916. *Some Synthetic Cysteinyl Peptides.*

By H. S. Bachelard and V. M. Trikojus.

The syntheses are described of a number of peptides of cysteine, suitable as substrates for studies of the specificity of the enzyme, thyroid " cysteinyl-tyrosinase."

During investigations in this Department on the separation of the components of the proteolytic system of the thyroid gland, a proteinase, substantially purified, was isolated.[1] This fraction also exhibited limited peptidase activity, when tested with a range of peptides, and appeared to hydrolyse specifically the peptide bond linking cysteine and tyrosine, in particular that of L-cysteinyl-L-tyrosine. However, unlike pepsin which also hydrolyses cysteinyltyrosine,[2] thyroid " cysteinyltyrosinase " activity did not seem to be a property of the proteinase molecule.[1] This has been confirmed by its separation from the proteinase by column electrophoresis.[3]

Apart from its interest in thyroid biochemistry, an enzyme specifically hydrolysing peptides at the cysteinyl (cystinyl) link should prove of value in the determination of protein structure. For the further elucidation of the specificity requirements of this enzyme several new peptides containing cysteine have been synthesised, of which the following are described in this paper: L-cysteinyl-L-phenylalanine; L-cysteinyl-L-tyrosine amide; L-leucyl-L-cysteine; L-leucyl-L-cysteinyl-L-tyrosine amide; glycyl-L-leucyl-L-cysteinyl-L-tyrosine amide.

As an additional check on the products obtained, two condensation procedures were usually employed. These were the azide method [4] and the mixed anhydride reaction.[5] Protection of free amino- and thiol groups followed the methods described by Bergmann and Zervas [6] and Wood and du Vigneaud; [7] protecting groups were removed by sodium and liquid ammonia,[8] and an *N*-benzyloxycarbonyl group preferentially by acetic acid saturated with hydrogen bromide.[9]

Isolation and purification of the basic peptides (those containing a terminal amide group) presented difficulties. They were very soluble in water and rapidly lost titratable thiol activity [10] during attempts to recrystallise them from this or other solvents. In consequence, after reductive removal of the protecting groups, the free peptides were precipitated as the mercaptides with modified Hopkins's reagent.[11] Benesch and Benesch [12] have described the formation of glutathione from its mercaptide in the electrolytic de-salter[13] as modified by Dent,[14] but the direct application of this technique was found to give low yields in the cases under discussion, largely owing to the considerable frothing. However, if the mercaptides were first decomposed with hydrogen sulphide and the filtered solutions de-salted and immediately dried in the frozen state, white powders were obtained, virtually uncontaminated by salts or trace metals and with high thiol titration values. This procedure may be of value in the isolation of other sensitive peptides.

[1] Laver and Trikojus, *Biochim. Biophys. Acta*, 1956, **20**, 444.
[2] Harington and Pitt Rivers, *Biochem. J.*, 1944, **38**, 417.
[3] McQuillan, unpublished experiments.
[4] Curtius, *Ber.*, 1902, **35**, 3226.
[5] Boissonas, *Helv. Chim. Acta*, 1951, **34**, 874.
[6] Bergmann and Zervas, *Ber.*, 1932, **65**, 1192.
[7] Wood and du Vigneaud, *J. Biol. Chem.*, 1939, **130**, 109.
[8] Sifferd and du Vigneaud, *ibid.*, 1935, **108**, 753.
[9] Ben Ishai and Berger, *J. Org. Chem.*, 1952, **17**, 1564.
[10] Benesch and Benesch, *Biochim. Biophys. Acta*, 1957, **23**, 643.
[11] Kendall, McKenzie, and Mason, *J. Biol. Chem.*, 1929, **84**, 657.
[12] Benesch and Benesch, *Biochim. Biophys. Acta*, 1957, **23**, 658.
[13] Consden, Gordon, and Martin, *Biochem. J.*, 1947, **41**, 590.
[14] Dent, " Recent Advances in Clinical Pathology," Churchill, London, 1951, 2nd edn., p. 252.

