

was crystallized from ethyl acetate-ether in 97% yield to afford the dipeptide free acid XXIX, mp 144–145°,  $[\alpha]_D^{25} -29.5^\circ$  (*c* 2.05, methanol).

*Anal.* Calcd for  $C_{20}H_{28}N_2O_7$ : C, 58.88; H, 6.86; N, 6.86. Found: C, 58.75; H, 6.78; N, 6.99.

**N-Carbobenzoxy- $\alpha$ -*t*-butyl-L-glutamyl-L-alanine Pentachlorophenyl Ester (XXX).** The dipeptide free acid XXIX (4.08 g, 0.01 mole) was converted into the pentachlorophenyl active ester as described for the preparation of the  $\alpha$ -dipeptide pentachlorophenyl ester III. Dipeptide active ester XXX was isolated in 50% yield after crystallization from methanol, mp 132–138°. Two recrystallizations from ethyl acetate-ether-petroleum ether raised the melting point to 141–142°,  $[\alpha]_D^{25} +3.86^\circ$  (*c* 0.88, chloroform).

*Anal.* Calcd for  $C_{26}H_{27}N_2O_7Cl_5$ : C, 47.50; H, 4.12; N, 4.27. Found: C, 47.68; H, 4.37; N, 4.24.

**N-Carbobenzoxy- $\alpha$ -*t*-butyl-L-glutamyl-L-alanyl- $\gamma$ -*t*-butyl-L-glutamic Acid Methyl Ester (XXXI).** Dipeptide active ester XXX (1.5 g, 0.0028 mole) was coupled with glutamic acid diester hydrochloride IV and the reaction mixture was worked up as described for the  $\alpha$ -tripeptide methyl ester V, yielding 72% of the  $\gamma$ -tripeptide methyl ester XXXI, after crystallization from ether-petroleum ether, mp 69–72°. Two recrystallizations from the same solvents raised the melting point to 72–73°,  $[\alpha]_D^{25} -6.8^\circ$  (*c* 2, chloroform).

*Anal.* Calcd for  $C_{30}H_{43}N_3O_{10}$ : C, 59.2; H, 7.3; N, 6.9. Found: C, 59.04; H, 7.46; N, 7.16.

**N-Carbobenzoxy- $\alpha$ -*t*-butyl-L-glutamyl-L-alanyl- $\gamma$ -*t*-butyl-L-glutamic Acid (XXXII).** Tripeptide methyl ester XXXI (0.607 g, 0.001 mole) was saponified as described previously and the saponified product, a white solid, was crystallized from ether-petroleum ether to yield 83% of the tripeptide free acid XXXII, mp 118–119°,  $[\alpha]_D^{25} +10^\circ$  (*c* 0.5, chloroform).

*Anal.* Calcd for  $C_{29}H_{43}N_3O_{10}$ : C, 58.71; H, 7.25; N, 7.09. Found: C, 58.60; H, 7.39; N, 7.35.

**Transpeptidation Studies.** N-Carbobenzoxy- $\gamma$ -*t*-butyl-L-glutamyl-L-alanyl- $\gamma$ -*t*-butyl-L-glutamic acid (VI, 0.030 g) was dissolved in 90% trifluoroacetic acid (1 ml) and left at room temperature for 50 min. The trifluoroacetic acid was removed under reduced pressure and the residue chromatographed on Whatman

No. 3MM paper, in different solvent systems, at concentrations ranging from 30 to 4000  $\mu$ g; in this case less than 1% of a tripeptide tricarboxylic acid could be detected when the chromatograms were sprayed with bromphenol blue. The solvent systems used were *n*-butyl alcohol saturated with water (descending),  $R_f$  0.85; *n*-butyl alcohol-water-acetic acid (4:1:1) (ascending),  $R_f$  0.89; *n*-butyl alcohol saturated with water and phenol-water (77:23) (two-dimensional ascending),  $R_f$  0.89 and 0.72; pyridine-water (4:1) (ascending),  $R_f$  0.79; phenyl-citric acid-disodium phosphate buffer, pH 7.6 (100:20) (ascending),  $R_f$  0.79. Similarly N-carbobenzoxy- $\alpha$ -*t*-butyl- $\gamma$ -L-glutamyl-L-alanyl- $\gamma$ -*t*-butyl-L-glutamic acid (XXXII) (0.030 g) was treated with 90% trifluoroacetic acid (1 ml) at room temperature for 50 min. After the removal of trifluoroacetic acid under reduced pressure, the residue was chromatographed on the same paper and in the solvent systems used for the  $\alpha$ -tripeptide, as described above. In most of the solvent systems, though a single spot was observed, the  $R_f$  values of the  $\gamma$ -tripeptide tricarboxylic acid were identical with those of the  $\alpha$ -tripeptide. The most satisfactory solvent systems were pyridine-water (4:1) and phenol-citric acid-disodium phosphate buffer, pH 7.6 (100:20). The  $R_f$  values with these solvent systems for the  $\gamma$ -tripeptide tricarboxylic acid were 0.68 and 0.62, respectively. In another experiment 3 mg each of  $\alpha$ - and  $\gamma$ -tripeptides VI and XXXII were treated separately with trifluoroacetic acid (0.5 ml) for 50 min at room temperature. After the removal of trifluoroacetic acid, the residues were chromatographed separately and as a mixture. The conditions of chromatography were identical with those described above; the  $R_f$  values of  $\alpha$ - and  $\gamma$ -tripeptide tricarboxylic acids were identical with those described above and they separated very well into two spots. Since only one spot was observed at concentrations ranging from 30 to 4000  $\mu$ g for both  $\alpha$ - and  $\gamma$ -tripeptide tricarboxylic acids, it was concluded that they were 99% pure  $\alpha$ - and  $\gamma$ -peptides.

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## A New Synthesis of Oxytocin Using S-Acyl Cysteines as Intermediates<sup>1,2</sup>

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**Abstract:** The fully S,N-protected derivatives (VI–IX) of the nonapeptide amide, L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutamyl-L-asparaginyl-L-cysteinyl-L-prolyl-L-leucylglycinamide, have been prepared by a step-by-step synthesis, using new N-protecting groups (the *t*-butyloxycarbonyl and the *o*-nitrophenylsulfenyl groups) in addition to the classical carbobenzoxy and new S-protecting groups (the S-benzoyl and S-carbobenzoxy groups). The selective removal of the S-protecting groups from compounds VI–IX by methanolysis afforded the N-protected oxytocines (X–XII). Oxidation of the free thiol groups of X and XII by 1,2-diiodoethane led to the formation of N-carbobenzoxy- (XIII), and N-*t*-butyloxycarbonyl-oxytocin, (XIV), respectively. The peptide obtained after decarboxylation of the N-carbobenzoxy-oxytocin (XIII) was purified by countercurrent distribution and partition chromatography on Sephadex. The isolated material exhibited the chemical, physical, and biological activities of oxytocin.

Oxytocin was isolated in highly purified form by du Vigneaud, *et al.*,<sup>3</sup> and its structure was postulated<sup>4</sup> and proved by synthesis.<sup>5</sup> Since the first,

classical synthesis of du Vigneaud, several other oxytocin syntheses were reported<sup>6–11</sup> from various labora-

(1) This investigation was supported by the Royal Hellenic Research Foundation, to which the author is greatly indebted.

(2) A preliminary communication of part of this work has already appeared: I. Photaki, *Experientia*, **20**, 487 (1964).

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(5) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis, and S. Gordon, *J. Am. Chem. Soc.*, **75**, 4879 (1953); V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, and P. G. Katsoyannis, *ibid.*, **76**, 3115 (1954).

