The Synthesis of Secretin. III. The Fragment-Condensation Approach

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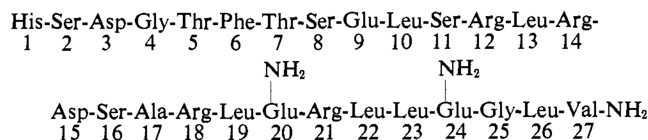
Abstract: A heptacosapeptide amide with the amino acid sequence proposed for porcine secretin has been synthesized by the condensation of four peptide fragments. The intermediate fragments were synthesized by the stepwise approach using, in most cases, active esters for the coupling reactions. The fragments were joined by the azide procedure. The final product showed all the physical, chemical, and biological properties of natural porcine secretin, confirming thereby the proposed structure.

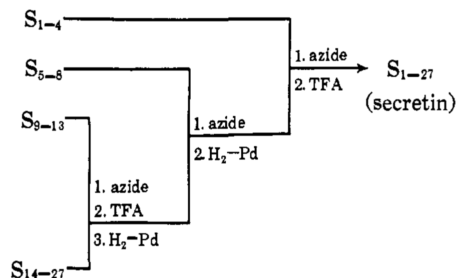
Previous communications of this series[2] have described the stepwise synthesis of porcine secretin. The structure of this intestinal hormone, as it stemmed from the investigations of Jorpes, Mutt, and their collaborators,[3] is shown in Scheme I.

Scheme I



Concurrently with the stepwise approach, an alternative pathway toward the preparation of the same heptacosapeptide amide was also explored. The present communication deals with this second approach, which consists of the coupling of four different fragments, as is schematically shown in Scheme II.[4]

Scheme II



Although the glycine residues would have been the most desirable C-terminal amino acids for fragment condensation,[5] the scarcity and irregular distribution of those residues demanded that the selection of fragments be guided by considerations of synthetic expediency. Initially, the preparation of the C-terminal fragment S_{14-27} was also attempted by the condensation of the two appropriate fragments: S_{14-22} and S_{23-27}. However, the lack of success in preparing the necessary hydrazide intermediate of fragment S_{14-22}, and the relative ease with which the desired tetradecapeptide amide S_{14-27} can be obtained by the stepwise procedure,[6] prompted us to abandon this particular fragment condensation.

The presence of C-terminal leucine and serine residues in fragments S_{9-13} and S_{5-8}, respectively, pointed toward the selection of the azide method of coupling to avoid the possibility of racemization. In the case of fragment S_{1-4} the C-terminal glycine residue afforded more latitude in the selection of the coupling procedure. Nevertheless, even in this case, the azide procedure turned out to be the method of choice for reasons that will be dealt with later on.

The hydrazide of fragment S_{9-13} was obtained by two different pathways. In the conventional approach the hydrazide was prepared by hydrazinolysis of the partially protected pentapeptide methyl ester. The other route utilized the principle of the "protected hydrazides" that was introduced by Hofmann in 1950.[7,8] Both procedures, which are schematically described in Scheme III, gave essentially the same material in comparable yields. Analytical data of the product obtained by hydrazinolysis of the methyl ester indicate the presence of a slight amount of hydrazine, possibly bound as a hydrazinium salt by the carboxyl of the glutamic acid residue. Eventually, both materials were obtained in crystalline form from methanol.

The synthesis of the hydrazide of fragment S_{5-8} is described in Scheme IV. In this synthesis, as in those of the other fragments, the stepwise approach from the C-terminal amino acid[9] was followed, and active esters[10] were used for practically all the coupling steps.

Two derivatives of the fragment S_{1-4} with different degrees of protection were prepared (Scheme V). In both of them the carboxyl group of the glycine residue was free, and the β-carboxyl of the aspartyl residue was esterified with benzyl alcohol. Attempts to couple these tetrapeptide derivatives to the rest of the secretin molecule by the mixed anhydride,[11] the 5-

(1) To whom all inquiries should be addressed.

(2) Paper II of this series: M. Bodanszky, M. A. Ondetti, S. D. Levine, and N. J. Williams, *J. Am. Chem. Soc.*, **89**, 6753 (1967).

(3) The sequence of Scheme I was presented by V. Mutt and J. E. Jorpes at the 4th International Symposium on the Chemistry of Natural Products, Stockholm, 1966; *cf.* also *Recent Progr. Hormone Res.*, **23**, 483 (1967).

(4) The abbreviations used in this and other figures are as follows: TFA, trifluoroacetic acid; OMe, methyl ester; ONP, *p*-nitrophenyl ester; ODNP, 2,4-dinitrophenyl ester; BZL, benzyl; Z, benzyloxycarbonyl; OBu^t, *t*-butyl ester; BOC, *t*-butyloxycarbonyl.

(5) M. Bodanszky and M. A. Ondetti, "Peptide Synthesis," John Wiley and Sons, Inc., New York, N. Y., 1966, p 161.

(6) M. Bodanszky and N. J. Williams, *J. Am. Chem. Soc.*, **89**, 685 (1967).

