tive tlc, using the same solvent. Removal and extraction (again with chloroform–acetone) of the bands yielded 20.6 mg (0.063 mmole, 16.2%) of hemiacetal **42**, mp 182°, and 31.4 mg (0.095 mmole, 25%) of recovered lactone **41**.

2-(2,3,3a,8a-Tetrahydro-2-acetoxy-4-hydroxy-6-methoxyfuro[2,3-*b*]-benzofuran-5-yl)-5-oxo-1-cyclopentene-1-carboxylic Acid δ-Lactone (43) (Racemic Aflatoxin B Hemiacetal Acetate). To synthetic hemiacetal **42** (21 mg, 0.064 mmole) dissolved in acetic acid (2 ml) and acetic anhydride (1.5 ml) were added several small crystals of *p*-toluenesulfonic acid. After 12 hr at ambient temperatures, the excess reagents were removed *in vacuo* and the product was isolated by preparative tlc. The racemic hemiacetal acetate **43** obtained (17 mg, 0.045 mmole, 70%) had mp 245–246° (from chloroform–ether. Its infrared spectrum was identical with that of **43** obtained from natural aflatoxin B_1 (**1**): ν_{max} ($CHCl_3$) 1760, 1750, 1685 (weak), 1625, 1600, 1555, 1485, 1440, 1380, 1310 cm^{-1}.

Optically Active Aflatoxin B Hemiacetal Acetate (43). A solution of aflatoxin B_1 (**1**) (115 mg, 0.37 mmole) in 10 ml of glacial acetic acid and 1 ml of acetic anhydride was stirred at 25° for 168 hr in the presence of 4 mg of toluenesulfonic acid. Sodium bicarbonate (70 mg) was added and the reaction mixture was concentrated. The resulting solid was washed with water, dried, and recrystallized from chloroform–cyclohexane and then ethyl acetate to afford 98 mg (0.26 mmole, 70%) of aflatoxin B hemiacetal acetate **43**: mp 233–235° (lit.[28] mp 227°); nmr ($CDCl_3$), 1.73 (3 H, s), 2.60 (2 H, t, J = 6) and 3.44 (2 H, t, J = 6, A_2B_2), 2.48 (2 H, d, J = 5), 4.00 (3 H, s), 4.22 (1 H, m), 6.41 (1 H, s), 6.47 (1 H, m), 6.56 (1 H, d, J = 6). The ultraviolet spectrum was identical with that of aflatoxin B_1 and the infrared spectrum in $CHCl_3$ was indistinguishable from that of the racemic acetate **43**.

Racemic Aflatoxin B_1 (1). Racemic aflatoxin B hemiacetal acetate **43** (16 mg, 0.043 mmole) was pyrolyzed at 240° for 15 min under reduced pressure (0.01 mm). The brown residue was applied to preparative tlc plate and the band corresponding to **1** was removed. Extraction of the silica gel with chloroform–methanol yielded 5.3 mg (0.017 mmole, 40%) of racemic aflatoxin B_1 (**1**), mp 255–256°, having infrared, ultraviolet, and mass spectra identical with those of the natural material.

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Synthesis of Secretin. II. The Stepwise Approach[1,2]

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Abstract: A heptacosapeptide amide with the amino acid sequence proposed by Jorpes and Mutt for porcine secretin was synthesized. The stepwise strategy was applied, active esters were used in the acylation reactions, and all the protected intermediates were isolated. After removal of the protecting groups and purification, the synthetic peptide showed the characteristic biological activities of natural (porcine) secretin.

Jorpes, Mutt, and their collaborators isolated porcine secretin in pure form[4] and proposed [5–7] sequence I for its amino acid constituents.

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His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-
 1   2   3   4   5   6   7   8   9   10  11  12  13

          Arg-Asp-Ser-Ala-Arg-Leu-Glu(NH2)-
           14  15  16  17  18  19  20

                  Arg-Leu-Leu-Glu(NH2)-Gly-Leu-Val-NH2
                   21  22  23  24       25  26  27
```

I[7]

The synthesis of a protected tetradecapeptide corresponding to sequence 14–27 has already been described.[1] The present paper reports the continuation of the stepwise synthesis to the completion of the entire chain of I.

Details of the synthesis are summarized in Chart I and here only its more important features are outlined. Nitrophenyl esters[8] were used in all the chain-lengthening steps with the exception of the last acylation, in which both the *p*-nitrophenyl ester of bisbenzyloxycarbonyl-L-histidine and the azide of benzyloxycarbonyl-L-histidine could be applied equally well. For the protection of this single histidine residue the benzyloxycarbonyl protecting group was used; for the masking of all other α-amino functions, the *t*-butoxycarbonyl group[9] was selected, since it could be removed under mild conditions with trifluoroacetic acid after each chain-lengthening step. In this manner the nitro groups on the arginine moieties, the benzyl ester groups on the side-chain carboxyls of aspartic acid and glutamic acid residues, and the benzyl–ether linkages on the serines were not affected and undesired acylation of the alcoholic hydroxyls in the threonine and serine side chains were avoided. The protected intermediates were isolated as solids, several of them in crystalline form, all in excellent yield.

