Anal. Calcd for $C_{20}H_{26}N_4NaO_{11}PS \cdot 3H_2O$: P, 4.85; S, 5.02. Found: P, 4.84; S, 4.97.

Preparation of 23. A solution of **13** (70 mg, barium salt) and the iodonucleoside **21** (340 mg) in 50% aqueous pyridine (3 ml) was heated at 70° for 7 hr. The purification procedure as described for **22** was used to obtain 60 mg (85%) of **23**; $\lambda_{max}^{H_2O}$ 267 mμ (ε 17,880).

Anal. Calcd for $C_{20}H_{26}N_4NaO_{11}PS$: C, 41.08; H, 4.48; S, 5.48. Found: C, 40.94; H, 5.27; S, 5.29.

Treatment of 3 with Aqueous Iodine. A solution of **3** (50 mg) in 50% aqueous acetone (2 ml) was treated with iodine (100 mg) for 17 hr, and then diluted with water (10 ml) and extracted with ether (three 5-ml portions). The residual ether was removed from the aqueous layer by evaporation, and the solution was applied to a DEAE column (50 × 2.3 cm, acetate form) which was eluted with a gradient of 2 l. of acetate buffer, pH 6 (0.005 *M*) in the mixing vessel, and 2 l. of the same buffer (0.2 *M*) in the reservoir. 3′-O-Acetylthymidine 5′-phosphate, 731 OD_{267} units (77%) was eluted from the column in fractions 72–83, and was identified by comparison with an authentic sample.[23]

Acknowledgments. Thanks are extended to Dr. V. Toome for ultraviolet spectra, to Dr. F. J. Scheidl for microanalyses, and to Dr. A. L. Nussbaum for helpful discussions.

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Synthesis of Cholecystokinin–Pancreozymin. I. The C-Terminal Dodecapeptide

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Abstract: A dodecapeptide amide with the structure proposed by Mutt and Jorpes for the C-terminal sequence of the intestinal hormone cholecystokinin–pancreozymin has been synthesized by the condensation of three fragments: di-, tetra-, and hexapeptide. All the fragments were prepared by the stepwise procedure using active esters, and the coupling of the intermediate peptides was carried out by the azide method. The synthetic dodecapeptide (XX) was identical with the natural counterpart isolated by partial tryptic digestion of the hormone. This C-terminal sequence has all the biological properties of cholecystokinin–pancreozymin.

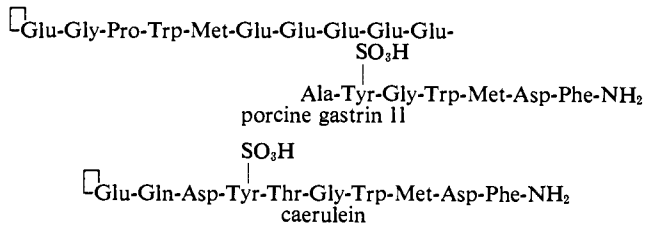
In 1928 Ivy and Oldberg[1] proposed the name cholecystokinin for the chemical entity present in the crude extracts of intestinal mucosa that was responsible for the contractile activity of these extracts on the gall bladder of the dog. Fifteen years later, Harper and Raper[2] named as pancreozymin the compound present in the same type of extracts and responsible for the stimulation of pancreatic enzyme release. The careful isolation carried out at the Karolinska Institute by Mutt and Jorpes[3] has shown that both biological activities are elicited by one compound that they have, at least temporarily, designated as cholecystokinin–pancreozymin (CCK–PZ). The structural studies have led to the conclusion that this hormone is a single-chain polypeptide of thirty-three amino acids. The partial sequence of Scheme I represents the latest published results of these investigations.[4] Through a personal communication from Professor J. E. Jorpes and Dr. V. Mutt we have learned that the specific sequence they have proposed for the C-terminal dodecapeptide is: Ile-Ser-Asp-Arg-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp Phe-NH_2. This dodecapeptide can be isolated by partial tryptic digestion of CCK–PZ.[4]

Scheme I

Lys-(Ala_1, Gly_1, Pro_1, Ser_1)-Arg-Val-(Ile_1, Met_1, Ser_1)-Lys-Asn-(Asx_1, Glx_1, His_1, Leu_2, Pro_1, Ser_2)-Arg-Ile-(Asp_1, Ser_1)-Arg-Asp-(Gly_1, SO_3H | Met_2, Trp_1, Tyr_1)-Asp-Phe-NH_2

The most notable feature of this C-terminal sequence is its resemblance to the similar portion of the gastric hormone gastrin[5] and also to the decapeptide caerulein[6] (Scheme II) isolated by Erspamer and his coworkers from the skin of the amphibian *Hyla caerulea.* While the similarity to gastrin extends strictly up to the C-terminal pentapeptide, in the case of caerulein it extends up to the octapeptide, except for the replacement of methionine by threonine. The presence of a tyrosine residue with the phenolic hydroxyl esterified with sulfuric acid is another interesting feature of this partial sequence. In the case of gastrin, both the sulfated and the unsulfated forms have been isolated, but in the case of cholecystokinin and caerulein only the sulfated forms have so far been found.