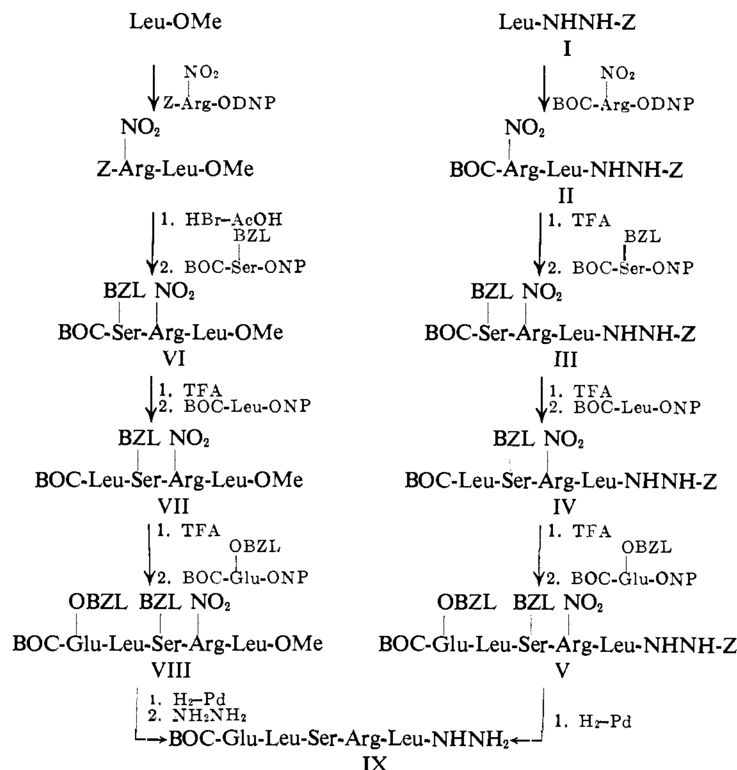
(7) K. Hofmann, M. Z. Magee, and A. Lindenmann, *ibid.*, **72**, 2814 (1950).

(8) K. Hofmann, A. Lindenmann, M. Z. Magee, and N. H. Khan, *ibid.*, **74**, 470 (1952).

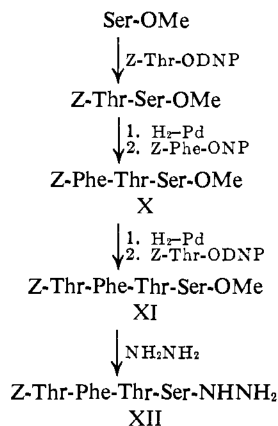
(9) M. Bondanszky, *Ann. N. Y. Acad. Sci.*, **88**, 655 (1960).

(10) M. Bondanszky, *Nature*, **175**, 685 (1955).

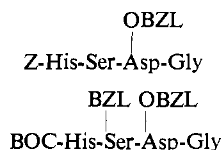
Scheme III



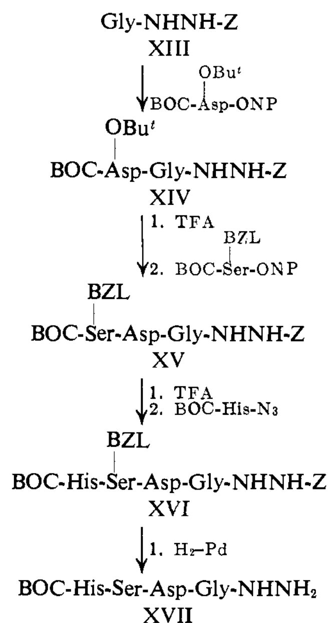
Scheme IV



Scheme V



Scheme VI



phenylisoxazolium-3′-sulfonate,[12] or the N,N′-carbonyldiimidazol[13] procedure, led to complicated mixtures that required extensive purification. Some of the side products seemed to arise from the elimination of the alcohol group of the side-chain protection of the aspartyl residue with the concomitant formation of cyclic succinimide derivatives.[14] When N,N′ carbonyldiimidazol was used as the coupling reagent, partial O-acylation of either the serine or threonine residues was also observed. To eliminate some of these side reactions we designed a derivative of fragment S_{1-4} which would allow the coupling of the glycine carboxyl while maintaining the carboxyl of the aspartyl side chain unaffected. The synthesis of this intermediate is described in Scheme VI.

In all the fragment condensations by the azide procedure, the technique described by Medzihradszky[15]

(11) Th. Wieland and H. Bernhard, *Ann.*, **572**, 190 (1951). R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951); J. R. Vaughan, Jr., and R. L. Osato, *J. Am. Chem. Soc.*, **74**, 676 (1952).

(12) R. B. Woodward, R. A. Olofson, and H. Mayer, *ibid.*, **83**, 1010 (1961).

(13) H. A. Staab, *Ann.*, **609**, 75 (1957); G. W. Anderson and R. Paul, *J. Am. Chem. Soc.*, **80**, 4423 (1958); **82**, 4596 (1960).

(14) A. R. Battersby and J. C. Robinson, *J. Chem. Soc.*, 259 (1955); E. Sondheimer and R. W. Holley, *J. Am. Chem. Soc.*, **76**, 2467 (1954); S. A. Bernhard, A. Berger, J. H. Carter, E. Katchalski, E. Sela, and Y. Shalitin, *ibid.*, **84**, 2421 (1962); G. Fölsch, *Acta Chem. Scand.*, **20**, 459, (1966).

(15) K. Medzihradszky, Communication at the Third European Peptide Symposium, Basle, 1960; *cf.* also M. Zaoral, *Collection Czech. Chem. Commun.*, **30**, 1853 (1965).

was employed. The azide intermediates were allowed to react *in situ* with the amino component, and the extent of the reaction was followed by spotting the reacting mixture on paper which was then sprayed with ninhydrin reagent. An excess of the acylating fragment was employed, which was added in several portions at regular intervals.

The coupling of fragments S_{9-13} and S_{14-27} was accomplished using either the partially protected or the completely unprotected form of the latter. In the first case the excess of S_{9-13} can be easily removed by washing the product with water after removal of the *t*-butyloxycarbonyl-protecting group. In the second case, this excess can be removed by ion exchange or countercurrent distribution. In the coupling of fragments S_{5-8} and S_{9-27} the unprotected form of the latter was employed, and the desired tricosapeptide amide (S_{5-27}) was isolated by countercurrent distribution after removal of the benzyloxycarbonyl protection by hydrogenolysis. The possibility of using, in the coupling step, the unprotected form of the larger molecular weight freagments permitted a more thorough purification of these intermediates, whenever needed.

