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A Convenient Method for the Synthesis of Peptide Trisulfides.

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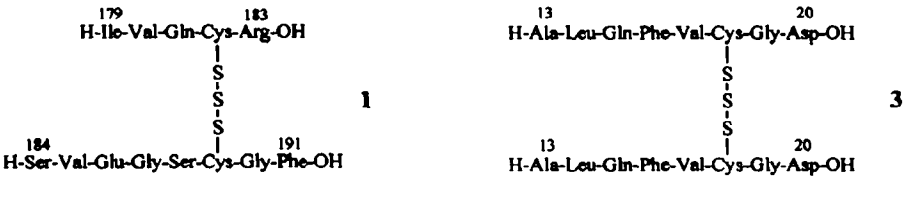
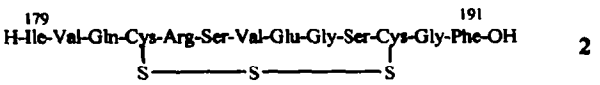
Abstract: *Symmetrical* **3**, *unsymmetrical* **1**, *and intramolecular* **2**, *peptide trisulfides have been prepared in good yields by reacting linear cysteine-containing peptides with* N,N'-*thiobisphthalimide in a mixture of acetonitrile-water.*

The recently reported discovery of a novel protein derivative, i. e. recombinant human growth hormone with a trisulfide bridge between two cysteins[1], calls for chemical methods for synthesizing peptide trisulfides in order to be able to identify peptide fragments convincingly in enzymatic peptide maps of this kind of new proteins. The synthesis of peptide trisulfides have to our knowledge not hitherto been described. Methods for synthesizing non-peptidic trisulfides however have been reported several times in the literature. The usefulness of *N,N'*-thiobisphthalimide as a sulfur-transfer reagent has in this context been demonstrated[2a-d].

We now wish to report the use of *N,N'*-thiobisphthalimide (TBPI)[3] in the synthesis of peptide trisulfides. We will describe the preparation of peptide trisulfides related to the C-terminal region of human growth hormone (hGH) : **1**, **2** and the B-domain of insulin-like growth factor 1 (IGF-1) : **3**

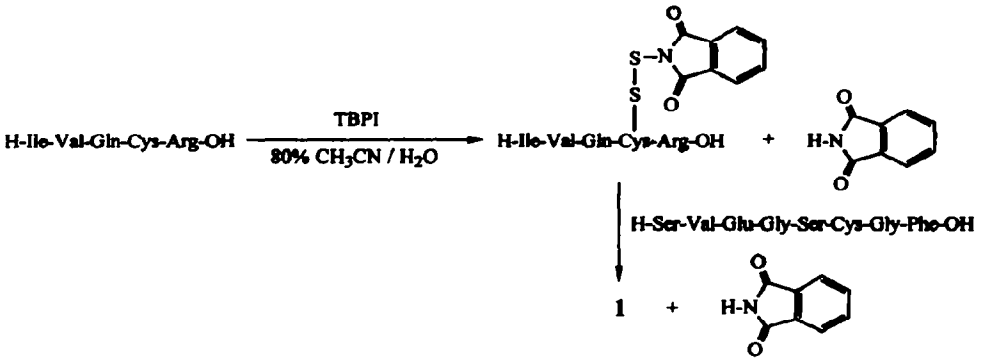




The three corresponding disulfides : **1'**, **2'**, and **3'** were synthesized for comparison[4].

The linear peptides were synthesized by using the *t*-Boc/ benzyl solid-phase technique. The amino acid coupling reactions were performed by using the HBTU/ *in situ* neutralization method[5]. The linear peptides were cleaved from the resins and the protecting groups were removed by treatment with liquid hydrogen fluoride/ anisole at 0°C for 1 hour. The resulting materials were purified by gel permeation chromatography.

For the preparation of **1**, 1 eq. of TBPI was dissolved in 80 % acetonitrile-water, 0.75 mM, at roomtemperature and 1 eq. of hGH 179-183 peptide was added to the stirred solution. After 6 hours 1 eq. of hGH 184-191 peptide was finally added and the solution was stirred at room temperature overnight (Scheme. 1). The preparation of **3** was performed similarly: 2 eq. of IGF-1 13-20 peptide were added to the 80 % acetonitrile-water solution of TBPI (1 eq.) and the solution was stirred at room temperature overnight.



