[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH]

Studies on the Synthesis of Insulin Peptides

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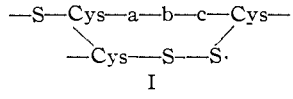
The use of dihydropyran for protecting the sulfhydryl group of cysteine during peptide synthesis has been investigated. 2-Tetrahydropyranyl sulfides were found to survive sodium–liquid ammonia reduction of carbobenzyloxy functions. The sulfhydryl is easily regenerated by silver ions at 0°. Initial attempts are reported on the use of a combination of sulfur-protecting groups for the synthesis of the cyclic hexapeptide portion of insulin.

The elucidation of the amino acid sequence of oxytocin[1] and of vasopressin[2] as well as that of insulin[3] has brought to light a structural element common to both hormonal species, a twenty-membered disulfide ring, but differing in amino acid composition. A comparison of insulin samples from various sources[3b] shows that species variation occurs only in three amino acids within the disulfide ring (I and Table I).



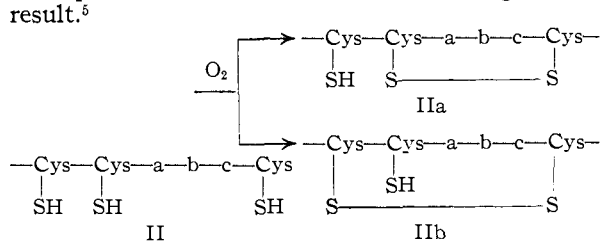
I

TABLE I

Source	a	b	c
Sheep	Ala	Gly	Val
Cattle	Ala	Ser	Val
Pig	Threo	Ser	Ileu
Whale	Threo	Ser	Ileu
Horse	Threo	Gly	Ileu

Since the hormonal activity of insulin appears to be independent of the animal source and the presence of an hydroxyl on a or b is not critical, it is tempting to assume that the over-all geometry of the cyclic disulfide is more significant to an enzyme or to a permeable membrane than the nature of the functional groups projecting from the ring.[4] In this Laboratory the synthesis of such a twenty-membered cyclic disulfide was considered a modest starting-point from which to explore some aspects of the nature of insulin action. Although the synthetic work is still in progress, we felt that a report of our early investigations would be of interest.

In addition to the work of du Vigneaud and his associates on the synthesis of large-ring disulfides[1,2] the oxidative cyclization of hexapeptides with terminal cysteine residues has been described.[5,6] In undertaking the synthesis of a peptide with the structural features of I, a new problem is introduced by the presence of an additional cysteine residue. Should the non-terminal sulfhydryl of II remain unprotected during the final oxidative cyclization, a competition between formation of a twenty- and a seventeen-membered ring might result.[5]



Since the penultimate step in the synthesis of a cyclic disulfide usually involves the removal of S-benzyl groups with sodium in liquid ammonia, a protective function was required for the non-terminal sulfhydryl which could survive reductive cleavage and yet be removable subsequently under conditions that would destroy neither a peptide nor a disulfide linkage. For this purpose we investigated the vinyl ether, dihydropyran. This reagent has been used for the protection of alcoholic and phenolic hydroxyls during peptide synthesis[7]; further, the addition of simple mercaptans to dihydropyran has been described.[8]

In preliminary experiments ethyl 2-tetrahydropyranyl sulfide (III) was found to undergo cleavage by sodium in liquid ammonia but at a rate sufficiently slow to warrant further study. A number of unsuccessful attempts were made to treat cysteine directly with dihydropyran. However, the vinyl ether added rapidly to cysteine methyl ester hydrochloride to form the adduct IV as a sirup or glass. By careful saponification of the ester, S-tetrahydropyranyl-L-cysteine (V) could be isolated as a crystalline amino acid. Conversion of the ester to its carbobenzyloxy derivative VI and saponification gave VII. When the carbobenzyloxy-amino acid was reduced with sodium in liquid ammonia, V was recovered in 35% yield.[9]

The removal of the blocking group from 2-tetrahydropyranyl sulfides can be effected by hydrolysis with dilute acid.[8] However, a preferable method was based on the observation that aqueous silver nitrate precipitated the silver mercaptide of cysteine quantitatively after several minutes at 0°. The other product of the cleavage, δ-hydroxyvaleraldehyde, remained in the filtrate. Under comparable conditions, cystine formed only a slight precipitate after 30 minutes.

(1) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyannis, THIS JOURNAL, **76**, 3115 (1954).

(2) M. F. Bartlett, A. Jöhl, R. Roeske, R. J. Stedman, F. H. C. Stewart, D. N. Ward and V. du Vigneaud, *ibid.*, **78**, 2905 (1956).

