the trifunctional amino acids. The easy removal of the different protecting groups during or after the cleavage of the peptide from the resin constitutes one of the main concerns from this point of view. Generally, side-chain peptide deprotection is performed using HF (Boc chemistry) or trifluoroacetic acid (TFA, Fmoc chemistry) [2], although other conditions are needed in some cases. For example, protection of methionine (Met) as sulfoxide (Met(O)) to prevent premature sulfur oxidation and alkylation [3], is compatible with both chemistries and is removed by reduction. Several methods have been reported in the literature to perform this process but none of them have been tested sufficiently to be considered of general use.

A few years ago our interest in the synthesis of a dimeric protein with a high Met content moved us to seek a suitable method for carrying out the deprotection of this amino acid in the presence of disulfide bridges. We focussed our attention on the use of NH_4I in TFA at low temperatures with the aim of using mild conditions to prevent side redox reactions [4,5].

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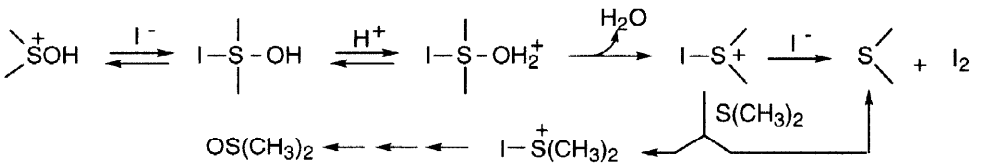
Scheme 1 shows the proposed mechanism for sulfoxide reduction with NH_4I in TFA [6].



Scheme 1

Acid-catalysis causes protonation of the sulfoxide promoting the attack of iodide at sulfur. Further acid catalyzed dehydratation of the species thus obtained followed by reaction with iodide yields the sulfide and iodine as the only by-product. One of the conclusions drawn from our study is that reaction rates can be increased using dimethylsulfide (Me_2S) as additive [7]. This result was explained in terms of competition of Me_2S with iodide for attack at the iodosulfonium cation. The additive would behave as a reducing agent in this case, affording dimethylsulfoxide (DMSO) by hydrolysis of the corresponding dimethylsulfonium cation. Regarding the scope of application of NH_4I in TFA as a general method for the deprotection of Met in peptides, we showed that these experimental conditions are mild enough to reduce the sulfoxide in the presence of disulfide bridges [7]. However, the main concern about the use of the NH_4I/TFA system is the temporary formation of iodosulfonium ions and the release of iodine, two species that can give rise to side reactions via electrophilic aromatic substitution or oxidation respectively (scheme 1). In this sense, special attention had to be paid to peptides containing free cysteine (Cys) or aromatic amino acid residues such as histidine (His), tyrosine (Tyr) or tryptophan (Trp). Here we present our results concerning the use of NH_4I in TFA in the presence of the amino acid residues mentioned above through the study of short peptide models.

Results and Dicussion

Cysteine

A study about the scope of application of the NH_4I/TFA reducing system for the deprotection of Met in peptides had to include Cys residues because of their propensity to oxidation. For example, it is well known that the use of iodine in the presence of acid is an excellent method for disulfide formation in peptides containing unprotected Cys or with acetamidomethyl protection (Acm) [8]. Therefore, the potential sensitivity of this residue to the experimental conditions that are to be used for the reduction of Met(O) cannot be discounted.

Two peptides (**1** and **2**, scheme 2) that were utilized as synthetic intermediates in our preceding work on the use of NH_4I in TFA for the reduction of Met(O) in disulfide

containing peptides were chosen to study this reaction in the presence of Cys residues. As described [7], Met(O) protected and unprotected Met peptides were considered with the idea of following the reductions and characterising the final products by high performance liquid chromatography (HPLC). The sequences of these peptides were assembled using Boc chemistry and a 4-(oxymethyl)phenylacetamidomethylpolystyrene solid support (PAM-polystyrene). Side chain protection for Cys was provided by *p*-methylbenzyl (Meb) and Acm groups. Commercially available Boc-Met(O)-PAM-polystyrene and Boc-Met-PAM-polystyrene were used as polymeric supports. Boc-amino acids were coupled manually using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) with diisopropylethylamine (DIEA) in dimethylformamide (DMF) [9-11] and the acidolytic cleavage of the peptide-resins thus obtained was performed with HF.