(6) R. A. Boissonnas, S. Guttman, P.-A. Jaquenoud, and J.-P. Waller, *Helv. Chim. Acta*, **38**, 1491 (1955).

tories including du Vigneaud's. The synthesis of oxytocin opened the way to the synthesis of a variety of "analogs" of the hormone, which were prepared in an attempt to establish the relationship between the chemical structure and the biological activity.

The characteristic features of du Vigneaud's synthesis of oxytocin are the following: (1) the preparation of an open-chain S,N-protected peptide, *i.e.*, an N-protected nonapeptide amide, bearing two S-protected cysteine residues at positions 1 and 6 of the molecule; (2) the use of the benzyl group exclusively for the S protection of the cysteine residues; (3) the use of the carbobenzoxy or the tosyl group for the N protection of the intermediate peptides and of the final open-chain S,N-protected nonapeptide; (4) the simultaneous removal of both the S-benzyl and the N-protecting groups from the S,N-protected nonapeptide by reduction with sodium in liquid ammonia; (5) the oxidation of the resulting "oxytoceine" (disulfhydryl nonapeptide), without its isolation, to oxytocin.

An inspection of the structure of oxytocin leads to the conclusion that for the moment it is not possible to use any other peptide precursor than the above described under 1. The alternative route of synthesis would be the preparation of unsymmetrical, open-chain cystine octapeptides (with the cystine bearing one amino group not combined in a peptide bond), and their subsequent cyclization to oxytocin by peptide bond formation. This route is not feasible, since unsymmetrical open-chain cystine derivatives are not stable but rearrange rapidly to the symmetrical ones.<sup>12,13</sup> Therefore, attempts to change the method of synthesis must be confined to points 2-5, *i.e.*, to the scheme of preparation of the S,N-protected nonapeptide and its conversion to oxytocin. In fact, until now almost all of the syntheses of oxytocin<sup>6-10</sup> and its analogs are similar in points 2-5, the only exception being a synthesis<sup>11</sup> in which the trityl group has been used, both as S- and N-protecting group, for the preparation of the nonapeptide. In this case hydrochloric acid was used for the removal of all the trityl groups in the final step, but no isolation of oxytocin and no quantitative results on the biological activity of the obtained solution were described.

In the present paper, a new synthesis of oxytocin is described which differs from du Vigneaud's scheme in the following points: (a) the use of new N-protecting groups in addition to the classical carbobenzoxy group; (b) the use of new S-protecting groups; (c) the selective removal of the S-protecting groups prior to the removal of the N-protecting group, thus allowing for the first time the isolation of N-protected oxytoceines; and (d) the oxidation of the resulting sulfhydryl groups in positions 1 and 6 of oxytoceine and the subsequent

splitting off of the N-protecting group without use of sodium in liquid ammonia.

For the synthesis of the new S,N-protected nonapeptides, in addition to the carbobenzoxy group of Bergmann-Zervas,<sup>14</sup> other N-protecting groups have been used, *e.g.*, the *o*-nitrophenylsulfenyl (NPS) group of Zervas, *et al.*,<sup>15</sup> which can be removed with 2-3 equiv of hydrogen chloride or the *t*-butoxycarbonyl group<sup>16</sup> which can be split off by means of trifluoroacetic acid or by hydrogen chloride.

In recent years, adequately N-protected S-trityl-, S-diphenylmethyl-, and S-acyl-L-cysteines have been widely used by Zervas, *et al.*,<sup>17-19</sup> as intermediates for the incorporation of cysteine residues into peptide chains including a fragment of insulin containing the intrachain disulfide bridge. These groups could be selectively removed under mild conditions without affecting sensitive parts of the molecule or any already existing S-S bridge. The S-trityl group was shown to be removable by the action of mercury chloride or silver nitrate,<sup>17</sup> and the benzhydryl<sup>17,20</sup> group by means of trifluoroacetic acid.<sup>17,21,21a</sup> The S-acyl groups were removed under the action of alkaline reagents and particularly by methanolysis in the presence of sodium methoxide.<sup>18</sup> From the S-protecting groups mentioned above we have used acyl<sup>22</sup> groups and in particular S-benzoyl<sup>18,19</sup> and S-carbobenzoxy-L-cysteines.<sup>18,23</sup>

The N- and the N,S-protected amino acids were incorporated into the peptide chain step-by-step beginning from its C-terminal amino acid to avoid racemization. The known tripeptide L-prolyl-L-leucylglycinamide<sup>24</sup>

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(21a) NOTE ADDED IN PROOF: The S-4,4'-dimethoxydiphenylmethyl group is also split off by trifluoroacetic acid and has been used for a synthesis of oxytocin: *cf.* R. W. Hanson and H. D. Law, *J. Chem. Soc.*, 7285 (1965).

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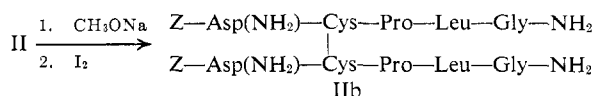
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was employed as starting material. Coupling of the tripeptide with *p*-nitrophenyl carbobenzoxy-S-benzoyl-L-cysteinate<sup>19</sup> led to the formation of crystalline N-carbobenzoxy-S-benzoyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (I) in good yield. The carbobenzoxy group was removed from the tetrapeptide by means of hydrogen bromide in acetic acid. Attempts to couple the peptide S-benzoyl-L-cysteinyl-L-prolyl-L-leucylglycinamide thus obtained with N-carbobenzoxy-L-asparagine by a variety of conventional methods, *e.g.*, the mixed anhydride with isobutyl<sup>25</sup> or ethylcarbonic<sup>26</sup> acid or the diphenylphosphoric acid,<sup>27</sup> the dicyclohexylcarbodiimide,<sup>28</sup> the *p*-nitrophenyl ester,<sup>9,29</sup> or using Woodward's reagent,<sup>30</sup> led to extremely poor yields of the pentapeptide II, which was obtained in crystalline form and good yield only by incorporating in the peptide chain the carbobenzoxy-L-asparagine *via* its mixed anhydride with pivalic acid.<sup>31</sup> Using *o*-nitrophenylsulfenyl-L-asparagine<sup>15b</sup> instead of carbobenzoxy-L-asparagine, the corresponding NPS pentapeptide IIa was also obtained.

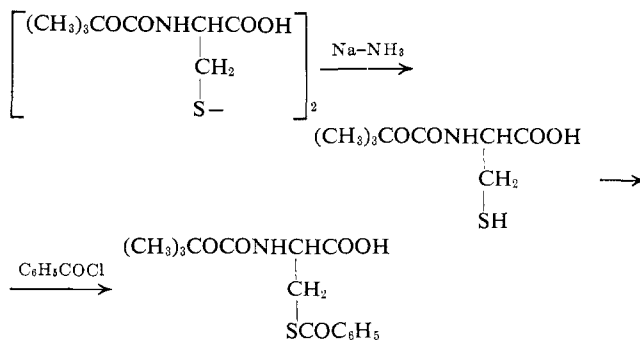
The possibility of an S → N migration in the presence of a base should be taken into account when using the S-acyl cysteine derivatives for peptide synthesis. Therefore, the course of this synthesis was checked by a simple method described in a recent publication of this laboratory.<sup>19</sup> The pentapeptide II was subjected to methanolysis followed by iodine titration of the resulting thiolpeptide to give the cystine peptide IIb. The hydrolysate of an analytically pure sample of compound IIb was found to contain asparagine, cystine, proline, leucine, and glycine. Should the pentapeptide II be an S-peptide, *i.e.*, N-benzoyl-S-(carbobenzoxy-L-asparaginyl)-L-cysteinyl-L-prolyl-L-leucylglycinamide, the hydrolysate of the methanolysis product would not contain asparagine.



