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Preparation of Trisulfide Derivatives of Cystine and their Formation as By-products during Peptide Synthesis.

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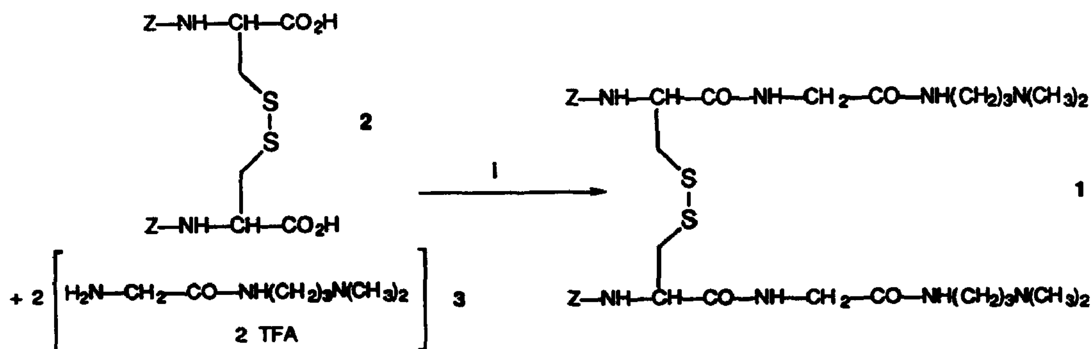
Abstract : The formation of a trisulfide derivative is observed when di-NH_2-protected cystine, is used during peptide synthesis. This side reaction occurs during the coupling step with several coupling reagents. A convenient method for the preparation of these derivatives in mild conditions is described.

In recent years, mass spectrometry has gained a widespread application to the systematic characterization of synthetic peptides and of their accompanying by-products[1]. Although most of the classical side reactions are now well documented, new side reactions are often detected as soon as less classical synthetic strategies are used. As an example, we report here an unexpected side reaction which was observed when a coupling step was performed on di-NH_2-protected cystine. Such an elongation procedure is not usual as, in general, peptide chemists tend to use S-protected cystein derivatives for chain assembly and form the disulfide by oxidation at the end of the synthesis.

In this case, we followed a procedure shown in scheme 1 previously described by Fairlamb[2] to prepare N,N'-bis(benzyloxycarbonyl)-L-cysteinylglycyl-3-dimethylaminopropylamide disulfide (1), an alternative substrate of trypanothione reductase, an enzyme specific for trypanosomatidae and a rational target for antiparasitic drug design. The last step of this synthesis involves coupling of N,N'-bis (benzyloxycarbonyl)-L-cystine (di-Z-cystine) (2) with two equivalents of glycine-3-dimethyl aminopropylamide ditrifluoroacetate (3) using two equivalents of dicyclohexylcarbodiimide (DCC) and of 1-hydroxybenzotriazole hydrate (HOBt) in the presence of ten equivalents of N,N-diisopropylethylamine (DIEA).



Reaction conditions : (i) Coupling agents, DIEA 10 eq., DMF, 24 h

Scheme 1.

Several syntheses of **1** were performed in high yield according to the described protocol, however, in each case, we detected the presence, in limited quantities of a by-product which eluted later than **1** in reversed phase HPLC (HPLC of the mixture : fig. 1a). Analysis of the crude product by Plasma Desorption Mass Spectrometry showed the presence, besides the expected Mass for 1 ($M+H^+=792$) of a peak ($M+H^+=824$) corresponding to an excess of 32 mass units (fig. 1b).

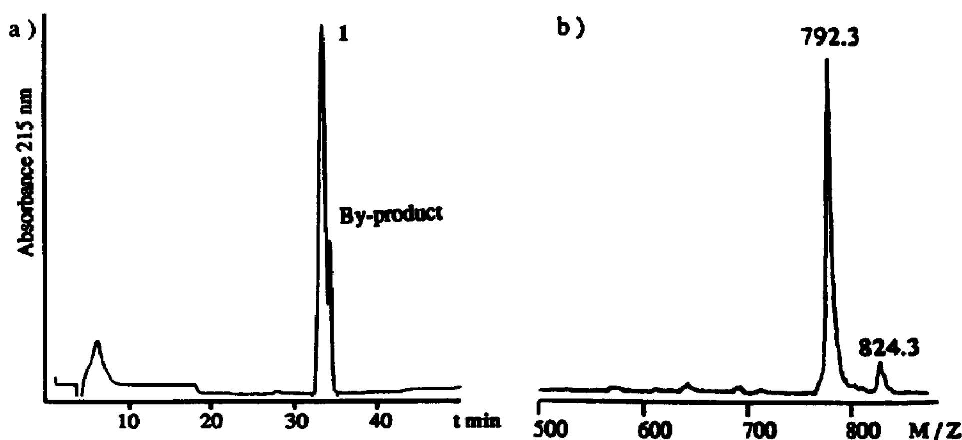


Figure 1 : Analytical characterization of **1** obtained according to scheme 1. (a) HPLC trace using a Nucleosil C18 reversed-phase column and eluting with a linear gradient from 0 to 100% of B in A where A is H_2O/0.05% TFA and B is 50% MeCN/H_2O 49.95%/0.05%TFA at a flow rate of 0.5 ml min^{-1} Detection at 215 nm by UV absorbance. b) PDMS/TOF spectrum of the crude product.