(3) (a) H. Brown, F. Sanger and R. Kitai, *Biochem. J.*, **60**, 556 (1955); (b) J. I. Harris, F. Sanger and M. A. Naughton, *Arch. Biochem.*, **65**, 427 (1956); (c) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463, 481 (1951); F. Sanger and E. O. L. Thompson, *ibid.*, **53**, 353, 366 (1953).

(4) *Cf.* C. Ressler and V. du Vigneaud, THIS JOURNAL, **79**, 4511 (1957).

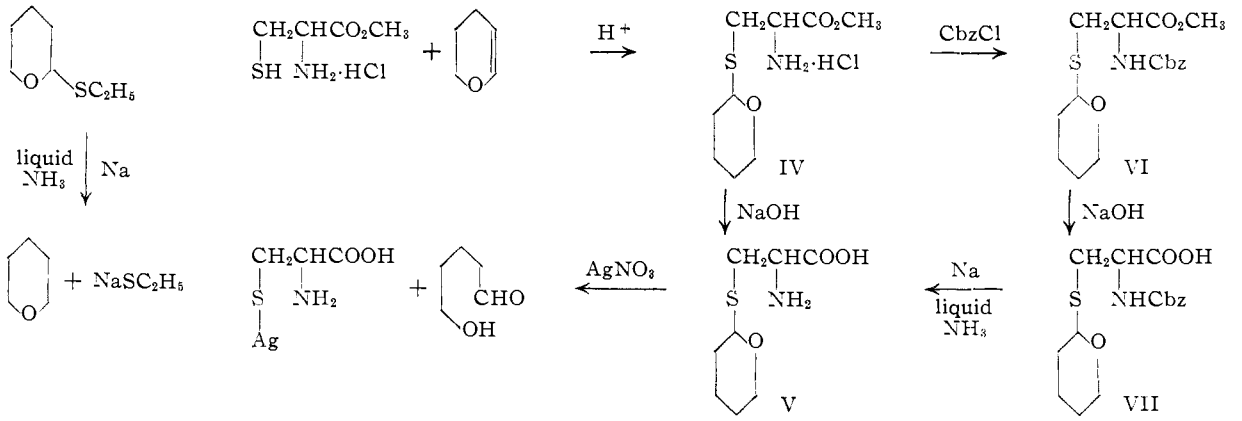
(5) G. S. Heaton, H. N. Rydon and J. A. Schofield, *J. Chem. Soc.*, 3157 (1956).

(6) W. Lautsch and H. Kraege, *Chem. Ber.*, **89**, 737 (1956); R. Wade, M. Winitz and J. P. Greenstein, THIS JOURNAL, **78**, 373 (1956).

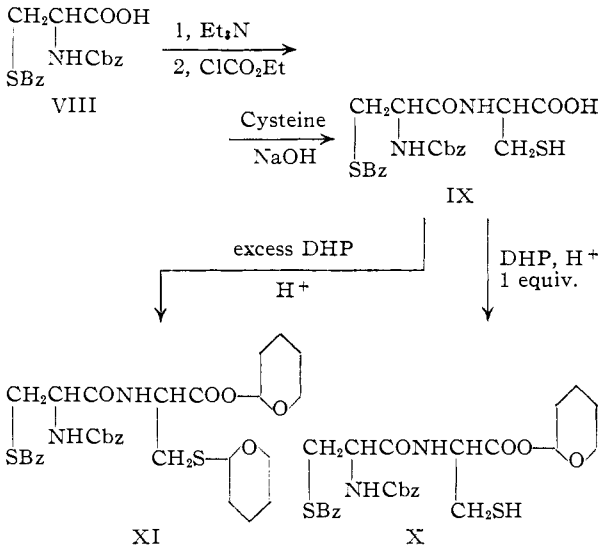
(7) B. Iselin and R. Schwyzer, *Helv. Chim. Acta*, **39**, 57 (1956).

(8) F. Kipnis and J. Ornfelt, THIS JOURNAL, **73**, 822 (1951); W. E. Parham and D. M. DeLaitsch, *ibid.*, **76**, 4962 (1954).

(9) The introduction of a new asymmetric center upon addition of dihydropyran provides for a pair of diastereoisomers. Presumably, this feature may hinder the crystallization of some intermediates. It is assumed that the crystalline amino acid V is a single optical isomer.