Scheme II



(1) A. C. Ivy and E. Oldberg, *Amer. Physiol.*, 86, 599 (1928).
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(3) J. E. Jorpes, V. Mutt, and K. Toczko, *Acta Chem. Scand.*, 18, 2408 (1964).
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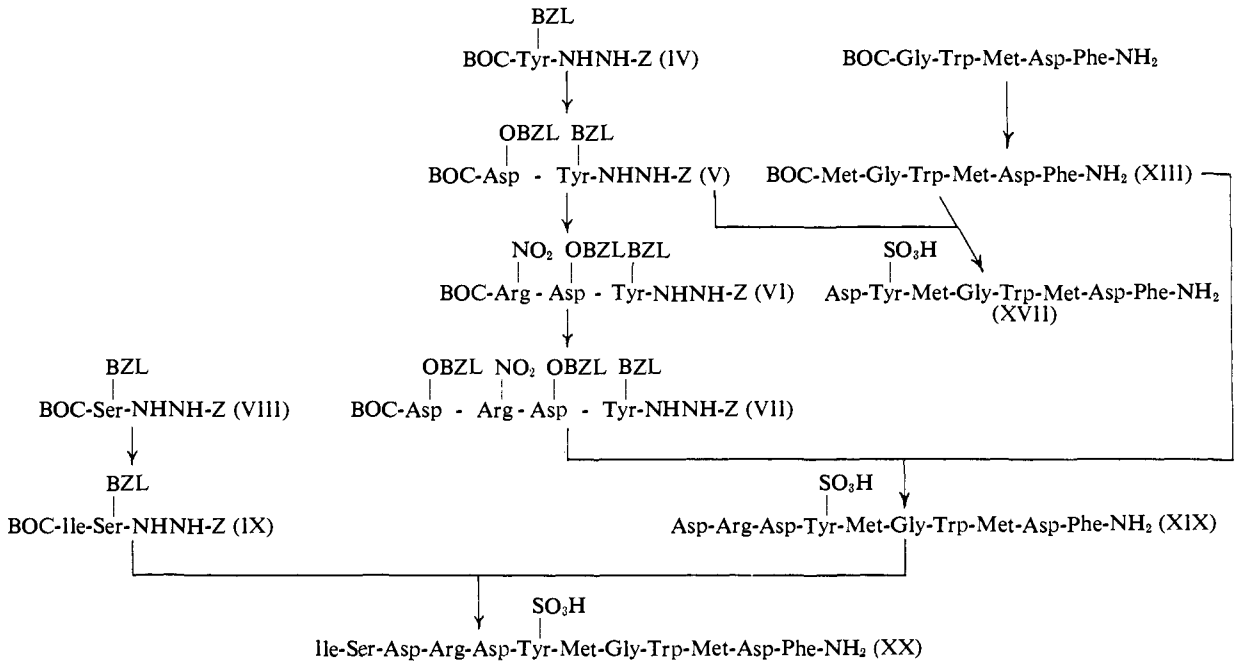
Scheme III



The first aim of these studies was the synthesis of the C-terminal octapeptide, because it is one of the fragments isolated after complete tryptic digestion of cholecystokinin.[4] A preliminary approach[7] to the synthesis of this octapeptide was that of the 4 + 4 type (coupling of two tetrapeptides) taking advantage of the presence of a glycine residue in the middle of the peptide chain. The carboxylic side chains were protected with tertiary butyl esters to enable the use of an acidolytic procedure, but the conditions required for this removal were such that the sulfate ester would be cleaved if it were already present in the molecule. This forced us to introduce the sulfate moiety directly on the free peptide after removal of all protecting groups,[8] as we shall describe below. Similar considerations had to be taken into account if a stepwise procedure was to be used, as we have done in the synthesis of analogs of this octapeptide sequence.[9] To avoid some of these limitations, an alternate pathway to the synthesis of this octapeptide, which could also be applied to the synthesis of the C-terminal decapeptide, was finally employed (Scheme III). The key intermediate of this route was the C-terminal hexapeptide XIII that might be converted either into the octa- or the decapeptide by coupling with the appropriate di- or tetrapeptide. All fragments were synthesized in the stepwise[10] manner from the respective C-terminal amino acid using different forms of the general active ester procedure.[11] For the couplings the azide procedure was selected to eliminate the possibility of racemization of the C-terminal tyrosine residue. The necessary hydrazides were prepared by way of the benzyloxycarbonyl hydrazides[12] in order to remove the carboxylic side chains protecting groups before the coupling step. The sulfate ester moiety was introduced at the octa- or decapeptide stage, and the conditions used for the removal of the *t*-butyloxycarbonyl group were found not to affect the sulfate ester linkage. The N-terminal dipeptide of the dodecapeptide sequence, isoleucyl–serine, was introduced after sulfation of the decapeptide to avoid the undesired sulfation of the serine moiety.

The introduction of the sulfate ester group on the tyrosine moiety presented a special set of problems. The use of a sulfur trioxide complex on peptides with free amino groups leads to the formation of the undesired sulfamic[13,14] acid derivatives; this prompted us to explore the application of concentrated sulfuric acid as a sulfating agent. The successful use of this reagent had been already described for tyrosine[15] itself, some simple synthetic peptides,[16] and for natural proteins.[15] While working with tyrosine alone, we found that temperature was a very important variable to control in order to achieve sulfation and not sulfonation. In the case of the C-terminal octapeptide it was very soon found that time is also of great importance in this connection. Reactions carried out at −15 or −20° (bath temperature) led almost exclusively to the sulfonate if allowed to proceed for extended periods. Fortunately, with chromatography on DEAE Sephadex or DEAE Cellulose we were able to obtain a very good separation between sulfated, sulfonated, and unreacted peptide. Infrared and ultraviolet spectroscopy distinguishes very clearly these three types of tyrosine peptides. The sulfated tyrosine peptides have a very strong sharp band at 1050 cm^{-1} on the ir spectrum,[17] and a practically negligible uv absorption in the 250–300