Enzyme–substrate studies with the above-mentioned and related peptides will be reported elsewhere.

Experimental

All chemicals were purified by the usual methods. Amino-acids were from Lights or B.D.H. Microanalyses were carried out by Dr. W. Zimmermann, C.S.I.R.O. Microanalytical Laboratory, University of Melbourne. Optical activity was measured in a tube 10 cm. × 3 mm., with a polarimeter of the Lippich type.

S-*Benzyl*-N-*benzyloxycarbonyl*-L-*cysteinyl*-L-*phenylalanine Methyl Ester*.—(a) *Mixed anhydride method*. To a cold solution of *S*-benzyl-*N*-benzyloxycarbonyl-L-cysteine [15] (4·13 g.) in chloroform (50 ml.) and triethylamine (1·58 ml.) was added freshly distilled ethyl chloroformate (1·135 ml.). The mixture was kept at 0° for 20 min., and a chilled solution of L-phenylalanine methyl ester (from 2·58 g. of the hydrochloride) in chloroform (50 ml.) was slowly added. After being kept at 0° for 30 min., overnight at room temperature, and finally at 50° for 3 hr., the solution was washed successively with dilute hydrochloric acid, saturated aqueous sodium hydrogen carbonate, and water, and dried (Na_2SO_4). Removal of solvents under reduced pressure resulted in an oil which produced white needles (from methanol) (60%), m. p. 103—105°. The *methyl ester*, recrystallised from methanol, had m. p. 105—105·5°, $[\alpha]_D^{24}$ −34·7° (*c* 1·5 in 95% ethanol) (Found: C, 66·7; H, 6·0; N, 5·5. $C_{28}H_{30}O_5N_2S$ requires C, 66·4; H, 6·0; N, 5·5%).

(b) *Azide method*. To an ethyl acetate solution of *S*-benzyl-*N*-benzyloxycarbonyl-L-cysteine azide, prepared from the hydrazide [2] (1·79 g.), was added a cold solution of L-phenylalanine methyl ester (from 1·08 g. of the hydrochloride) in chloroform (20 ml.). The mixture was kept at 3° for 2 days and the solvents were removed under reduced pressure, leaving an oil which produced needles (from methanol) (60%), m. p. 103—104°. The products from (*a*) and (*b*) proved identical by mixed melting points.

S-*Benzyl*-N-*benzyloxycarbonyl*-L-*cysteinyl*-L-*phenylalanine*.—The above methyl ester (2·2 g.) was saponified in methanol (200 ml.) with N-sodium hydroxide (14 ml.) for 24 hr. at 20°, after which the solution was adjusted to pH 6 with 5N-hydrochloric acid. On removal of most of the methanol and acidification to pH 3, the product was obtained as white needles (80%), m. p. 147—151°. The *peptide*, recrystallised twice from methanol, had m. p. 155·5°, $[\alpha]_D^{21}$ −16·5° (*c* 2 in ethanol) (Found: C, 65·9; H, 5·75; N, 5·7. $C_{27}H_{28}O_5N_2S$ requires C, 65·8; H, 5·7; N, 5·7%).

L-*Cysteinyl*-L-*phenylalanine*.—*S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-L-phenylalanine (2·0 g.) was dissolved in liquid ammonia, and small pieces of sodium were added until a permanent blue colour was obtained (0·7 g. being required). Ammonium chloride (1·8 g.) was added and the ammonia was removed. The residue was dissolved in 0·5N-sulphuric acid (35 ml.) and filtered. The mercaptide was precipitated, washed, and decomposed according to the techniques described by Harington and Pitt Rivers.[2] The aqueous peptide solution from the treatment with hydrogen sulphide was concentrated *in vacuo* to 20 ml., filtered, and electrolytically de-salted. Crystals began to grow in clusters immediately and were recrystallised three times from hot oxygen-free glass-distilled water and had m. p. >300° (65%), $[\alpha]_D^{24}$ +8·5° (*c* 2 in N-HCl) (lit.,[16] $[\alpha]_D^{25}$ −8·9°) (Found: C, 51·5; H, 6·2. Calc. for $C_{12}H_{16}O_3N_2S,\frac{1}{2}H_2O$: C, 52·0; H, 6·2%). Thiol titration [10] gave 98% of SH. The dipeptide was dried *in vacuo* at 120° to constant weight (Found: C, 53·6; H, 6·1. Calc. for $C_{12}H_{16}O_3N_2S$: C, 53·7; H, 6·0%).