(1) For the first part of the stepwise synthesis, *cf.* M. Bodanszky and N. J. Williams, *J. Am. Chem. Soc.*, **89**, 685 (1967).

(2) The present synthesis was reported in a preliminary form: M. Bodanszky, M. A. Ondetti, S. D. Levine, V. L. Narayanan, M. V. Saltza, J. T. Sheehan, N. J. Williams, and E. F. Sabo, *Chem. Ind.* (London), 1757 (1966).

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

(5) J. E. Jorpes, V. Mutt, S. Magnusson, and B. B. Steele, *Biochem. Biophys. Res. Commun.*, **9**, 275 (1962).

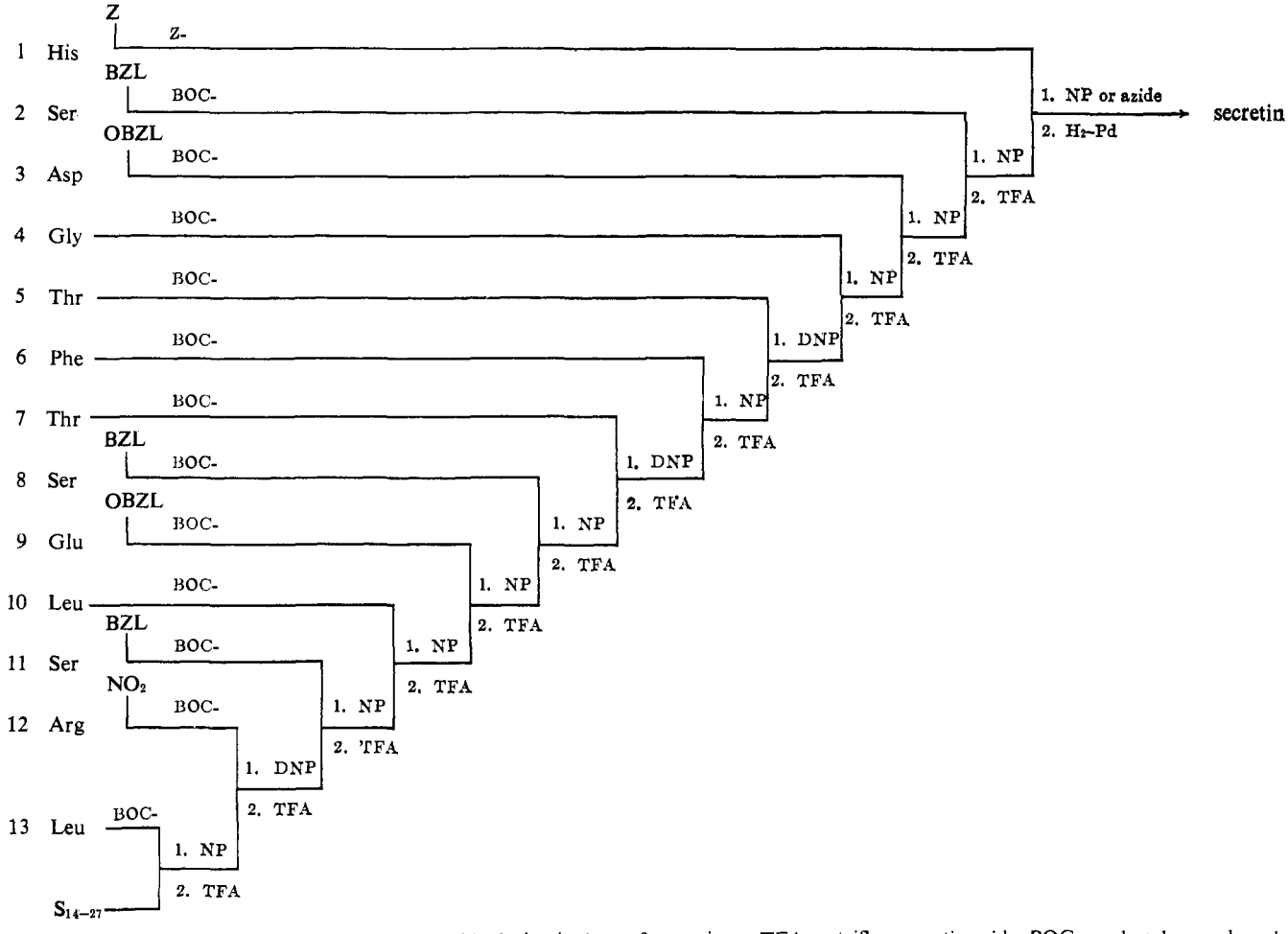
(6) V. Mutt, S. Magnusson, J. E. Jorpes, and E. Dahl, *Biochemistry*, **4**, 2358 (1965).

(7) Sequence I was presented by V. Mutt and J. E. Jorpes at the 4th International Symposium on the Chemistry of Natural Products, Stockholm, Sweden, 1966.