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(10) M. Bodanszky, *Ann. N. Y. Acad. Sci.*, **88**, 655 (1960).
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mμ region, which in our case shows only the typical absorption pattern of the tryptophan moiety. The sulfonated peptides show in the ir two sharp bands of medium intensity at 1020 and 1090 cm^{-1}, and a very intense uv absorption in the 247–306 mμ range. None of these bands are present in the ir of tyrosine peptides. Acid hydrolysis under the usual conditions (110°, 16 hr) led to complete hydrolysis of the sulfated tyrosine moiety. In the case of the sulfonated derivatives approximately 25% of 3′-sulfonyl tyrosine was recovered after the same treatment.

With the N-protected octapeptide and decapeptide, sulfation was better accomplished using the sulfur trioxide–pyridine complex. A pretreatment of the solvent (dimethylformamide) with molecular sieves was found to be an essential requirement for the success of this reaction. Practically no sulfation was observed when reagent grade untreated dimethylformamide was used.

Comparison of natural and synthetic samples of the C-terminal dodecapeptide by different procedures (paper chromatography, electrophoresis, and enzymatic degradation)[18] showed that they are identical and confirmed the sequence proposed by Mutt and Jorpes.

The C-terminal octapeptide XVII is capable of eliciting all the biological responses of pure cholecytokinin–pancreozymin at dosages $^1/_5$–$^1/_8$ that of the whole molecule.[19] The threshold dose for contraction of the guinea pig gall bladder *in situ* is 2 ng/kg. The deca- and dodecapeptide are also very powerful stimulators of the guinea pig gall bladder.[20] However, the unsulfated octapeptide has only $^1/_{300}$ of the activity of its sulfated counterpart. These results, which are similar to those observed with sulfated and unsulfated caerulein,[21] might tend to indicate that the sulfated tyrosine moiety is *per se* the active site of the hormone. That this is not the case was shown by the fact that tyrosine-O-sulfate and two sulfated tyrosine tetrapeptides

$$\begin{matrix} SO_3H \\ | \\ \text{Asp-Tyr-Met-Gly} \end{matrix} \quad \text{and} \quad \begin{matrix} SO_3H \\ | \\ \text{Asp-Tyr-Met-Gly-NH}_2 \end{matrix}$$

have little or no activity in the same biological systems. Evidently, both a strong anionic grouping and a specific peptide backbone are indispensable for a successful interaction with the receptor site.

When the sulfuric acid residue is attached to the aromatic ring of the tyrosine moiety, the biological potency of the sulfonated peptide is $^1/_5$ that of the corresponding sulfated derivatives.

Experimental Section[22]

***t*-Butyloxycarbonyl-methionyl-glycine (I).** Glycine (1.87 g, 25 mmol) was acylated in a 1:1 mixture of pyridine–water (90 ml) with *t*-butyloxycarbonylmethionine 2,4,5-trichlorophenyl ester (10.70 g, 25 mmol) maintaining the pH at 8.6 by addition of 2 *N* sodium hydroxide. The reaction mixture was diluted with water (200 ml), extracted with ethyl acetate, acidified to pH 3 with 6 *N* hydrochloric acid, and extracted with ethyl acetate. This last ethyl acetate extract was dried over $MgSO_4$. The solvent was removed *in vacuo*, and the residue crystallized from ethyl acetate–hexane: yield, 6.1 g (81%); mp 125–126°; $[\alpha]^{23}D$ −12° (*c* 1.2, dimethylformamide).

Anal. Calcd for $C_{12}H_{22}N_2O_5S$: C, 47.1; H, 7.2; N, 9.1. Found: C, 47.5; H, 7.7; N, 8.4.

***t*-Butyloxycarbonyl-tyrosyl-methionyl-glycine (II).** Methionyl-glycine hydrochloride (2.42 g, 10 mmol, obtained from 3.1 g of I with 2 *N* hydrochloric acid in acetic acid) was dissolved in cold dimethylformamide (50 ml) and neutralized with triethylamine (2.8 ml). *t*-Butyloxycarbonyltyrosine 2,4,5-trichlorophenyl ester (5.1 g, 11 mmol) was added and the mixture was mechanically stirred for 5 hr at room temperature. After dilution with ethyl acetate (400 ml) and extraction with 20% citric acid (100 ml) and water (3 × 50 ml), the organic layer was dried ($MgSO_4$) and the solvent was removed *in vacuo*. The residue was dissolved in ethyl acetate (45 ml) and dicyclohexylamine (2 ml) was added. The crystalline tripeptide salt was filtered, washed with ethyl acetate, and dried: yield, 5.1 g (79%); mp 139–141°; $[\alpha]^{24}D$ −12.6° (*c* 1.9 dimethylformamide).

Anal. Calcd for $C_{33}H_{54}N_4O_7S$: C, 60.9; H, 8.4; N, 8.6. Found: C, 60.5; H, 8.3; N, 8.5.

