Cholecystokinin (Pancreozymin). 4.[1] Synthesis and Properties of a Biologically Active Analogue of the C-Terminal Heptapeptide with ε-Hydroxynorleucine Sulfate Replacing Tyrosine Sulfate

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The influence of tyrosine *O*-sulfate, the 27th residue in the sequence of cholecystokinin (pancreozymin) (CCK-PZ), on the contraction of gall bladder of guinea pigs and on the release of amylase in isolated pancreatic cells of the same animal was studied with an analogue of the biologically active C-terminal heptapeptide, CCK-PZ-(27–33). In the new analogue, tyrosine *O*-sulfate was replaced by ε-hydroxynorleucine *O*-sulfate. The synthetic peptide was found a full agonist in these tests, not quite as potent as the unaltered heptapeptide, but much more active than the previously prepared and studied serine *O*-sulfate containing analogue. Thus, the distance of the sulfate ester group from the peptide backbone has a major influence on the biological activity of CCK-PZ.

Cholecystokinin, a gastrointestinal hormone which stimulates the contraction of the gall bladder, was discovered by Ivy and Oldberg[3] in 1928. More than a decade later, a factor causing the release of digestive enzymes from the pancreas was detected by Harper and Raper[4] in the intestinal mucosa of the hog. More recently, Jorpes and Mutt[5] demonstrated that a single compound is responsible for both kinds of activities. The sequence of cholecystokinin (pancreozymin) (CCK-PZ), a 33-peptide,[6] was elucidated by Mutt and Jorpes[7] (Figure 1). A shorter peptide, caerulein, with very similar activities and with a sequence closely related to the C-terminal portion of CCK-PZ was isolated from the skin of hyla caerulea by Anastasi and her associates.[8] The analogy between CCK-PZ and caerulein can be extended also to the C-terminal sequence of gastrin.[9] Hence, these peptides can be regarded as members of the gastrin family.

Synthetic efforts[10] produced biologically highly active peptides corresponding to C-terminal sequences of caerulein, while the group led by Ondetti[11] prepared and studied peptides with C-terminal sequences of CCK-PZ. Synthesis of CCK-PZ-(1–8) was reported by Bodanszky and his associates[12] and that of CCK-PZ-(17–23) by Klausner and Bodanszky.[13] The entire 33-peptide chain was assembled by Yajima and his co-workers[14] but without the sulfate ester group on tyrosine-27. The omission of this sulfate ester group resulted in an ~250-fold reduction of the biological activities of the hormone. The major contribution of the sulfate ester group to potency emerged also from the studies cited in ref 10 and 11. It was, therefore, of considerable interest to investigate the influence of tyrosine *O*-sulfate on the potency of CCK-PZ in more detail. Particularly, it was tempting to replace the tyrosine *O*-sulfate residue with moieties which would result in less complicated synthesis.[15]

In the preceding paper in this series,[1] we reported the replacement of L-tyrosine *O*-sulfate by L-serine *O*-sulfate in the potent C-terminal heptapeptide portion of the CCK-PZ chain. The definite, albeit weak, activity of the serine *O*-sulfate analogue revealed that the nature of the residue that occupies this position has a major influence on the hormone–receptor interaction and, hence, on potency. It became necessary to explore the significance of the aromatic nucleus in the side chain of this residue. To wit, it seemed to be possible that this ring is not necessary as such, that it serves merely as a spacer between the more crucial sulfate ester group and the peptide backbone. The greatly reduced activity found in the serine *O*-sulfate analogue[1] could have been due to the short distance determined by the one-carbon side chain in serine rather than to the absence of aromatic character or ring structure. We decided, therefore, to prepare a new analogue in which the side chain of the residue replacing tyrosine has approximately the length of the tyrosine side chain. An inspection of molecular models suggested a four- or five-carbon aliphatic chain. Because of previous experience[16] in this laboratory with ε-hydroxynorleucine in peptide synthesis, this hydroxyamino acid was chosen as a constituent of the new analogue of CCK-PZ-(27–33).

Synthesis. Difficulties experienced[17] in the synthesis of C-terminal segments of CCK prompted a new approach. The heptapeptide derivative, *tert*-butyloxycarbonyl-*O*-acetyl-L-ε-hydroxynorleucyl-L-methionylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide, was built by the stepwise approach[18] with benzyloxycarbonylamino acids[19] up to the pentapeptide stage and with *tert*-butyloxycarbonyl protection[20] in the case of the two last added residues (Chart I). A characteristic feature of the synthesis is the removal of the benzyloxycarbonyl group by hydrogenolysis,[19] even though the protected peptides contained methionine. The conditions proposed by Medzihradszky-Schweiger and Medzihradszky[21] were applied, but for better efficiency they had to be modified.[22] Thus, Pd on $BaSO_4$ was found to be a more suitable catalyst, in this case, than Pd on charcoal. It should be noted here that the reduction proceeded smoothly and within a few hours, only if water was added to the solution of the protected peptides in DMF. Often a black (colloidal?) solution resulted from this step. The color (precipitate?) could be eliminated by filtration through a short column of activated neutral alumina. This was best done after the next acylation step was completed. The incorporation of the second methionine residue produced a protected peptide which acted as a catalyst poison even under the conditions just described. The benzyloxycarbonyl group could be removed only by repeated additions of the catalyst, and it had to be applied in impractically large amount. Therefore, for the final execution of the synthesis, the *tert*-butyloxycarbonyl group was used at this point and also in the incorporation of the ε-hydroxynorleucine residue. The DL form of the latter was obtained as described earlier.[16] For resolution with acylase,[23] the *N*-acetyl derivative was applied. The L-amino acid was acetylated by a method known for serine[24] and then protected on the α-amino group by reaction with