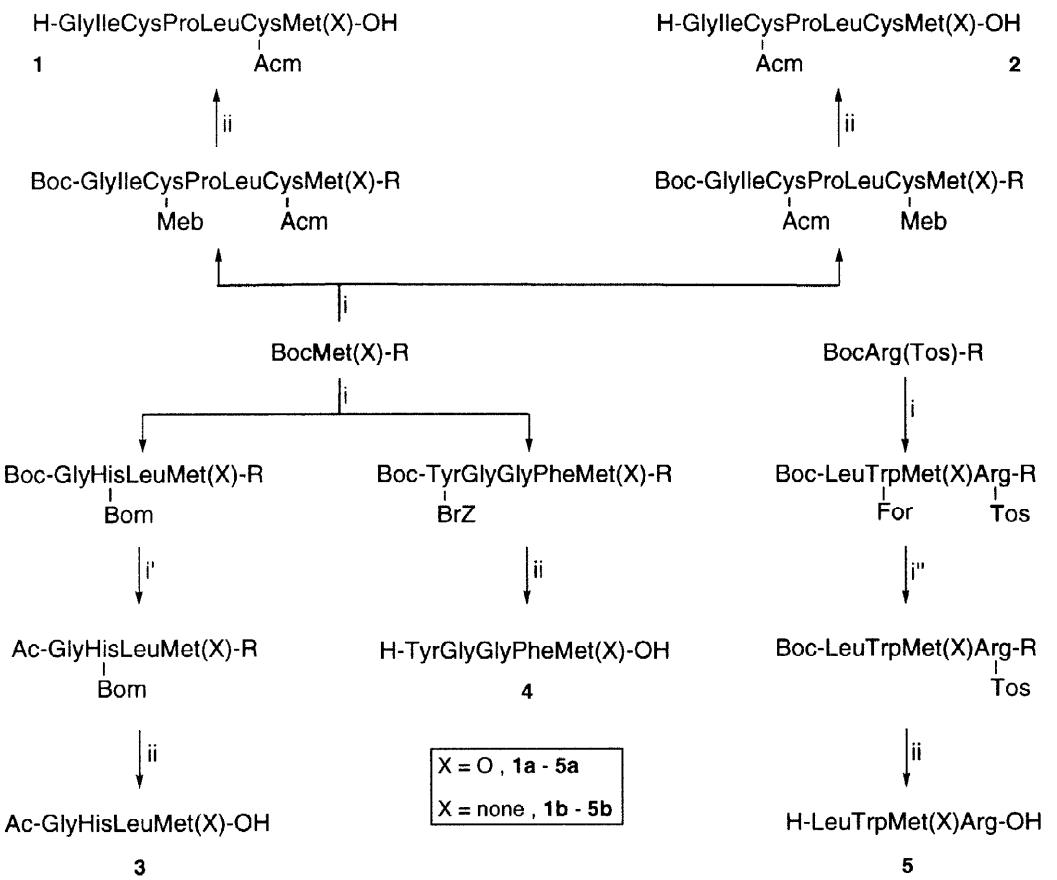
Met deprotections were carried out under similar conditions to those that were used to study the same reaction in the presence of disulfide bridges (neat TFA at 0°C, 1.6-2.2 mM solution of peptide and 30 eq of NH_4I). Reductions of **1a** and **2a** were achieved in 75% yield after 1 h and were quantitative in the presence of Me_2S (30 eq). However, instead of obtaining the desired products (**1b** and **2b**, respectively), concomitant oxidation of the free Cys residues afforded the dimeric peptides in quantitative yields. The analysis of the crude with electrospray mass spectrometry (ESMS) revealed that dimerisation was provoked by oxidation of the free Cys residues while Acm groups remained stable (figure 1). The participation of iodine in the process was ruled out since **1b** did not afford the corresponding dimer by treatment with this reagent under similar conditions to those used for the reduction of Met(O) residues. In the light of these results, thiol activation promoted by the intermediate species that are formed during the reduction of the sulfoxide and further reaction with another thiol group can be suggested as the probable mechanism for this process.

According to these results, the method for the reduction of Met studied in this work is incompatible with the presence of free Cys residues, but it has to be pointed out that this limitation might be considered as an advantage in some particular cases. Thus, simultaneous Met(O) reduction and Cys oxidation is being used in our laboratory for the preparation of disulfide containing homodimeric peptides to carry out structural studies [12].

Histidine and Tyrosine

As mentioned above, the reduction of Met(O) with NH_4I in TFA involves the formation of an iodosulfonium salt as an intermediate species and iodine as a final by-product. When the reaction is carried out in the presence of Me_2S, the possibility of DMSO formation has also to be considered. Under these conditions, aromatic amino acid residues might undergo electrophilic aromatic substitution and/or oxidation processes resulting in their irreversible chemical modification or degradation. In this sense, reducing agents have been utilized as additives in order to avoid the presence of these by-products. Thus, the reduction of Met(O) using NH_4I with an excess of 2-mercaptoacetic acid in 70% aqueous TFA has been described

in the synthesis of motilin [4] and the 8-11 fragment of [Tyr]^8-substance P [5]. The desired peptides were obtained quantitatively according to HPLC and TLC analyses of the reaction crude products, respectively. On the other hand, no degradation of Tyr and His residues was detected by amino acid analysis after the final deprotection of the Met residue using similar conditions in the synthesis of human calcitonin [4]. However, Büllesbach *et al.* did not detect the formation of by-products during the deprotection of the Met residue using the same reagents in the absence of any additive (aqueous 90% TFA in this case) for the preparation of the Tyr containing peptide human relaxin [13].