After the coupling of fragments S_{1-4} and S_{5-27} and removal of protecting groups, the heptacosapeptide amide (secretin) was isolated by countercurrent distribution in the 1-butanol–0.1 *M* phosphate buffer system,[16] where it has the same distribution coefficient as natural porcine secretin. Chromatographic and electrophoretic behavior of the two materials are also identical. The synthetic heptacosapeptide amide possesses the same potency as natural porcine secretin in stimulating the pancreatic bicarbonate secretion in the cat[17] and in the dog.[18] This potency amounts to approximately 4000 clinical units per milligram. These results confirm those obtained by the stepwise approach[3] and support the correctness of the amino acid sequence of porcine secretin proposed by Mutt and Jorpes.[4]

Experimental Section[19]

1. Synthesis of S_{9-13}. L-Leucine Benzyloxycarbonylhydrazide Trifluoroacetate (I). Dicyclohexylcarbodiimide (2.06 g, 10 mmol) was added to an ice-cold stirred solution of *t*-butyloxycarbonyl-L-leucine hydrate (2.5 g, 10 mmol) and benzyloxycarbonyl hydrazide[8] (1.66 g, 10 mmol) in dichloromethane (25 ml). The mixture was stirred for 1 hr in the ice bath and 5 hr at room temperature. The precipitate of dicyclohexylurea was filtered off, and the filtrate was concentrated to dryness *in vacuo*. The oily residue was redissolved in ethyl acetate (150 ml), and the solution was washed once with 20% citric acid, once with water, twice with saturated sodium bicarbonate, and three times with water. The organic phase was dried ($MgSO_4$), and the solvents were removed *in vacuo*. The residue (3.7 g) of *t*-butyloxycarbonyl-L-leucine benzyloxycarbonylhydrazide was dissolved in ice-cold trifluoroacetic acid (8 ml) and the solution kept for 15 min at room temperature. Most of the trifluoroacetic acid was removed *in vacuo*, and the residue was crystallized from ether (10 ml)–hexane (300 ml), yield: 3.65 g (93%); mp 181–183°; $[\alpha]^{24}D$ −23.7° (*c* 1.1, dimethylformamide).

Anal. Calcd for $C_{16}H_{22}F_3N_3O_5$: C, 49.0; H, 5.6; F, 14.5; N, 10.7. Found: C, 48.4; H, 5.5; F, 14.3; N, 10.6.

***t*-Butyloxycarbonylnitro-L-arginyl-L-leucine Benzyloxycarbonylhydrazide (II).** *t*-Butyloxycarbonylnitro-L-arginine 2,4-dinitrophenyl ester[3] (7.15 g, 14.4 mmol) was added to an ice-cold solution of L-leucine benzyloxycarbonylhydrazide trifluoroacetate (4.75 g, 12 mmol) and triethylamine (1.68 ml, 12 mmol) in tetrahydrofuran (24 ml). Two more portions of the dinitrophenyl ester (0.7 g each) were added at 1-hr intervals. The mixture was kept at room temperature overnight and then dimethylaminopropylamine[20] (0.75 ml) was added. After 1 hr, the reaction mixture was diluted with ethyl acetate (200 ml), and the resulting solution was washed once with 20% citric acid, once with water, twice with 0.5 *N* ammonium hydroxide, and four times with water. The organic layer was dried ($MgSO_4$), and the solvents were evaporated *in vacuo*. The oily residue was triturated under ether until it became solid. This crude product was crystallized from an ethyl acetate–ether mixture (1:9), yield: 6.15 g (88%); mp (softens 120°) 124–128°; $[\alpha]^{24}D$ −40.2° (*c* 1, methanol).

Anal. Calcd for $C_{25}H_{40}N_8O_8$: C, 51.7; H, 6.9; N, 19.3. Found: C, 51.5; H, 7.2; N, 19.2.

***t*-Butyloxycarbonyl-O-benzyl-L-serylnitro-L-arginyl-L-leucine Benzyloxycarbonylhydrazide (III).** *t*-Butyloxycarbonylnitro-L-arginyl-L-leucine benzyloxycarbonylhydrazide (7 g, 12 mmol) was dissolved in ice-cold trifluoroacetic acid (25 ml), and the solution was kept at room temperature for 15 min. The trifluoroacetic acid was removed *in vacuo* at room temperature, and the residue was triturated with ether. The solid was filtered, washed with ether, and dried *in vacuo* (KOH). This trifluoroacetate was dissolved in ice-cold dimethylformamide (36 ml), neutralized with triethylamine (1.68 ml, 12 mmol) and allowed to react with *t*-butyloxycarbonyl-O-benzyl-L-serine *p*-nitrophenyl ester[7] (prepared from 4.4 g (15 mmol) of *t*-butyloxycarbonyl-O-benzyl-L-serine). After standing at room temperature overnight, the reaction mixture was diluted with ethyl acetate (300 ml) and washed once with 20% citric acid and twice with water. The organic phase was dried ($MgSO_4$), the solvent was removed *in vacuo*, and the residue was crystallized from ethyl acetate, yield: 6.3 g (70%); mp 115–118°; $[\alpha]^{24}D$ −37.7° (*c* 1, methanol).

Anal. Calcd for $C_{35}H_{51}N_9O_{10}$: C, 55.5; H, 6.8; N, 16.6. Found: C, 55.3; H, 7.2; N, 16.5.

***t*-Butyloxycarbonyl-L-leucine-O-benzyl-L-serylnitro-L-arginyl-L-leucine benzyloxycarbonylhydrazide (IV)** was prepared in the manner described for III and crystallized from absolute ethanol, yield: 82%; mp 202–203°; $[\alpha]^{24}D$ −21° (*c* 1.4, dimethylformamide).

Anal. Calcd for $C_{41}H_{62}N_{10}O_{11}$: C, 56.6; H, 7.2; N, 16.1. Found: C, 55.9; H, 7.1; N, 16.1.

***t*-Butyloxycarbonyl-γ-benzyl-L-glutamyl-L-leucyl-O-benzyl-L-serylnitro-L-arginyl-L-leucine benzyloxycarbonylhydrazide (V)** was prepared by essentially the same procedure described for III, and crystallized from methanol, yield: 84%; mp 220–221°; $[\alpha]^{24}D$, −19.3° (*c* 1.3, dimethylformamide).