In attempting the synthesis of a hexapeptide of type IIb we chose to couple a suitable derivative of cysteinylcysteine with a derivative of alanylglycylvalylcysteine. Carbobenzyloxy-S-benzyl-L-cysteine (VIII) was coupled with cysteine by the mixed anhydride method to give the dipeptide derivative IX. Treatment with one equivalent of dihydropyran (DHP) resulted in blocking of the carboxyl in preference to the sulfhydryl group (X). Both functional groups reacted with excess reagent to form XI, which was resistant to mild alkaline saponification.

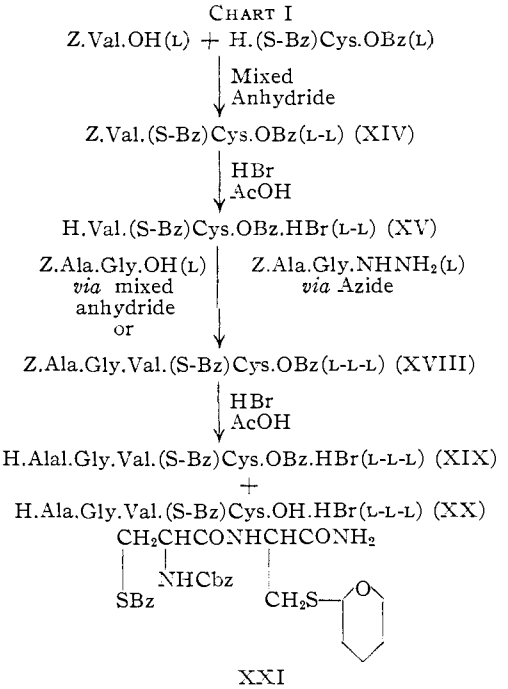


When VIII was coupled with the tetrahydropyranyl ester IV, the corresponding dipeptide ester XII was obtained in 87% yield. As in the case of XI, alkaline saponification could not be effected under the usual conditions.[10] However, XII was readily converted to the hydrazide XIII which was used in subsequent steps.

Carbobenzyloxy-L-alanylglycine (XVI) was coupled with L-valyl-S-benzyl-L-cysteine benzyl ester hydrobromide (XV) by the mixed anhydride method to obtain the tetrapeptide derivative XVIII, as outlined in Chart I. Azide coupling led to a product with the same optical rotation and slightly higher melting point. Removal of the carbobenzyloxy group of XVIII with hydrogen bromide in acetic acid led to the hydrobromide XIX together with varying amounts of the corresponding tetrapeptide XX.

A number of attempts were made to couple the azide corresponding to XIII with crude tetrapeptide benzyl ester hydrobromide XIX. However, the only identifiable products were the amide XXI and small amounts of XX. Similar cases of reduction of an azide to an amide have been reported.[11] Such reductions may be due to the presence of oxides of nitrogen in the organic layer which have survived alkaline extraction. This possibility is currently being studied.

CHART I



Experimental[12]

Reduction of Ethyl 2-Tetrahydropyranyl Sulfide (III).—To a solution of 2.9 g. (0.02 mole) of III in 100 ml. of liquid ammonia was added 0.28 g. (0.012 mole) of sodium while the solution was stirred magnetically. Twelve minutes was

(10) *Cf.* K. C. Hooper, H. N. Rydon, J. A. Schofield and G. S. Heaton, *J. Chem. Soc.*, 3148 (1956).

(11) V. Prelog and P. Wieland, *Helv. Chim. Acta*, **29**, 1130 (1946); B. Hegedüs, *ibid.*, **31**, 740 (1948); C. W. Roberts, THIS JOURNAL, **76**, 6203 (1954); R. Roeske, F. H. C. Stewart, R. J. Stedman and V. du Vigneaud, *ibid.*, **78**, 5884 (1956).

(12) Melting points were measured on the Kofler block and are corrected.

required for the total disappearance of the blue color. When the reaction was repeated with the addition of 0.92 g. (0.04 mole) of sodium, 30 minutes was necessary for complete reduction. After evaporation of the solvent, sodium ethyl mercaptide was identified in the residue by conversion to its dinitrophenyl derivative.

Methyl S-(2-Tetrahydropyranyl)-L-cysteinate Hydrochloride (IV).—Finely powdered L-cysteine (24.2 g., 0.2 mole) was suspended in 200 ml. of methyl alcohol and the mixture was saturated with hydrogen chloride at 0°. The reaction mixture was stored for 12 hours at 25°, decanted from a slight residue and concentrated under reduced pressure. The ester hydrochloride crystallized during concentration; it was triturated with ether, filtered with suction and dried *in vacuo* to give an almost quantitative yield of needles, m.p. 142–143° (lit. 137–138.5°).[13]

The product was suspended in 100 ml. of methylene chloride and 16.8 g. (0.02 mole) of freshly distilled dihydropyran added. Solution was essentially complete after the mixture had been refluxed for 1–2 hours.[14] After removal of the solvent, the residual sirup was triturated with petroleum ether and with ether and dried. It gave no nitroprusside test for sulfhydryl and a very faint test after treatment with sodium cyanide. The product resisted a number of attempts at crystallization.