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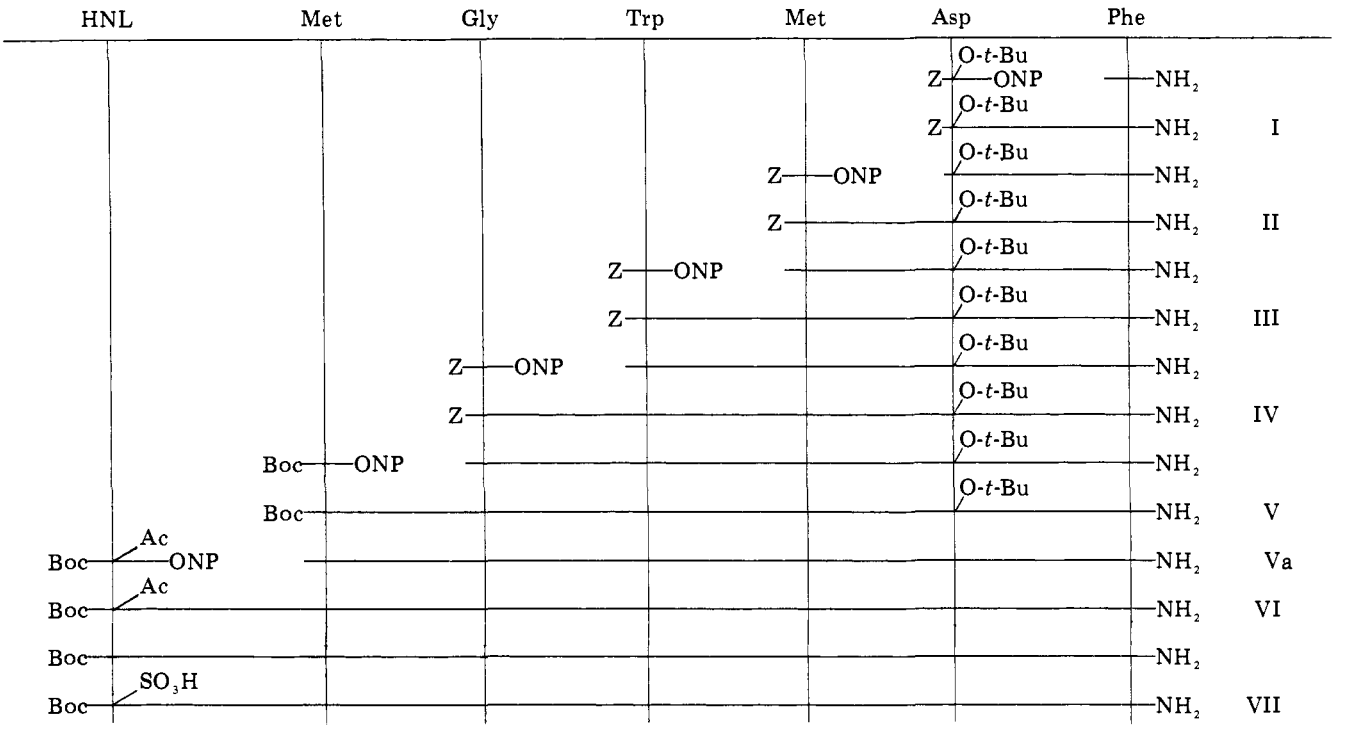
Chart I[a]



[a] HNL = ε-hydroxynorleucine.

2 4 6 8 10 12 14
Lys-Ala-Pro-Ser-Gly-Arg-Val-Ser-Met-Ile-Lys-Asn-Leu-Gln-
16 18 20 22 24 26
Ser-Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-
28 30 32
Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2

Figure 1. The amino acid sequence of cholecystokinin (pancreozymin). The sequence of the originally studied (ref 7) 33-peptide is shown. Subsequently, a 39-peptide (with an additional six residues at the N terminus) was also identified.[41]

Boc-ON.[25] Finally, the *tert*-butyloxycarbonyl-*O*-acetyl-L-ε-hydroxynorleucine was converted to its crystalline *p*-nitrophenyl ester, which was used for the acylation of the completely deprotected hexapeptide amide. Removal of the *O*-acetyl group with ammonia in a mixture of DMF and methanol and sulfation with SO_3–pyridine complex[26] completed the synthesis. The final product was obtained in homogeneous and analytically pure form by chromatography on a column of silica gel with ethyl acetate–pyridine–acetic acid–water as eluent.