Anal. Calcd for $C_{53}H_{75}N_{11}O_{14}$: C, 58.4; H, 6.9; N, 14.1. Found: C, 58.8; H, 7.1; N, 14.5.

***t*-Butyloxycarbonyl-O-benzyl-L-serylnitro-L-arginyl-L-leucine methyl ester (VI)** was prepared by essentially the same procedure described for II starting from nitro-L-arginyl-L-leucine methyl ester hydrobromide.[21] It was obtained as an amorphous solid from ether–hexane, yield: 83%; $[\alpha]^{24}D$ −25.5° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{28}H_{45}N_7O_9$: C, 53.9; H, 7.3; N, 15.7. Found: C, 54.1; H, 7.7; N, 15.4.

***t*-Butyloxycarbonyl-L-leucyl-O-benzyl-L-serylnitro-L-arginyl-L-leucine methyl ester (VII)** was prepared by essentially the same procedure described for II, and crystallized from absolute ethanol, yield: 80%; mp 169–170°; $[\alpha]^{24}D$ −32° (*c* 1.2, methanol).

Anal. Calcd for $C_{34}H_{56}N_8O_{10}$: C, 55.4; H, 7.7; N, 15.2. Found: C, 55.3; H, 7.9; N, 15.0.

***t*-Butyloxycarbonyl-γ-benzyl-L-glutamyl-L-leucyl-O-benzyl-L-serylnitro-L-arginyl-L-leucine methyl ester (VIII)** was prepared by essentially the same procedure described for II, and crystallized from

(16) J. E. Jorpes and V. Mutt, *Acta Chem. Scand.*, **15**, 1790 (1961).

(17) This comparison was carried out by Professor J. E. Jorpes and Dr. V. Mutt (Karolinska Institute, Stockholm).

(18) This comparison was carried out by Dr. M. I. Grossman (Veterans Administration Center, Los Angeles, Calif.).

(19) Melting points were taken in capillary tubes and are uncorrected. Chromatographies were carried out on a thin layer of cellulose (Eastman Chromagram Sheet) by the ascending technique with the following solvent systems: R_f^1, 1-butanol–pyridine–acetic acid–water (30:20:6:24); R_f^2, 1-butanol–acetic acid–water (4:1:5); R_f^3, 2-butanol–3% ammonia (3:1); R_f^4, methyl ethyl ketone–pyridine–water (40:10:16). R_f values are expressed as multiples of the distance traveled by an arginine marker. Paper electrophoreses were carried out according to the technique described by L. N. Werum, H. T. Gordon, and W. Thornburg, *J. Chromatog.*, **3**, 125 (1960). Quantitative amino acid analyses were carried out in a modified Technicon Autoanalizer.

(20) M. Löw and L. Kisfaludy, *Acta. Chim. Acad. Sci. Hung.*, **44**, 61 (1965).

(21) Obtained by deprotection of Z-Arg(NO_2)-Leu-OMe with HBr-AcOH; *cf.* H. Gibian and E. Schröder, *Ann.*, **642**, 145 (1961).

absolute ethanol, yield: 79%; mp 184–186°; $[\alpha]^{24}D$ −30° (*c* 1.1, methanol).

Anal. Calcd for $C_{46}H_{69}N_9O_{18}$: C, 57.8; H, 7.3; N, 13.2. Found: C, 57.6; H, 7.6; N, 13.1.

***t*-Butyloxycarbonyl-L-glutamyl-L-leucyl-L-seryl-L-arginyl-L-leucine Hydrazide (IX).** A. Palladium on charcoal (10%, 100 mg) was added to a suspension of V (0.5 g) in a mixture of methanol–acetic acid–water (2:1:1). The suspension was stirred under a hydrogen atmosphere for 48 hr. The catalyst was filtered, and the filtrate was concentrated *in vacuo*. The oily residue was triturated with ethyl acetate until a solid was obtained, yield: 339 mg (100%); $[\alpha]^{24}D$ −27.5° (*c* 2, dimethylformamide).

Anal. Calcd for $C_{31}H_{58}N_{10}O_{10}$: C, 50.9; H, 8.0; N, 19.2; hydrazide N, 3.8.[22] Found: C, 51.3; H, 8.1; N, 18.9; hydrazide N, 3.7.

B. A solution of VIII (5 g) in 80% aqueous acetic acid (75 ml) was hydrogenated for 24 hr with 10% palladium on charcoal (500 mg) as catalyst. Another portion of fresh catalyst (500 mg) was added and the hydrogenation continued for 24 hr more. The catalyst was filtered off, and the filtrate was freeze dried. The oily residue was triturated under ethyl acetate, filtered, and dried. This material was dissolved in a 5% solution of anhydrous hydrazine in methanol (200 ml). The solution was stored at room temperature for 3 hr and concentrated to *ca.* 80 ml, and ethyl acetate was added (400 ml). The precipitate was filtered, washed with ethyl acetate, and dried, yield: 3.62 g (95%); $[\alpha]^{24}D$ −26.2° (*c* 2, dimethylformamide).

Anal. Calcd for $C_{31}H_{58}N_{10}O_{10} \cdot 2H_2O$: C, 48.5; H, 8.1; N, 18.3; hydrazide N, 3.7. Found: C, 47.5; H, 7.9; N, 18.9; hydrazide N, 4.4.

The materials obtained by procedures A and B behaved identically on tlc (silica gel; methanol–chloroform, 8:2; R_f 0.29). They were used without any further treatment in the coupling steps. However, they can be obtained in crystalline form, as a dihydrate, from methanol; mp (softens at 178°) 191–193°; $[\alpha]^{24}D$ −26.7° (*c* 2, dimethylformamide).