***t*-Butyloxycarbonyl-β-*t*-butyl-aspartyl-tyrosyl-methionyl-glycine (III).** Tyrosyl-methionyl-glycine trifluoroacetate (2.6 g, 5.3 mmol, obtained from 3.6 g of II with trifluoroacetic acid) was acylated with *t*-butyloxycarbonyl-β-*t*-butyl-aspartic acid *p*-nitrophenyl ester (2.5 g, 6.1 mmol) in the way described for II. The product was obtained as an amorphous solid from ether: yield, 2.3 g.

Anal. Calcd for $C_{29}H_{44}N_4O_{10}S$: C, 54.4; H, 6.9; N, 8.7. Found: C, 54.8; H, 7.3; N, 8.3.

N-(*t*-Butyloxycarbonyl-O-benzyl-tyrosyl)-N′-benzyloxycarbonyl hydrazine (IV). Dicyclohexylcarbodiimide (824 mg, 4 mmol) was added to an ice-cold solution of *t*-butyloxycarbonyl-O-benzyl tyrosine (1.5 g, 4 mmol) and benzyloxycarbonyl hydrazine (688 mg, 4 mmol). After 2 hr of stirring in the ice–water bath and 2 hr at room temperature, the substituted urea was filtered off, and the filtrate was concentrated to dryness. The residue was crystallized from ether: yield, 1.7 g (85%); mp 140–141°; $[\alpha]^{22}_{365}$ +20.7° (*c* 1,2, dimethylformamide).

Anal. Calcd for $C_{29}H_{33}N_3O_6$: C, 67.0; H, 6.4; N, 8.1. Found: C, 66.9; H, 6.4; N, 8.1.

N-(*t*-Butyloxycarbonyl-β-benzyl-aspartyl-O-benzyl-tyrosyl)-N′-benzyloxycarbonyl Hydrazine (V). This product was obtained by the reaction of *t*-butyloxycarbonyl-β-benzyl-aspartic acid *p*-nitrophenyl ester (1.47 g, 3.3 mmol) and N-(O-benzyl-tyrosyl)-N′-benzyloxycarbonyl hydrazine (1.56 g, 3 mmol) following the general procedure described for the preparation of II. The product was crystallized from ether: yield, 1.7 g (85%); mp 135–137°; $[\alpha]^{23}D$ −19.1° (*c* 1.2, dimethylformamide).

Anal. Calcd for $C_{40}H_{44}N_4O_9$: C, 66.3; H, 6.12; N, 7.7. Found: C, 66.0; H, 6.2; N, 7.8.

N-(N^{α}-*t*-Butyloxycarbonyl-N^{ω}-nitroarginyl-β-benzyl-aspartyl-O-benzyl-tyrosyl)-N′-benzyloxycarbonyl Hydrazine (VI). This material was prepared as described for II, using *t*-butyloxycarbonyl-nitroarginine N-hydroxysuccinimide ester.[23] The product was obtained as an amorphous solid from ether: yield, 95%; $[\alpha]^{23}D$ −14.9° (*c* 1.4, dimethylformamide).

Anal. Calcd for $C_{46}H_{55}N_9O_{12}\cdot H_2O$: C, 58.5; H, 6.1; N, 13.4. Found: C, 58.5; H, 5.9; N, 13.6.

N-(*t*-Butyloxycarbonyl-β-benzyl-aspartyl-nitroarginyl-β-benzyl-aspartyl-O-benzyl-tyrosyl)-N′-benzyloxycarbonyl Hydrazine (VII). This material was prepared by the general active ester procedure described for II. The product was obtained as an amorphous solid

(18) We thank Dr. Viktor Mutt for this comparison.

(19) B. Rubin and S. Engel, *J. Pharm. Sci.*, in press.

(20) The shortest C-terminal sequence capable of displaying all the biological properties of CCK–PZ is the heptapeptide. This compound has already been synthesized as an analog of the C-terminal heptapeptide of caerulein, *cf.* ref 21.

(21) A. Anastasi, L. Bernardi, G. Bertaccini, G. Bosisio, R. De Castiglione, V. Erspamer, O. Goffredo, and M. Impicciatore, *Experientia* **24**, 771 (1968).

(22) Melting points were taken in capillary tubes and are uncorrected. Chromatographies were carried out on a thin layer of cellulose (Eastman chromagram sheet) with the following solvents: R_f^A, 1-butanol–pyridine–acetic acid–water (30:20:6:24); R_f^B, 2-butanol–3% ammonia (100:44). Electrophoreses were carried out according to the technique described by L. N. Werum, H. T. Gordon, and W. Thornburg, *J. Chromatogr.*, **3**, 125 (1960); mobilities are expressed as a function of the migration of the standard dyes for the five pH's: 3.3, 4.7, 7.2, 8.0, and 9.3. Acid hydrolyses were run at 110° for 16 hr in glass ampoules sealed under vacuum. Enzymatic hydrolyses were performed as described by K. Hofmann, H. Yajima, T. Y. Liu, N. Yanaihara, C. Yanaihara, and J. Humes, *J. Amer. Chem. Soc.*, **84**, 4481 (1962). Leucine aminopeptidase was obtained from Boehringer Mannheim Corp., New York, N. Y. Quantitative amino acid analyses were carried out with a modified Technicon autoanalyzer. All amino acids are of L configuration.

(23) This active ester was obtained in crystalline form from 2-propanol, mp 115–117°; $[\alpha]^{23}D$ −24.7° (*c* 2.3, dimethylformamide). *Anal.* Found: C, 43.3; H, 5.7; N, 20.0.

from ether: yield, 85%; $[\alpha]D^{23}$ − 16.6° (*c* 2.3, dimethylformamide).