(8) M. Bodanszky, *Nature*, **175**, 685 (1955).

(9) F. C. McKay and N. F. Albertson, *J. Am. Chem. Soc.*, **79**, 4686 (1957); L. A. Carpino, *ibid.*, **79**, 98, 4427 (1957); G. W. Anderson and A. C. McGregor, *ibid.*, **79**, 6180 (1957); L. A. Carpino, A. Giza, and B. A. Carpino, *ibid.*, **81**, 955 (1959); L. A. Carpino, *ibid.*, **82**, 2725 (1960); R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622 (1959).

Chart I[a]



[a] S_{14-27} represents the C-terminal tetradecapeptide derivative[1] as a free amine. TFA = trifluoroacetic acid; BOC = *t*-butyloxycarbonyl; NP, coupling by the *p*-nitrophenyl ester method; DNP, coupling with 2,4-dinitrophenyl esters; BZL = benzyl.

The acylation reactions were carried out in dimethylformamide and triethylamine was used for the liberation of the amines from their trifluoroacetates. Completion of the acylation was tested by spotting the reaction mixture on paper and spraying the spot with ninhydrin. After no more reaction with ninhydrin could be observed, a liberal quantity of ethyl acetate was added to the mixture. Excess active ester, nitrophenol, and triethylammonium trifluoroacetate are all freely soluble in this solvent, which, on the other hand, precipitated the desired protected peptides. A characteristic feature of the present synthesis is that the excess of the active ester is increased with the growth of the chains. For a practical execution of acylation with active esters, it is preferable to start the reaction with the reactants in not less than 0.1 *M* concentration. The molecular weights and solubilities of the partially protected peptide intermediates are often such as to prevent the preparation of solutions of desirable concentration; however, the excess of the active ester can be raised to the point that, at least in respect of this component, the concentration reaches the desirable range. In this way, practical rates are maintained, and undesirable side reactions that are often monomolecular and, therefore, independent of concentration, can be kept at a minimum. The possibility of counterbalancing the difficulties that originate from the increasing molecular weight during the synthesis of long chains is a valuable feature of the stepwise approach.[10]

Since the N-terminal amino acid, histidine, was added as a benzyloxycarbonyl derivative, all the protecting groups could simultaneously be removed by catalytic hydrogenation. While both palladium on charcoal and palladium on barium sulfate proved to be efficient catalysts, the reduction of the nitro groups proceeded better in 80% (aqueous) acetic acid than in glacial acetic acid. Removal of the catalyst by filtration and of the solvent by evaporation *in vacuo* and lyophilization of the residue from glacial acetic acid resulted in crude preparations that showed a potency of about 1000–1500 clinical units/mg.[11] Like natural secretin, the synthetic peptide stimulates the pancreas to release sodium bicarbonate and also inhibits the action of gastrin, enhances the flow of bile, releases insulin, and reduces the spontaneous motor activity of the stomach and duodenum.[12]

The purification of the crude product was seriously hindered by its instability. For example, after countercurrent distribution in the system 1-butanol–pyridine–acetic acid–water (4:2:1:7), the highest specific activity

(10) M. Bodanszky, *Ann. N. Y. Acad. Sci.*, **88**, 655 (1960).

(11) J. E. Jorpes and V. Mutt, *Acta Physiol. Scand.*, **66**, 316 (1966); *cf.* also V. Mutt and U. Söderberg, *Arkiv Kemi*, **15**, 63 (1954).

(12) Inhibition of gastrin and enhancement of bile flow was reported by Dr. M. I. Grossman, Veterans Administration, Los Angeles, Calif., personal communication; *cf.* also K. G. Wormsley and M. I. Grossman, *Gastroenterology*, 47, 72 (1964). The insulin-releasing activity was tested by Dr. H. Elrick (San Diego, Calif.). The inhibition of stomach and duodenum motility was studied by Dr. W. Y. Chey (Temple University, School of Medicine, Philadelphia, Pa.).