2. Synthesis of S_{5-8}. Benzyloxycarbonyl-L-phenylalanyl-L-threonyl-L-serine Methyl Ester (X). Palladium on charcoal (10%, 2.1 g) was added to a solution of benzyloxycarbonyl-L-threonyl-L-serine methyl ester[23] (21 g, 60 mmol) in a mixture of absolute ethanol (200 ml) and 1 *N* hydrochloric acid (60 ml). The suspension was stirred under a hydrogen atmosphere for 7 hr. The catalyst was filtered, and the filtrate was concentrated *in vacuo*. The oily residue was dissolved in dimethylformamide (300 ml) containing triethylamine (8.4 ml, 60 mmol) and allowed to react with benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (29 g, 72 mmol). The mixture was stored at room temperature for 6 hr. The solvents were removed *in vacuo*, and the residue was digested with ethyl acetate. The crystalline solid was collected, dried, and recrystallized from methanol–water (2:1), yield: 16.5 g (55%); mp 182–184°; $[\alpha]^{24}D$ −6.8° (*c* 0.84, dimethylformamide).

Anal. Calcd for $C_{25}H_{31}N_3O_8$: C, 59.9; H, 6.2; N, 8.4. Found: C, 59.7; H, 6.1; N, 8.3.

Benzyloxycarbonyl-L-threonyl-L-phenylalanyl-L-threonyl-L-serine Methyl Ester (XI). To a solution of L-phenylalanyl-L-threonyl-L-serine methyl ester hydrochloride [prepared by hydrogenolysis of 15 g of X (30 mmol)] in dimethylformamide (100 ml) containing triethylamine (5.04 ml, 36 mmol) benzyloxycarbonyl-L-threonine 2,4-dinitrophenyl ester[24] (15 g, 36 mmol) was added. The mixture was kept at room temperature overnight. After removing the solvent *in vacuo* the residue was digested with ethyl acetate. The crystalline solid was collected, dried, and recrystallized from 95% ethanol, yield: 15.4 g (85%); mp 198–200°; $[\alpha]^{25}D$ −10.4° (*c* 0.6, dimethylformamide).

Anal. Calcd for $C_{29}H_{38}N_4O_{10} \cdot H_2O$: C, 56.1; H, 6.5; N, 9.0; OMe, 5.0. Found: C, 55.7; H, 6.4; N, 9.1; OMe, 5.2.

Benzyloxycarbonyl-L-threonyl-L-phenylalanyl-L-threonyl-L-serine Hydrazide (XII). Hydrazine hydrate (1.7 ml) was added to a solution of benzyloxycarbonyl-L-threonyl-L-phenylalanyl-L-threonyl-L-serine methyl ester (2.1 g, 3.5 mmol) in methanol (170 ml). The mixture was stored at room temperature for 20 hr, and the precipitate formed was collected by filtration, dried, and crystallized from methanol, yield: 1.56 g (74%); mp (softens 205°) 234–235°; $[\alpha]^{24}D$ +1.4° (*c* 1, dimethyl sulfoxide).

Anal. Calcd for $C_{28}H_{38}N_6O_9$: C, 55.8; H, 6.4; N, 13.9; hydrazide N, 4.7. Found: C, 56.3; H, 6.3; N, 14.4; hydrazide N, 5.3.

3. Synthesis of S_{1-4}. Glycine benzyloxycarbonylhydrazide trifluoroacetate (XIII) was prepared by the procedure described for I and crystallized from ether–hexane, yield: 90%; mp 174–175°.

Anal. Calcd for $C_{12}H_{14}N_3F_3O_5$: C, 42.7; H, 4.2; N, 12.4. Found: C, 43.2; H, 4.7; N, 12.2.

***t*-Butyloxycarbonyl-β-*t*-butyl-L-aspartylglycine Benzyloxycarbonylhydrazide (XIV).** Triethylamine (1.54 ml, 11 mmol) and *t*-butyloxycarbonyl-β-*t*-butyl-L-aspartic acid *p*-nitrophenyl ester[25] (3.69 g, 0.9 mmol) were added, in that order, to an ice-cold solution of glycine benzyloxycarbonylhydrazide trifluoroacetate (3.7 g, 11 mmol) in dimethylformamide (18 ml). The mixture was kept at room temperature for 4 hr, diluted with ethyl acetate (200 ml), and washed once with 20% citric acid and four times with water. The organic layer was dried ($MgSO_4$), and the solvent was removed *in vacuo*. The residue was freeze dried from benzene, yield: 5.4 g. This material was used in the next step without further treatment. Countercurrent distribution of a small sample in the system toluene–chloroform–methanol–water (5:5:8:2:) showed one major peak (*K* 0.8) corresponding to the protected dipeptide hydrazide [$[\alpha]^{24}D$ −18.7° (*c* 1.2, dimethylformamide)], and a small peak (*K* 1.5) of *p*-nitrophenol.

Anal. Calcd for $C_{23}H_{34}N_4O_8$: C, 55.8; H, 6.9; N, 11.3. Found: C, 55.3; H, 7.2; N, 11.3.

***t*-Butyloxycarbonyl-O-benzyl-L-seryl-L-aspartylglycine Benzyloxycarbonylhydrazide (XV).** *t*-Butyloxycarbonyl-β-*t*-butyl-L-aspartylglycine benzyloxycarbonylhydrazide (10.8 g) was dissolved in ice-cold trifluoroacetic acid (50 ml), and the solution was kept at room temperature for 1 hr. The trifluoroacetic acid was removed *in vacuo* at room temperature and the residue was triturated under ether. The solid was filtered, washed with ether, and dried *in vacuo* K(OH), yield: 7.1 g (16 mmol). This trifluoroacetate was dissolved in ice-cold dimethylformamide, neutralized with triethylamine (4.62 ml, 33 mmol) and allowed to react with *t*-butyloxycarbonyl-O-benzyl-L-serine *p*-nitrophenyl ester [prepared from 7.1 g (24 mmol) of *t*-butyloxycarbonyl-O-benzyl-L-serine]. The reaction mixture was kept at room temperature for 2 hr, diluted with ethyl acetate (400 ml), and extracted once with 20% citric acid and three times with water. The organic layer was dried ($MgSO_4$), and the solvent was removed *in vacuo*. The residue was crystallized from ethyl acetate, yield: 7.7 g (80%); mp 83–84°; $[\alpha]^{24}D$ −12.8° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{29}H_{37}N_5O_{10}$: C, 56.6; H, 6.1; N, 11.4. Found: C, 56.4; H, 6.1; N, 11.1.