Anal. Calcd for $C_{57}H_{67}N_{10}O_{15}$: C, 60.5; H, 6.0; N, 12.4. Found: C, 60.2; H, 5.9; N, 12.1.

N-(*t*-Butyloxycarbonyl-O-benzyl-seryl)-N′-benzyloxycarbonyl Hydrazine (VIII). This compound was prepared by the same procedure as described for IV. The product was crystallized from ether–hexane: yield, 78%; mp 77–79°; $[\alpha]D^{23}$, −3.3° (*c* 1.0, dimethylformamide).

Anal. Calcd for $C_{23}H_{29}N_3O_6$: C, 62.3; H, 6.6; N, 9.5. Found: C, 62.7, H, 6.5; N, 9.5.

N-(*t*-Butyloxycarbonyl-isoleucyl-O-benzyl-seryl)-N′-benzyloxycarbonyl Hydrazine (IX). This compound was prepared by the general active ester procedure described for II, using *t*-butyloxycarbonyl-isoleucine N-hydroxysuccinimido ester.[24] The product was crystallized from ether: yield, 72%; mp 140–141°; $[\alpha]^{23}D$ −10.2° (*c* 1.5, dimethylformamide).

Anal. Calcd for $C_{29}H_{40}N_4O_7$: C, 62.7; H, 7.2; N, 10.1. Found: C, 62.5; H, 7.2; N, 10.2.

***t*-Butyloxycarbonyl-aspartyl-tyrosine Hydrazide (X).** Palladium on charcoal (10%, 200 mg) was added to a solution of V(1 g) in a mixture of methanol, acetic acid, and water (2:1:1; 30 ml). The suspension was stirred in a hydrogen atmosphere at normal pressure for 4.5 hr. The catalyst was removed by filtration and the filtrate was concentrated to dryness *in vacuo*. The crystalline residue was suspended in ethyl acetate, filtered, and dried: yield, 650 mg; mp 160–162°; $[\alpha]^{23}D$ −38.4° (*c* 1.8, dimethylformamide).

Anal. Calcd for $C_{18}H_{26}N_4O_7$: C, 52.7; H, 6.4; N, 13.6; hydrazide N, 6.8.[25] Found: C, 51.8, H, 6.5; N, 13.2; hydrazide N, 6.4.

***t*-Butyloxycarbonyl-aspartyl-arginyl-aspartyl-tyrosine Hydrazide (XI).** This compound was obtained by catalytic hydrogenolysis of VII (5 g) for 24 hr, following the procedure described for X. The product was obtained as an amorphous powder from ether: yield, 3.3 g; $[\alpha]^{23}D$ −14.8° (*c* 1.3, dimethylformamide).

Anal. Calcd for $C_{28}H_{43}N_9O_{11}\cdot CH_3COOH\cdot H_2O$: C, 47.5; H, 6.5; N, 16.6; hydrazide N, 3.8. Found: C, 47.2; H, 6.2; N, 16.9; hydrazide N, 4.1.

***t*-Butyloxycarbonyl-isoleucyl-serine Hydrazide (XII).** This hydrazide was obtained by catalytic hydrogenolysis of IX (1 g) following the procedure described for X. The product was crystallized from methanol–ether: yield, 433 mg; mp 170–171°; $[\alpha]^{23}D$ −9.6° (*c* 1.3, dimethylformamide).

Anal. Calcd for $C_{14}H_{28}N_4O_5$: C, 50.5; H, 8.4; N, 16.8; hydrazide N, 8.4. Found: C, 50.3; H, 8.5; N, 16.6; hydrazide N, 8.6.

***t*-Butyloxycarbonyl-methionyl-glycyl-tryptophyl-methionyl-aspartyl-phenylalanine Amide (XIII).** Glycyl-tryptophyl-methionyl-aspartyl-phenylalanine amide trifluoroacetate[26] (15.4 g, 20 mmol) was acylated with *t*-butyloxycarbonyl-methionine 2,4,5-trichlorophenyl ester (9.8 g, 23 mmol) following the procedure described for the synthesis of II. The product was isolated by precipitation with ethyl acetate and crystallization from 95% ethanol: yield, 10.2 g (60%); mp 184–185°; $[\alpha]^{23}D$ −19.7° (*c* 1.4, dimethylformamide).

Anal. Calcd for $C_{41}H_{56}N_8O_{10}S_2$: C, 55.6; H, 6.4; N, 12.7; S, 7.2. Found: C, 56.0; H, 6.9; N, 13.3; S, 6.9.