After completion of this work, the synthesis of L-cysteinyl-L-phenylalanine by an alternative route, involving the catalytic hydrogenation of the corresponding cystinyl peptide, was reported.[16] The compound, however, gave the negative value for $[\alpha]_D$ cited above.

S-*Benzyl*-L-*cysteinyl*-L-*phenylalanine*.—*S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-L-phenylalanine (0·2 g.) and glacial acetic acid saturated with hydrogen bromide (2 ml.) were kept under anhydrous conditions for 30 min. at room temperature. Addition of dry ether deposited needles which were collected, washed with ether, and dissolved in water (4 ml.). Addition of ammonia (*d* 0·88) to pH 5 and concentration *in vacuo* to 2 ml. yielded an amorphous product

[15] Harington and Mead, *Biochem. J.*, 1936, **30**, 1598.
[16] Berse, Boucher, and Piche, *J. Org. Chem.*, 1957, **22**, 805.

(80%), m. p. 233—235° (decomp.). For analysis, S-*benzyl*-L-*cysteinyl*-L-*phenylalanine* was recrystallised twice from methanol to give platelets, m. p. 236° (decomp.), $[\alpha]_D^{27}$ +14·0° (*c* 1 in dimethylformamide) (Found: N, 7·5. $C_{19}H_{22}O_3N_2S$ requires N, 7·8%).

S-*Benzyl*-N-*benzyloxycarbonyl*-L-*cysteinyl*-L-*tyrosine Methyl Ester*.—*S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteine azide (from the hydrazide, 8·0 g.) and L-tyrosine methyl ester (4·3 g.) were condensed by the azide method. The resultant oil was triturated with successive small aliquot parts of ether, to give needles, m. p. 92—97°. The *methyl ester*, recrystallised twice from ethanol (charcoal) (60%), had m. p. 110—111° (lit.,[17] m. p. 89°), $[\alpha]_D^{24}$ −30·5° (*c* 2 in 95% ethanol) (Found: C, 64·35; H, 5·85; N, 5·2. $C_{28}H_{30}O_6N_2S$ requires C, 64·35; H, 5·8; N, 5·35%) (lit.,[17] C, 63·5%).

S-*Benzyl*-N-*benzyloxycarbonyl*-L-*cysteinyl*-L-*tyrosine Amide*.—(*a*) *S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-L-tyrosine methyl ester (4·5 g.) was dissolved in methanol which had previously been saturated with ammonia at 0° (50 ml.). After 2 days at room temperature under anhydrous conditions, the flask contained a solid mass of white needles (70%), m. p. 196—197° (decomp.).

(*b*) *S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteine hydrazide (5 g.) and L-tyrosine amide (2·55 g.) were condensed by the azide method, to give white needles (80%), m. p. 195—196° (decomp.). The *amide*, recrystallised from ethanol, had m. p. 196—196·5° (decomp.), $[\alpha]_D^{23}$ −36·0° (*c* 2 in dimethylformamide) (Found: C, 64·1; H, 5·9; N, 8·1. $C_{27}H_{29}O_5N_3S$ requires C, 63·9; H, 5·8; N, 8·3%).

(*c*) The protected dipeptide amide was also prepared by the mixed anhydride method, but the yield was only 20%. The products from all three methods were found to be identical by mixed melting points.