***t*-Butyloxycarbonyl-L-histidyl-O-benzyl-L-seryl-L-aspartylglycine Benzyloxycarbonylhydrazide (XVI).** Sodium nitrite (840 mg, 12 mmol) was added to a solution of *t*-butyloxycarbonyl-L-histidine hydrazide[26] (3.22 g, 12 mmol) in a mixture of ethyl acetate (48 ml) and 1 *N* hydrochloric acid (36 ml), while stirring in an ice–salt bath. After 3 min, an aqueous (50%) solution of potassium carbonate (9.6 ml) was added, and after another 3 min the stirring was interrupted, and the two phases were allowed to separate. The aqueous phase was extracted with a second portion of ethyl acetate (12 ml) and discarded. The combined organic phases were pooled and dried at 0° with magnesium sulfate.

This ethyl acetate solution of *t*-butyloxycarbonyl-L-histidine azide was added to an ice-cold solution of O-benzyl-L-seryl-L-aspartylglycine benzyloxycarbonylhydrazide trifluoroacetate [prepared from 5.0 g of XV (8 mmol)] in a mixture of dimethylformamide (24 ml) and triethylamine (2.24 ml). The reaction mixture was kept at 5°. After 24 hr another portion of *t*-butyloxycarbonyl-L-histidine azide (prepared from 1.07 g of the corresponding hydrazide) was added. Twenty-four hours after this addition, the reaction mixture was concentrated to dryness *in vacuo*, and the residue was crystallized twice from 50% aqueous ethanol, yield: 4.4 g (70%); mp 174–176°; $[\alpha]^{24}D$ −12.1° (*c* 1.1, dimethylformamide).

Anal. Calcd for $C_{35}H_{44}N_8O_{11} \cdot 2H_2O$: C, 53.3; H, 6.1; N, 14.2. Found: C, 53.4; H, 5.9; N, 14.5.

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(25) Prepared according to the general procedure described in M. J. Coon, Ed., "Biochemical Preparations," Vol. 9, John Wiley and Sons, Inc., New York, N. Y., 1962, p 110: mp 77–79°; $[\alpha]^{22}D$ −42.5° (*c* 2, dimethylformamide containing 1% acetic acid).
(26) E. Schröder and H. Gibian, *Ann.*, **656**, 190 (1962).

t-Butyloxycarbonyl-L-histidyl-L-seryl-L-aspartylglycine Hydrazide (XVII). t-Butyloxycarbonyl-L-histyl-O-benzyl-L-seryl-L-aspartylglycine benzyloxycarbonyl hydrazide (900 mg) was dissolved in a mixture of methanol–acetic acid–water (2:1:1) (90 ml) and hydrogenated for 5 hr over 10% palladium on charcoal (300 mg). The catalyst was filtered, and the filtrate was concentrated to dryness *in vacuo*. The residue was triturated with ethyl acetate, filtered, and dried, yield: 650 mg (100%); $[\alpha]^{24}D$ −18.8° (*c* 1.1, dimethylformamide).

Anal. Calcd for $C_{20}H_{32}N_8O_9 \cdot 1.5H_2O$: C, 43.2; H, 6.3; N, 20.2; hydrazide N, 5.0. Found: C, 43.7; H, 6.3; N, 19.6; hydrazide N, 5.0.

4. **Synthesis of S_{9-27} ($S_{9-13} + S_{11-27}$). L-Glutamyl-L-leucyl-L-seryl-L-arginyl-L-leucylnitro-L-arginyl-β-benzyl-L-aspartyl-O-benzyl-L-seryl-L-alanylnitro-L-arginyl-L-leucyl-L-glutaminylnitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valine Amide Trifluoroacetate (XVIII).** Concentrated hydrochloric acid (0.9 ml) was added to a solution of t-butyloxycarbonyl-L-glutamyl-L-leucyl-L-seryl-L-arginyl-L-leucine hydrazide (1.35 g, 1.8 mmol) in dimethylformamide (13.5 ml) cooled in a Dry Ice–acetone bath at −20°. The temperature of the bath was allowed to rise to −15°, and an aqueous 14% solution of sodium nitrite (1.35 ml, 2.7 mmol) was added. After 5 min, the bath temperature was lowered to −25° and N-ethylpiperidine was added (1.25 ml). To this mixture containing the t-butyloxycarbonyl pentapeptide azide, a solution of nitro-L-arginyl-β-benzyl-L-aspartyl-O-benzyl-L-seryl-L-alanylnitro-L-arginyl-L-leucyl-L-glutaminylnitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucylvaline amide trifluoroacetate[6] (2.8 g, 1.4 mmol) in dimethylformamide (13.5 ml) was added. The reaction mixture was stored at 5° and after 48 hr another portion of t-butyloxycarbonyl pentapeptide azide (prepared from 150 mg of the corresponding hydrazide) was added. After a total of 4 days the solvents were removed *in vacuo*, and the residue was disintegrated with ethyl acetate. The solid was collected by filtration and dried. The material thus obtained was dissolved in cold trifluoroacetic acid (30 ml) and the solution kept at room temperature for 15 min. The trifluoroacetic acid was removed *in vacuo* and the residue was disintegrated with ether, filtered, and dried. This trifluoroacetate was washed twice with water (30 ml) to remove salts and excess pentapeptide. The insoluble material was collected by centrifugation and dried *in vacuo* (KOH), yield: 2.9 g (80%).

Anal. Calcd for $C_{109}H_{180}N_{37}O_{33} \cdot CF_3COOH$: C, 50.3; H, 6.9; N, 19.5; F, 2.1. Calcd for $C_{109}H_{180}N_{37}O_{33} \cdot 0.5CF_3COOH$: C, 50.9; H, 7.0; N, 20.0; F, 1.1. Found: C, 51.0; H, 7.6; N, 20.3; F, 1.3.