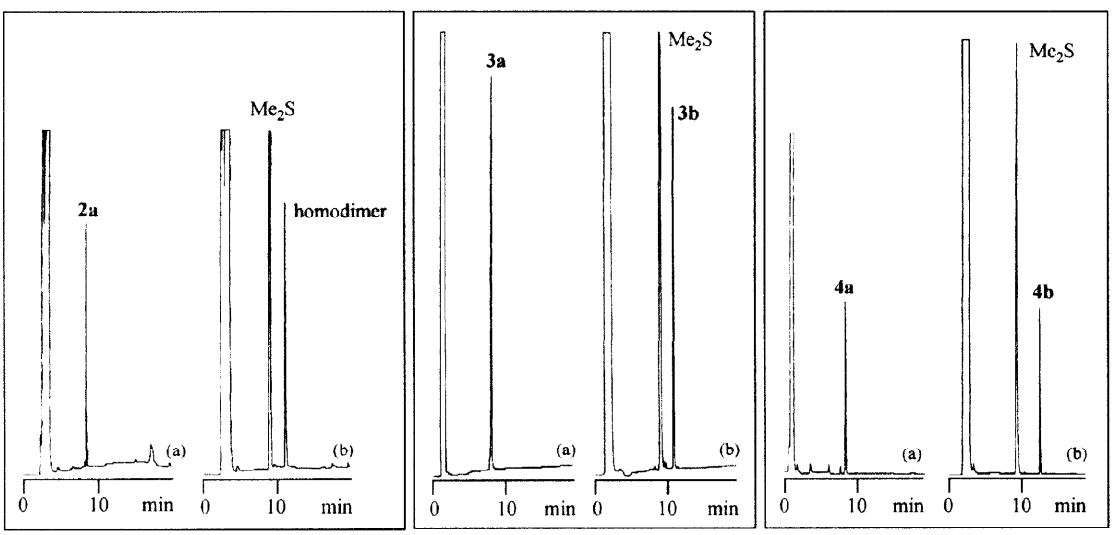


R: oxymethyl-PAM-polystyrene. i) Activation: Boc-amino acid, 3 eq; TBTU, 2.96 eq; DIEA, 8.5 eq; DMF, 8 min. Deprotection: neat TFA, 2 + 8 min. Coupling: DMF, 12 min. i') Deprotection: neat TFA, 2 + 8 min. Acetilation: Ac_2O, 10 eq; DIEA, 10 eq; 10 min. i'') Deformilation: piperidine/DMF (1:1), 10 min. ii) HF (10% anisole, X = O; 10% p-cresol, X = none).

scheme 2

The main reason why we became interested in the use of NH_4I in TFA for the reduction of Met(O) was due to the possibility of performing this reaction with disulfide containing

peptides. In such conditions, the presence of thiols or mercapto derivatives has to be avoided in order to preserve disulfides from reduction or scrambling processes [8]. Therefore, we carried out a study of the compatibility of the reducing system with His and Tyr using the experimental conditions that were utilized in our preceding work, that is, those used with the Cys containing peptides. The peptides Met-enkephalin (**3b**) [14] and a tetrapeptide corresponding to the fragment 11-14 of bombesin (**4b**) [15] were chosen as models in this case. These peptides and the corresponding analogues with the Met residue protected as the sulfoxide (**3a** and **4a**, respectively) were prepared by solid phase methodology, following similar protocols to those used for the synthesis of **1** and **2**. The protecting groups 2-bromobenzyloxycarbonyl (BrZ) and benzyloxymethyl (Bom) were utilized for Tyr and His, respectively.



Cromatographic conditions: Nucleosil C_{18} Reverse-phase columns were used (25x0.5 cm, 10 μm). **2a/homodimer**, linear gradient from 10% to 100% of CH_3CN in 20 min; **3a/3b** and **4a/4b**, linear gradient from 5% to 65% of CH_3CN in 20 min; 1 mL/min, 220 nm. Reaction conditions: 1.60 mM of **2a**, 2.00 mM of **3a**, 2.23 mM of **4a**; 30 eq of NH_4I in all cases, TFA at 0°C. Retention times: **2a**, 8.6 min and homodimer, 11 min; **3a**, 7.9 min and **3b**, 10.7 min; **4a**, 8.1 min and **4b**, 12.1 min. (a) Starting material, (b) after 1 h.

Figure 1. Reverse phase chromatographic profiles for reductions of peptides **2a**, **3a** and **4a** with NH_4I in TFA

The phenol and imidazole rings proved to be fairly stable under the experimental conditions studied. Thus, treatment of **3a** and **4a** with NH_4I in TFA at 0°C afforded **3b** and **4b** as the only products detected by HPLC. While reactions were incomplete after 1 h (68% yield for **3b** and 44% yield for **4b**), yields were quantitative in the presence of Me_2S at the

same reaction time (figure 1). On the other hand, no changes were observed in the corresponding chromatographic profiles after leaving the reaction mixture for 24 h.