Decarbobenzoylation of protected pentapeptide II and incorporation of the sixth amino acid afforded N-carbobenzoxy-L-glutamyl-L-asparaginyl-S-benzoyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (III) in good yield. The same protected hexapeptide III was obtained by removal of the NPS group from compound IIa and coupling of the resulting pentapeptide hydrochloride with *p*-nitrophenyl carbobenzoxy-L-glutamate.<sup>9b</sup>

The peptide chain was lengthened from six to eight amino acid residues by successive reactions with the *p*-nitrophenyl esters of carbobenzoxy-L-isoleucine<sup>9b</sup> and N-carbobenzoxy-O-benzyl-L-tyrosine.<sup>9b</sup> From the protected octapeptide V the N-carbobenzoxy and O-benzyl groups were removed by means of hydrogen bromide in acetic acid. Coupling of the appropriate S-benzoyl or S-carbobenzoxy cysteine derivatives, as shown in Scheme I with the octapeptide, afforded the

N,S-protected nonapeptides VI-IX. The N-*t*-butoxycarbonyl-S-benzoyl-L-cysteine used in this synthesis was prepared by reduction of bis-*t*-butoxycarbonyl-L-cystine with sodium in liquid ammonia followed by S-benzoylation in sodium bicarbonate solution.



The next step consisted in the methanolysis of the N,S-protected nonapeptides VI-IX in the presence of sodium methoxide.<sup>18</sup> The dithiol compounds X-XIII thus formed may be called N-protected oxytoceines according to the nomenclature of du Vigneaud. S-Benzoylation of compound X afforded du Vigneaud's N-carbobenzoxy-S,S'-dibenzyl-oxytoceine.<sup>5</sup>

In this paper the conversion to oxytocin of only one of these three N-protected oxytoceines, *i.e.*, carbobenzoxy-oxytoceine is described. As was expected the N-protected oxytoceines were stable in air. du Vigneaud, *et al.*, have found that the oxidation of the reduced form of deamino-oxytocin could not be completed even after aeration for 5 days.<sup>32</sup> It has also been our experience that various N-acyl or N-aminoacyl peptides such as carbobenzoxy-L-cysteinylglycine ethyl ester<sup>17</sup> and carbobenzoxy-L-phenylalanyl-L-cysteinylglycine ethyl ester<sup>17</sup> did not change after storage for several months at room temperature. Therefore the oxidation of N-carbobenzoxy-oxytoceine was accomplished by Weygand's method<sup>33</sup> using 1,2-diiodoethane as the oxidizing agent, and N-carbobenzoxy-oxytocin was isolated in microcrystalline form. The carbobenzoxy group was removed by hydrogen bromide in acetic acid and the resulting hydrobromide was dissolved together with a few drops of 2 *N* ammonia in 50 ml of the lower phase of the solvent system 1-butanol-1-propanol-0.5% acetic acid containing 0.1% pyridine (6:1:8).<sup>34</sup> This solution was subjected to countercurrent distribution<sup>35</sup> in the same solvent system. The oxytocin so obtained, gave the expected amino acid and elementary analyses, and possessed a specific rotation which agreed with that quoted in the literature. No differences were detected in the infrared patterns of the two synthetic products.<sup>5</sup> Furthermore our synthetic oxytocin has been shown to be identical with a highly purified sample of du Vigneaud's oxytocin<sup>36</sup> by the criteria of thin layer chromatography and electrophoresis at three different

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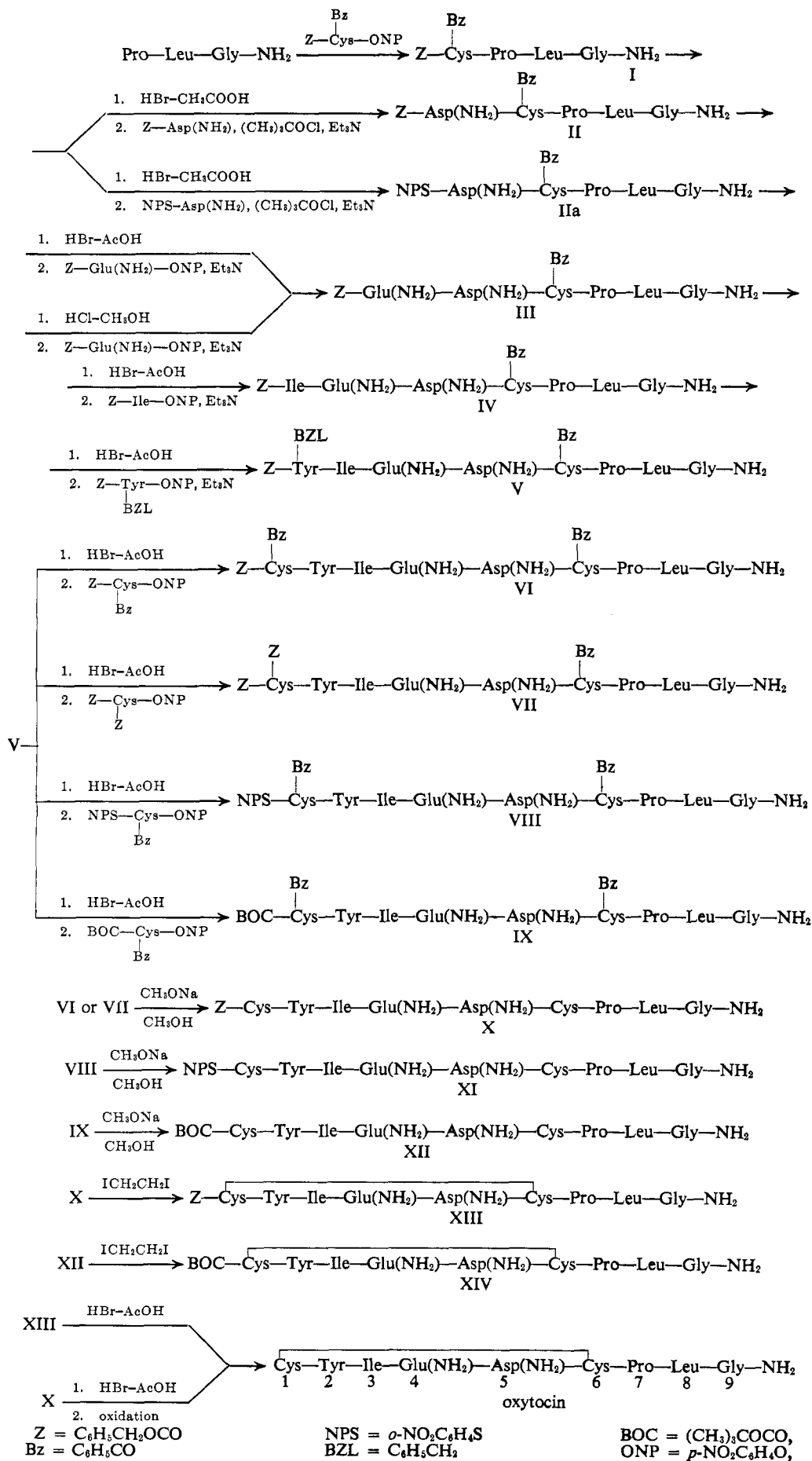
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Scheme I



hydrogen ion concentrations. Oxytocin prepared as described above possesses an avian depressor activity<sup>37</sup> of approximately 380 IU/mg as measured by Dr. W. Chan at the Department of Biochemistry, Cornell University.<sup>36</sup> This value may be increased by *ca.* 15% if the water and acetic acid content of the lyophilized product are taken into account. The same sample of oxytocin after storage for some months at  $-10^{\circ}$  was further purified by partition chromatography on Sephadex according to the procedure of Yamashiro.<sup>38</sup> The material so obtained exhibited an avian depressor activity of *ca.* 380 IU/mg and an oxytocic activity<sup>39</sup> of *ca.* 400 IU/mg as measured by Dr. I. Krejci at the Research Institute for Natural Drugs, Prague.<sup>40</sup>

Oxytocin has also been obtained from N-carbobenzoxymethionine by another route. The N-carbobenzoxymethionine was decarbobenzoxylated by hydrogen bromide in acetic acid. The resulting amorphous hydrobromide has been dissolved in water and after adjustment of the pH to *ca.* 6.8 it was oxidized by aeration. The oxidized solution was concentrated to a volume of approximately 50 ml and subjected to countercurrent distribution as described above. Oxytocin obtained by this route showed the same chemical, physical, and biological properties compared with the product described above.