L-*Cysteinyl*-L-*tyrosine Amide*.—*S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-L-tyrosine amide (3·5 g.) was dissolved in liquid ammonia (300 ml.) and small pieces of sodium were added until a blue colour persisted for 30 sec. (0·95 g. being required). Ammonium chloride (2·6 g.) was added and the ammonia was removed. After dissolution of the residue in 0·5N-sulphuric acid (60 ml.) the mercaptide was precipitated with modified Hopkins's reagent [11] and decomposed with hydrogen sulphide.[2] The aqueous solution of L-*cysteinyl*-L-*tyrosine amide* was then electrolytically de-salted and freeze-dried (80%); it had m. p. 83—84° (decomp.), $[\alpha]_D^{24}$ +12·0° (*c* 2·5 in N-hydrochloric acid) (Found: C, 49·7; H, 6·1. $C_{12}H_{17}O_3N_3S$ requires C, 50·9; H, 6·05%). Thiol titration [10] gave 96% of SH. The peptide amide proved difficult to purify. White prisms obtained from methanol–ether contained only 35% of SH (by titration [10]). Attempted purifications by formation of the hydrochloride, picrate, and picrolonate were unsuccessful. When protected with *N*-ethylmaleimide,[18] the peptide behaved as a single substance on paper chromatograms in butan-1-ol–acetic acid–water (4 : 1 : 1).

S-*Benzyl*-L-*cysteinyl*-L-*tyrosine Amide*.—*S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-L-tyrosine amide (3·4 g.) and glacial acetic acid saturated with hydrogen bromide (35 ml.) were allowed to react at room temperature under anhydrous conditions for 45 min. On addition of ether (200 ml.) an oil was deposited which was washed with ether by decantation and dissolved in water (30 ml.). The product obtained on the addition of ammonia (*d* 0·88) to pH 8 had m. p. 94—96° (decomp.). The *amide* was purified by dissolving it in 0·2N-acetic acid and adjusting the pH to 8 {yield 80%, m. p. 98—98·5° (decomp.), $[\alpha]_D^{28}$ +6·0° (*c* 1 in dimethylformamide)} (Found: C, 61·1; H, 6·1. $C_{19}H_{23}O_3N_3S$ requires C, 61·1; H, 6·2%).

N-*Benzyloxycarbonyl*-L-*leucyl*-S-*benzyl*-L-*cysteinyl*-L-*tyrosine Amide*.—(a) *Mixed anhydride method*. A solution of *N*-benzyloxycarbonyl-L-leucine [19] (1·43 g.) in chloroform (25 ml.) and triethylamine (0·725 ml.) was cooled to 0°, freshly distilled ethyl chloroformate (0·525 ml.) was added dropwise with stirring, and the mixture was kept at 0° for 30 min. Then was added a cold solution of *S*-benzyl-L-cysteinyl-L-tyrosine amide (2·0 g.) in dimethylformamide (20 ml.). The whole was kept at 0° for 30 min., overnight at room temperature, and finally at 60° for 1 hr. After removal of the chloroform under reduced pressure, the crude product was precipitated with water. The *amide* recrystallised from ethanol as white needles (90%), m. p. 192—193°, $[\alpha]_D^{24}$ −21·0° (*c* 2 in dimethylformamide) (Found: C, 63·4; H, 6·5; N, 8·8. $C_{33}H_{40}O_6N_4S$ requires C, 63·8; H, 6·5; N, 9·0%).

(b) *Azide method*. An ethyl acetate extract of *N*-benzyloxycarbonyl-L-leucine azide was

[17] Boissonas, Guttman, Jaquenoud, and Waller, *Helv. Chim. Acta*, 1955, **38**, 1491.
[18] Hanes, Hird, and Isherwood, *Nature*, 1950, **166**, 288.
[19] Bergmann, Zervas, and Fruton, *J. Biol. Chem.*, 1936, **115**, 593.

prepared at 0° from the hydrazide [20] (0·5 g.), and a cold solution of *S*-benzyl-L-cysteinyl-L-tyrosine amide (0·7 g.) in dimethylformamide (5 ml.) was added. After 2 days at 3°, the product (70%), m. p. 192—193°, proved identical with that from (*a*) by mixed melting points.