Aspartyl-O-sulfate-tyrosyl-methionyl-glycine (XIV). The protected tetrapeptide III (150 mg) was dissolved in cold trifluoroacetic acid (5 ml), and after 1 hr at room temperature the solution was concentrated to dryness *in vacuo* and the residue was triturated with ether, filtered, washed with ether, and dried *in vacuo* over potassium hydroxide. The free tetrapeptide (135 mg) was added to concentrated sulfuric acid (8 ml) stirred and chilled in a Dry Ice–acetone bath at −5°. After 20 min the reaction mixture was poured into Dry Ice–cold ether (150 ml) with vigorous stirring. The precipitate was centrifuged and washed several times with cold ether. It was finally dissolved in 0.1 *M* ammonium carbonate and applied to a column of DEAE-Sephadex A-25 (11-mm diameter, 14 cm long) in the OH-cycle, previously washed with the same concentration of ammonium carbonate. The elution was carried out with a linear gradient of ammonium carbonate (300 ml of 0.1 *M* and 300 ml of 1.5 *M*). Fractions of 5 ml were collected and scanned by ultraviolet absorption at 256 mμ. The tubes corresponding to the peak of the sulfated tetrapeptide (fractions 48–64) were pooled, concentrated to dryness, and freeze dried several times from water to eliminate the ammonium carbonate: yield, 44 mg; ir (KBr) 1050 and 1250 cm^{-1}; partition chromatography, R_f^A 0.47, R_f^B 0.08; paper electrophoresis, 3.3(−26), 4.7(−61), 7.2(−67), 8.0 (−71), 9.3(−87); quantitative amino acid analysis, acid hydrolysis, Asp (0.95), Gly (1.0), Met (0.90), Tyr (1.07); enzymatic hydrolysis, Asp (0.92), Gly (1.02), Met (1.00).

Aspartyl-O-sulfate-tyrosyl-methionyl-glycine Amide (XV). *t*-Butyloxycarbonyl-β-*t*-butyl-aspartyl-tyrosyl-methionyl-glycine *p*-nitrophenyl ester (500 mg) was dissolved in cold trifluoroacetic acid (10 ml) and after 1 hr at room temperature the solution was concentrated to dryness *in vacuo*. The oily residue was treated with ether and the insoluble solid residue was filtered, washed with ether, and dried. This tetrapeptide nitrophenyl ester trifluoroacetate (450 mg) was dissolved in methanol (3 ml) and added with vigorous stirring to a 9 *M* solution of ammonia in methanol (200 ml). The solution was kept for 4 hr at room temperature, the solvent was removed *in vacuo*, and the residue triturated with ether: yield, 244 mg; neutralization equivalent (acid), calcd, 483; found, 482. This tetrapeptide amide (244 mg) was sulfated with concentrated sulfuric acid (15 ml) at −5° for 15 min. The isolation of the product was carried out as described for XIV: yield, 113 mg; ir (KBr) 1050 and 1250 cm^{-1}; partition chromatography, R_f^A 0.49; R_f^B 0.24; paper electrophoresis, 3.3(−21), 4.7(−35), 7.2(−45), 8.0(−46), 9.3(−65); quantitative amino acid analysis, acid hydrolysis, Asp (1.00), Gly (1.00), Met (1.00), Tyr (1.03); enzymatic hydrolysis, Asp (1.00), Gly (0.62), Met (1.00).

Aspartyl-tyrosyl-methionyl-glycyl-tryptophyl-methionyl-aspartyl-phenylalanine-amide (XVI). A. Dicyclohexylcarbodiimide (206 mg, 1 mmol) was added to an ice-cold solution of III (640 mg, 1 mmol) and *p*-nitrophenol (154 mg, 1.1 mmol) in a mixture of acetonitrile (3.2 ml) and dimethylformamide (0.3 ml). The reaction mixture was stirred for 2.5 hr at room temperature, the urea derivative was filtered off, and the filtrate was concentrated to dryness *in vacuo*. The residue was distributed for 60 transfers in the system chloroform–toluene–methanol–water (5:5:8:2). The protected tetrapeptide nitrophenyl ester was isolated from a peak with *K*, 0.6: yield, 316 mg; ir (Nujol) 1770 (C=O) cm^{-1}.

This nitrophenyl ester (547 mg, 0.72 mmol) was added to a solution of tryptophyl-methionyl-aspartyl-phenylalanine amide,[27] (428 mg, 0.6 mmol) in dimethylformamide (3.6 ml) and triethylamine (0.17 ml, 1.2 mmol). After 2 hr the reaction mixture was poured into ethyl acetate (170 ml) with vigorous stirring. The precipitate was collected by centrifugation, washed with ethyl acetate, and dried: yield, 694 mg. The protecting groups were removed by a 1 hr treatment with trifluoroacetic acid (20 ml) under a blanket of nitrogen. The trifluoroacetate (698 mg) was distributed for 300 transfers in the system 1-butanol–pyridine–acetic acid–water (4:2:1:7). The free octapeptide was isolated from a peak with K = 3.2: yield, 265 mg; $[\alpha]^{23}D$ −24.6° (*c* 0.6, 1 *N* NH_3); partition chromatography, R_f^A 0.77, R_f^B 0.51; paper electrophoresis, 3.3 (+2), 4.7 (−17), 7.2 (−22), 8.0 (−23), 9.3 (−32); quantitative amino acid analysis, acid hydrolysis, Asp (2.00), Gly (0.98), Met (1.93), Tyr (1.20), Phe (1.11); enzymatic hydrolysis, Asp (2.00), Gly (0.96), Met (2.03), Tyr (1.15), Phe (1.04).