S-(2-Tetrahydropyranyl)-L-cysteine (V).—To a stirred solution of 2.6 g. (0.01 mole) of IV in 50 ml. of water was added dropwise over one hour 20 ml. of *N* sodium hydroxide. After 3 hours at 25° the basicity had dropped to *p*H 9 and remained constant for a fourth hour. The solution was adjusted to *p*H 7 with *N* hydrochloric acid and concentrated to dryness at 25°. The residue, a mixture of sirup and sodium chloride, was triturated with ether and ethyl acetate and the residual material was extracted with warm acetone. Upon slow evaporation of the acetone solution, a deposit of colorless, granular crystals was obtained, 0.7 g., m.p. 170–171°, to a brown liquid with gas evolution. It was recrystallized twice from methanol–ethanol, raising the melting point to 186–187°. The nitroprusside test was negative before or after treatment with cyanide ion; $[\alpha]^{26}D$ +11.6° (*c* 1.1, water).

Anal. Calcd. for $C_8H_{15}O_3NS$: C, 46.82; H, 7.37; N, 6.83. Found: C, 46.76; H, 7.16; N, 6.86.

When 0.5 *N* silver nitrate was added to a dilute aqueous solution of V at 0°, a flocculent white precipitate of the silver mercaptide of cysteine was formed immediately. After 10 minutes the precipitate was removed by centrifugation. No additional mercaptide could be obtained from the supernatant solution, and it gave a negligible ninhydrin test compared to the original solution. δ-Hydroxyvaleraldehyde was recovered from solution as its dinitrophenylhydrazone, m.p. 110–111° (lit.109°),[15] accounting for 80% of the theoretical amount.

N-Carbobenzyloxy-S-(2-tetrahydropyranyl)-L-cysteine (VII).—Acylation of IV with carbobenzoxy chloride and triethylamine was performed in methylene chloride solution. The reaction mixture was washed with *cold N* hydrochloric acid, dried and concentrated to a sirup which was saponified directly to the free acid VII with *N* sodium hydroxide. The alkaline solution was washed with ether, chilled in ice, acidified to *p*H 3 with *N* hydrochloric acid and rapidly extracted with 2 × 100 ml. of ether. The dried solution was concentrated to a colorless sirup which could not be crystallized.

Anal. Calcd. for $C_{16}H_{21}O_5NS$: C, 56.63; H, 6.24; N, 4.13. Found: C, 56.34, 56.49; H, 6.66, 6.83; N, 3.71, 4.20.

Reduction of VII to V.—A solution of 1.7 g. (0.005 mole) of VII in 50 ml. of liquid ammonia was stirred magnetically while small pieces of sodium (approximately 25 mg. each) were added, waiting for a disappearance of blue color between additions. Addition of the eleventh portion resulted in a blue color which persisted for at least 5 minutes. Thus, 60–70% of the theoretical amount of sodium necessary to cleave the carbobenzyloxy group was consumed.[16] After evaporation of the solvent, the residue was dissolved in a small volume of water, chilled to 5°, acidified to *p*H 3 and extracted with ether. The aqueous phase was adjusted to *p*H 6 and concentrated to dryness at 25°. The residue was triturated with warm acetone and the amino acid isolated as described above. Comparison was made by melting point, mixed melting point, optical rotation and formation of the silver mercaptide. Approximately 0.35 g. (35%) of crystalline material was obtained. The ether extract gave no silver nitrate test for tetrahydropyranyl–sulfur linkages. The acetone-insoluble residue gave a faint nitroprusside test after cyanide reduction. From this evidence it was concluded that removal of the carbobenzyloxy group had been complete and that loss of the tetrahydropyranyl group had proceeded to a relatively minor extent.