L-Glutamyl-L-leucyl-L-seryl-L-arginyl-L-leucyl-L-arginyl-L-aspartyl-L-seryl-L-alanyl-L-arginyl-L-leucyl-L-glutaminyl-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valine Amide (XIX). A solution of the partially protected nonadecapeptide amide (1.4 g) in aqueous 80% acetic acid (50 ml) was hydrogenated over 10% palladium on charcoal for 48 hr. The catalyst was removed by filtration, and the filtrate was freeze dried, yield: 1.25 g. This material was used without any further purification in the following step. Countercurrent distribution in the system 1-butanol–pyridine–acetic acid–water (4:2:1:7) shows a major peak (*K* 1.3) and two other minor peaks which were not investigated. Ion-exchange chromatography on carboxymethyl cellulose shows also one major peak. Chromatography: single-spot ninhydrin and Sakaguchi positive; R_f^1 2.4 × Arg: R_f^2 2.5 × Arg; R_f^3 2.8 × Arg; R_f^4 6.6 × Arg. Paper electrophoresis: single-spot ninhydrin and Sakaguchi positive at pH's 3.3 and 4.7; $[\alpha]^{24}D$ −56.3° (*c* 1.0, 1 *N* acetic acid). Amino acid analysis after acid hydrolysis gave the following molar ratios: Asp (1.03); Ser (1.65); Glu (2.90); Gly (1.00); Ala (1.00); Val (1.00); Leu (5.90); Arg (3.95). Amino acid molar ratios in the leucine aminopeptidase (LAP) digest:[27] Asp (0.90); Ser (2.04); Glu (1.13); Gly (1.20); Ala (0.95); Val (0.96); Leu (6.35); Arg (3.60).

5. **Synthesis of S_{5-27} ($S_{5-8} + S_{9-27}$). L-Threonyl-L-phenylalanyl-L-threonyl-L-seryl-L-glutamyl-L-leucyl-L-seryl-L-arginyl-L-leucyl-L-arginyl-L-aspartyl-L-seryl-L-alanyl-L-arginyl-L-leucyl-L-glutaminyl-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valine Amide (XX).** Aqueous 14% sodium nitrite (0.45 ml, 0.9 mmol) was added to a solution of benzyloxycarbonyl-L-threonyl-L-phenylalanyl-L-threonyl-L-serine hydrazide (450 mg, 0.75 mmol) in dimethylformamide (5.6 ml) containing concentrated hydrochloric acid (0.37 ml) cooled in a Dry Ice–acetone bath at −15°. After 15 min the temperature of the bath was lowered to −25°, and N-ethylpiperidine (0.53 ml) was added. To this mixture, containing the benzyloxycarbonyl tetrapeptide azide, a solution of the free nonadecapeptide amide XIX (1.2 g, 0.5 mmol) in dimethylformamide (3.3 ml) and water (1 ml) was added. The reaction mixture was stored at 5°. A second portion of benzyloxycarbonyl tetrapeptide azide (prepared from 90 mg of the corresponding hydrazide) was added after 24 hr. After a total of 48 hr, the solvents were removed *in vacuo*, and the residue was dissolved in aqueous 90% acetic acid (70 ml) and hydrogenated over 10% palladium on charcoal (500 mg) for 7 hr. The catalyst was removed by filtration and the filtrate was freeze dried. This lyophillizate was distributed for 250 transfers in the system: 1-butanol–pyridine–acetic acid–water (4:2:1:7). Only one major peak (*K* 3.7) was observed, yield: 740 mg (50%), $[\alpha]^{24}D$ −48.3° (*c* 1, 1 *N* acetic acid). Chromatography: single-spot ninhydrin and Sakaguchi positive; R_f^1 2.6 × Arg; R_f^2 2.8 × Arg; R_f^3 4 × Arg; R_f^4 9.1 × Arg. Paper electrophoresis: single-spot ninhydrin and Sakaguchi positive at pH's 3.3 and 4.7.

Quantitative amino acid analysis after acid hydrolysis gave the following molar ratios: Asp (1.10); Thr (2.00); Ser (2.60); Glu (3.00); Gly (1.06); Ala (1.00); Val (1.00); Leu (6.10); Phe (1.08); Arg (4.00). Quantitative amino acid analysis after digestion with aminopeptidase M[28] gave the following molar ratios: Asp (1.01); Thr (2.01); Ser (2.70); Glu (1.00); Gly (1.02); Ala (0.93); Val (1.01); Leu (6.10); Phe (0.95); Arg (3.60).

6. **Synthesis of S_{1-27} ($S_{1-4} + S_{5-27}$). L-Histidyl-L-seryl-L-aspartylglycyl-L-threonyl-L-phenylalanyl-L-threonyl-L-seryl-L-glutamyl-L-leucyl-L-seryl-L-arginyl-L-leucyl-L-arginyl-L-aspartyl-L-seryl-L-alanyl-L-arginyl-L-leucyl-L-glutaminyl-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valine Amide (XXI).** Concentrated hydrochloric acid (0.09 ml) was added to a stirred solution of t-butyloxycarbonyl-L-histidyl-L-seryl-L-aspartylglycine hydrazide (96 mg, 0.18 mmol) in dimethylformamide (1.5 ml) cooled in a Dry Ice–acetone bath at −20°. The temperature of the bath was allowed to rise to −15°, and an aqueous 14% solution of sodium nitrite (0.15 ml, 0.3 mmol) was added. After 5 min the temperature of the bath was lowered to −25°, and N-ethylpiperidine (0.13 ml) was added. To this mixture, containing the t-butyloxycarbonyl tetrapeptide azide, a solution of the free tricosapeptide amide XX (168 mg, 0.06 mmol) in dimethylformamide (2.1 ml) was added. The reaction mixture was stored at 5°. A second portion of t-butyloxycarbonyl tetrapeptide azide (prepared from 32 mg of the corresponding hydrazide) was added after 24 hr. After a total of 48 hr, the solvents were removed *in vacuo*, and the residue was dissolved in cold trifluoroacetic acid (6 ml). The solution was kept at room temperature for 15 min, and the heptacosapeptide trifluoroacetate was precipitated with ether (100 ml). The solid was collected by centrifugation, washed with ether and dried, yield: 330 mg. This material was distributed for 200 transfers in the system 1-butanol–0.1 *M* phosphate buffer pH 7 (1:1). The desired heptacosapeptide amide was found in a clearly separated peak of *K* 0.74, and it was recovered from the two-phase system by the alginic acid adsorption procedure,[29] yield: 62 mg (33%); $[\alpha]^{24}D$ −61.6° (*c* 0.25, 1 *N* acetic acid). Chromatography: single-spot ninhydrin, Sakaguchi, and Pauly positive; R_f^1 2.1 × Arg; R_f^2 2.3 × Arg; R_f^4 5.2 × Arg. Paper electrophoresis: single-spot ninhydrin, Sakaguchi, and Pauly positive at pH's 3.3 and 4.7. The same mobilities were observed with natural secretin and the synthetic heptacosapeptide amide obtained by the stepwise approach.[2]