B. Concentrated hydrochloric acid (1.92 ml) was added to a solution of X (1.6 g, 4 mmol) in dimethylformamide (16 ml) cooled and mechanically stirred in a Dry Ice–acetone bath at −20°. After 5 min a 14% aqueous solution of sodium nitrite (2 ml) was added and the temperature of the bath was kept at −15° for 5 min. After lowering the temperature to −30°, N-ethylpiperidine (4.16 ml) was added, followed by a solution of methionyl-glycyl-tryptophyl-methionyl-aspartyl-phenylalanine amide trifluoroacetate (prepared from 2.9 g of XIII with trifluoroacetic acid) in dimethylformamide (16 ml). The reaction mixture was stored at 5° for 18 hr and a second portion of dipeptide azide was added (prepared from 0.4 g of X). After another 24 hr storage at 5° the reaction mixture was poured into 360 ml of 3% aqueous acetic acid with vigorous stirring. The precipitate was filtered, washed with dilute acetic acid and dried *in vacuo:* yield, 3.3 g (XVIa). The *t*-butyloxycarbonyl protecting group was removed by a brief treatment (15 min) with trifluoroacetic acid (25 ml) under a blanket of nitrogen. The octapeptide trifluoroacetate was precipitated with ether: yield, 3.06 g

(24) This active ester was obtained in crystalline form from ethyl acetate–hexane; mp 101–103°; $[\alpha]^{23}D$ −25.3 (*c* 1.5, dimethylformamide). *Anal.* Found: C, 54.5; H, 7.1; N, 8.4. G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **86**, 1839 (1964), reported mp 92–93°; $[\alpha]^{25}D$ −26.5 (*c* 2, dioxane).

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(XVIb). These materials (XVIa, XVIb) were used as such for the sulfation experiments described below. A portion of XVIb (300 mg) was chromatographed in DEAE-Sephadex A-25 using a gradient of ammonium carbonate as described for XIV. The product obtained (120 mg) was identical with the preparation of A: paper chromatography, R_f^A 0.77; R_f^B 0.51; $[\alpha]^{23}D$ −22.9° (*c* 0.5, 1 *N* NH_3).

Aspartyl-O-sulfate-tyrosyl-methionyl-glycyl-tryptophyl-methionyl-aspartyl-phenylalanine Amide (XVII). A. The octapeptide trifluoroacetate XVIb (984 mg) was added to concentrated sulfuric acid (60 ml) cooled at −5° in a Dry Ice–acetone bath. After 15 min the reaction mixture was poured into Dry Ice–cold ether (850 ml) with vigorous stirring. The precipitate was separated by centrifugation and washed several times with cold ether. This residue, without drying, was dissolved in 0.1 *M* ammonium carbonate (20 ml) and the pH readjusted with 5 ml of 1.1 *M* ammonium carbonate. The resulting solution was applied to a column of DEAE-Sephadex A-25 in OH form (85 ml) previously washed with 0.1 *M* ammonium carbonate. The elution was carried out with a linear gradient from 900 ml of 0.1 *M* and 900 ml of 1.5 *M* ammonium carbonate. Ten-milliliter fractions were collected. Three major peaks were observed: tube 75–105 (unreacted octapeptide), tube 106–125 (sulfonated octapeptide), and tubes 150–212 (sulfated octapeptide). This last material is isolated by repeated freeze-drying from water to eliminate the ammonium carbonate: yield, 231 mg; $[\alpha]^{23}D$, −18.4° (*c* 0.7, 1 *N* NH_3); ir (KBr) 1050 and 1250 cm^{-1}; uv max (0.1 *N* NaOH) 280 (ε 4850) and 288 mμ (ε 4230), sh 273 mμ (ε 4650); partition chromatography, R_f^A 0.69, R_f^B 0.48; paper electrophoresis, 3.3 (−16), 4.7 (−36), 7.2 (−39), 8.0 (−44), 9.3 (−55); quantitative amino acid analyses, acid hydrolysis, Asp (2.04), Gly (1.00), Met (1.85), Tyr (1.09), Phe (1.04); enzymatic hydrolysis, Asp (2.02), Gly (1.00), Met (2.02), Phe (1.00). Two-dimensional thin-layer electrophoresis and chromatography on cellulose of the enzymatic hydrolysate showed the presence of a strongly acidic amino acid with the same mobility of tyrosine-O-sulfate.

B. The *t*-butyloxycarbonyl octapeptide XVIa (1 g) was dissolved in a mixture of dimethylformamide (55 ml, dried over molecular sieve Linden type 5A) and pyridine (55 ml, distilled over sodium hydroxide). The resulting solution was mixed with another solution of pyridine–sulfur trioxide complex (2.7 g) in dimethylformamide (55 ml). After 17 hr standing at room temperature, the solvent was removed *in vacuo* and the residue was taken up with water (20 ml). The solid precipitate was collected by centrifugation and washed with water. The *t*-butyloxycarbonyl group was removed by a brief treatment (15 min) with trifluoroacetic acid (10 ml). The trifluoroacetate (730 mg) was precipitated with ether and the octapeptide sulfate was isolated by chromatography on DEAE-Sephadex as described in A: yield, 333 mg. This material is identical with the preparation of A.

Aspartyl-3′-sulfonyl-tyrosyl-methionyl-glycyl-tryptophyl-methionyl-aspartyl-phenylalanine Amide (XVIII). The octapeptide trifluoroacetate XVIb (100 mg) was added to concentrated sulfuric acid (6 ml) cooled in a Dry Ice–acetone bath at −20°. The solution was stored at −20° for 66 hr. The sulfonated octapeptide was isolated and purified by chromatography on DEAE-Sephadex as described for XVII A: yield, 55 mg; $[\alpha]^{23}D$ −33.0° (*c* 1.1; 1 *N* NH_3); ir (KBr) 1020, 1.00 cm^{-1}; uv (0.1 *N* NaOH) max 247 (ε 12,700), 279 (ε 6300), and 283 mμ (ε 6300), sh 306 mμ (ε 4939); quantitative amino acid analyses, acid hydrolysis, Asp (2.00), Gly (1.05), Met (2.00), Tyr (0.75), Phe (1.04); enzymatic hydrolysis, Asp (2.00), Gly (1.04), Met (2.00), Phe (0.95).