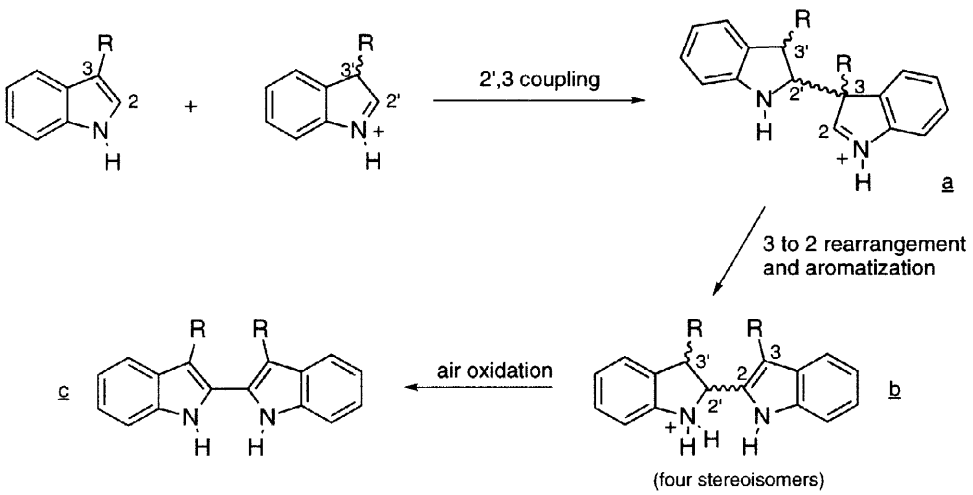
Tryptophan

Several side reactions have been reported for Trp in strong acidic media [3,16]. Thus, atmospheric oxygen [17] and DMSO [18] oxidize this residue to oxindolylalanine (2-hydroxytryptophan), which can undergo further degradation to other products resulting from the heterocyclic ring opening. Moreover, the TFA catalyzed cyclization to pyrrolidino[2,3-*b*]indoline systems [19] and electrophilic aromatic substitution processes such as cyclization to 3,4-dihydro-*b*-carboline rings [20] or 2,2' dimerization to indolylindole derivatives [21,22] have been reported.

The use of NH_4I in aqueous TFA for the deprotection of Met in Trp containing peptides has been documented, but the results are controversial. While Büllesbach *et al.* [13] did not observe decomposition of the Trp residue by UV and amino acid analysis in the synthesis of human relaxin, Izeboud et al. [4b] detected the presence of by-products by TLC after the reduction of the tripeptide H-Trp-Leu-Met(O)-NH_2. In order to draw conclusions about the convenience of this methodology for the deprotection of Met in the presence of Trp residues, we chosed the tetrapeptide H-Leu-Trp-Met-Arg-OH, used as a substrate in aminopeptidase mediated hydrolysis studies [23,24], to carry out this study under the same experimental conditions that were utilized with other peptide models in this work. Met protected and fully unprotected peptides (**5a** and **5b**, respectively; scheme 2) were prepared by solid phase methodology as before. Side chain protections for Arg and Trp were the tosyl (Tos) and formyl (For) groups, respectively. The latter was removed with piperidine prior to the cleavage of the peptide from the resin with HF [25].

The reduction of **5a** with NH_4I in neat TFA at 0°C was particularly slow, with 35% of conversion to **5b** after 1 h. Longer reaction times to increase the yield of the product resulted in the formation of by-products. Thus, this undesired material was the major product after 4 h (60% overall) while a 2% yield of **5a** could still be detected. The presence of Me_2S accelerated the process of reduction in such a way that no starting material was observed after 1 h, but neither was the formation of **5b** quantitative (95%). The HPLC of this reaction mixture after 24 h showed the same impurities that were observed in the absence of the additive. The analysis by HPLC/ESMS of the crude indicated that all by-products had the same mass, a value that was twice of the desired peptide **5b** (m/z 1208). Indole dimerization in strong acidic media has been reported to proceed by electrophilic aromatic substitution at position 3 of one of the rings (a, scheme 3), followed by a Wagner-Meerwein type rearrangement to position 2 to afford 2,2'-indolylindoline derivatives (b, scheme 3) [26]. Experiments carried out with Trp derivatives in neat TFA have shown that the reaction is stereoselective, yielding the 2,2'-indolylindolines with the *trans(2',3')* relative

stereochemistry [27,28]. These cyclic systems can undergo further air oxidation to the fully aromatic 2,2'-bisindole derivative (c, scheme 3) [29,30].



Scheme 3

In light of these results, the fact that the by-products obtained in the reduction of **5a** have the same molecular weight would be in agreement with a 2,2'-indole dimerization process considering that a maximum of four diastereoisomers would be obtained in this particular case, since the R group is chiral (b, scheme 3). With the object of testing this hypothesis, the major dimer was purified by medium pressure liquid chromatography (MPLC) and was studied by high field 1D and 2D ^{1}H-NMR spectroscopy. The monodimensional 500 MHz spectrum registered in D_2O/H_2O (8.5/1.5) showed only one signal in the zone where the indolyl NH protons of Trp containing peptides usually appear (9.8 ppm), which indicated that one of the indole rings was modified in the dimer. That was comfirmed by the TOCSY spectrum carried out in D_2O, which showed the typical set of signals for a Trp side chain with the absence of the signal corresponding to the proton at position 2, and another set with a doublet at 5.2 ppm (2') and a multiplet at 3.7 ppm (3') that revealed the presence of an indolenilalanine residue in the dimer (figure 2). The ^{1}H-NMR experiments and the analysis by HPLC/ESMS proved that the three by-products that were detected after the reduction of **5a** are diastereoisomeric dimers of **5b** that were formed by a 2,2' indole coupling of two Trp residues through an electrophilic aromatic substitution process, the major product probably having *trans* stereochemistry according to literature precedent [27,28].

HPLC analysis of the reaction mixture at different reaction times indicated that the formation of the side-products was favoured with an overexposure of the peptide to the acidic conditions needed to carry out the reduction of methionine. Therefore, a strict control of the

process is required in order to minimize this problem. In this sense, the short reaction time needed for the synthesis of human relaxin by reduction of the corresponding peptide sulfoxide (15 min; 90% aqueous TFA, 3.3 mM of peptide, 15 eq of iodide) would explain that Büllesbach *et al.* did not detect the formation of by-products. However, the results achieved by Izeboud *et al.* suggest that other factors such as a higher concentration of iodine and/or the presence of an important quantity of water might have been responsible for the low stability of the Trp residue under the conditions that were used (70% aqueous TFA, 25.3 mM of peptide, 23 eq of iodide; 5 min).