Release of Amylase from Isolated Pancreatic Cells of Guinea Pigs. Release of amylase from dispersed pancreatic acini increased significantly by 3×10^{-11} M CCK-(26–33) and was maximal with 3×10^{-10} M (Figure 2). Concentrations of CCK-(26–33) greater than 3×10^{-10} M caused submaximal stimulation of enzyme secretion (Figure 2). The *tert*-butyloxycarbonyl derivative of CCK-(27–33) (A) was identical with CCK-(26–33) in its ability to increase amylase secretion. Each of the analogues of compound A, when tested at sufficiently high concentrations, stimulated amylase secretion as effectively as did the parent peptide and their dose–response curves were parallel (Figure 2). Absence of the sulfate ester group from tyrosine caused a 600-fold reduction in the potency of compound A. Replacing the tyrosine sulfate residue in this heptapeptide derivative by ε-hydroxynorleucine sulfate (compound B) caused about a tenfold reduction in potency, while replacing tyrosine sulfate with serine sulfate (compound C) caused a 1000-fold reduction.

Contraction of the Gall Bladder of the Guinea Pig. The effect of the three peptides, A = *N*-*tert*-butyloxycarbonyl(*O*-sulfate)-L-tyrosyl-L-methionylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide, B = *N*-*tert*-butyloxycarbonyl(*O*-sulfate)-L-ε-hydroxynorleucyl-L-methionylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide, and C = *N*-*tert*-butyloxycarbonyl(*O*-sulfate)-L-seryl-L-methionylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide, on the guinea pig gall bladder in situ was assayed according to the method of Ljungberg.[27] It is evident from Figure 3 (upper row) that the injection of 7 ng of A gives approximately the same contraction as the injection of 0.07 Ivy dog units (IDU) of standard cholecystokinin, whereas in the same amounts B gives a weaker but nevertheless quite marked response and C no noticeable response. From the middle row of Figure 3, it is evident that B is slightly more than one-third and C somewhat less than $^1/_{200}$th as active as A. Finally, the lower row demonstrates that A is slightly more than twice as potent as B and 250 times as active as C. Although the patterns of the responses are somewhat different for B and C than for A (shorter duration of contraction at the same or stronger *initial* degree of contraction), it may be stated that in this assay system the activity of B is about 40% that of A and the activity of C not more than 0.4% of A.

Conclusions

The action of CCK on its target tissues is mediated by specific cell surface receptors which also interact with naturally occurring, structurally related peptides such as gastrin and caerulein.[28] Our present results indicate that in CCK, altering the chemical nature of the side chain in position 27 influences the affinity of the peptide for its receptors but does not alter qualitatively its intrinsic

