New Method of Linking Tryptophan to Cysteine Sulphydryl Groups in Peptides and Proteins

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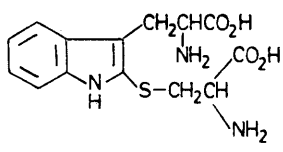
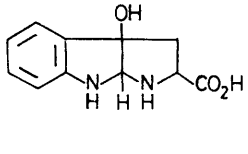
Summary A new simple method for establishing a crosslink between tryptophan and cysteine leading to tryptathionine (**1**), involving the reaction of 3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole-2-carboxylic acid (**2**) with cysteine in 25% trifluoroacetic acid, is described.

TRYPTATHIONINE residues (**1**) occur in phalloidin and related toxic peptides of *Amanita phalloides*, where they provide a crosslink between a tryptophan and a cysteine residue.[1] A synthetic route to these residues involves conversion of the sulphydryl group of a suitable cysteine derivative into the corresponding sulphenyl halide,[2] which leads to a sulphide derivative at the 2-position of the indole nucleus of tryptophan.[3] It has recently been reported by one of us[4] that the acid (**2**), obtained by peroxyacetic acid oxidation of tryptophan,[5] reacts with simple aliphatic thiols in 20% acetic acid at 60—80 °C to give the corresponding 2-tryptophanyl sulphide. We have now investigated the reactivity of (**2**) towards the sulphydryl group of cysteine residues in peptides and proteins, and have found that the use of this reagent provides a new effective method for the covalent binding of tryptophan to cysteine residues.

L-Cysteine was treated with 1·2 equiv. of (**2**) in 25% trifluoroacetic acid at room temperature for 2 days, the product being isolated by gel filtration on a Sephadex LH-20 column, equilibrated and eluted with water. The yield of analytically pure compound {monohydrate, crystallized from water; m.p. 245 °C (decomp.) $[\alpha]_D^{20}$ +8·8°, *c* 0·1 in 0·5M HCl} was 80%, based on cysteine. The product had u.v. spectral properties [λ_{max} (H_2O) 219 (ϵ 25,900), 289 (ϵ 11,250), and 282sh (ϵ 10,900) nm] similar to those described for tryptathionine obtained by the sulphenyl halide method.[2] On acid hydrolysis with 3M toluene-*p*-sulphonic acid[6] tryptathionine was converted quantitatively into



(1)



(2)

oxindolylalanine (2-hydroxytryptophan) and cysteine, the latter amino-acid being recovered on the analyser partly as cystine.† Glutathione (γ-L-glutamyl-L-cysteinyl-glycine) reacted analogously with (**2**) in 25% trifluoroacetic acid to give *S*-tryptophanylated glutathione in 85% yield. The spectral properties of the derivative resembled those of tryptathionine. Amino-acid analysis of an acid hydrolysate of the compound gave the following values: glutamic acid, 1·00; glycine, 0·98; oxindolylalanine, 1·08; cysteine, 0·70; and traces of cystine.

The reaction has been further extended to a protein. Reduced ribonuclease[7] (6 mg), containing eight cysteine residues per molecule,[8] was treated with (**2**) (6 equiv. based on protein SH content) in 1 ml of 25% trifluoroacetic acid for 24 h at room temperature. The mixture was evaporated *in vacuo* at 37 °C, diluted with water, and then excess of reagent and its degradation products were removed by gel filtration on a Sephadex G-25 column in 10% acetic acid. The modified protein was located in the effluent by spectrophotometry and recovered by lyophilization (4 mg). Amino-acid analysis of the *S*-tryptophanylated protein gave an oxindolylalanine value of 7·6 residues per molecule, in good agreement with the expected value (8·0). In addition, all other amino-acids were recovered unchanged on the analyser. The absorption spectrum of the product in water (λ_{max} 283 nm), while differing markedly from that of reduced ribonuclease (λ_{max} 275 nm, similar to tyrosine), resembled closely both in λ_{max} and shape the spectrum obtained with a solution containing 8 equiv. of tryptathionine and 6 equiv. of tyrosine, as would be required, in theory, for fully *S*-tryptophanylated protein.

Native ribonuclease, which does not contain any free cysteine, showed an unchanged u.v. spectrum after reaction with (**2**) and unchanged amino-acid composition after hydrolysis. The selectivity of the reaction was checked additionally by treating an amino-acid mixture containing all common amino-acids except cysteine with 20 equiv. of (**2**) in 20% trifluoroacetic acid. All amino-acids were recovered unchanged on the analyser.

The reaction described here is of practical use in the field of peptide synthesis, providing a more simple method for establishing a crosslink between tryptophan and cysteine, as a basic step in the chemical synthesis of toxic peptides of *Amanita phalloides*.[1] In addition, the ease, completeness and selectivity of the reaction for SH groups of proteins make the use of (**2**) of value also in protein modification studies.

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† Hydrolysis of tryptathionine in 20% HCl at 100 °C for 20 h produces cysteine and oxindolylalanine, but the yields of the latter are not quantitative owing to the instability of the amino-acid in hot HCl (Th. Wieland and G. Schmidt, *Annalen*, 1952, **577**, 215). Hot H_2SO_4 (H. Wieland and B. Witkop, *Annalen*, 1940, **543**, 171) or 20% aqueous AcOH at 110 °C produced higher yields of oxindolylalanine than HCl [Th. Wieland, O. Weiberg, W. Dilger, and E. Fischer, *Annalen*, 1955, **592**, 69; F. M. Veronese, A. Fontana, E. Boccu', and C. A. Benassi, *Z. Naturforsch.* (*B*), 1968, **23**, 1319]. We have found that oxindolylalanine is stable under hydrolytic conditions in 3M toluene-*p*-sulphonic acid and in presence of 0·2% of 3-(2-aminoethyl)-indole (ref. 6) and that tryptathionine residues can be quantitatively estimated by measuring the oxindolylalanine content of an acid hydrolysate by an automatic aminoacid analysis.

[1] Th. Wieland, 'Progress in the Chemistry of Organic Natural Compounds,' ed. L. Zechmeister, Springer Verlag, Heidelberg–New York, 1967, vol. 25, p. 214; Th. Wieland, 'Peptides 1972,' Proc. 12th European Symp., Reinhardsbrunn Castle, 1972, eds. H. Hanson and H.-D. Jakubke, North Holland, Amsterdam, 1972, p. 38.
[2] Th. Wieland and R. Sarges, *Annalen*, 1962, **658**, 181; Th. Wieland, C. Jochum, and H. Faulstich, *ibid.*, 1969, **727**, 13.
[3] A. Fontana and E. Scoffone, 'Methods in Enzymology,' eds. C. H. W. Hirs and S. N. Timasheff, Academic Press, New York–London, 1972, vol. 25, p. 482.
[4] W. E. Savige, presented at the 5th Int. Wool Textile Res. Conference, Aachen, September 1975, *Schriftenreihe* (*German Wool Res. Inst.*), in the press.
[5] W. E. Savige, *Austral. J. Chem.*, 1975, **28**, 2275.
[6] T. Y. Liu and Y. H. Chang, *J. Biol. Chem.*, 1971, **246**, 2842.
[7] C. B. Anfinsen and E. Haber, *J. Biol. Chem.*, 1961, **236**, 1361.
[8] C. H. W. Hirs, S. Moore, and W. H. Stein, *J. Biol. Chem.*, 1960, **235**, 633.