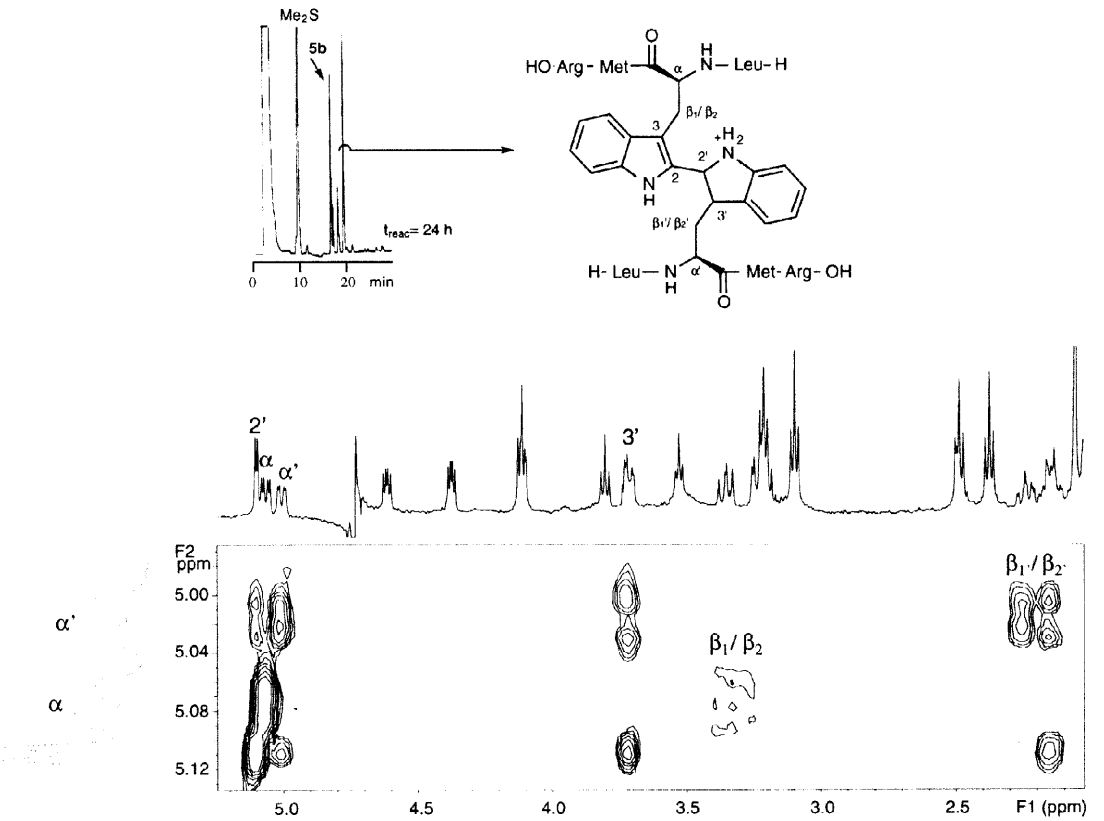


Figure 2. Tryptophan and indolenylalanine regions of a 500 MHz TOCSY spectrum of the 2,2'-indolylindoline dimer in D_2O at room temperature; mixing time: 80 ms.

Conclusions

The use of NH_4I in neat TFA for the reduction of Met(O) containing peptides in the presence of Cys, His ,Tyr or Trp residues has been studied. Free Cys residues undergo oxidation to yield disulfide derivatives. Thiol activation by the iodosulfonium cation that is obtained as an intermediate of the reduction process has been suggested as the probable cause for this side reaction. On the other hand, while His and Tyr residues have proved to be stable

under the experimental conditions, the strong acidic media catalyzes the 2,2' coupling between unprotected Trp residues to afford 2-indolylindoline derivatives. The use of additives such as Me_2S increase the reduction rates and minimizes the problem.

According to the mechanism of dimerization, protonation of an indole ring is required for the 2,2' coupling of two Trp residues. The tendancy of the indole ring to be protonated and its nuclephilicity would be considerably reduced by acylation of the nitrogen atom [16]. Thus, the possibility to avoid the side reaction using the Trp residue protected with the formyl group is under investigation.

Experimental

Boc-amino acids were supplied by Novabiochem AG (Läufelfingen, Switzerland), Bachem Feinchemikalien AG (Bubendorf, Switzerland), Advanced ChemTech (Maidenhead, England) or Propeptide (Vert-le-Petit, France). Starting resins for this work (Novabiochem) were either BocMetOCH2Pam-polystyrene (0.60 mmol/g), BocMet(O)OCH2Pam-polystyrene (0.60 mmol/g) or BocArg(Tos)OCH2Pam-polystyrene (0.50 mmol/g). TBTU, NH4I and Me2S were supplied by Fluka Chimie AG (Buchs, Switzerland) and were used without purification. DMF was supplied by Scharlau (Spain), and was bubbled with nitrogen to remove volatile contaminants and kept stored over activated 4 Å molecular sieves. MeCN (Scharlau, Spain) was HPLC grade, DCM (Scharlau, Spain), and TFA (Solvay, Germany) were peptide synthesis grade and were used directly.