## Experimental Section

For the coupling reactions anhydrous reactants and dry solvents were used; the ether was dry and free of peroxides. Evaporations were carried out *in vacuo* at  $35-40^{\circ}$ . Capillary melting points were determined for all compounds and are not corrected. Prior to analysis the compounds were dried between  $20$  and  $80^{\circ}$  under high vacuum over phosphorus pentoxide.

Thin layer chromatography was performed on silica gel G (Merck)<sup>41</sup> using the following solvent systems: (a) 1-butanol-acetic acid-water (100:10:30),<sup>42</sup> (b) 1-butanol-acetic acid-water-pyridine (30:6:24:20),<sup>43</sup> (c) 1-propanol-ammonia 33% (67:33),<sup>41</sup> and (d) 1-propanol-water (64:36).<sup>41</sup> Identification of the amino acids contained in the intermediate protected peptides was performed by means of "two-dimensional" thin layer chromatography of their hydrolysates in the solvent systems c and d. These chromatograms were compared to that of an equivalent mixture of the expected amino acids.

**N-Carbobenzoxymethionine-S-benzoyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (I).** To a cold solution of 3.2 g (0.011 mole) of L-prolyl-L-leucylglycinamide in 8 ml of dimethylformamide 5.5 g of *p*-nitrophenyl N-carbobenzoxymethionine-S-benzoyl-L-cysteinate was added. The solution was allowed to stand at room temperature for 4 days. Ethyl acetate was added and the solution was washed with cold 1 *N* hydrochloric acid and with water until the water extracts were neutral to congo red paper. The ethyl acetate layer was dried over sodium sulfate and evaporated to dryness. The residue was dissolved in 6 ml of dimethylformamide and crystalline compound I separated out upon addition of 400 ml of ether. The yield was 5.8 g (82%), mp  $160-161^{\circ}$ , unchanged after recrystallization from 70% methanol,  $[\alpha]_D^{25} -73.2^{\circ}$  (*c* 2.5, dimethylformamide).

*Anal.* Calcd for  $C_{31}H_{49}N_5O_7S$ : C, 59.50; H, 6.28; N, 11.19; S, 5.12. Found: C, 59.73; H, 6.23; N, 11.12; S, 5.20.

**N-Carbobenzoxymethionine-L-asparaginyl-S-benzoyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (II).** A solution of the mixed anhydride of

carbobenzoxymethionine-L-asparagine (2.65 g, 0.01 mole) and pivalic acid in 30 ml of chloroform was prepared as described in the literature.<sup>31</sup> After allowing the anhydride solution to stand for 3 min at  $5-10^{\circ}$ , it was added with stirring at  $-10^{\circ}$  to a chloroform (30 ml) suspension of S-benzoyl-L-cysteinyl-L-prolyl-L-leucylglycinamide hydrobromide prepared by decarbobenzoxylation<sup>44</sup> of 0.01 mole of compound I. Then triethylamine (0.01 mole plus an amount necessary to neutralize the excess<sup>44b</sup> of hydrogen bromide) was added dropwise and the stirring was continued at room temperature until the solution was transformed to a gelatinous mass. After allowing the mixture to stand overnight the solvent was removed *in vacuo*. The residue was triturated twice in a mortar with cold 1 *N* hydrochloric acid, then with cold water, and twice with a cold 5% solution of sodium hydrogen carbonate and water, alternately. The residue was dried in a desiccator over phosphorus pentoxide and was recrystallized by dissolving it in 200-250 ml of hot methanol, followed by the addition of an equal volume of water. After allowing the solution to cool at room temperature and seeding, the product separated out in prisms. The yield was 4.5 g (61%), mp  $214-215^{\circ}$ ,  $[\alpha]_D^{19} -71.5^{\circ}$  (*c* 2.5, dimethylformamide).

*Anal.* Calcd for  $C_{35}H_{46}N_7O_9S$ : C, 56.82; H, 6.13; N, 13.25; S, 4.33. Found: C, 56.97; H, 6.33; N, 13.09; S, 4.19.

**N-*o*-Nitrophenylsulfenyl-L-asparaginyl-S-benzoyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (IIa)** was prepared by coupling N-*o*-nitrophenylsulfenyl-L-asparagine with S-benzoyl-L-cysteinyl-L-prolyl-L-leucylglycinamide in the same manner as described for compound II. After being allowed to stand for 18 hr at room temperature the mixture was triturated with ethyl acetate. The precipitate was filtered off and washed on the filter with water and with a solution of potassium hydrogen carbonate. The crude product IIa was dissolved in dimethylformamide and precipitated with ether. The yield was 55%, mp  $208-210^{\circ}$ ,  $[\alpha]_D^{19} -126^{\circ}$  (*c* 1, dimethylformamide).

*Anal.* Calcd for  $C_{38}H_{42}N_8O_9S_2$ : N, 14.76; S, 8.45. Found: N, 14.95; S, 8.29.

**N,N'-Bis(carbobenzoxymethionine-L-asparaginyl)-L-cystinylbis(L-prolyl-L-leucylglycinamide) (IIb).** To a suspension of 0.185 g of protected pentapeptide II in 2.5 ml of absolute methanol, 0.51 ml of methanolic 0.5 *N* sodium methoxide was added in an atmosphere of hydrogen with stirring at room temperature.<sup>18</sup> The substance dissolved completely during the first few minutes of stirring which was maintained for 20 min. On acidification with 0.4 ml of acetic acid and titration with 0.1 *N* iodine,<sup>18</sup> 2.45 ml of iodine solution (98% of the theoretical amount) was consumed. The mixture was concentrated *in vacuo* until most of the methanol was removed; it was diluted with water and extracted with chloroform. Precipitation of the cystine peptide was achieved upon neutralization of the aqueous layer with a saturated solution of sodium hydrogen carbonate. The yield was 0.098 g (61%), mp  $191-192^{\circ}$  dec (after softening at  $164^{\circ}$ ), unchanged after recrystallization from 2 ml of ethanol,  $[\alpha]_D^{19} -129^{\circ}$  (*c* 1, dimethylformamide). Thin layer chromatography of an hydrolysate showed the presence of all the expected amino acids, *i.e.*, aspartic acid, cystine, proline, leucine, and glycine.

*Anal.* Calcd for  $C_{56}H_{80}N_{14}O_{16}S_2$ : C, 52.97; H, 6.35; N, 15.44; S, 5.05. Found: C, 52.76; H, 6.62; N, 15.30; S, 4.86.

**N-Carbobenzoxymethionine-L-glutaminyl-L-asparaginyl-S-benzoyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (III).** A solution of pentapeptide hydrobromide prepared by decarbobenzoxylation<sup>44b</sup> of 5.18 g (0.007 mole) of compound II in 10 ml of dimethylformamide was cooled to  $0^{\circ}$  and 2.96 g of *p*-nitrophenyl N-carbobenzoxymethionine-

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(38) D. Yamashiro, *Nature*, **201**, 76 (1964).