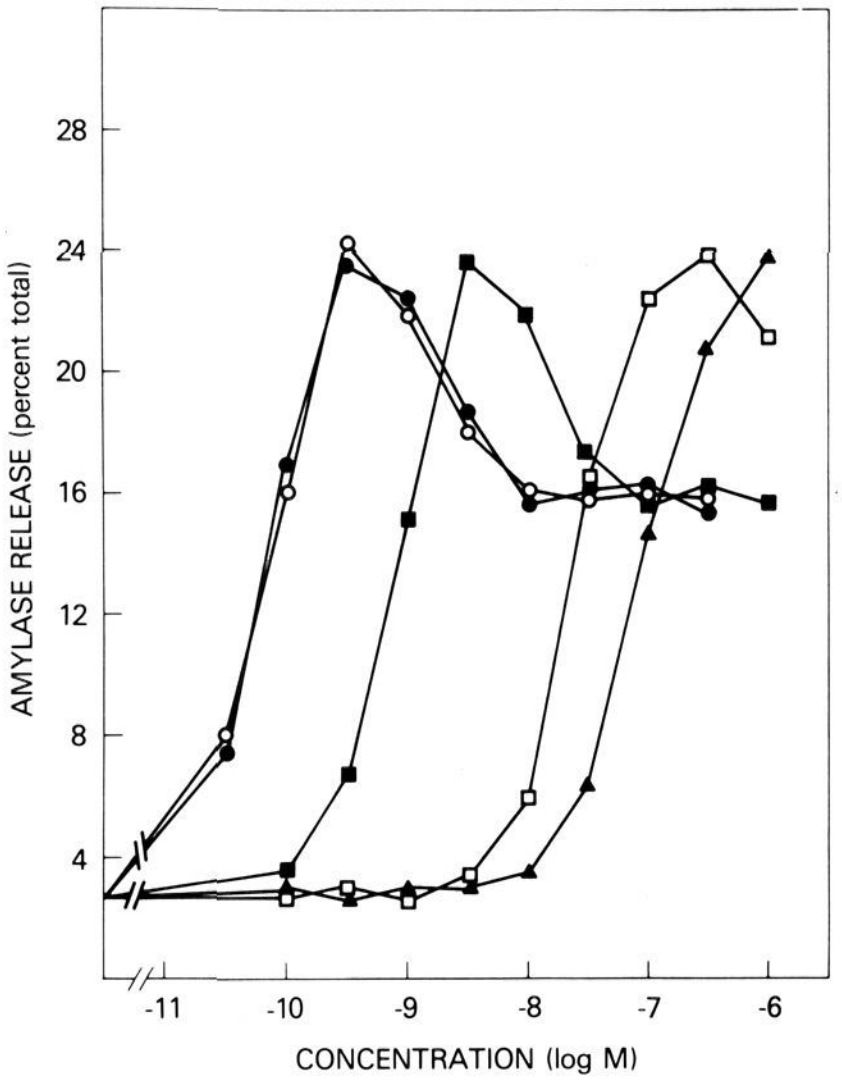


Figure 2. Release of amylase from acinar cells of guinea pigs: (-●—●-) CCK-(26–33) [Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2]; (-○—○-) Boc-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2 (A); (-■—■-) Boc-HNL(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2 (B); (-□—□-) Boc-Try-Met-Gly-Trp-Met-Asp-Phe-NH_2; (-▲—▲-) Boc-Ser(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2 (C).

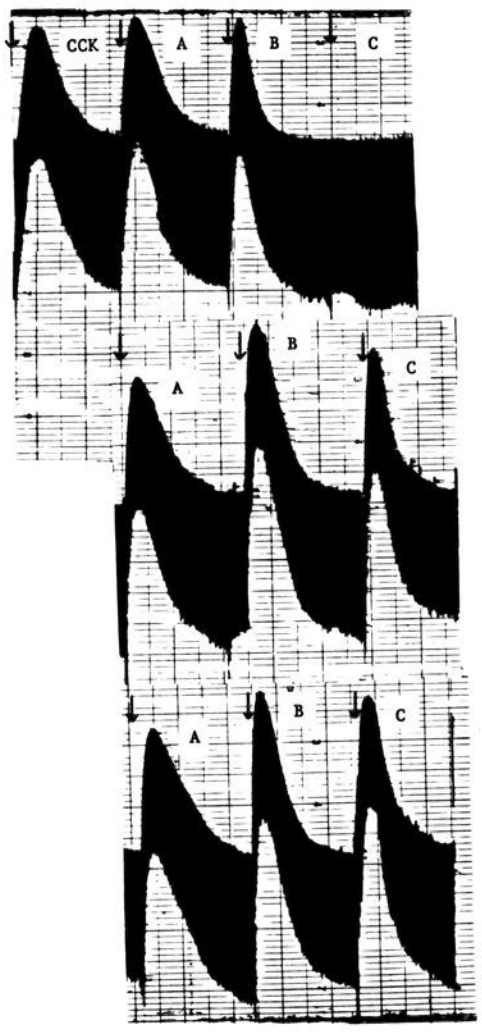


Figure 3. Showing effects on the guinea pig gall bladder in situ *of three peptides, A–C, structurally related to cholecystokinin* (see text). Assay technique according to Ljungberg.[27] From left to right: (top row) 0.07 IDU of CCK, 7 ng of A, 7 ng of B, 7 ng of C; (middle row) 7 ng of A, 21 ng of B, 1400 ng of C; (bottom row) 7 ng of A, 14 ng of B, 1900 ng of C.

biologic activity. The activity of the ϵ-hydroxynorleucine analogue of CCK-(27–33) is somewhat less than that of the unaltered *tert*-butyloxycarbonyl heptapeptide in the stimulation of amylase release from the acinar cells of guinea pigs. Their dose–response curves are parallel and, when compared at the half-maximal response, the sulfated ϵ-hydroxynorleucine peptide is about ten times less potent than the one that contains tyrosine sulfate in the corresponding position. In the same experiments, the ϵ-hydroxynorleucine sulfate analogue is about 100 times more potent than the serine sulfate analogue.[1] The relative potencies are somewhat different in the ability of these peptides to contract the gall bladder of guinea pigs; yet, in this test also, substitution of tyrosine by ϵ-hydroxynorleucine results only in a moderate decrease in potency, while the replacement of tyrosine by serine caused a very substantial loss in activity. These comparisons clearly demonstrate that the distance between the sulfate ester group and the peptide backbone is an important structural feature of CCK. The difference between the activity of the unsulfated tyrosine-containing peptide (Figure 2) and that of the serine sulfate analogue demonstrates that the aromatic ring of tyrosine is also not without effect in the hormone–receptor interaction. The receptors in the pancreas and in the gall bladder were stimulated by the ϵ-hydroxynorleucine derivative, but for a perfect molecular fit and, thus, for full potency, the presence of the tyrosine residue *and* the sulfate ester group seems to be necessary.

Experimental Section

Capillary melting points are reported uncorrected. Thin-layer chromatography (TLC) was performed on (Merck) silica-gel plates in the following solvent systems (ratios by volume): A, 1-butanol–AcOH–H_2O (4:1:1); B, chloroform–methanol (9:1); C, chloroform–methanol (8:2); D, chloroform–methanol (30:1); E, EtOAc–pyridine–AcOH–H_2O (60:20:6:11); F, EtOAc–pyridine–AcOH–H_2O (120:20:6:11). Spots were revealed with iodine vapor, ninhydrin, and fluorescamine and also by charring and by their absorption in the UV. Unless otherwise stated, samples for amino acid analysis were hydrolyzed with constant boiling HCl in evacuated, sealed ampules at 110 °C for 16 h. Analyses were by the Spackman–Stein–Moore method on a Beckman-Spinco instrument. The following abbreviations are used: DMF, dimethylformamide; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid.