L-*Leucyl*-L-*cysteinyl*-L-*tyrosine Amide*.—*N*-Benzyloxycarbonyl-L-leucyl-*S*-benzyl-L-cysteinyl-L-tyrosine amide (1·0 g.) was reduced in liquid ammonia with sodium (0·45 g.), and the product (65%) isolated as described for L-cysteinyl-L-tyrosine amide. The freeze-dried peptide, m. p. 171—174° (softens at 153°), $[\alpha]_D^{24}$ −1·8° (*c* 2 in N-hydrochloric acid), contained 95% of SH by titration.[10] Satisfactory analytical figures were not obtained. The difficulties of purification encountered with L-cysteinyl-L-tyrosine amide were also experienced with this peptide, which, however, behaved as a single entity, after protection with *N*-ethylmaleimide,[18] on paper chromatograms in butan-1-ol–acetic acid–water (4 : 1 : 1).

S-*Benzyl*-L-*cysteinyl*-L-*tyrosine*.—*S*-Benzyl-*N*-benzyloxycarbonyl-L-cysteinyl-L-tyrosine [2] (0·5 g.) was treated as described previously for *S*-benzyl-L-cysteinyl-L-phenylalanine. S-*Benzyl*-L-*cysteinyl*-L-*tyrosine*, recrystallised from water (80%), had m. p. 114—115°, $[\alpha]_D^{27}$ +26·5° (*c* 1 in dimethylformamide) (Found: N, 7·3. $C_{19}H_{22}O_4N_2S$ requires N, 7·5%).

N-*Benzyloxycarbonyl*-L-*leucyl*-S-*benzyl*-L-*cysteine Ethyl Ester*.—*N*-Benzyloxycarbonyl-L-leucine [19] (1·5 g.) and *S*-benzyl-L-cysteine [2] (1·58 g.) were coupled by the mixed anhydride procedure, and the *ethyl ester* was recrystallised from ethanol (75%); it had m. p. 103·5—104°, $[\alpha]_D^{23}$ −66·5° (*c* 2 in 95% ethanol) (Found: C, 64·2; H, 7·05; N, 5·5. $C_{26}H_{34}O_5N_2S$ requires C, 64·2; H, 7·0; N, 5·75%).

N-*Benzyloxycarbonyl*-L-*leucyl*-S-*benzyl*-L-*cysteine*.—The ethyl ester was saponified as previously described; the *peptide*, recrystallised from aqueous ethanol (80%), had m. p. 157—158°, $[\alpha]_D^{22}$ −41·5° (*c* 2 in 95% ethanol) (Found: N, 5·9. $C_{24}H_{30}O_5N_2S$ requires N, 6·1%).

L-*Leucyl*-L-*cysteine*.—*N*-Benzyloxycarbonyl-L-leucyl-*S*-benzyl-L-cysteine (2·0 g.) was reduced in liquid ammonia with sodium (0·6 g.), and the product isolated as previously described for L-cysteinyl-L-phenylalanine. (Twice the expected volume, 14 ml., of the modified Hopkins' reagent [11] was required to give complete precipitation of the mercaptide.) The resultant freeze-dried powder was dissolved in oxygen-free glass-distilled water and placed over a dish of ethanol in a partially evacuated desiccator, to give spherical crystalline aggregates of L-*leucyl*-L-*cysteine* (45%), m. p. >250° (softens at 153°, darkens at 185°), $[\alpha]_D^{26}$ +19·5° (*c* 2 in N-hydrochloric acid) (Found: C, 44·5; H, 8·0. $C_9H_{18}O_3N_2S,\frac{1}{2}H_2O$ requires C, 44·4; H, 7·9%). The peptide was dried at 120° *in vacuo* to constant weight (Found: C, 46·5; H, 7·7. $C_9H_{18}O_3N_2S$ requires C, 46·1; H, 7·7%). Thiol titration [10] gave 95% of SH.