Peptide-resins were hydrolysed using 12M HCl/propionic acid (1:1) at 155°C for 2 h and peptides were hydrolysed in 6M aqueous HCl solution at 155°C for 1 h. BocMet(O)OCH2Pam-polyestirene was hydrolysed with 4N aqueous MSA solution at 110°C for 22 h. Amino acid analysis was performed on a Beckman System 6300 analyser. HPLC was carried out on a Shimadzu apparatus comprising two solvent delivery pumps model LC-6A, automatic injector model SIL-6B69A, variable wavelength detector model SPD-6A, system controller model SCL-6B and plotter model C-R6A. Nucleosil C_{18} Reverse-phase columns were used (25x0.5 cm, 10 μm). In general, peptides were eluted at a flow rate of 1 mL/min (A: water, 0.045% of TFA; B: MeCN, 0.035% of TFA) and detection was carried out at 220 nm. Reverse phase MPLC was carried out using a CFG-Prominent/Duramat pump, a 757 ABI variable wavelength detector, an automatic fraction collector model Gilson FC 203 and an Omniscribe B-5000 plotter. A glass column (2.5 cm x 26 cm) packed with reverse phase Vydac-C_{18} was used. A flow rate of 125 mL/h was utilized (A and B: mixtures of water/MeCN with 0.1% of TFA) and the products were detected at 220 nm. Gel filtration chromatography was performed using a LDC/ MiltonRoy pump, an LKB 2158 Uvicord SD variable wavelength detector, an automatic fraction collector model LKB Ultrorac II 2070 and a Servoscribe 1s plotter. Two glass columns (2.5 cm x 90 cm and 1.5 cm x 60 cm)

packed with Sephadex G-15 (Pharmacia) were used. Peptides were eluted at 25 mL/h (0.1% of aqueous AcOH) and the detection was carried out at 206 nm in all cases.

General procedure for the solid-phase assembly of peptides and their acidolytic cleavage from the resin

Peptide synthesis was performed manually in a 20 mL polypropylene syringe fitted with a polyethylene disc. Boc-amino acids were assembled using the following protocol: 1) CH_2Cl_2, 2 x 4 min; 2) neat TFA, 1 x 2 min, 1 x 8 min; 3) DMF, 4 x 2 min; 4) Boc-amino acid (3 eq) in DMF, 12 min; 5) DMF, 4 x 2 min. Boc-amino acids were activated separately as follows: to 3 eq of Boc-amino acid was added 2.96 eq of TBTU (0.5 M solution in DMF) and 8.5 eq of DIEA consecutively; the mixture was left for 6-8 min and was added to the peptidyl-resin.

Peptides were cleaved from the resins with HF (UCAR) on a Kel-F Toho-Kasei Ltd (Tokio, Japan) apparatus. Peptidyl-resins (650 mg - 800 mg batches) were treated with 4.5 mL of HF and 500 μL of anisole (Met(O)) or *p*-cresol (Met) during 1 h at 0°C. The resins were washed with 10-15 mL of Et_2O and the crude materials were dissolved with 10-20 mL of 10% aqueous AcOH and liophilyzed. For the preparation of peptides **5a** and **5b** the deformylation of the Trp residue was performed previously to the cleavage from the resin using the following protocol: 1) CH_2Cl_2, 3 x 1 min and DMF, 3 x 1 min; 2) 50% piperidine in DMF, 15 min; 3) DMF, 3 x 1 min. Products were purified by MPLC, volatiles were removed under vacuum and the remaining solutions were lyophilized.

H-Gly-Ile-Cys-Pro-Leu-Cys(Acm)-Met(O)-OH (**1a**).

672 mg of peptidyl-resin afforded 152 mg (185 μmol) of crude material (95%), which was eluted with a 15% (A, 300 mL) to 20% (B, 300 mL) convex gradient of organic component. 53 mg (64 μmol, 35% recovery). HPLC: rt, 8.7 min; 10% to 100% of B over 20 min. Amino acid composition Gly: 0.98, Pro: 0.97, Cys: 0.68, Met: 0.80, Ile: 0.90, Leu: 1.00; FABMS *m/z* 844.9 $[M+Na]^+$, 823.6 $[M+H]^+$, 752.0 $[M+2\text{-Acm}]^+$; $C_{33}H_{58}N_8O_{10}S_3$ requires 822.3.

H-Gly-Ile-Cys-Pro-Leu-Cys(Acm)-Met-OH (**1b**).

661 mg of peptidyl-resin afforded 134 mg (166 μmol) of crude material (74%), which was eluted with a 15% (A: 400 mL) to 25% (B: 400 mL) convex gradient of organic component. 46 mg (56 μmol, 34% recovery). HPLC: rt, 10.3 min; 10% to 100% of B over 20 min. Amino acid composition Gly: 1.00, Pro: 0.99, Cys: 0.44, Met: 0.65, Ile: 0.90, Leu: 0.98; FABMS *m/z* 829.8 $[M+Na]^+$, 807.8 $[M+H]^+$, 736.8 $[M+2\text{-Acm}]^+$; $C_{33}H_{58}N_8O_9S_3$ requires M806.3.

H-Gly-Ile-Cys(Acm)-Pro-Leu-Cys-Met(O)-OH (**2a**).