Scheme 1

For the preparation of **2**, 1 eq. of hGH 179-191 peptide, which contains two sulfhydryl groups, was dissolved in 80 % acetonitrile-water, 0.07 mM, 1 eq. of TBPI was added and the solution was stirred at room temperature overnight. The completion of the reactions were monitored at 214 nm by reversed phase-FPLC®-analysis[6]. The acetonitrile was removed

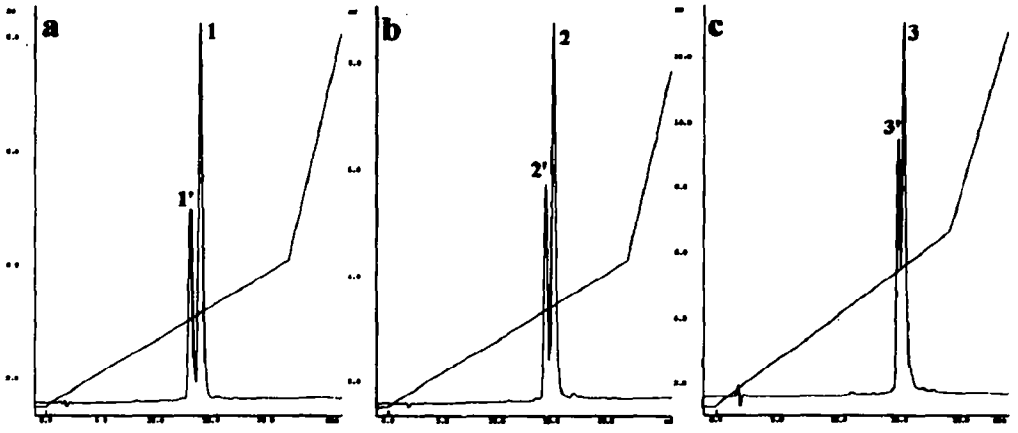


Figure 1. Analytical FPLC® [6] of a mixture of a)**1** and **1'**, b) **2** and **2'**, and c) **3** and **3'**.
Gradients: a) and b): 0 % B to 38 % B within 23 min, c): 0 % B to 45 % B within 20 min.

from the reaction mixtures by evaporation and the remaining water solutions were filtered in order to remove the precipitated phthalimide. The resulting filtrates were lyophilized and then

subjected to further purification by gel permeation chromatography[7]. The yields after purification were 55 %, 52 % and 54 % for 1, 2 and 3 respectively and the purity was > 95 % according to FPLC-analysis. The trisulfides all showed longer retention times in FPLC than the corresponding disulfides (Fig. 1a-c).

The amino acid analyses of 1, 2 and 3 showed the correct amino acid composition and there were no significant divergences from the amino acid analyses of the corresponding disulfides 1', 2' and 3'.