***N*-Benzyloxycarbonyl-β-*tert*-butyl-L-aspartyl-L-phenylalanine Amide (I).** A solution of L-phenylalanine amide hydrochloride[17a] (3.8 g, 19 mmol) in DMF (50 mL) was cooled in an ice–water bath. Then, DIEA (6.4 mL, 40 mmol) and 1-hydroxybenzotriazole monohydrate[29] (3.0 g, 19 mmol) were added, followed by a solution of *p*-nitrophenyl *N*-benzyloxycarbonyl-β-*tert*-butyl-L-aspartate[30] (8.7 g, 19.6 mmol) in DMF (50 mL). After 30 min, the mixture was allowed to come to room temperature and was stirred overnight. The fluorescamine reaction was negative at that time. The solvent was removed in vacuo and the residue triturated and stirred with ether until a white powder was obtained. This was collected and washed with ether. After drying, the product weighed 8.5 g (95.5%): mp 156–158 °C; single spot on TLC, R_f(B) 0.5 (lit.[31] mp 158.5–159.5 °C).

***N*-Benzyloxycarbonyl-L-methionyl-β-*tert*-butyl-L-aspartyl-L-phenylalanine Amide (II).** A sample of compound I (8.4 g, 18 mmol) was hydrogenated in methanol (300 mL) in the presence of a 10% Pd-on-charcoal catalyst. After removal of the catalyst and the solvent, the oily residue was triturated with ether. The crystals, which formed, were filtered, washed with ether, and dried: 5.7 g (95%); mp 122–124 °C (lit.[31] mp 123–124 °C). The major part of the dipeptide amide (5.5 g, 16.5 mmol) was dissolved in DMF (100 mL), cooled in an ice–water bath, and treated with *N*-benzyloxycarbonyl-L-methionine *p*-nitrophenyl ester[32] (7.33 g,

18.6 mmol). After 0.5 h at 0 °C, the mixture was kept at room temperature until the fluorescamine reaction became negative. The solvent was evaporated in vacuo, and the crystalline residue was triturated with EtOAc, filtered, washed with ether, and dried in vacuo. The protected tripeptide amide (9.39 g, 95%), mp 198–201 °C, gave one spot on TLC, R_f(A) 0.9, R_f(B) 0.55; $[\alpha]^{25}_D$ −39.4° (*c* 1.1, DMF). Anal. ($C_{30}H_{40}N_4O_7S$) C, H, N, S.

***N*-Benzyloxycarbonyl-L-tryptophyl-L-methionyl-*β*-*tert*-butyl-L-aspartyl-L-phenylalanine Amide (III).** Compound II (8.5 g, 14 mmol) was dissolved in DMF (320 mL); water (80 mL) was added, followed by DIEA (12 mL, 75 mmol) and a 10% Pd-on-$BaSO_4$ catalyst (0.85 g). The mixture was hydrogenated in a closed system overnight. The calculated amount of H_2 was absorbed, and examination by TLC showed the absence of the starting material and the formation of a single compound: R_f(A) 0.6, R_f(C) 0.5. After the removal of the catalyst, the black solution was concentrated in vacuo to about 50 mL to remove water and the tertiary base. The volume was adjusted, with DMF, to 300 mL; the solution was cooled in an ice–water bath and treated with *N*-benzyloxycarbonyl-L-tryptophan *p*-nitrophenyl ester[33] (7.3 g, 16 mmol). After 0.5 h at 0 °C and overnight at room temperature, the fluorescamine reaction was negative. The solution was concentrated in vacuo to ca. 150 mL and passed through a column of neutral alumina (100 g). Elution with DMF gave a clear, colorless solution that was evaporated in vacuo. Trituration of the residue with ether gave a solid, which was resuspended in EtOAc, stirred, filtered, washed with EtOAc and then with ether, and dried in vacuo. The product (compound III) weighed 9.9 g (90%): mp 192–196 °C; $[\alpha]^{25}_D$ −35.2° (*c* 1, DMF); R_f(B) 0.45. A second crop (0.3 g, 3%) was recovered from the EtOAc washings. Anal. ($C_{41}H_{50}N_6O_8S$) C, H, N, S.