Carbobenzyloxy-S-benzyl-L-cysteinyl-L-cysteine (IX).[17]—To a solution of 10.4 g. (0.03 mole) of carbobenzyl-S-benzyl-L-cysteine (VIII) in 150 ml. of tetrahydrofuran was added 4.2 ml. (0.03 mole) of triethylamine. After the solution had been cooled to −5°, 3.9 ml. (0.03 mole) of isobutyl chlorocarbonate was added and the mixture kept at this temperature for 10 minutes. Finely powdered cysteine (7.3 g., 0.06 mole) was added; the solution was stirred and maintained at −5° while 120 ml. of *N* sodium hydroxide was added over 30 minutes. The reaction mixture was allowed to come to room temperature slowly and kept there for 10 hours. It was acidified to *p*H 2 with concd. HCl at 0°, concentrated under reduced pressure and the residue extracted with 2 × 100 ml. of methylene chloride. The dried extract was concentrated to an oil which was dissolved in 25 ml. of ethyl acetate, centrifuged from a small residue and reconcentrated. The residual oil was taken up in 150 ml. of dry ether and chilled overnight at 5°. The precipitate which formed was filtered with suction, washed several times with ether and dried for 6 hours at 55° and 0.1 mm. The product consisted of 6 g. of an almost colorless powder, softening at 125° and melting at 137–139°. Further purification was not attempted.

Anal. Calcd. for $C_{21}H_{24}O_5N_2S_2$: C, 55.24; H, 5.39; N, 6.25. Found: C, 56.45; H, 5.86; N, 6.51.

Reaction of IX with Dihydropyran.—To a solution of 1.34 g. (0.003 mole) of IX in 50 ml. of methylene chloride was added 0.25 g. (0.003 mole) of dihydropyran and 2 ml. of 2% hydrogen chloride in ether. After 10 hours at 25°, the solution still gave a strong sulfhydryl test. Extraction of the solution with sodium bicarbonate and acidification failed to reveal any free carboxyl material. The preparation was repeated using two grams of dihydropyran. A neutral product was isolated which was negative to nitroprusside before or after cyanide treatment. Analysis indicated the addition of dihydropyran to both the sulfhydryl and carboxyl groups.

Anal. Calcd. for $C_{31}H_{40}O_7N_2S_2$: C, 60.4; H, 6.5. Found: C, 59.8; H, 6.61.

Methyl Carbobenzyloxy-S-benzyl-L-cysteinyl-S-(2-tetrahydropyranyl)-L-cysteinate (XII).—To a suspension of 6.9 g. (0.02 mole) of carbobenzyloxy-S-benzyl-L-cysteine in 100 ml. of methylene chloride was added 2.8 ml. (0.02 mole) of triethylamine. The solution was cooled to −5° and 1.9 ml. (0.02 mole) of ethyl chlorocarbonate added. After 10 minutes at −5°, there was added 7.7 g. (0.03 mole) of methyl tetrahydropyranyl-L-cysteinate hydrochloride (IV) and 7 ml. of triethylamine (0.05 mole). The mixture was stored at 25° for 8 hours, washed with water, *cold N* HCl, 5% sodium bicarbonate, dried and concentrated to give 9.5 g. (87%) of a sirup which resisted attempts at crystallization. When the reaction was run on a 0.1 mole scale, the yield was approximately the same.

A solution of XII in aqueous methanol was brought to *p*H 11 with sodium hydroxide. The consumption of alkali

(13) M. X. Sullivan, W. C. Hess and H. W. Howard, *J. Wash. Acad. Sci.*, **32**, 285 (1942).

(14) In one preparation the ester hydrochloride was obtained as a sirup; its reaction with dihydropyran was complete after 15 minutes at 25°.

(15) G. F. Woods and H. Sanders, THIS JOURNAL, **68**, 2111 (1946).

(16) It has been observed previously by Dr. A. Patchornik (private communication) that the cleavage of N-benzyl groups could be effected with somewhat less than the calculated amount of sodium. That a regeneration of reducing agent may be occurring was demonstrated by the isolation of dibenzyl from the reaction. The isolation of dibenzyl has been reported previously by R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 756 (1935). A similar phenomenon may have occurred in the present case.

(17) Wherever possible the synthesis was conducted in a nitrogen atmosphere.

over several hours was negligible. Raising the temperature to 50° failed to effect saponification. Even in 2 *N* alkali, a negligible acidic fraction was obtained.

Carbobenzyloxy-S-benzyl-L-cysteinyl-S-(2-tetrahydropyranyl)-L-cysteine Hydrazide (XIII).—To a solution of 10.9 g. (0.02 mole) of the above ester XII in 50 ml. of ethyl alcohol was added 2 g. (0.04 mole) of 100% hydrazine hydrate. After storage for 20 hours at 25°, the solution was heated on steam for 30 minutes and diluted to turbidity with water. On slow cooling the product separated as a white powder (rapid cooling resulted in gel formation). After recovery of the product, further material was obtained by dilution of the filtrate with water. The air dried material was washed with ether and dried *in vacuo*, m.p. 155–160°. It was recrystallized from ethanol–water to give 9.0 g. (82%) of colorless powder, m.p. 165–166°.