Table I. Protected Intermediates

Protected peptide	Formula	C, % Calcd	C, % Found	H, % Calcd	H, % Found	N, % Calcd	N, % Found	Dec pt, °C	$[\alpha]^{25}D$, deg (c 2, AcOH)
Pentadecapeptide XV	$C_{94}H_{154}N_{30}O_{28}$	52.4	52.3	7.2	7.9	19.5	19.3	245	−32
Hexadecapeptide XVI	$C_{100}H_{165}N_{35}O_{31}$	51.0	50.7	7.1	7.4	20.8	20.4	240	−30
Heptadecapeptide XVII	$C_{110}H_{176}N_{36}O_{33}$	52.0	52.2	7.0	7.1	19.9	19.4	240	−27
Octadecapeptide XVIII	$C_{116}H_{187}N_{37}O_{34}$	52.7	52.4	7.1	7.0	19.6	19.6	305	−20
Nonadecapeptide XIX	$C_{128}H_{200}N_{38}O_{37}$	53.6	53.6	7.0	7.5	18.6	18.4	315	−23
Eicosapeptide XX	$C_{138}H_{211}N_{39}O_{39}$	54.5	54.1	7.0	7.4	18.0	18.0	320	−24
Heneicosapeptide XXI	$C_{142}H_{218}N_{40}O_{41}$	54.3	54.2	7.0	7.1	17.8	17.8	315	−18
Docosapeptide XXII	$C_{151}H_{227}N_{41}O_{42}$	55.1	55.4	6.9	7.2	17.5	17.0	315	−15
Tricosapeptide XXIII	$C_{155}H_{234}N_{42}O_{44}$	54.9	54.0	7.0	6.8	17.4	17.5	310	−14
Tetracosapeptide XXIV	$C_{157}H_{237}N_{43}O_{45}$	54.7	54.7	6.9	7.0	17.5	17.5	305	−15
Pentacosapeptide XXV	$C_{168}H_{248}N_{44}O_{48}$	55.2	54.7	6.9	6.9	16.9	16.9	310	−14
Hexacosapeptide XXVI	$C_{178}H_{259}N_{45}O_{50}$	55.8	55.8	6.8	7.4	16.5	16.6	310	−14
Heptacosapeptide XXVIIa	$C_{195}H_{270}N_{48}O_{53}$	56.6	56.6	6.6	6.8	16.3	16.8	310	−12

was found under a peak corresponding to a distribution coefficient (K) of 1.7. The potency of the recovered material, however, was still only about 1000–1500 clinical units/mg and considerable loss in the total activity was observed. Finally, distribution in 1-butanol–0.1 M phosphate buffer of pH 7 (1:1)[4] led to the separation of a peak with $K = 0.7$ and from this band a product with a potency of about 4000 units/mg was obtained. The synthetic product from the last-mentioned distribution was indistinguishable from natural secretin in paper chromatographic behavior and quantitative amino acid analysis. The latter gave ratios of amino acids in good agreement with the expected values. No differences were found in the cleavage products when natural secretin and the purified synthetic preparation were hydrolyzed with thrombin or with trypsin.[6,13]

The danger of racemization was not overlooked either during the chain-lengthening procedure or during the removal of the protecting groups and the subsequent purifications. The use of protected amino acids (and not protected peptides) bearing urethan-type amino protecting groups more or less excludes racemization by the azlactone mechanism.[14] Racemization by reversible β elimination had to be considered mainly in connection with the O-benzyl-L-serine residues. By avoiding the presence of excess base,[15] this cause of racemization and also the probability of simple abstraction of the proton from the α-carbon atom were kept at a minimum. The possibility of contamination by undesired diastereoisomers was examined by digestion of the purified synthetic products with trypsin and leucine aminopeptidase. No evidence was found for the presence of D-amino acid residues: quantitative amino acid analysis of the digest gave the expected ratios of the component amino acids.[16]

(13) We thank Dr. Mutt for some of these comparisons.

(14) For a review on mechanisms of racemization, *cf.* M. Bodanszky and M. A. Ondetti, "Peptide Synthesis," Interscience Publishers, Inc., New York, N. Y., 1966, p 137.

(15) Triethylamine was added to the reaction mixture until a weak alkaline reaction was shown on wet universal indicator paper held closely above the surface of the solution.

(16) A preparation assaying only about 1000 units/mg was treated with trypsin, and the five peptides corresponding to sequence 1–12 (b), 13–14 (d), 15–18 (a), 19–21 (c), and 22–27 (e) (*cf.* ref 6) were separated. Digestion of peptide a with leucine aminopeptidase followed by quantitative amino acid analysis of the digest gave the expected molar ratios for aspartic acid, serine, alanine, and arginine. Therefore, no rearrangement of the aspartyl residue took place in this part of the molecule. The dodecapeptide b was digested with chymotrypsin. The N-terminal hexapeptide, with the aspartyl residue in its position 3, was isolated and digested with leucine aminopeptidase. Amino acid analysis of the digest gave indication of partial rearrangement of an α-aspartyl moiety. These findings offer some explanation for the low activity of the crude synthetic preparations and also help to explain the instability of secretin. The aspartyl–glycyl sequence is probably more susceptible to rearrangements than other aspartyl sequences, since the cyclic intermediate in the α-aspartyl → β-aspartyl rearrangement involves an N-diacyl group, and it is well known that glycine shows a remarkable readiness for the formation of N-diacyl derivatives (Th. Wieland and B. Heinke, *Ann.*, **599**, 70 (1956); K. D. Kopple and R. J. Renick, *J. Org. Chem.*, **23**, 1565 (1958)). The complete digestibility of the purified synthetic preparations indicates that in these products no β-aspartyl derivatives or amino succinyl residues are present.

(17) The potency of pure porcine secretin was reported[4] to be 20,000 clinical units/mg; however, several subsequent preparations obtained in the Karolinska Institutet, although showing no difference in their amino acid composition, assayed only about 4000 units/mg (*cf.* ref 2). Such materials were used in the comparisons that are discussed in the present paper.