675 mg of peptidyl-resin afforded 137 mg (166 μmol) of crude material (88%), which was eluted with a 10% (A, 300 mL) to 20% (B, 300 mL) convex gradient of organic component.

41 mg (50 μmol, 30% recovery). HPLC: rt, 8.6 min; 10% to 100% of B over 20 min. Amino acid composition Gly: 0.97, Pro: 0.97, Cys: 0.62, Met: 0.82, Ile: 0.82, Leu: 1.06; FABMS *m/z* 845.3 $[M+Na]^+$, 823.3 $[M+H]^+$, 752.0 $[M+2\text{-Acm}]^+$; $C_{33}H_{58}N_8O_{10}S_3$ requires 822.3.

H-Gly-Ile-Cys(Acm)-Pro-Leu-Cys-Met-OH (**2b**).

681 mg of peptidyl-resin afforded 132 mg (164 μmol) of crude material (73%), which was eluted with a 15% (A: 400 mL) to 20% (B: 400 mL) convex gradient of organic component. 45 mg (55 μmol, 34% recovery). HPLC: rt, 10.6 min; 10% to 100% of B over 20 min. Amino acid composition Gly: 0.95, Pro: 1.05, Cys: 0.19, Met: 0.92, Ile: 0.74, Leu: 1.00; FABMS *m/z* 829.3 $[M+Na]^+$, 807.3 $[M+H]^+$, 736.3 $[M+2\text{-Acm}]^+$; $C_{33}H_{58}N_8O_9S_3$ requires 806.3.

Ac-Gly-His-Leu-Met(O)-OH (**3a**).

1.09g of peptidyl-resin afforded 193 mg (375 μmol) of crude material (64%), which was eluted with a 5% (A, 300 mL) to 50% (B, 300 mL) convex gradient of organic component. 147 mg (277 μmol, 75% recovery). HPLC: rt, 7.9 min; 5% to 65% of B over 20 min. Amino acid composition: Gly: 0.99, His: 0.90, Leu: 0.97, Met: 0.5. ESMS: *m/z* 515.4 $[M+H]^+$; $C_{21}H_{35}N_6O_7S_1$ requires 514.6.

Ac-Gly-His-Leu-Met-OH (**3b**).

1.18 g of peptidyl-resin afforded 137 mg (270 μmol) of crude material (46%), which was eluted isocratically with 5% of CH3CN over H_2O (+0.05% TFA) (B, 600 mL total volume). 64 mg (126.μmol, 47% recovery). HPLC: rt, 10.7 min; 5% to 65% of B over 20 min. Amino acid composition: Gly: 0.99, His: 0.90, Leu: 0.97, Met: 0.5. ESMS: *m/z* 499.4 $[M+H]^+$, 454.0 $[M\text{-Ac}]^+$; $C_{21}H_{35}N_6O_6S_1$ requires 498.6.

H-Tyr-Gly-Gly-Phe-Met(O)-OH (**4a**).

1.16 g of peptidyl-resin afforded 110 mg (186 μmol) of crude material (36%), which was eluted with a 0% (A, 350 mL) to 50% (B, 350 mL) convex gradient of organic component. 66 mg (111 μmol, 60% recovery). HPLC: rt, 8.1 min; 5% to 65% of B over 20 min. Amino acid composition: Gly: 2.07, Met: 0.71, Tyr: 0.88, Phe: 0.92. ESMS: *m/z* 590.7 $[M+H]^+$; $C_{27}H_{36}N_5O_8S_1$ requires 589.7.

H-Tyr-Gly-Gly-Phe-Met-OH (**4b**).

1.32 g of peptidyl-resin afforded 173 mg (301 μmol) of crude material (51%), which was eluted with a 0% (A, 350 mL) to 50% (B, 350 mL) convex gradient of organic component. 69 mg (120 μmol, 40% recovery). HPLC: rt, 12.1 min; 5% to 65% of B over 20 min. Amino

acid composition: Gly: 2.06, Met: 0.41, Tyr: 0.93, Phe: 1.03. ESMS: *m/z* 574.70 $[M+H]^+$; $C_{27}H_{36}N_5O_7S_1$ requires 573.7.

H-Leu-Trp-Met(O)-Arg-OH (**5a**).

380 mg of peptidyl-resin were deformylated as described above. The acidolytic treatement of the resulting material afforded 76 mg (124 μmol) of the crude peptide (70%), which was eluted with a 0% (A, 350 mL) to 30% (B, 350 mL) convex gradient of organic component. 22 mg (37 μmol, 30% recovery). HPLC: rt, 11.9 min; 5% to 65% of B over 20 min. Amino acid composition: Leu: 1.05; Met: 1.00; Arg: 1.05. FABMS: *m/z* 621.6 $[M+H]^+$, 643.6 $[M+Na]^+$; $C_{28}H_{45}N_8O_6S_1$ requires 620.7.

H-Leu-Trp-Met-Arg-OH (**5b**).

147 mg of peptidyl-resin were deformylated as described above. The acidolytic treatment of the resulting material afforded 27 mg (45 μmol) of the crude peptide (62%), which was eluted with a 10% (A, 400 mL) to 30% (B, 400 mL) convex gradient of organic component. 8.4 mg (14 μmol, 20% recovery). HPLC: rt, 13.9 min; 5% to 65% of B over 20 min. Amino acid composition: Leu: 0.91; Met: 1.01; Arg: 1.07. ESMS: m/z 605 $[M+H]^+$, 303 $[M+2H]^{2+}$; $C_{28}H_{45}N_8O_5S_1$ requires 604.7.