(39) Oxytocic assays were performed on isolated rat uterus suspended in Munsick's solution: R. H. Munsick, *Endocrinology*, **66**, 451 (1960).

(40) Appreciation is expressed to Dr. I. Krejci (Research Institute for Natural Drugs, Prague) for the measurement of both the avian depressor and the oxytocic activities of our synthetic oxytocin.

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(44) (a) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952). (b) Decarbobenzoxylation of the N-protected peptides was performed throughout this work as follows. To the solution of 0.01 mole of carbobenzoxymethionine peptide in anhydrous acetic acid, sufficient hydrogen bromide in acetic acid was added so that a final volume of 35 ml, and a concentration of 2.5 *N* hydrogen bromide in acetic acid were obtained. After standing for 30-35 min at room temperature the mixture was poured with stirring into five times its own volume of cold ether. The hydrobromide which separated out was allowed to settle and was washed several times with ether, each washing being followed by decantation. The hygroscopic residue was then filtered off and washed with ether; after drying *in vacuo* over potassium hydroxide and phosphorus pentoxide, it was quickly weighed. The homogeneity of the peptide hydrobromides was tested by thin layer chromatography in the solvent systems a and b. These hydrobromides are soluble in water and they usually retain more than 1 equiv of hydrogen bromide showing acidic reaction on congo red paper. The excess of hydrogen bromide was calculated on the basis of the weight of the peptide hydrobromide assuming a quantitative yield in the decarbobenzoxylation step.

glutamate was added. This was followed by the addition of triethylamine (0.007 mole plus an amount necessary to neutralize the excess<sup>44b</sup> of hydrogen bromide). After allowing the mixture to stand overnight at room temperature, 100 ml of ethyl acetate was added. The solid was filtered off, washed with ethyl acetate, and dried *in vacuo*. The product thus obtained was ground in a mortar, triturated with cold water, filtered off, and washed with cold water. This procedure was repeated using alcohol and then ether. For purification the crude product was dissolved in 60 ml of hot dimethylformamide, filtered, and precipitated upon addition of the triple quantity of ether. The yield was 5.2 g (85%), mp 214–216°,  $[\alpha]_D^{25} - 66.2^\circ$  (c 2.5, dimethylformamide).

*Anal.* Calcd for  $C_{40}H_{58}N_9O_{11}S$ : C, 55.35; H, 6.15; N, 14.52; S, 3.69. Found: C, 55.22; H, 6.28; N, 14.24; S, 3.54.

**B.** To a suspension of *o*-nitrophenylsulfenylpentapeptide IIa (0.415 g) in 10 ml of absolute methanol, 1 ml of 2 *N* methanolic hydrogen chloride was added with stirring. The solid dissolved within 15 min and the solution was immediately added to ether. The ether was decanted and the residue was dissolved in methanol and reprecipitated with ether. After filtration 0.280 g (80%) of L-asparaginyl-S-benzoyl-L-cysteinyl-L-prolyl-L-leucylglycinamide hydrochloride was obtained, mp 138–145°,  $R_f$  0.38 (solvent system a). This hydrochloride was coupled with *p*-nitrophenyl N-carbobenzoxy-L-glutamate as described under A for the corresponding hydrobromide. The yield of compound III was 85%, mp 214°,  $[\alpha]_D^{18} - 67.0^\circ$  (c 2, dimethylformamide).

**N-Carbobenzoxy-L-isoleucyl-L-glutamyl-L-asparaginyl-S-benzoyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (IV).** Protected hexapeptide III (5.2 g, 0.006 mole) was decarbobenzoxylated<sup>44b</sup> and the resulting hydrobromide was dissolved in 14 ml of dimethylformamide and cooled to 0°. *p*-Nitrophenyl N-carbobenzoxy-L-isoleucinate (2.45 g) and triethylamine (0.006 mole plus an amount necessary to neutralize the excess<sup>44b</sup> of hydrogen bromide) were added. The mixture was treated as described for compound III. The yield of IV was 4.5 g (77%), mp 222–223° dec,  $[\alpha]_D^{18} - 52.9^\circ$  (c 1, dimethylformamide).

*Anal.* Calcd for  $C_{46}H_{64}N_{10}O_{12}S$ : C, 56.31; H, 6.57; N, 14.27; S, 3.26. Found: C, 56.14; H, 6.98; N, 14.44; S, 3.22.

**N-Carbobenzoxy-O-benzyl-L-tyrosyl-L-isoleucyl-L-glutamyl-L-asparaginyl-S-benzoyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (V).** A. Protected heptapeptide IV (3.9 g, 0.004 mole) was decarbobenzoxylated in the usual manner<sup>44b</sup> except that 20 ml of 2.5 *N* hydrogen bromide in acetic acid was used. To a solution of the resulting hydrobromide in 14 ml of dimethylformamide, cooled to 0°, *p*-nitrophenyl N-carbobenzoxy-O-benzyl-L-tyrosinate (2.21 g) and triethylamine (0.004 mole plus an amount necessary to neutralize the excess<sup>44b</sup> of hydrogen bromide) were added. The mixture was treated as described for compound III giving 4.1 g (83%) of V, mp 225–228° dec,  $[\alpha]_D^{17} - 44.6^\circ$  (c 1, dimethylformamide).

*Anal.* Calcd for  $C_{62}H_{79}N_{11}O_{13}S \cdot H_2O$ : C, 59.45; H, 6.51; N, 12.30; S, 2.56. Found: C, 59.67; H, 6.74; N, 12.40; S, 2.49.

**B.** The heptapeptide hydrobromide, prepared by decarbobenzoxylation of compound IV (0.001 mole) as described under A, was dissolved in 10 ml of methanol and passed through a column of ion-exchange resin IR-400 (OH form). The column was washed twice with small portions of methanol. The eluate and washings were combined and evaporated *in vacuo*. The crystalline residue was recrystallized from water to give 0.150 g of free heptapeptide amide, mp 192–195°,  $[\alpha]_D^{18} - 54.5^\circ$  (c 1, dimethylformamide),  $R_f$  0.35 (solvent system a),  $R_f$  0.78 (solvent system b). A second fraction of 0.260 g showing the same physical constants was obtained by concentration of the filtrate and addition of acetone. The first fraction of the free heptapeptide amide was coupled with *p*-nitrophenyl N-carbobenzoxy-O-benzyl-L-tyrosinate in dimethylformamide and octapeptide V was obtained (70% yield), mp 224–228°,  $[\alpha]_D^{18} - 45.3^\circ$  (c 1, dimethylformamide).

**N-Carbobenzoxy-S-benzoyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutamyl-L-asparaginyl-S-benzoyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (VI).** Protected octapeptide V (1.23 g, 0.001 mole) was decarbobenzoxylated in the usual manner<sup>44b</sup> except that 8 ml of 2.5 *N* hydrogen bromide in acetic acid was used, and the solution was allowed to stand for 45 min at room temperature. The hydrobromide so obtained was dissolved in 3.5 ml of dimethylformamide cooled to 0°, and mixed with 0.5 g of *p*-nitrophenyl N-carbobenzoxy-S-benzoyl-L-cysteinate followed by addition of triethylamine (0.001 mole plus an amount necessary to neutralize the excess<sup>44b</sup> of hydrogen bromide). The mixture was left for 36 hr at room temperature and worked up as described for the preparation of compound III. The crude product was purified by dissolving it in

8 ml of hot dimethylformamide, filtering off some undissolved material, and precipitating with ether. The yield was 1.1 g (83%), mp 227–229°,  $[\alpha]_D^{25} - 64.5^\circ$  (c 1, dimethylformamide).