(18) Synthesis of secretin by fragment condensation will be reported in a separate paper.

(19) Since the lengthening by one amino acid of the already existing peptide chain was done under practically the same experimental conditions in each step, only a few such steps are reported here in detail. Physical constants and analytical values for the protected intermediates are summarized in Table I.

(20) Melting points were taken in capillary tubes and are uncorrected. R_f^A refers to the system 1-butanol–acetic acid–water 4:1:5 (S. M. Partridge, *Biochem. J.*, **42**, 238 (1948)); R_f^B refers to the system 1-butanol–pyridine–acetic acid–water 30:20:6:24 (S. G. Waley and G. Watson, *ibid.*, **55**, 328 (1953)); both were on Whatman No. 1 paper in descending chromatography.

In conclusion, it can be stated that a synthetic heptacosapeptide amide showing the various biological activities of secretin and roughly as potent as the natural hormone[17] was obtained. The fact that a peptide with 27 amino acids was synthesized entirely by the stepwise strategy[10,18] through isolated intermediates (Table I) can serve as indication that this approach may be applicable to even more ambitious endeavors.

Experimental Section[19,20]

Protected Pentadecapeptide. ***t*-Butoxycarbonyl-L-leucyl-nitro-L-arginyl-β-benzyl-L-aspartyl-O-benzyl-L-seryl-L-alanyl-nitro-L-arginyl-L-leucyl-L-glutaminyl-nitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide** (XV).[1] The C-terminal tetradecapeptide trifluoroacetate amide (8.1 g) was dissolved with stirring in dimethylformamide (40 ml). Triethylamine (0.60 ml) and *t*-butoxycarbonyl-L-leucine *p*-nitrophenyl ester (2.8 g) were added; after 2 hr at room temperature, the reaction mixture became ninhydrin negative. It was allowed to stand at room temperature for an additional 3 hr, then diluted with ethyl acetate (*ca.* 1.3 l.). After standing overnight, the precipitate was filtered and washed with ethyl acetate (500 ml). The product was air dried; (8.4 g, 98%), mp, *ca.* 125° (softens), 230° (darkens), 245° dec; $[\alpha]^{21}D$ −32°, $[\alpha]^{21}_{436}$ −76° (*c* 2, AcOH).

The protected pentadecapeptide amide (8.2 g) was dissolved in trifluoroacetic acid (70 ml). After about 15 min at room temperature, most of the trifluoroacetic acid was removed *in vacuo*, the residue triturated with ether, filtered, and washed with ether (8.15 g); R_f^A 0.65, R_f^B 0.90; mp 135° (softens), 160° (darkens), 240–245° dec.

Anal. Calcd for $C_{89}H_{146}N_{30}O_{26}\cdot CF_3COOH$: N, 19.4; F, 2.7. Found: N, 19.4; F, 2.9.

Protected Nonadecapeptide. *t*-Butoxycarbonyl-γ-benzyl-L-glutamyl-L-leucyl-O-benzyl-L-seryl-nitro-L-arginyl-L-leucyl-nitro-L-arginyl-β-benzyl-L-aspartyl-O-benzyl-L-seryl-L-alanyl-nitro-L-arginyl-L-leucyl-L-glutaminyl-nitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (XIX). The free amine trifluoroacetate (7.1 g) from the protected octadecapeptide amide XVIII was dissolved in dimethylformamide (85 ml). Triethylamine (0.40 ml) and *t*-butoxycarbonyl-γ-benzyl-L-glutamic acid *p*-nitrophenyl ester[21] (3.85 g) were added. After 5 hr the reaction mixture was very slightly ninhydrin positive. After standing overnight at room temperature, the reaction mixture became ninhydrin negative; and it was then diluted with ethyl acetate (2.6 l.). The precipitate was filtered, washed with ethyl acetate (1.3 l.) and air dried (7.4 g, 92%); mp 135° (softens), 240° (darkens), 315° dec; $[\alpha]^{25}D$ −22.5°, $[\alpha]^{25}_{436}$ −57° (*c* 2, AcOH).

Anal. Calcd for $C_{128}H_{200}N_{38}O_{37}$: C, 53.6; H, 7.0; N, 18.6. Found: C, 53.6; H, 7.5; N, 18.4.

The *t*-butoxycarbonyl group was removed from the protected nonadecapeptide amide (7.2 g) with trifluoroacetic acid (72 ml) as described above. The trifluoroacetate was dried in a vacuum desiccator over sodium hydroxide (7.3 g); mp 145° (softens), 245° (darkens), 312° dec; R_f^A close to the front, R_f^B at the front, single spots.