***N*-Benzyloxycarbonylglycyl-L-tryptophyl-L-methionyl-*β*-*tert*-butyl-L-aspartyl-L-phenylalanine Amide (IV).** To a solution of compound III (6.8 g, 8.7 mmol) in DMF (400 mL), water (100 mL) and DIEA (14 mL) were added. The mixture was hydrogenated in the presence of a 10% Pd-on-$BaSO_4$ catalyst (0.7 g) as described above. After the removal of the catalyst and most of the solvent, the black solution (ca. 80 mL) was diluted with DMF to ca. 150 mL, cooled in an ice–water bath, and treated with *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester[34] (3.2 g, 9.6 mmol). After the acylation was complete, the solution was further diluted with DMF (ca. 250 mL) and passed through a column of neutral alumina (50 g). The product was isolated as described in the previous paragraph. The protected pentapeptide (6.8 g, 93%), mp 188–190 °C, showed a single spot on TLC, R_f(A) 0.85, R_f(B) 0.6; $[\alpha]^{25}_D$ −24.4° (*c* 1.3, DMF). Anal. ($C_{43}H_{53}N_7O_9S$) C, H, N, S.

***N*-*tert*-Butyloxycarbonyl-L-methionylglycyl-L-tryptophyl-L-methionyl-*β*-*tert*-butyl-L-aspartyl-L-phenylalanine Amide (V).** This intermediate was prepared similarly to compound IV. A sample of compound IV (2.2 g, 2.6 mmol) in DMF (100 mL) and water (30 mL) was hydrogenated in the presence of DIEA (4 mL) and 10% Pd-on-$BaSO_4$ catalyst (0.22 g). Acylation was carried out in DMF (50 mL) with *N*-*tert*-butyloxycarbonyl-L-methionine *p*-nitrophenyl ester[35] (1.1 g, 3 mmol). After filtration through alumina (20 g) and concentration and trituration with ether and then with EtOAc, the protected hexapeptide amide V (2.24 g, 92%), mp 196–200 °C, was homogeneous on TLC, R_f(A) 0.75, R_f(B) 0.5; $[\alpha]^{25}_D$ −26.2° (*c* 1, DMF). Anal. ($C_{45}H_{64}N_8O_{10}S_2$) C, H, N, S.

The corresponding benzyloxycarbonyl derivative was prepared in essentially the same way. It was secured in 91% yield: mp 208–211 °C dec; $[\alpha]^{25}_D$ −27.3° (*c* 1, DMF); TLC R_f(B) 0.5. Anal. ($C_{48}H_{62}N_8O_{10}S$) C, H, N, S.

L-Methionylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine Amide (Va). A solution of acetyl-L-methionine *n*-butyl ester[36] (1 g) in a mixture of TFA (20 mL) and 2-mercaptoethanol (2 mL) was cooled in an ice–water bath, while a stream of N_2 was passed over it. A sample of the protected hexapeptide V (2.5 g, 2.67 mmol) was added and the solution was stirred at 0 °C under N_2 for 7 h. Ether (200 mL) was added to the well-stirred reaction mixture. The precipitate that formed was collected and washed with ether and then with EtOAc. This product was dissolved, with gentle heating, in a mixture of EtOAc, pyridine, AcOH, and H_2O (60:20:6:11) (50 mL). The solvent mixture was evaporated with a stream of N_2 and this process repeated once more. The residue was treated with more (30 mL) of the same solvent mixture, and the suspension was allowed to cool to room temperature and then placed in the refrigerator. The next day the precipitate was collected and then suspended in hot water. After standing at room temperature and then in the refrigerator, a white powder was obtained, which was filtered, washed with water, and dried in vacuo. The purified free hexapeptide amide Va weighed 1.54 g. With a second crop obtained from the washings, a total yield of 1.75 g (83%) was secured: mp 214–216 °C dec; $[\alpha]^{25}_D$ −20.2° (*c* 1.1, DMF); a single spot on TLC, R_f(A) 0.45, R_f(E) 0.25. Anal. ($C_{36}H_{48}N_8O_8S_2 \cdot 2H_2O$) C, H, N.

L-ε-Hydroxynorleucine. Triethylamine (42.5 mL, 306 mmol) and *p*-nitrophenyl acetate (30.5 g, 169 mmol) were added to a solution, in water (500 mL), of crude DL-ε-hydroxynorleucine[16] (22.5 g, 153 mmol). After being stirred overnight, the solution was made acid to congo red paper, with 2 M HCl, and extracted three times with ether. The separated aqueous layer was concentrated in vacuo, acetone was added, and the precipitated triethylamine hydrochloride was removed by filtration. On evaporation of the filtrate, and addition of acetone to the residue, more salt separated. It was removed and the evaporated filtrate yielded a light brown oil.

The oil was dissolved in water (800 mL). Following adjustment of the pH to 7.5 with LiOH solution (first 2 M and then 0.1 M), hog kidney acylase[23] (100 mg) was added. The mixture was incubated at 37 °C. Further slight adjustment of the pH to 7.5 was made twice during the next 48 h, and on each occasion acylase (100 mg) was added. After that time, glacial acetic acid was added to pH 5.0, and the solution was boiled for 1 min with a mixture (1:1 w/w) of charcoal and Celite (1 g). Filtration, followed by evaporation of the filtrate, and addition of ethanol to the oily residue produced the required amino acid. This was collected, washed well with ethanol, and dried: 6.4 g; mp 250–251 °C; $[\alpha]^{21}_D$ +22.8° (*c* 2, 5 M HCl) [lit.[37] $[\alpha]^{27}_D$ +23.7° (*c* 2, 6 M HCl)]; TLC R_f(A) 0.23.