*Anal.* Calcd for  $C_{66}H_{84}N_{12}O_{16}S_2$ : C, 57.76; H, 6.11; N, 12.43. Found: C, 57.92; H, 6.40; N, 12.14.

**N-Carbobenzoxy-S-carbobenzoxy-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutamyl-L-asparaginyl-S-benzoyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (VII)** was prepared from 0.280 g of *p*-nitrophenyl N,S-dicarbobenzoxy-L-cysteinate<sup>18</sup> and the octapeptide hydrobromide (prepared by decarbobenzoxylation of 0.0005 mole of compound V) using the procedure described for compound VI. The crude product was purified by dissolving it in 5 ml of hot dimethylformamide, and reprecipitating with ether. The yield was 0.44 g (64%), mp 224–226°, unchanged after trituration with 5 ml of hot methanol,  $[\alpha]_D^{25} - 60.6^\circ$  (c 1, dimethylformamide).

*Anal.* Calcd for  $C_{66}H_{84}N_{12}O_{16}S_2$ : C, 57.37; H, 6.12; N, 12.11; S, 4.64. Found: C, 57.05; H, 6.08; N, 12.24; S, 4.69.

**N-*o*-Nitrophenylsulfenyl-S-benzoyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutamyl-L-asparaginyl-S-benzoyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (VIII)** was prepared from 0.265 g of *p*-nitrophenyl N-*o*-nitrophenylsulfenyl-S-benzoyl-L-cysteinate<sup>15c</sup> and the octapeptide hydrobromide (prepared by decarbobenzoxylation of 0.0005 mole of compound V). The procedure used was as described for compound VI except that half of the triethylamine was added to the hydrobromide solution prior to the addition of the *p*-nitrophenyl ester. The crude product (0.405 g, 60%) was recrystallized by dissolving it in 2 ml of hot dimethylformamide and adding 10 ml of ethanol, mp 226–229°,  $[\alpha]_D^{25} - 56.6^\circ$  (c 1, dimethylformamide).

*Anal.* Calcd for  $C_{68}H_{79}N_{13}O_{16}S_3$ : C, 55.20; H, 5.81; N, 13.28; S, 7.01. Found: C, 55.23; H, 6.05; N, 13.53; S, 6.97.

**Bis-*t*-butyloxycarbonyl-L-cystine.** To a cold solution of 10.5 g of L-cystine in a mixture of 435 ml of dioxane and 175 ml of 0.5 *N* sodium hydroxide, 25 ml of *t*-butyloxycarbonyl azide was added with stirring; stirring was continued for 48 hr at room temperature. During the first 9 hr 90 ml of 1 *N* sodium hydroxide was added in nine equal portions. The mixture was filtered to remove a precipitate of cystine which did not react. The dioxane was removed by evaporation *in vacuo*. The remaining solution was extracted with ethyl acetate and the aqueous layer was acidified with cold 1 *N* sulfuric acid. The product separated out and was filtered off after allowing the mixture to stand for a few hours in the refrigerator. The yield was 13 g of small prisms, mp 141–142°. Recrystallization from ethyl acetate-petroleum ether (bp 50–70°) raised the melting point to 145–146°,  $[\alpha]_D^{18} - 138^\circ$  (c 2.5, methanol).

*Anal.* Calcd for  $C_{16}H_{28}N_2O_8S_2$ : C, 43.62; H, 6.40; N, 6.36; S, 14.55. Found: C, 43.80; H, 6.61; N, 6.29; S, 14.70.

**N-*t*-Butyloxycarbonyl-S-benzoyl-L-cysteine.** Bis-*t*-butyloxycarbonyl-L-cystine (2.2 g) was dissolved in 100 ml of liquid ammonia (freshly distilled from sodium) and treated at the boiling point with 0.8 g of sodium in portions until a blue color persisted for 5 min. Some drops of glacial acetic acid were added to discharge the color and the ammonia was evaporated *in vacuo*. The solid residue was suspended in 60 ml of cold 1 *N* sulfuric acid and was extracted twice into ether. The ethereal solution was washed with water and was then extracted three times with cold 10% potassium hydrogen carbonate solution; immediately afterwards and while the temperature of the aqueous layer was kept between 0 and 5°, 3 ml of benzoyl chloride was added and the mixture was vigorously stirred for 5 min. The stirring was continued for an additional 15 min at room temperature and then the solution was acidified with sulfuric acid. The precipitate was collected by filtration. Contaminating benzoic acid was removed by repeated treatment of the product with boiling water followed by decantation. Finally the crude product was purified (1.1 g, mp 105–110°) by dissolving in 5 ml of hot methanol and reprecipitating by addition of 50 ml of boiling water; recovery 0.9 g, mp 110–112° dec,  $[\alpha]_D^{20} - 52.5^\circ$  (c 2, dimethylformamide),  $R_f$  0.25 in toluene-pyridine-acetic acid (80:10:1), chromatogram developed in iodine vapor.<sup>45</sup>

*Anal.* Calcd for  $C_{15}H_{19}NO_5S$ : C, 55.36; H, 5.88; N, 4.30. Found: C, 55.25; H, 5.76; N, 4.20.

The purification of the above substance was also achieved by converting it to its dicyclohexylammonium salt. To prepare this salt 1.2 ml of dicyclohexylamine was added to a solution of 1.3 g of crude product (mp 105–110°) in 10 ml of ethyl acetate. The yield of crystalline salt was 1.7 g (85%), mp 147°. After recrystallization

(45) A. C. Barrett, *Nature*, **194**, 1171 (1962).



from ethyl acetate (recovery 80%) the melting point was raised to 151–153°,  $[\alpha]^{25}_D +18^\circ$  (c 1, dimethylformamide).

*Anal.* Calcd for  $C_{27}H_{42}N_2O_5S$ : C, 63.99; H, 8.35; N, 5.54; S, 6.28. Found: C, 63.78; H, 8.48; N, 5.25; S, 5.96.

To a suspension of 0.750 g of the above dicyclohexylammonium salt in cold water, 2 ml of 1 *N* sulfuric acid was added and the mixture was immediately extracted with ether. The ethereal layer was repeatedly washed with water until the water extracts were neutral to congo red paper, dried over sodium sulfate, and evaporated *in vacuo* to dryness. The residue was recrystallized from methanol-water to give 0.415 g (86%) of *N*-*t*-butyloxycarbonyl-S-benzoyl-L-cysteine, mp 111–113°.

***N*-*t*-Butyloxycarbonyl-S-benzoyl-L-cysteine *p*-Nitrophenyl Ester.**

To a cold solution of 0.405 g of *N*-*t*-butyloxycarbonyl-S-benzoyl-L-cysteine and 0.2 g of *p*-nitrophenol in 12 ml of ethyl acetate, 0.275 g of dicyclohexylcarbodiimide was added. The mixture was stirred for 30 min at 0° and then allowed to stand overnight in the refrigerator and for 5 hr at room temperature. The precipitated *N,N'*-dicyclohexylurea was removed by filtration and the filtrate was evaporated to dryness *in vacuo*. The crystalline residue was recrystallized from 10 ml of ethanol. The yield was 0.475 g (84%), mp 131–132°,  $[\alpha]^{25}_D -38.3^\circ$  (c 2, dimethylformamide),  $R_f$  0.83 in toluene-pyridine-acetic acid (80:10:1), chromatogram developed in iodine vapor.

*Anal.* Calcd for  $C_{31}H_{40}N_2O_7S$ : C, 56.49; H, 4.96; N, 6.27. Found: C, 56.42; H, 4.97; N, 6.35.