Protected Heneicosapeptide. *t*-Butoxycarbonyl-L-threonyl-O-benzyl-L-seryl-γ-benzyl-L-glutamyl-L-leucyl-O-benzyl-L-seryl-nitro-L-arginyl-L-leucyl-nitro-L-arginyl-β-benzyl-L-aspartyl-O-benzyl-L-seryl-L-alanyl-nitro-L-arginyl-L-leucyl-L-glutaminyl-nitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (XXI). The trifluoroacetate of the α-amino-deprotected eicosapeptide amide XX (6.8 g) was dissolved in dimethylformamide (45 ml). Triethylamine (0.31 ml) and *t*-butoxycarbonyl-L-threonine 2,4-dinitrophenyl ester[22] (2.6 g) were added. The basicity of the reaction mixture was maintained by the addition of small portions of triethylamine. After 3 hr, the mixture remained alkaline;[15] a total of 1.3 ml of triethylamine was used. After 7.5 hr, the reaction mixture became ninhydrin negative. It was diluted with ethyl acetate (3 l.), the precipitate was collected by filtration, washed with ethyl acetate (1 l.), and air dried (6.7 g, 95%); mp 140° (softens), 235° (darkens), 315° dec; $[\alpha]^{25}D$ −17.5°, $[\alpha]^{25}_{436}$ −52° (*c* 2, AcOH).

The free amine trifluoroacetate was prepared from the protected heneicosapeptide (6.3 g) as described above, weight 6.5 g, mp 140° (softens), 240° (darkens), 315° dec; R_f^A 0.95.

Protected Heptacosapeptide (by the Active Ester Method). Bis-benzyloxycarbonyl-L-histidyl-O-benzyl-L-seryl-β-benzyl-L-aspartylglycyl-L-threonyl-L-phenylalanyl-L-threonyl-O-benzyl-L-seryl-γ-benzyl-L-glutamyl-L-leucyl-O-benzyl-L-seryl-nitro-L-arginyl-L-leucyl-nitro-L-arginyl-β-benzyl-L-aspartyl-O-benzyl-L-seryl-L-alanyl-nitro-L-arginyl-L-leucyl-L-glutaminyl-nitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (XVIIa). The trifluoroacetate of the α-amino deprotected XXVI (1.54 g) was dissolved in dimethylformamide (8 ml). Triethylamine (0.28 ml of a 20% v/v solution) and bisbenzyloxycarbonylhistidine *p*-nitrophenyl ester (0.87 g) were added. After 6 hr, the reaction mixture became ninhydrin negative. After standing overnight at room temperature, ethyl acetate (400 ml) was added, the precipitate collected by filtration, washed with ethyl acetate (200 ml), and air dried (1.55 g, 93%), mp 140° (softens), 235° (darkens), 310° dec.

Protected Heptacosapeptide (by the Azide Method). Benzyloxycarbonyl-L-histidyl-O-benzyl-L-seryl-β-benzyl-L-aspartylglycyl-L-threonyl-L-phenylalanyl-L-threonyl-O-benzyl-L-seryl-γ-benzyl-L-glutamyl-L-leucyl-O-benzyl-L-seryl-nitro-L-arginyl-L-leucyl-nitro-L-arginyl-β-benzyl-L-aspartyl-O-benzyl-L-seryl-L-alanyl-nitro-L-arginyl-L-leucyl-L-glutaminyl-nitro-L-arginyl-L-leucyl-L-leucyl-L-glutaminylglycyl-L-leucyl-L-valinamide (XXVIIb). The trifluoroacetate (195 mg) of the α-amino deprotected XXVI was dissolved in a mixture of dimethylformamide (1.6 ml) and triethylamine (0.014 ml). To this solution, which was cooled in an ice–water bath, was added a 0.2 *M* solution of benzyloxycarbonyl-L-histidine azide in ethyl acetate (0.75 ml).[23] After 32 hr at 5°, a second portion of azide solution (0.25 ml) was added. After about 48 hr, the ninhydrin reaction became completely negative. The mixture was diluted with ethyl acetate, the precipitate filtered, washed with ethyl acetate, and dried (170 mg), mp 140° (softens), 230° (darkens), 312° dec; $[\alpha]^{25}D$ −11.5° (*c* 2, AcOH). After removal of the protecting groups, as described below, the resulting free heptacosapeptide was indistinguishable (amino acid analysis, chromatographic behavior, potency) from the product obtained from XXVIIa.

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-valinamide (Salt with Acetic Acid) (XXVIII). a. Removal of Protecting Groups. The protected heptacosapeptide amide XXVIIa (170 mg) was dissolved in 80% aqueous acetic acid (55 ml), and the solution was stirred with 10% palladium on charcoal (150 mg) under a stream of hydrogen. After 48 hr, the catalyst was removed by filtration and the filtrate was freeze-dried, yield 146 mg. The potency of such preparations varied between 1000 and 1500 clinical units/mg.[11]

b. Attempted Purification by Countercurrent Distribution in a 1-Butanol–Pyridine–Acetic Acid–Water System. The crude free heptacosapeptide amide (520 mg) was distributed through 250 transfers in the 1-butanol–pyridine–acetic acid–water (4:2:1:7) system. An automatic Craig apparatus with 10-ml phases was used. The contents of the tubes corresponding to the peak with $K = 1.7$ were pooled, concentrated to dryness *in vacuo* (bath temperature below 30°) and the residue was freeze-dried from glacial acetic acid, yield 212 mg; potency 1000 clinical units/mg; amino acid analysis: Asp, 1.8; Thr, 2.2; Ser, 3.8; Glu, 3.0; Gly, 2.0; Ala, 1.1; Val, 1.1; Leu, 6.3; Phe, 0.9; NH_3, 5.7; His, 1.0; Arg, 3.8.