***O*-Acetyl-L-ε-hydroxynorleucine.** A mixture of perchloric acid (3.4 g of a 70% solution, 24 mmol of $HClO_4$) and acetic anhydride (1.9 mL, 20 mmol) was diluted to about 40 mL with glacial acetic acid; L-ε-hydroxynorleucine (2.9 g, 20 mmol) and further acetic anhydride (8 mL) were added. The mixture was stirred at room temperature for 1.5 h. Water (1 mL) was added and stirring continued for 1 h. Then, the addition of *tert*-butylamine (3.3 mL, 32 mmol) and ether (200 mL) produced a white precipitate that was isolated by filtration and washing with ether. The product was used without further purification: yield 3.4 g (91%); mp 234–241 °C; $[\alpha]^{22}_D$ +19.6° (*c* 2, 6 M HCl); TLC R_f(A) 0.28 [lit.[16] mp 233–237 °C; $[\alpha]^{25}_D$ +18.9° (*c* 2.2, 6 M HCl)].

***N*-*tert*-Butyloxycarbonyl-*O*-acetyl-L-ε-hydroxynorleucine *p*-Nitrophenyl Ester.** A solution, in DMF, of *O*-acetyl-L-ε-hydroxynorleucine (3.0 g, 15.8 mmol), DIEA (2.53 mL, 15.8 mmol), and 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile[25] (Boc-ON) (4.3 g, 17.4 mmol) was stirred at room temperature overnight. The solvent was evaporated, water and ether were added, the layers were separated, and the organic layer was washed twice more with water. The aqueous phases were combined and acidified with 1 M $KHSO_4$. Extraction with ether, drying, and evaporation of solvent in vacuo afforded the protected amino acid (3.0 g, 65%): an oil; TLC R_f(A) 0.85.

Dicyclohexylcarbodiimide (2.06 g, 10 mmol) was added to a cooled (0 °C), stirred solution in EtOAc (40 mL) of the amino acid derivative (3.0 g, 10.4 mmol) and *p*-nitrophenol (1.58 g, 11.4 mmol). Stirring continued at 0 °C for 1 h and then for 16 h at room temperature. The dicyclohexylurea was removed by filtration. Evaporation of the solvent in vacuo yielded an oil, which solidified on trituration with hexane. The active ester (3.62 g, 88%), mp 47–51 °C, R_f(D) 0.81, still contained a trace of *p*-nitrophenol, R_f(D) 0.5. Recrystallization of a portion of the product, from EtOAc and hexane, produced chromatographically homogeneous material: mp 54–56 °C; $[\alpha]^{22}_D$ −25.8° (*c* 1.1, EtOAc). Anal. ($C_{19}H_{26}N_2O_8$) C, H, N.

***N*-*tert*-Butyloxycarbonyl-*O*-acetyl-L-ε-hydroxynorleucyl-L-methionylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine Amide (VI).** A solution, in DMF (1 mL), of the foregoing active ester (51 mg, 0.125 mmol) and L-methionylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenyl-

alanine amide (Va) (78 mg, 0.1 mmol) was stirred at room temperature for 20 h. Evaporation of the solvent, followed by trituration and washing with EtOAc, yielded the protected heptapeptide VI: 98 mg (93%); mp 205–208 °C; $[\alpha]^{21}_D$ −27.3° (c 1.04, DMF); TLC R_f(A) 0.79, R_f(E) 0.72. A sample was hydrolyzed for amino acid analysis: Asp, 1.06; Met, 2.05; Phe, 1.00. As expected,[16] hydroxynorleucine eluted with Gly and hydroxynorleucine lactone appeared just before the peak corresponding to Phe. Anal. ($C_{49}H_{69}N_6O_{13}S_2$) C, H, N. From a larger scale preparation, the title compound VI (465 mg) was obtained in 96% yield.

***N*-*tert*-Butyloxycarbonyl(*O*-sulfate)-L-ε-hydroxynorleucyl-L-methionylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine Amide (VII).** Methanol (20 mL) was added to a solution in DMF (4 mL) of the protected heptapeptide VI (500 mg, 0.47 mmol). Ammonia gas was blown onto the surface of the resulting solution cooled in an ice bath. After 1.5 h the flask was stoppered and the mixture allowed to stand at room temperature. Each subsequent day, the progress of the reaction was checked by TLC and the mixture resaturated with ammonia. After 5 days, all starting material had disappeared, the solvents were evaporated, and the residue was triturated and washed with ether. The deacetylated peptide (517 mg, in excess in 100%), R_f(F) 0.19, was dried in a vacuum desiccator.