In order to get more information on the structure of this by-product, it was isolated by preparative HPLC and submitted to several analytical methods. One possibility to account for a 32 mass units excess, (not in agreement with increased retention time in reversed phase HPLC) is an oxidation of the disulfide, resulting in the incorporation of two oxygen atoms. Fast Atom Bombardment (FAB) spectrum of the by-product (fig. 2)

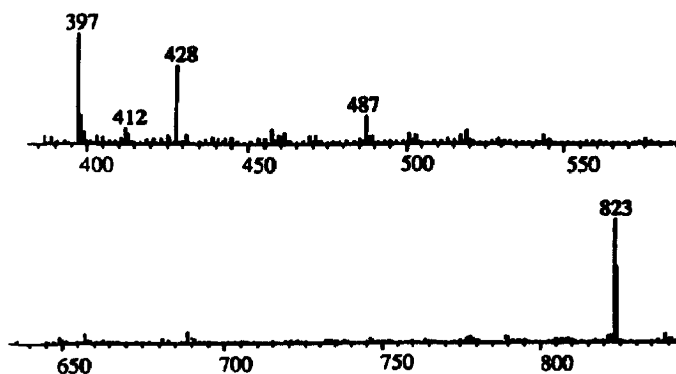
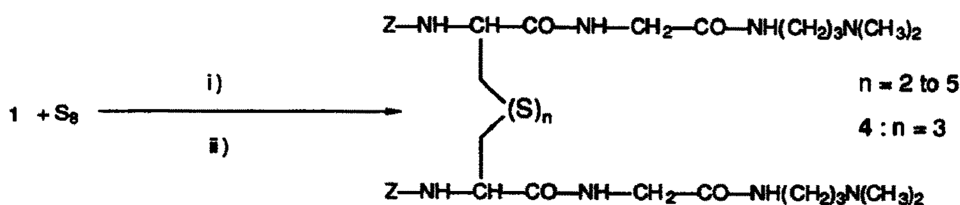


Figure 2 : FAB mass spectrum of the by-product.

did not support the hypothesis of a symmetrical S,S'-dioxide (—SO—SO—) as no fragment corresponding to a cleavage between the two sulfur atoms ($M^+=[(791/2)+16]$) could be detected. By contrary, strong peaks corresponding to the half of **1** with a 32 mass units excess ($M^{\circ+}=[(791/2)+32]$) or without mass excess ($M+H^+=[(791/2)+1]$) were observed.

On the contrary, both ^1H and ^{13}C NMR spectra[3] did not support the hypothesis of an unsymmetrical S,S-dioxide (—S—SO_2—) as no splitting of the signals was observed. The major difference with the spectrum of 1 being a 0.1 ppm upfield shift of the βCH_2 proton of cystein in the ^1H spectrum. This led us to speculate that the 32 mass unit excess could correspond to an additional sulfur atom incorporated by formation of the symmetrical trisulfide derivative **4**, this hypothesis being consistent with the fragmentation pattern observed in mass spectrometry, with the symmetrical nature of the NMR spectrum and with the increase in hydrophobicity in HPLC. In order to confirm this hypothesis, several attempts were made to synthesize **4**. Among the different possibilities[4,5], a modification of the method described by Fletcher[6], which offers the advantage of being compatible with peptide structures was developed. It consists in reacting **1** at pH 10 with a solution of elemental sulfur (10 equivalents) in CS_2 at room temperature (scheme 2). After 2 hours of vigourous mixing, several products, eluting after **1** in reversed phase HPLC were isolated (fig. 3).



Reaction conditions : (i) $EtOH/CHCl_3/CS_2/NH_4OH$ (45/5/2/2) (v/v) (ii) AcOH pH=2

Scheme 2. Preparation of polysulfide derivatives.