c. Purification by Countercurrent Distribution in a 1-Butanol–0.1 *M* Phosphate Buffer (pH 7) System.[5] The crude free heptacosapeptide amide (0.1 g) was distributed through 150 transfers in the system 1-butanol–0.1 *M* phosphate buffer pH 7 (1:1). The contents of the tubes corresponding to the peak with $K = 0.6$ were pooled and diluted with 3.1 l. of double-distilled water. The pH of the solution was adjusted to 2.5 with concentrated hydrochloric acid and stirred at 5° with wet alginic acid (E. Mendell Co., Yonkers, N. Y.; 5 g) for 40 min. The alginic acid was collected by filtration (Whatman paper no. 541) and washed with ice-cold 0.001 *N* hydrochloric acid. The heptacosapeptide amide was eluted from the alginic acid with seven 2-ml portions of ice-cold 0.2 *N* hydrochloric acid. The combined eluent was passed through a column (1.6 × 16 cm) of DEAE-Sephadex A-25 in acetate cycle, and the heptacosapeptide amide acetate was eluted with 0.2 *M* acetic acid. Based on the peptide content (recovery of amino acids in the analysis) of the lyophilized material, 15 mg of purified material was obtained. Its potency is 4000 clinical units/mg; amino acid analysis: Asp, 2.0; Thr, 1.8; Ser, 3.1; Glu, 3.0; Gly, 2.0; Ala, 1.1; Val, 1.1; Leu, 5.8; Phe, 0.9; NH_3, 3.8; His, 0.95; Arg, 4.2.

The heptacosapeptide amide traveled as a single spot on paper chromatograms, and its R_f value was identical with that of natural secretin. Similarly, homogeneity was shown in paper electrophoresis at pH 2.0 (31.2 ml of 90% formic acid, 59.2 ml of glacial acetic acid diluted with distilled water to 1 l.).

Tryptic Digestions.[6] The heptacosapeptide amide acetate XXVIII (2 mg) was dissolved in water (0.5 ml), and the solution was diluted with 2% aqueous ammonium bicarbonate (0.5 ml). A slight turbidity was observed, which disappeared after incubation at room temperature with 0.02 ml of trypsin solution[24] for 20–30 min. After 2 and 4 hr, two more portions of trypsin solution (0.02 ml each) were added. The solution was freeze-dried after a total of 6 hr. The residue was dissolved in 1 ml of water, heated for 6

(21) Prepared according to the general procedure described in "Biochemical Preparations," Vol. 9, M. J. Coon, Ed., John Wiley and Sons, Inc., New York, N. Y., 1962, p 110; mp 119–120°; $[\alpha]^{25}D$ −32° (*c* 1.2, dimethylformamide containing 1% acetic acid). *Cf.* also C. H. Li, B. Gorup, D. Chung, and J. Ramachandran, *J. Org. Chem.*, **28**, 178 (1963).

(22) This active ester was prepared in the same way as described for the corresponding benzyloxycarbonyl derivative (R. Rocchi, I. Marchiori, and E. Scoffone, *Gazz. Chim. Ital.*, **93**, 823 (1963)), mp 72–74°, $[\alpha]^{25}D$ −32° (*c* 1.2, dimethylformamide containing 1% acetic acid).

(23) R. W. Holley and E. Sondheimer, *J. Am. Chem. Soc.*, **76**, 1326 (1954).

(24) Worthington TRL trypsin; the solution was prepared as described in ref 6.

min and freeze-dried. Paper chromatography of this tryptic hydrolysate showed the expected mixture[6] of the five tryptic peptides (R_f^B "a" = 0.12, "b" = 0.3, "c" = 0.3, "d" = 0.45, and "e" = 0.89). In some experiments also a small amount of peptide a and d (R_f^B 0.20) was observed. Better separation of this tryptic hydrolysate could be achieved by a two-dimensional combination of electrophoresis (pH 2; formic acid–acetic acid) and chromatography in the same solvent system.

Isolation of Peptide A from the Tryptic Digest. Samples (20 mg) of the heptacosapeptide amide (1000 clinical units/mg) and the free C-terminal tridecapeptide amide (sequence 15–27)[1] were digested with trypsin as described above. The mixtures were chromatographed on Whatman 3 MM sheets, and the band corresponding to peptide A (R_f^B 0.12) eluted with 0.1 *M* acetic acid. The materials thus obtained were compared by paper electrophoresis at three different pH values: 2.0 (formic acid, acetic acid), 6.4 (pyridine, acetic acid), and 8.5 (N-ethylmorpholine, acetic acid). A single band of identical mobility and ninhydrin color was observed in the three experiments for both preparations of peptide A. The purple ninhydrin color indicated α-aspartyl peptides rather than β-aspartyl derivatives.