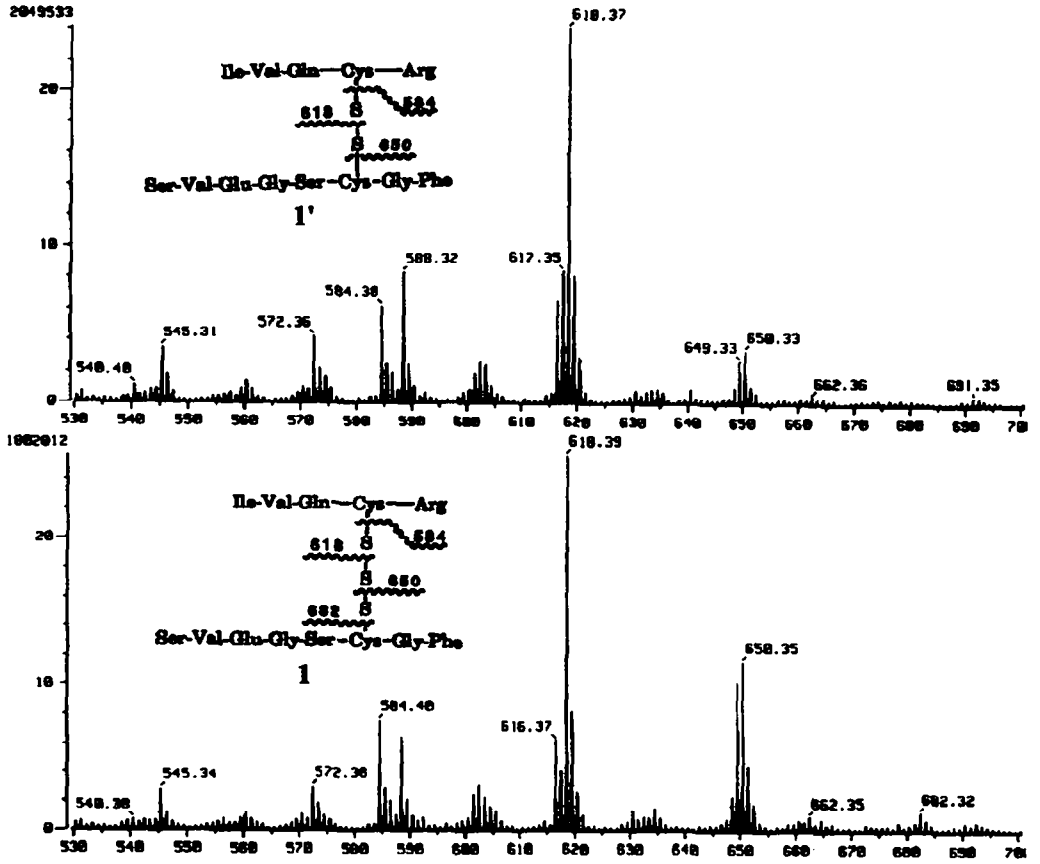


Figure 2. Positive FAB spectra in the mass range 530-700 Da of 1' (upper) and 1(lower). Fragment ions originating from the clevages of the di- and trisulfide bridges are indicated.

Positive ion fast atom bombardment, FAB (Xe 6kV, glycerol matrix) mass spectrometry verified the molecular masses of the products. The observed monoisotopic masses were in good agreement with the theoretical masses (in parenthesis). The observed masses were for 1 1432.7 (1432.6) Da, 1' 1400.6 (1400.6) Da, 2 1414.6 (1414.6) Da, 2' 1382.7 (1382.6) Da, 3 1733.8

(1733.7) Da, and 3' 1701.8 (1701.8) Da. Fragment ions due to cleavage of the di- and trisulfide bridges of 1 and 1' clearly distinguished the structures, verifying the trisulfide bridges of 1.See Figure 2, which show the FAB mass spectra in the mass range 530-700 Da of 1 and 1'. The fragment ion at 682 Da cannot be formed from cleavage of the disulfide and is thus indicative of the trisulfide. Due to their cyclic structures 2 and 2' could not exhibit the similar fragmentation ions from the cleavages of the -SS- and -SSS- bridges. However, treatments of 2 and 2' with DTT at pH 8.3 yielded the same reduced product having the same monoisotopic molecular masses 1384.7 and 1384.6 Da, respectively (theoretical mass 1384.6 Da). At the reduction of 2 the trisulfide bond broke, H_2S was eliminated, and the reduced form of 2' was produced. In spite of their double-chain structures 3 and 3' did not show the same simple fragmentation of the -SS- and -SSS- bridges as did 1 and 1'. The reduced forms of 3 and 3', however, did have the same molecular mass, 851.4 (851.4) Da, which confirmed their structures.

The liberation of hydrogen sulfide from the trisulfides in the presence of a thiol could also be demonstrated by adopting the procedure stated by Jespersen *et al.*[1]: When 1, 2, and 3 were reduced with cysteine the released hydrogen sulfide could easily be detected by the blackening of a filter paper soaked in lead acetate solution.

References and Notes

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2. a) Sullivan, A. B.; Boustany, K. *Int. J. Sulfur Chem. A* **1971**,*1:3*, 207-210. b) Harpp, D. N.; Ash, D. K. *Int. J. Sulfur Chem. A* **1971**,*1:3*, 211-214. c) Harpp, D. N.; Back, T. G. *Tetrahedron Lett.* **1972**, *15*, 1481-1484. d) Harpp, D. N.; Back, T. G. *J. Labelled Compd.* **1975**,*XI:1*, 95-98.
3. Kalnins, M. V. *Can. J. Chem.* **1966**, *44*, 2111-2113.
4. 1' was prepared by reacting the hGH 184-191 peptide with the Cys *S*-Npys protected hGH 179-183 peptide in water at pH 7.5, 2' by oxidizing the hGH 179-191 peptide with $K_3Fe(CN)_6$ in water, pH 7.5, at high dilution (0.07 mM), and 3' by oxidizing IGF-1 13-20 peptide with $K_3Fe(CN)_6$ in 0.1 M ammonium acetate at pH 8.
5. Schnölzer, M.; Alewood, P.; Jones, A.; Alewood, D.; Kent, S. B. H. *Int. J. Peptide Protein Res.* **1992**, *40*, 180-193.
6. FPLC®-system (Pharmacia Biotech AB), pep-RPC HR 5/5 C2-C18 column (5 μm, 5 x 50 mm). Eluents: A) 0.1 % TFA-water, B) 0.1 % TFA-acetonitrile. Linear gradients from 0% B to 100 % B. Detection 214 nm. Flow rate 0.6 mL/min.
7. Fractogel® PGM 2000, 0.032-0.063 mm (Merck). 2.5 x 80 cm column. Eluent 0.1 % TFA-water.

Abbreviations.

t-Boc, *tert*-butyloxycarbonyl; DTT, 1,4-dithiothreitol; FPLC, fast protein liquid chromatography; HBTU, benzotriazole-1-yl-tetramethyluronium tetrafluoroborate; Npys, 3-nitro-2-pyridinesulfenyl: TFA, trifluoroacetic acid.

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