Anal. Calcd. for $C_{26}H_{34}O_5N_4S_2$: C, 57.11; H, 6.27; N, 10.25; S, 11.73. Found: C, 57.23; H, 6.57; N, 10.09; S, 11.61.

Benzyl Carbobenzyloxy-L-valyl-S-benzyl-L-cysteinate (XIV).—A solution of 0.5 g. (0.002 mole) of carbobenzyloxy-L-valine[18] and 0.28 ml. (0.002 mole) of triethylamine in 10 ml. of chloroform was cooled to 0° and 0.19 ml. (0.002 mole) of ethyl chlorocarbonate added with stirring. After 20 minutes a second solution of 1.15 g. (0.003 mole) of benzyl S-benzyl-L-cysteinate hydrobromide[19] and 0.42 ml. (0.003 mole) of triethylamine in 20 ml. of chloroform was added dropwise over a period of 10 minutes. After one hour at 25° the reaction mixture was washed with *N* hydrochloric acid, *N* sodium bicarbonate and water. The dried solution was concentrated under reduced pressure to a white solid. Recrystallization from ethyl acetate gave 0.8 g. (75%) of XIV, m.p. 155–156°. In larger-scale preparations, the yield was essentially the same. For analysis the compound was recrystallized twice from ethyl acetate, m.p. 155.5–156.5°, $[\alpha]^{20}D$ −17.4° (*c* 1.0, chloroform).

Anal. Calcd. for $C_{30}H_{34}O_5N_2S$: C, 67.39; H, 6.41; N, 5.24. Found: C, 67.63; H, 6.19; N, 5.13.

Benzyl L-Valyl-S-benzyl-L-cysteinate Hydrobromide (XV).—To a flask protected by a drying tube and containing 1.0 g. (0.0019 mole) of XIV was added 6 ml. of a saturated solution of hydrogen bromide in glacial acetic acid. After 20 minutes the solution was poured into 100 ml. of absolute ether and the mixture refrigerated for 2 hours. The crystalline product was collected by filtration, 0.7 g., m.p. 182–184°. An analytical sample was prepared by two recrystallizations from ethyl alcohol–petroleum ether, m.p. 184.5–185.5°, $[\alpha]^{20}D$ −37.1° (*c* 1.0, methanol).

Anal. Calcd. for $C_{22}H_{29}N_2O_3SBr$: C, 54.88; H, 6.13; N, 5.82. Found: C, 54.74; H, 6.13; N, 5.69.

Carbobenzyloxy-L-alanylglycine (XVI).—Carbobenzyloxy-L-alanine[20] was converted to carbobenzyloxy-L-alanylglycine ethyl ester *via* mixed anhydride coupling in 80% yield, m.p. 98° (lit. m.p. 100°,[21] prepared *via* the acid chloride). Alkaline saponification gave an 82% yield of XVI, m.p. 130° (lit. m.p. 132°).[22]

Carbobenzyloxy-L-alanylglycine Hydrazide (XVII).—Carbobenzyloxy-L-alanylglycine ethyl ester was converted to the hydrazide by refluxing with hydrazine hydrate for 1 hour. A 95% yield was obtained, melting at 146–147° after recrystallization from water (lit. 145–147°[23a]; 157°[23b]).

Benzyl Carbobenzyloxy-L-alanylglycyl-L-valyl-S-benzyl-L-cysteinate (XVIII). A. By Mixed Anhydride Coupling.—To a solution of 1.6 g. (0.0045 mole) of XVI and 0.62 ml. (0.0045 mole) of triethylamine in 10 ml. of chloroform was added at 0°, 0.42 ml. (0.0045 mole) of ethyl chlorocarbonate. After 20 minutes at 0° a solution of 2.14 g. (0.0045 mole) of XV and 0.62 ml. of triethylamine in 10 ml. of chloroform was added. The mixture was stirred overnight at 25°, washed with *N* hydrochloric acid, *N* sodium bicarbonate and water, dried and concentrated under reduced pressure to an oil. Crystallization from chloroform–petroleum ether yielded 1.9 g. of XVIII, m.p. 183–184°. An analytical sample was prepared by two recrystallizations from ethanol–petroleum ether, m.p. 186–187°; $[\alpha]^{20}D$ −53.8° (*c* 1.0, acetic acid).

Anal. Calcd. for $C_{35}H_{42}O_7N_4S$: C, 63.44; H, 6.34; N, 8.46. Found: C, 63.24; H, 6.32; N, 8.24.