An aliquot of these two preparations of peptide A was digested with leucine aminopeptidase[25] (Boehringer, Mannheim Corp., New York, N. Y.). Quantitative amino acid analyses of the digests gave the following ratios: peptide A from the tridecapeptide Asp, 1.10; Ser, 1.05; Ala, 1.00; Arg, 1.10; peptide A from the heptacosapeptide Asp, 1.05; Ser, 1.00; Ala, 0.90; Arg, 1.10.

Isolation of Peptide B from the Tryptic Digest. A sample (20 mg) of heptacosapeptide amide (1000 clinical units/mg) was digested with trypsin, as described above. The peptide mixture was fractionated by electrophoresis at pH 2 on Whatman 3 MM paper. The Pauly-positive band (peptide B) was eluted with 0.1 *M* acetic acid and freeze-dried (6.5 mg) To an aliquot of this preparation (3 mg) in 1% aqueous ammonium bicarbonate (0.3 ml), a 0.25% solution (24 μl) of chymotrypsin[26] in 1% ammonium bicarbonate was added, and the mixture incubated at 40° for 5 hr. A second 24-μl portion of chymotrypsin solution was added, and the digestion was allowed to proceed for a total of 24 hr. The digest was fractionated by preparative paper electrophoresis at pH 7 (collidine, acetic acid), and the single Pauly-positive band, which showed the mobility of an authentic sample of His-Ser-Asp-Gly-Thr-Phe,[27] was eluted with 0.1 *M* acetic acid. After removal of the solvent by freeze-drying, the residue was digested with leucine aminopeptidase.[25] Quantitative amino acid analysis of the digest gave the following molar ratios of amino acids: His, 1.00; Ser, 1.10; Asp, 0.51; Gly, 0.46; Thr, 0.60; Phe, 0.40.[16]

Digestion with Trypsin and Leucine Aminopeptidase. Samples (2 ml) of the crude and of the purified heptacosapeptide amide preparations were digested with trypsin as described above. The mixture of tryptic peptides thus obtained was then digested with leucine aminopeptidase.[25] Quantitative amino acid analyses of the resulting digests gave the following molar ratios: crude preparation (1000–1500 clinical units/mg): Asp, 1.32; Thr, 1.45; Ser, 2.7; Glu, 0.45; Gly, 1.45; Ala, 1.0; Val, 1.1; Leu, 5.2; Phe, 0.32; His, 0.73; Arg, 3.05. The purified preparation (about 4000 clinical units/mg) gave the following ratios: Asp, 1.9; Thr, 2.1; Ser, 3.6; Glu, 1.2; Gly, 1.9; Ala, 1.1; Val, 1.05; Leu, 5.6; Phe, 0.8; His, 1.06; Arg, 3.5.

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(26) Prepared from Worthington CD 1 α-chymotrypsin.

(27) This hexapeptide was prepared by Dr. John T. Sheehan of this laboratory.

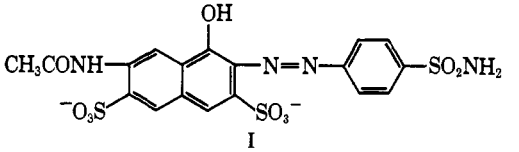
Communications to the Editor

Carbonic Anhydrase–Azosulfonamide Complexes. Optical Rotatory Dispersion and Circular Dichroism

Sir:

A variety of sulfonamides inhibit the zinc metalloenzyme carbonic anhydrase. The requirement for inhibition is the presence of an unsubstituted sulfonamide group attached to a ring system the structure of which can be varied widely without losing the relatively high affinity of the inhibitor for the enzyme.[1–3] These compounds have played an important role in studies of the active site of this enzyme since sulfonamide binding has been shown to be: (1) metal ion dependent,[4,5] (2) accompanied by changes in energy and optical activity of the d–d transitions of the Co(II) derivative of the enzyme,[6,7] and (3) limited to the metallocarbonic anhydrases containing Co(II) and Zn(II), the only first transition and IIb metal ions which restore significant enzymatic activity to apocarbonic anhydrase.[5,6]

The present communication reports spectral, optical rotatory dispersion (ORD), and circular dichroism (CD) studies of carbonic anhydrase–azosulfonamide complexes employing 2-(4-sulfamylphenylazo)-7-acetamido-1-hydroxynaphthalene-3,6-disulfonate (I) as the inhibitor. On binding of this sulfonamide to carbonic anhydrase, the strong absorption band of the azochromophore near 500 mμ (ϵ_{max} ~25,000) shows both marked



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spectral shifts and induced optical activity. These features make this molecule an effective detector for changes in the environment or conformation of the bound sulfonamide and a sensitive probe for conformational features of the active center of carbonic anhydrase.

Carbonic anhydrase, isozyme B, was prepared from human or monkey erythrocytes by the methods previously reported.[7,8] The azosulfonamide was purchased

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