L-*Leucyl*-S-*benzyl*-L-*cysteinyl*-L-*tyrosine Amide*.—*N*-Benzyloxycarbonyl-L-leucyl-*S*-benzyl-L-cysteinyl-L-tyrosine amide (1·8 g.) was treated as described for *S*-benzyl-L-cysteinyl-L-tyrosine amide to give L-*leucyl*-S-*benzyl*-L-*cysteinyl*-L-*tyrosine amide* (85%), m. p. 192—193° (decomp.), $[\alpha]_D^{20}$ −40·5° (*c* 2 in dimethylformamide) (Found: N, 11·2. $C_{25}H_{34}O_4N_4S$ requires N, 11·5%).

N-*Benzyloxycarbonylglycyl*-L-*leucyl*-S-*benzyl*-L-*cysteinyl*-L-*tyrosine Amide*.—(a) *Mixed anhydride method.* A solution of *N*-benzyloxycarbonylglycine [6] (0·23 g.) in chloroform (15 ml.) and triethylamine (0·14 ml.) was cooled to 0°, and freshly distilled ethyl chloroformate (0·10 ml.) was added dropwise. The mixed anhydride solution was kept at 0° for 20 min. and a cold solution of L-leucyl-*S*-benzyl-L-cysteinyl-L-tyrosine amide (0·5 g.) in dimethylformamide (5 ml.) was added. The mixture was kept at 0° for 30 min., then overnight at room temperature, and finally at 60° for 1 hr. The chloroform was removed under reduced pressure and the product was precipitated with water. Recrystallisation from acetone gave m. p. 181—183° (70%). The *amide* was recrystallised twice from ethanol to give needles, m. p. 187—188°, $[\alpha]_D^{21}$ −57·0° (*c* 2 in dimethylformamide) (Found: N, 10·4; S, 4·5. $C_{35}H_{43}O_7N_5S$ requires N, 10·35; S, 4·7%).

(b) *Azide method.* An ethyl acetate extract of *N*-benzyloxycarbonylglycyl-L-leucine azide (from 1·0 g. of the hydrazide [21]) was condensed at 0° with *S*-benzyl-L-cysteinyl-L-tyrosine amide (1·1 g.) in dimethylformamide (10 ml.). After 2 days at 3°, the product (90%) proved identical with that obtained from the mixed anhydride procedure.

Glycyl-L-*leucyl*-L-*cysteinyl*-L-*tyrosine Amide*.—*N*-Benzyloxycarbonylglycyl-L-leucyl-*S*-benzyl-L-cysteinyl-L-tyrosine amide (0·45 g.) was reduced in liquid ammonia with sodium (0·18 g.), and the product was isolated as described for L-cysteinyl-L-tyrosine amide, to give glycyl-L-leucyl-L-cysteinyl-L-tyrosine amide (60%), m. p. 179—181° (softens at 168°, decomp.), $[\alpha]_D^{20}$ −39·5°

[20] Bergmann, Zervas, Fruton, Schneider, and Schleich, *J. Biol. Chem.*, 1935, **109**, 325.
[21] Harris and Fruton, *ibid.*, 1951, **188**, 251.

(*c* 2 in N-hydrochloric acid). Thiol titration [10] gave 94% of SH. Satisfactory analytical figures were not obtained. Similar difficulties of purification to those with L-cysteinyl-L-tyrosine amide were experienced. The peptide behaved as a single substance, after protection with *N*-ethylmaleimide,[18] on paper chromatograms in butan-1-ol–acetic acid–water (4 : 1 : 1). The peptide must be stored under anhydrous conditions to prevent deterioration.

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DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF MELBOURNE,
CARLTON, N.3, VICTORIA, AUSTRALIA.

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