B. **By Azide Coupling.**—A solution of 0.5 g. (0.0017 mole) of XVII in a mixture of 5 ml. of acetic acid, 3 ml. of 5 *N* hydrochloric acid and 20 ml. of water was cooled to −5°. A cold, concentrated solution of sodium nitrite (0.14 g., 0.002 mole) was added and the azide extracted immediately with a cooled mixture of 20 ml. of ether and 20 ml. of ethyl acetate. The organic layer was washed successively with water, 3% sodium bicarbonate and water, all chilled before use. The dried ether solution was added in one portion to a cold ethereal solution of L-valyl-S-benzyl-L-cysteine benzyl ester (prepared by neutralizing an aqueous solution of 1.2 g. (0.0025 mole) of XV with triethylamine, extracting with ether and drying with sodium sulfate). The reaction mixture was stored at −5° overnight and the crystalline precipitate separated by suction filtration to give 0.83 g. of XVIII. On recrystallization from ethanol–petroleum ether, needles were obtained, m.p. 191–192°, $[\alpha]^{20}D$ −53.4° (*c* 1.0, acetic acid).

Benzyl L-Alanylglycyl-L-valyl-S-benzyl-L-cysteinate Hydrobromide (XIX).—To a flask protected by a drying tube and containing 0.5 g. (0.00076 mole) of XVIII was added 4 ml. of a saturated solution of hydrogen bromide in glacial acetic acid. After 15 minutes the solution was poured into 100 ml. of dry ether. The mixture was stored at −5° for several hours and the ether decanted from an oily residue. Crystallization of the oil from ethanol–petroleum ether yielded 0.23 g. of the hydrobromide, m.p. 148–149°. After two recrystallizations from the same solvent it melted at 178–179°.

Anal. Calcd. for $C_{27}H_{37}O_5N_4SBr$: C, 53.20; H, 6.08; N, 9.20. Found: C, 52.56; H, 6.28; N, 9.23.

L-Alanylglycyl-L-valyl-S-benzyl-L-cysteine (XX).—During several of the preparations of XIX, a high-melting by-product was obtained, particularly when exposure to hydrogen bromide was longer than 15 minutes. This was apparently the hydrobromide of the free tetrapeptide. Neutralization of an ethanol solution of the cleavage mixture with triethylamine resulted in the separation of needles, m.p. 210–220°. The peptide was soluble in water but separated as clusters of fine needles from methanol, m.p. 229–231° with decomposition. A ninhydrin test was positive and acid hydrolysis followed by paper chromatography revealed four spots corresponding to the appropriate amino acids (including S-benzyl-L-cysteine); $[\alpha]^{20}D$ −32° (*c* 0.66, water).

Anal. Calcd. for $C_{20}H_{30}N_4O_5S$: C, 54.77; H, 6.90; N, 12.78; S, 7.31. Found: C, 54.85; H, 6.66; N, 12.95; S, 7.17.

Attempted Coupling to Hexapeptide.—To a chilled mixture of 1 ml. of water, 2 ml. of *N* hydrochloric acid and 0.1 g. of sodium nitrite was added 0.55 g. (0.001 mole) of hydrazide XIII. The mixture was extracted immediately with 2 × 5 ml. of chilled methylene chloride, the extract dried briefly over sodium sulfate and added in one portion to a cold, dry chloroform solution containing the crude product resulting from the hydrogen bromide cleavage of 0.54 g. (0.00082 mole) of XVIII and 0.2 ml. of triethylamine. After storage at 25° overnight, the solution was filtered from a small amount of XX, washed with water, *N* hydrochloric acid, *N* sodium bicarbonate and water. The chloroform solution was dried and concentrated to a solid mass which was extracted with ethyl alcohol. Upon addition of petroleum ether to the extract and chilling, a crystalline product separated, m.p. 140° (softening at 110–115°). After two recrystallizations from 95% ethanol, the compound melted at 142–144°, resolidified and melted at 170–172°. The material analyzed for carbobenzyloxy-S-benzyl-L-cysteinyl-S-(2-tetrahydropyranyl)-L-cysteine-amide hemihydrate (XXI).

Anal. Calcd. for $C_{26}H_{33}O_5N_3S_2 \cdot {}^1/_2H_2O$: C, 57.72; H, 6.29; N, 7.77. Found: C, 57.74; H, 6.05; N, 7.77.

(18) J. R. Vaughan, Jr., and J. A. Eichler, THIS JOURNAL, **75**, 5556 (1955).

(19) The hydrobromide was prepared according to D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952), from the carbobenzyloxy derivative (C. R. Harington and T. H. Mead, *Biochem. J.*, **30**, 1598 (1936)) of S-benzyl-L-cysteine (J. L. Wood and V. du Vigneaud, *J. Biol. Chem.*, **130**, 109 (1939)).