A product with an excess of 32 mass units which coeluted with **4** and had identical NMR and mass spectra was isolated. In addition, derivatives corresponding to the incorporation of 2 and 3 sulfur atoms were also detected in the slower eluting fractions. Initially, we suspected that the trisulfide derivative could be an impurity contained in di-Z-cystine (**2**) as the presence of bis-(2 amino-2-carboxyethyl) trisulfide (cystine trisulfide) has been reported as a contaminant of cystine in wool hydrolysates[6]. Following the previously described protocol, di-Z-cystine trisulfide was prepared from di-Z-cystine. Using HPLC conditions suitable to separate these two derivatives, no trace of di-Z-cystine trisulfide could be detected in the different commercial (Bachem) batches of di-Z-cystine which were examined, indicating that the formation of the trisulfide occured during the synthesis of the peptide. This led us to examine the coupling reaction. When achieved in standard conditions and monitored by reversed phase HPLC[7], 5% of **4** could be detected after 4 hours of coupling and 10% after 24 hours indicating that the trisulfide is formed during the coupling reaction.

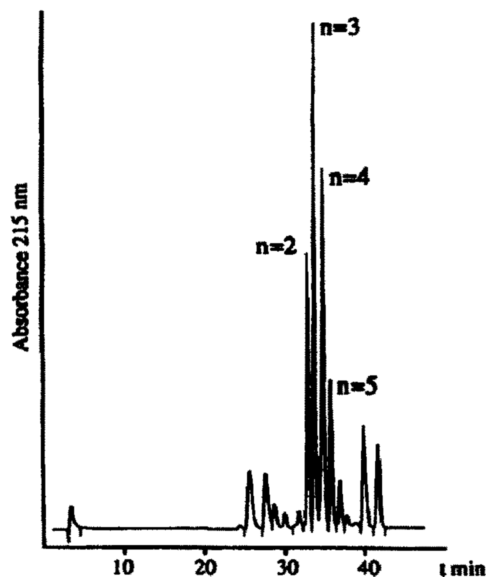


Figure 3 : HPLC trace of the mixture of polysulfides obtained according to scheme 2. n indicates the number of sulfur atoms. The product corresponding to n=3 was isolated and proved to be identical to **4**. Conditions as for figure 1a.

In the aim to determine whether the formation of the trisulfide is caused by the reaction medium (solvent or alkaline solution) or requires the presence of coupling reagents, we have mixed di-Z-cystine (2) with 3 in DMF with ten equivalents of DIEA without adding the coupling agents. In these conditions, even after 20 h, no conversion of 2 to the trisulfide was observed using HPLC or mass spectrometry. After this time, the coupling agents were added and, 4 h later the presence of 4 (5%) was observed, demonstrating that addition of coupling agents is necessary for the formation of trisulfide derivatives. In order to get more information about the influence of the coupling agents, the reaction was performed using only DCC and DIEA (2 to 10 eq. of DIEA) and other reagents such as Benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP)/HOBt or [2-(1H-benzotriazol-1-yl)-1,1,3,3tetramethyluronium hexafluorophospate] (HBTU)/HOBt). The results were similar but, in the last two cases mentioned above, 4 was formed in larger quantities (15-20 %) after 24 h of coupling. As Hirst[8] has shown that addition of hydroquinone inhibits the photochemical degradation of cystine which produces cystine trisulfide among different products ; the coupling reaction was performed in presence of this radical quencher and no trace of 4 was detected. By contrary, the formation of 4 was not prevented when the coupling reactions were performed in the dark.

To the best of our knowledge, this is the first time that a trisulfide derivative of cystein has been observed in a synthetic peptide. While this work was in progress, the presence of cystine trisulfide has been demonstrated in a by-product isolated from recombinant human growth hormone produced in *Escherichia coli*[9]. As these compounds appear to be fairly stable in aqueous solution as well as in more drastic conditions such as trifluoroacetic acid treatment, their presence should be more carefully searched in cystine containing peptides.

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3. **compound 1** ^{1}H NMR (D_2O, 250 MHz) : δppm 7.5(10H,s), 5.2(4H,s), 4.8(2H,m), 4.6(2H,m), 4.0(4H,m), 3.4(8H,m), 3.2(4H,t), 3.0(12H,s), 2.0(4H,m).
   ^{13}C NMR (D_2O, 250 MHz) : δppm 176.2, 174.3, 160.8, 139.2, 131.7, 131.4, 130.5, 70.2, 58.2, 57.2, 45.7, 41.8, 38.9, 27.0.
   compound 4 ^{1}H NMR (D_2O, 250 MHz) : δppm 7.5(10H,s), 5.2(4H,s), 4.9(2H,m), 4.6(2H,m), 3.9(4H,m), 3.3(8H,m), 3.1(4H,t), 2.9(12H,s), 1.9(4H,m).
   ^{13}C NMR (D_2O, 250 MHz) : δppm 176.2, 174.3, 160.8, 139.2, 131.7, 131.4, 130.6, 70.3, 58.1, 57.1, 45.7, 41.5, 38.9, 27.0.
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