Two-dimensional cellulose thin-layer electrophoresis and chromatography of the enzymatic hydrolyzate showed that the sulfonated tyrosine is identical with the material obtained by direct sulfonation of tyrosine.[28]

Aspartyl-arginyl-aspartyl-O-sulfate-tyrosyl-methionyl-glycyl-tryptophyl-methionyl-aspartyl-phenylalanine Amide (XIX). Concentrated hydrochloric acid (1.16 ml) was added to a solution of XI (1.6 g, 2.2 mmol) in dimethylformamide (9.6 ml) cooled in Dry Ice–acetone bath at −20°. The temperature of the bath is allowed to rise to −15° and a 14% aqueous sodium nitrite (1.3 ml) is added. After 5 min the temperature of the bath is lowered to −25° and N-ethylpiperidine (2.5 ml) is added, followed by a solution of hexapeptide trifluoroacetate (prepared from 1.06 g, 1.2 mmol of XIII) in dimethylformamide (6 ml). The reaction mixture was stored at 5° and two more portions of tetrapeptide azide (prepared from 750 and 375 mg of XI) were added after 48 and 96 hr. After a total of 120 hr the reaction mixture was poured into 200 ml of 0.2 *M* acetic acid with vigorous stirring. The precipitate was collected by filtration, washed with dilute acetic acid, and dried: yield, 1.1 g. The *t*-butyloxycarbonyl decapeptide (250 mg) was dissolved in a mixture of dimethylformamide (12 ml, dried over molecular sieve Linden type 5A) and pyridine (12 ml, distilled over sodium hydroxide) with gentle heating. The resulting solution was cooled down to room temperature and was mixed with a solution of pyridine–sulfur trioxide complex (544 mg) in dimethylformamide (12 ml). The reaction was stored for 17 hr at room temperature, the solvent was removed *in vacuo*, and the residue was taken up with water (8 ml). The solid precipitate was collected by centrifugation, washed with water, and dried *in vacuo:* yield, 247 mg. The *t*-butyloxycarbonyl group was removed by a brief treatment (15 min) with trifluoroacetic acid (2 ml) under a blanket of nitrogen. The trifluoroacetate (225 mg) was precipitated with ether and the decapeptide sulfate was isolated by chromatography on DEAE-Sephadex as described for XVII A: yield, 97 mg; $[\alpha]^{23}D$ −25.7° (*c* 0.7, 1 *N* NH_3); ir (KBr) 1050 and 1250 cm^{-1}; uv (0.1 *N* NaOH) max 280 (ε 4750) and 288 mμ (ε 4250); sh 273 mμ (ε 4600); partition chromatography, R_f^A 0.62, R_f^B 0.33: paper electrophoresis, 3.3 (+1), 4.7 (−27), 7.2 (−27), 8.0 (−37), 9.3 (−45); quantitative amino acid analysis, acid hydrolysis, Asp (2.90), Gly (1.00), Met (1.86), Tyr (1.09), Phe (1.05), Arg (1.00), enzymatic hydrolysis, Asp (2.96), Gly (1.07), Met (1.90), Phe (1.00), Arg (0.93).

Isoleucyl-seryl-aspartyl-arginyl-aspartyl-O-sulfate-tyrosyl-methionyl-glycyl-tryptophyl-methionyl-aspartyl-phenylalanine Amide (XX). Concentrated hydrochloric acid (0.12 ml) was added to a solution of XII (82 mg) in dimethylformamide (1 ml) cooled on Dry Ice–acetone bath at −20°. After 5 min the temperature was allowed to rise to −15°, and 14% aqueous sodium nitrite solution (0.125 ml) was added. The temperature was kept at −15° for 5 min and then lowered to −25° and N-ethylpiperidine (0.30 ml) was added. To this solution of the dipeptide azide a solution of XIX (224 mg) in dimethylformamide (2 ml) cooled at −20° was added. The reaction mixture was stored at 5° for 24 hr, and another portion of dipeptide azide (prepared from 82 mg of XII) was added. After another 24 hr at 5°, the reaction mixture was taken to dryness *in vacuo*. The *t*-butyloxycarbonyl groap was removed by a brief treatment (15 min) with trifluoroacetic acid (3 ml) under a blanket of nitrogen. The free dodecapeptide was chromatographed on a column of DEAE-Sephadex with a gradient of ammonium carbonate: yield, 132 mg; $[\alpha]^{22}D$ −22.8 (*c* 0.4 1*N* NH_3), ir (KBr) 1050 and 1250 cm^{-1}; uv (0.1 *N* NaOH) max 282 (ε 4940) and 289 mμ (ε 4460), sh 276 mμ (ε 4980); partition chromatography, R_f^A 0.66, R_f^B 0.57; paper electrophoresis, 3.3 (+5), 4.7 (−23), 7.2 (−25), 8.0 (−33), 9.3 (−41); quantitative amino acid analysis, acid hydrolysis, Asp (3.00), Ser (0.95), Gly (1.00), Met (1.93), Ile (1.00), Tyr (1.06), Phe (1.18), Arg (1.0); enzymatic hydrolysis, Asp (2.93), Ser (1.05), Gly (1.02), Met (1.76), Ile (1.08), Phe (0.95), Arg (1.00).

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