(20) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).

(21) M. Bergmann, L. Zervas, J. Fruton, F. Schneider and H. Schleich, *J. Biol. Chem.*, **109**, 342 (1935).

(22) W. H. Stein, S. Moore and M. Bergmann, *J. Biol. Chem.*, **154**, 191 (1944).

(23) (a) M. Bergmann and J. S. Fruton, *ibid.*, **117**, 189 (1937); (b) E. L. Smith and M. Bergmann, *ibid.*, **153**, 627 (1944)

The anhydrous compound was obtained by drying *in vacuo* at 140° for two days, m.p. 170°, $[\alpha]^{20}D$ −68.6° (*c* 1.0, dimethylformamide).

Anal. Calcd. for $C_{26}H_{33}O_5N_3S_2$: C, 58.75; H, 6.26; N, 7.91; S, 12.06; amide N, 2.63. Found: C, 58.46; H, 6.47; N, 7.90; S, 12.32; amide N, 2.08.[24]

(24) A portion of the ammonia was lost by foaming; insufficient material was available for a second determination.

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Antihypertensively Active Amidoximes

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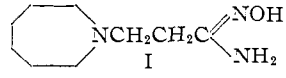
Hexahydro-1-azepinepropionamidoxime and structurally related compounds were prepared and were found to have prolonged antihypertensive properties. Maximum activity was noted with the hexahydroazepine ring compound; this activity diminished as the ring size was altered. Variation of the propionamidoxime side chain likewise resulted in a lessening of activity.

The chemotherapy of the amidoximes has been the subject of several recent papers. Lamb and White[3] have studied the antitrypanosomal activity of alkylene diamidoximes and diamidoximes derived from biphenyl, diphenylmethane and related compounds.

The literature[4] also reports that *p*-sulfamylbenzamidioxime exhibits pronounced antirickettsial activity on experimental typhus infections in mice; acetamidoxime thionocarbamates of morpholine and piperidine have been studied for their antibacterial and antifungal properties.[5]

Buu-Hoï,[6] *et al.*, found that halogenated salicylamidoximes display considerable tuberculostatic properties *in vitro;* the pyridineamidoximes were found to be inactive.[7]

The literature does not disclose a similar interest in the pharmacology of the amidoximes. In the case of hexahydro-1-azepinepropionamidoxime (I), however, a unique antihypertensive activity has been noted.[8]



I

A study of its effects on the cardiovascular system of the dog revealed that a single intravenous dose of 30 mg./kg. lowered the arterial pressure of neurogenic and renal hypertensive dogs. However, in normotensive animals 30 mg./kg. of the compound given intravenously eliminated the severe hypertension elicited by high doses of amphetamine and ephedrine and also markedly antagonized carotid occlusion reflex pressor responses. These antihypertensive effects were slow in onset and lasted for approximately two to six weeks following single injection. The compound was found to be orally active and had a cumulative action when given in small daily doses.

The relationship of ring size to antihypertensive activity in this class of compounds was found to be quite critical. For example, the 1-pyrrolidine and 1-piperidinepropionamidoximes were almost devoid of antihypertensive activity, whereas the corresponding hexahydroazepine derivative was most active. With further ring enlargement activity gradually diminished and was totally absent in the eleven-membered ring compound, 1-azacycloundecanepropionamidoxime. Modification of the azepine ring by substitution or formation of tetrahydro-3,1H-benzazepine failed to give compounds with increased activity. Frequently these alterations caused reduction of activity. Replacement of the hexahydroazepinyl moiety by piperazinyl, di-2-pyridylamino, carbazolyl and other ring systems did not disclose any interesting pharmacological properties. Dialkylaminopropionamidoximes as well as other aliphatic amidoximes were prepared, but in all cases activity was absent. Consideration also was given to the amidoxime derivatives; in the case of the O-acyl compounds there was a noticeable retention of activity. Loss of activity occurred too when the amidoxime function in the hexahydroazepine side chain was replaced by a variety of other functional groups.

The most active member of the series was prepared by cyanoethylation of hexahydroazepine to give hexahydro-1-azepinepropionitrile.[9] Treatment of the latter compound with hydroxylamine in ethanol yielded the desired amidoxime which could be converted to an appropriate salt. The feasibility of preparing this compound from the hexahydro-2-oxo-1-azepinepropionamidoxime by lithium aluminum hydride reduction was investigated. It was found that in ether the reduction occurred without alteration of the amidoxime func-

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