***N*-*t*-Butyloxycarbonyl-S-benzoyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutamyl-L-asparaginyl-S-benzoyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (IX)** was prepared from *p*-nitrophenyl *N*-*t*-butyloxycarbonyl-S-benzoyl-L-cysteinate (0.490 g) and the octapeptide hydrobromide (prepared by decarboxylation of 0.001 mole of compound V) in the same manner as described for compound VIII. The yield was 1.05 g (80%), mp 225–228° dec,  $[\alpha]^{25}_D -55.3^\circ$  (c 1, dimethylformamide).

*Anal.* Calcd for  $C_{62}H_{84}N_{12}O_{16}S_2$ : C, 56.51; H, 6.42; N, 12.75; S, 4.86. Found: C, 56.24; H, 6.52; N, 12.74; S, 4.76.

***N*-Carbobenzoxy-oxytocine (X).** A. To a suspension of 0.4 g (0.0003 mole) of compound VI in 4 ml of dimethylformamide, 1.8 ml of methanolic 0.5 *N* sodium methoxide<sup>18</sup> and 2 ml of absolute methanol were added in an atmosphere of hydrogen with stirring at ca. 20°. The protected peptide dissolved completely during the first few minutes of stirring. After 15 min the solution was acidified with 0.75 ml of acetic acid, 0.5 ml of methanol was added, and the product was precipitated upon addition of 100 ml of cold, oxygen-free water. The mixture was cooled and was centrifuged. The supernatant liquid was decanted and the solid was washed twice with water. Methanol was added to the residue repeatedly and evaporated to remove water. The residue was triturated with 10 ml of hot methanol, and after cooling was centrifuged and washed with methanol giving 0.3 g (88%) of compound X, mp 234–235°,  $[\alpha]^{25}_D -46.4^\circ$  (c 1, dimethylformamide). The nitroprusside test for the sulfhydryl group was strongly positive.

*Anal.* Calcd for  $C_{51}H_{74}N_{12}O_{14}S_2$ : C, 53.57; H, 6.52; N, 14.70; S, 5.60. Found: C, 53.71; H, 6.43; N, 14.79; S, 5.49.

B. Upon methanolysis of 0.1 g of compound VII for 30 min, 0.05 g of *N*-carbobenzoxy-oxytocine (X) was obtained, mp 232–233°,  $[\alpha]^{25}_D -45.6^\circ$  (c 1, dimethylformamide).

***N*-*o*-Nitrophenylsulfenyl-oxytocine (XI)** was prepared and purified starting from the protected nonapeptide VIII (0.274 g) in the same way as compound X was prepared from the corresponding nonapeptide VI. The yield was 0.15 g (65%), mp 213–214° dec.

*Anal.* Calcd for  $C_{49}H_{71}N_{13}O_{14}S_3$ : C, 50.63; H, 6.15; N, 15.66; S, 8.27. Found: C, 50.58; H, 5.98; N, 15.33; S, 7.74.

***N*-*t*-Butyloxycarbonyl-oxytocine (XII).** This compound was prepared by methanolysis of compound IX (0.261 g) in the same manner as compounds X and XI were prepared from their corresponding protected nonapeptides VI and VIII. After the addition of water, the methanol was removed *in vacuo*, the solution was extracted with ethyl acetate, and from the water layer the product was precipitated by addition of sodium sulfate to a final concentration of ca. 1%. The yield was 0.1 g (40%), mp 224–227°,  $[\alpha]^{25}_D -48^\circ$  (c 1, dimethylformamide).

*Anal.* Calcd for  $C_{48}H_{70}N_{12}O_{14}S_2$ : C, 51.96; H, 6.90; N, 15.15. Found: C, 51.81; H, 6.61; N, 15.33.

***N*-Carbobenzoxy-S-benzyl-L-cysteinyl-L-isoleucyl-L-glutamyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide.** A solution of *N*-carbobenzoxy-oxytocine (X, 0.06 g) in 1 ml of dimethylformamide was benzylated by addition of 0.1 ml of tri-

ethylamine and 0.4 ml of freshly distilled benzyl chloride, the latter being added in two portions within 5 min. After stirring for 40 min, water was added, the mixture was cooled to 0°, and the product was collected by centrifugation. The precipitate was washed with water and with acetone, and then dried and triturated with hot 95% tetrahydrofuran. The yield was 0.05 g, mp 235–236°,  $[\alpha]^{25}_D -52.5^\circ$  (c 1, dimethylformamide) (lit. mp 224–225°,  $[\alpha]^{25}_D -241^\circ$ ,<sup>8</sup> 237–239°,<sup>8</sup> 243–245°,<sup>9a</sup> 245–248°,<sup>9b</sup>  $[\alpha]^{25}_D -46^\circ$  (c 0.6, dimethylformamide),<sup>8</sup>  $[\alpha]^{25}_D -43^\circ$  (c 2, dimethylformamide),<sup>9a</sup>  $[\alpha]^{25}_D -50.5^\circ$  (c 1, dimethylformamide)<sup>9b</sup>).

*Anal.* Calcd for  $C_{66}H_{88}N_{12}O_{14}S_2$ : C, 58.98; H, 6.55; N, 12.70. Found: C, 59.08; H, 6.36; N, 12.34.

***N*-Carbobenzoxy-oxytocin (XIII).** A solution of 0.572 g (0.0005 mole) of freshly prepared compound X in 40 ml of dimethylformamide and another solution of 0.162 g of freshly recrystallized 1,2-diiodoethane<sup>38</sup> in 40 ml of absolute methanol were prepared. Both of the above solutions were added simultaneously, dropwise, and with stirring, into a mixture of 100 ml of absolute methanol and 20 ml of dimethylformamide, under hydrogen, and within a period of 1 hr. Upon removal of the methanol *in vacuo* at room temperature and addition of ethyl acetate, the oxidation product precipitated; it was collected by centrifugation and washed with ethyl acetate. The yield was 0.34 g (60%). This substance was purified by dissolving in 1 ml of dimethylformamide and reprecipitating with 10 ml of ethyl acetate (recovery 90%); it melted with decomposition between 220 and 227° after shrinking at 165°. This material was further used for the preparation of oxytocin,  $[\alpha]^{25}_D -76.8^\circ$  (c 0.5, dimethylformamide). For analysis a sample was dissolved in dimethylformamide, precipitated by water, collected by centrifugation, washed with acetone and ethyl acetate, redissolved in dimethylformamide, and reprecipitated with acetone; recovery 70%, mp 225–226° after shrinking at 175°,  $[\alpha]^{25}_D -78^\circ$  (c 0.5, dimethylformamide).

*Anal.* Calcd for  $C_{51}H_{72}N_{12}O_{14}S_2$ : C, 53.67; H, 6.36; N, 14.71; S, 5.61. Found: C, 53.32; H, 6.76; N, 14.61; S, 5.57.

***N*-*t*-Butyloxycarbonyl-oxytocin (XIV)** was prepared by oxidation of compound XII in the same manner as described for the preparation of compound XIII. The yield was 54%, mp 220–225° dec,  $[\alpha]^{25}_D -54.3^\circ$  (c 1, dimethylformamide).

*Anal.* Calcd for  $C_{48}H_{74}N_{12}O_{14}S_2$ : C, 52.06; H, 6.73; N, 15.18. Found: C, 52.40; H, 6.95; N, 15.25.