^{1}H-RMN (500 MHz, D_2O-H_2O (15:85) (δ in ppm)): Leu residue: 3.99 (αH), 1.68 (βH), 1.60 (γCH_2), 0.90 (δCH_3); Trp residue: 8.68 (NH), 4.90 (αH), 3.25 (βH), 7.25 (2H), 7.65 (4H), 7.17 (5H), 7.24 (6H), 7.50 (7H), 9.85 (NH); Met residue: 8.12 (NH), 4.35 (αH), 1.95/1.83 (βH), 2.42 (γCH_2), 2.10 (εCH_3); Arg residue: 7.93 (NH), 4.07 (αH), 1.79/1.65 (βH), 1.52 (γCH_2), 3.14 (δCH_3), 7.15 (NH).

H-Leu-Trp(For)-Met(O)-Arg-OH (**6a**).

380 mg of peptidyl-resin afforded 96 mg (148 μmol) of crude material (85%), which was eluted with a 0% (A, 350 mL) to 30% (B, 350 mL) convex gradient of organic component; 38 mg (59 μmol, 40% recovery). HPLC: rt, 12.4 min; 5% to 65% of B over 20 min. Amino acid composition: Leu: 0.93; Met: 1.01; Arg: 1.05. FABMS: *m/z* 649.6 $[M+H]^+$, 671.5 $[M+Na]^+$; $C_{29}H_{45}N_8O_7S_1$ requires 648.7.

H-Leu-Trp(For)-Met-Arg-OH (**6b**).

147 mg of peptidyl-resin from the total 295 mg obtained afforded 34 mg (54 μmol) of crude material (75%), which was eluted with a 10% (A, 350 mL) to 30% (B, 350 mL) convex gradient of organic component; 8.2 mg (13 μmol, 25% recovery). HPLC: rt, 15.9 min; 5% to 65% of B over 20 min. Amino acid composition: Leu: 0.90; Met: 1.01; Arg: 1.08. FABMS: *m/z* 633.6 $[M+H]^+$, 655.6 $[M+Na]^+$; $C_{29}H_{45}N_8O_6S_1$ requires 632.7.

General Procedure for the Reduction of sulfoxides

Peptides were dissolved in neat TFA (**1a**, 1.67 mM; **2a**, 1.60 mM; **3a**, 2.00 mM; **4a**, 2.23 mM; **5a**, 2.24 mM i **6a**, 2.24 mM) and the solutions were cooled to 0°C. Then, Me_2S, when used (1:1 molar ratio respect to the reducing agent), and NH_4I (26-30 eq.) were added and the mixtures were stirred vigorously. HPLC monitoring of the reactions was performed as follows: samples were taken from the reaction mixtures and a saturated aqueous solution of ascorbic acid was added to quench the reductions and remove iodine; analysis were carried out using linear gradients from 20% to 40% of B over 20 min or 5% to 65% of B over 20 min (1 mL/min, 220 nm). The final products were characterized by HPLC , ESMS or FABMS and amino acid analysis of collected samples.

Isolation and characterization of one of the dimers obtained as a subproduct during the Met(O) reduction in ***5a***

10 mg of crude product from the reduction with NH_4I/Me_2S of peptide **5a** were eluted with a 10% (A, 400 mL) to 30% (B, 400 mL) convex gradient of organic component and the fractions containing the desired product were joinned. The volatiles were removed under vacuum and the remaining solution was lyophilized. 2.2 mg of dimer were obtained (25% yield). HPLC: rt, 14.9 min; 5% to 65% of B over 20 min. ESMS: *m/z* 605 $[M+2H]^{2+}$, 403 $[M+3H]^{3+}$ (1207); $C_{56}H_{88}N_{16}O_{10}S_2$ requires 1207.4. ^{1}H-RMN (500 MHz, D_2O-H_2O (15:85) and D_2O; (δ in ppm)): Leu residue: 4.12 (αH), 1.73 (βH), 1.58 (γCH_2), 0.96/0.92 (δCH_3); Leu' residue: 3.82 (αH), 1.56 (βH), 1.47 (γCH_2), 0.90/0.86 (δCH_3); Trp residue: 8.85 (NH), 5.07 (αH), 3.35/3.25 (βH), 7.41 (4H), 7.06 (5H), 7.13 (6H), 7.28 (7H), 9.80 (NH); Trp' residue: 8.84 (NH), 5.01 (αH), 2.25/2.15 (βH), 5.10 (2H), 3.72 (3H), 7.38 (4H), 7.02 (5H), 7.24 (6H), 6.82 (7H); Met residue: 8.57 (NH), 4.62 (αH), 2.16/1.96 (βH), 2.50 (γCH_2), 2.08 (εCH_3); Met' residue: 8.04 (NH), 4.38 (αH), 2.00/1.76 (βH), 2.38 (γCH_2), 2.04 (εCH_3); Arg residue: 8.43 (NH), 4.12 (αH), 1.80/1.68 (βH), 1.56 (γCH_2), 3.10 (δCH_3), 7.15 (NH); Arg' residue: 8.10 (NH), 3.54 (αH), 1.66 (βH), 1.62 (γCH_2), 3.22 (δCH_3), 7.23 (NH).

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