Quantitative amino acid analysis of an acid hydrolyzate gave the following molar ratio of amino acids: Asp (2.0); Thr (1.84); Ser (3.70); Glu (3.13); Gly (2.05); Ala (0.94); Val (1.04); Leu (6.20); Phe (0.97); His (0.94); Arg (4.05). Quantitative amino acid analysis of a trypsin-LAP hydrolyzate[2] gave the following molar ratio: Asp (1.86); Thr (2.03); Ser (3.65); Glu (1.10); Gly (2.05); Ala (1.10); Val (1.10); Leu (5.80); Phe (0.92); His (1.00); Arg (4.00).

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The Structure Determination of Antibiotic Compounds from *Hypericum uliginosum*. I[1]

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Abstract: Structures for two antibiotic compounds, uliginosin A (I) and uliginosin B (II), have been deduced from a detailed study of the nmr, ir, uv, and mass spectra of these substances and of simple derivatives of them. The compounds contain phloroglucinol and filicinic acid residues and are closely related chemically to fern constituents such as aspidin and flavaspidic acid. The structures also resemble that of rottlerin which, like I and II, occurs in a higher plant.

Hypericum uliginosum HBK is a woody herb which is widely distributed in Mexico and Central America. In Mexico, where the plant is used for the treatment of diarrhea, it is called Tzotzil and also "rabbit plant." An investigation of the chemical constituents of the plant was undertaken as part of a general search for natural products with potentially useful biological activity. An extract of the plant showed antibacterial properties and purification of the extract by chromatography and countercurrent distribution led to the isolation of two pale yellow crystalline compounds, both of which showed good activity against gram-positive bacteria.[2] We have undertaken the elucidation of the structures of these antibiotics and have called the higher melting material (161.5°) uliginosin A (I) and the lower melting one (142.0°) uliginosin B (II). The spectra of these compounds indicate that they are closely related, but since the spectra of uliginosin B appeared more tractable, our initial efforts were directed to this substance.

Elemental analysis of uliginosin B was consistent with both $C_{31}H_{38}O_9$ and $C_{28}H_{34}O_8$ but an ebullioscopic molecular weight determination gave a value of 512 ± 25, more in agreement with the latter formula (MW = 498.60) rather than the former (MW = 554.66). Confirmation of this choice was obtained from the high-resolution mass spectrum of II (to be discussed in more detail later) which not only showed a parent ion peak at 498.2261 mass units but also revealed an impurity present to the extent of ~20% and having a molecular weight of 512.2406 ($C_{29}H_{36}O_8$). Efforts to remove this impurity by recrystallization were to no avail. Fortunately, the presence of this substance, once recognized, did not prove too much of a hindrance to the elucidation of the structure of II.

Catalytic reduction of uliginosin B gives rise to a dihydro derivative III ($C_{28}H_{36}O_8$), whereas bromination followed by pyridine dehydrobromination leads to a monobromo compound, $C_{28}H_{33}BrO_8$ (IV). Neither of these compounds could be freed completely from the corresponding derivatives of the impurity present in II. However, they were sufficiently pure for spectroscopic studies. Attempts to acetylate or methylate II did not lead to clean products, and these experiments were abandoned.

The infrared spectrum of II showed broad absorption in the 3-μ region and this, coupled with intense peaks from 1600 to 1650 cm^{-1}, suggested the presence of an enolic 1,3-diketo system or a 2-hydroxyaryl ketone.[3,4] Although II did not give a distinct ferric chloride test (brown precipitate) the formation of a copper chelate confirmed the presence of such a group. It is noteworthy that recrystallization of the chelate followed by regeneration of II by treatment with sodium sulfide did not lead to an improvement in the purity of II.

The nmr spectrum (Figure 1) of uliginosin B proved most informative since it contains several very interesting features that reveal a number of important structural details. First and foremost, the multiplet at 4 ppm is well resolved at 100 Mc and is easily recognized as two overlapping septets, one centered at 4.19 ppm (J = 6.6 cps) and the other at 3.85 ppm (J = 6.6 cps). Although the high-field septet appears the more intense, integration indicates that to all intents and purposes, each is caused by one proton. These multiplets are coupled with superimposed doublets at 1.17 ppm (J = 6.7 cps) which integrate for 12 protons. Taken in conjunction, these features indicate two isopropyl groups. The septets occur in a region that is typical for an isopropoxy group. However, since the latter function rarely occurs in natural products, it seemed more reasonable to regard these peaks as being due to isopropyl ketone moieties even though the >C*H*– of this group generally absorbs at higher fields.[5a,6a]

(1) Paper I in a series of two papers.

(2) The isolation work was carried out by Drs. R. M. Brooker and H. L. Taylor of the Dow Human Health and Development Center, Zionsville, Ind., who will report their procedure and their biological test results in a separate publication.

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