**Oxytocin.** A. *N*-Carbobenzoxy-oxytocin (XIII, 0.266 g) was finely ground and dissolved in 2 ml of freshly prepared, bromine-free 2.5 *N* hydrogen bromide in glacial acetic acid. After 40 min at room temperature the solution was poured into 50 ml of cold ether. The supernatant liquor was decanted and the hydrobromide was washed three times with cold ether, each washing being followed by decantation. The residue was then dried *in vacuo* over potassium hydroxide and phosphorus pentoxide. The hydrobromide was dissolved in 50 ml of the lower phase of the solvent system 1-butanol-1-propanol-0.5% acetic acid containing 0.1% pyridine (6:1:8),<sup>34</sup> and 0.3 ml of 2 *N* ammonium hydroxide was added. The solution and washings were placed in the first ten tubes of a 200-tube countercurrent distribution machine. After 400 transfers determination of the Folin-Lowry color values<sup>46</sup> indicated a main peak between tubes 110 and 165 ( $K = 0.5$ ), as well as two other minor peaks with  $K$  values of approximately 0.2 and 0.04, respectively. The contents of tubes 120–155 were concentrated and lyophilized to give 65 mg of a fluffy material,  $[\alpha]^{25}_D -24.0^\circ$  (c 0.5, 1 *N* acetic acid) (lit.<sup>47</sup>  $[\alpha]^{25}_D -23.1^\circ$  (c 0.51, 1 *N* acetic acid)).

For analysis a sample was dried at 100° over phosphorus pentoxide *in vacuo* (0.1 torr) and a loss in weight of 6% was observed.

*Anal.* Calcd for  $C_{48}H_{76}N_{12}O_{12}S_2 \cdot CH_3COOH \cdot H_2O$ : C, 49.80; H, 6.68; N, 15.48. Found: C, 50.03; N, 6.73; H, 15.44.

A sample was hydrolyzed in 6 *N* hydrochloric acid at 110° and then analyzed for amino acids<sup>36</sup> in the 30–50° system according to the method of Spackman, Stein, and Moore<sup>48</sup> on a Beckman Spinco amino acid analyzer. The following molar ratios of amino acids and ammonia were obtained, with the value of glycine taken as 1.0: aspartic acid, 1.0; proline, 1.0; glycine, 1.0; glutamic acid, 1.0; cystine, 1.0; isoleucine, 1.0; leucine, 1.0; tyrosine, 0.8; and ammonia, 3.

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Oxytocin prepared as described above is identical with a sample of highly purified oxytocin prepared in the laboratory of du Vigneaud<sup>36</sup> as compared by paper or thin layer chromatography and by paper electrophoresis. Paper chromatography on Whatman No. 4 paper and thin layer chromatography were performed in butanol-acetic acid-water (4:1:5, ascending) and the chromatograms were developed with ninhydrin. Paper electrophoresis was carried out on Schleicher and Schüll 2043-B paper for 4.75 hr in pyridine-acetate buffer of pH 4 at 300 v, or for 16 hr in pyridine-acetate buffer of pH 5.6 at 80 v, or for 2 hr in glycine buffer of pH 9.25 at 300 v, and bromophenol blue-mercuric chloride reagent<sup>49</sup> was used for development.

The oxytocin obtained as described above was submitted to partition chromatography on Sephadex.<sup>38</sup> A sample of 14 mg of oxytocin was dissolved in 1 ml of the upper phase of the solvent system 1-butanol-benzene-pyridine-0.1% acetic acid (6:2:1:9) and applied to a Sephadex G-25 column (1.1 × 112 cm) which had been equilibrated with both phases. Elution with the upper phase was performed and 137 fractions (1.5 ml) were collected at a flow rate of approximately 5 ml/hr. The Folin-Lowry color values showed a main peak having a  $R_f$  of 0.27 (reported  $R_f$  for oxytocin under the same conditions 0.24<sup>38</sup>). The recovery of oxytocin from the central portion of this peak was 9.5 mg. Materials taken before and after partition chromatography exhibited the same behavior electrophoretically and chromatographically.

**B.** N-Carbobenzoxy-oxytocine (X, 0.154 g) was finely ground and dissolved in 2 ml of freshly prepared, bromine-free 2.5 *N* hydrogen bromide in glacial acetic acid. After 30 min at room temperature 100 ml of cold ether was added. The supernatant liquor was decanted and the hydrobromide was washed five times with cold ether, each washing being followed by decantation. The residue was dried *in vacuo* over potassium hydroxide and calcium chloride. The hydrobromide was dissolved in 150 ml of air-free water and was brought to a pH of *ca.* 7 by addition of a few drops of 2 *N* ammonium hydroxide. After adjustment of the pH to *ca.* 6.8 with 1 *N* acetic acid, the solution was aerated with CO<sub>2</sub>-free air for 5 hr. The pH of the solution was adjusted to *ca.* 4, before concentrating it in a flash evaporator to a volume of 50 ml and submitting it to countercurrent distribution as described under A. After 388 transfers, determination of the Folin-Lowry color values indicated a main peak with a partition coefficient (*K*) of approximately 0.5. The contents of tubes 110-150 were concentrated and lyophilized to give 30 mg of oxytocin which was identical with preparation A in its chromatographic (paper and thin layer) and electrophoretic behavior. A sample was hydrolyzed in 6 *N* HCl at 110° and analyzed<sup>38</sup> according to the method of Spackman, Stein, and Moore. The following molar ratios of amino acids and ammonia were obtained, with the value of glycine taken as 1.0: aspartic acid, 1.0; proline, 1.0; glycine, 1.0; glutamic acid, 1.0; cystine, 1.0; isoleucine, 1.0; leucine, 1.0; tyrosine, 0.8; and ammonia, 3.

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## Coenzyme A Analogs. Synthesis of D-Oxypantetheine-4' Phosphate and Oxy-Coenzyme A<sup>1a,b</sup>

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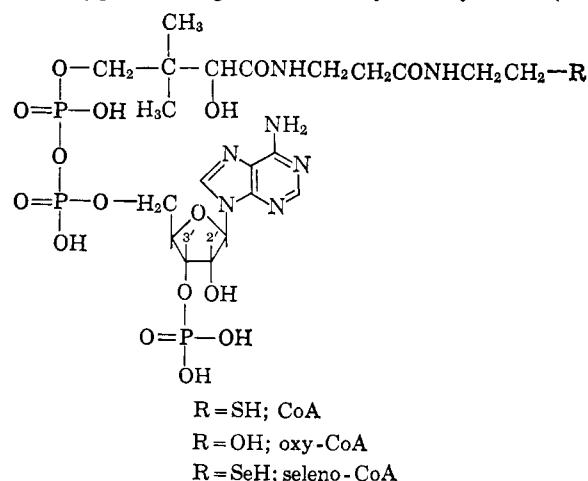
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**Abstract:** The syntheses of D-oxypantetheine-4' phosphate, 3'-dephosphooxy-coenzyme A, isooxy-coenzyme A, and oxy-coenzyme A are described. Oxy-coenzyme A is shown to be a competitive inhibitor of coenzyme A in the phosphotransacetylase reaction with a  $K_i = 3.5 \times 10^{-7}$  M.

The ubiquitous role that coenzyme A (CoA) plays in intermediary metabolism is well known.<sup>4</sup> It is desirable to have available a specific CoA antagonist in order to facilitate the study of cofactor binding effects on the various apoenzymes and advance one's knowledge of the reaction mechanisms of CoA-dependent reactions. The successful syntheses of CoA by Moffatt and Khorana,<sup>5</sup> Michelson,<sup>6</sup> and Gruber and Lynen<sup>7</sup> have provided routes for the synthesis of CoA analogs; *e.g.*, the synthesis of seleno-coenzyme A

(seleno-CoA) has been reported recently by Günther and Mautner.<sup>8</sup>

The oxygen analog of CoA, oxy-coenzyme A (oxy-



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