SO_3–pyridine complex (1.2 g) was added to a stirred solution of the heptapeptide derivative (200 mg) in dry pyridine (2 mL) and DMF (5 mL). Stirring continued for 24 h; then the solvents were evaporated in vacuo. Cold water (5 mL) was added, followed by concentrated ammonia solution, dropwise, to maintain the pH at about 7 (indicator paper). After about 30 min, the solution remained neutral. Some product (127 mg), in suspension, was collected by centrifugation, washed with water, and dried in a vacuum desiccator over P_2O_5. A further crop (61 mg) was isolated by extraction of the aqueous solutions with 1-butanol. A portion (120 mg) of the crude product was purified by chromatography on a column of silica gel (15 g, 40 × 1 cm) with EtOAc–pyridine–AcOH–H_2O (60:20:6:11) as eluent. Fractions were examined by TLC: no. 8–20 contained the required material but also a trace of a faster impurity. Fractions 21–28 containing the pure sulfated heptapeptide (VII) were evaporated with a stream of N_2, pooled in 0.1 M NH_4OH, and lyophilized to yield VII (43 mg): mp 175–178 °C dec; homogeneous on TLC, R_f(A) 0.55, R_f(E) 0.31. After hydrolysis with 6 N HCl, amino acid analysis showed Asp, 0.93; Met, 1.95; Phe, 1.00 (Gly and hydroxynorleucine coincided, the hydroxynorleucine lactone appears before Phe). On hydrolysis with leucine aminopeptidase (after removal of the Boc group), amino acid analysis showed Asp, 1.02; Gly, 1.00; Met, 2.07; Phe 1.04 (ε-hydroxynorleucine sulfate appeared before Asp and had about the same area). Anal. ($C_{47}H_{67}N_9O_{15}S_3 \cdot 4H_2O$) C, H, N. This material was used in the biological tests. Residues from fractions 8–20 (and some beyond 28) were pooled similarly to give slightly contaminated VII (55 mg).

Release of Amylase from Pancreatic Acinar Cells of Guinea Pigs. A. Materials. Male NIH strain guinea pigs (150–200 g) were obtained from the Small Animals Section, Veterinary Resources Branch, National Institutes of Health, Bethesda, Md.; Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) from Calbiochem. La Jolla, Calif.; carbamylcholine and soybean trypsin inhibitor from Sigma Chemical Co., St. Louis, Mo.; Basal medium (Eagle) amino acids (100 times concentrated) from Grand Island Biological Co., Grand Island, N.Y.; essential vitamin mixture (100 times concentrated) from Microbiological Associates, Bethesda, Md.; glutamine from Research Plus Laboratories, Inc., Denville, N.J.; bovine plasma albumin (fraction V) from Armour Pharmaceutical Co., Pheonix, Ariz.; purified collagenase (*Clostridium histolyticium*) from Worthington Biochemical Corp., Freehold, N.J.; and Phadebas amylase test from Pharmacia Diagnostics, Piscataway, N.J. The synthetic C-terminal octapeptide of cholecystokinin (CCK 26-33) was a gift from Dr. Miguel A. Ondetti, Squibb Institute for Medical Research, Princeton, N.J.

Unless otherwise stated, the standard incubation solution contained 24.5 mM Hepes (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM NaH_2PO_4, 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 11.5 mM glucose, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 2 mM glutamine, 0.2% (w/v) albumin, 0.01% (w/v) trypsin inhibitor, 2% (v/v) amino acid mixture, and 1% (v/v) essential vitamin mixture. The pH of the incubation solution was 7.4. The incubation solution was equilibrated with 100% O_2 and all incubations were performed with 100% O_2 as the gas phase.

B. Methods. Tissue Preparation. A guinea pig was killed by a blow to the head. The pancreas was removed immediately and trimmed of fat and mesentery. A suspension of dispersed acini was prepared by injecting the pancreas five times with 5 mL of digestion solution composed of standard incubation solution plus purified collagenase (0.18 mg/mL). The pancreas was incubated for 15 min at 37 °C in a Dubnoff incubator at 160 oscillations per minute. The gas phase was 100% O_2. The digestion solution was discarded and the tissue was washed three times with 5 mL of standard incubation solution. The pancreas was incubated with 5 mL of digestion solution for 10 min at 37 °C and washed once with standard incubation solution. This step was repeated once and the tissue was dispersed by passing it five times through each of a series of siliconized glass pipets of decreasing bore (3, 1, and 0.5 mm). Large fragments of tissue and the duct system were discarded. The suspension of dispersed acini was layered over standard incubation solution containing 4% (w/v) albumin and centrifuged for 5 s at 800 rpm. The supernatant was discarded and the acini were washed twice with standard incubation solution containing 4% (w/v) albumin. Dispersed acini from one pancreas were suspended in 200 mL of standard incubation solution containing 0.5 mM calcium and 5 mM theophylline.

C. **Amylase release** was measured, using the procedure reported previously.[38] At appropriate times, 500 μL of cell suspension was centrifuged at 10000g for 15 s in a Beckman Model 153 microcentrifuge. Supernatant (200 μL) was added to 500 μL of diluent solution [0.01 M sodium phosphate, pH 7.8, 0.1% (w/v) albumin, and 0.1% (w/v) sodium dodecylsulfate]. Duplicate 100-μL samples were assayed for amylase activity by the method of Ceska et al.,[39,40] using the Phedabas reagent. To assay total amylase activity, 1.5 mL of cell suspension was added to 15 mL of diluent solution and after vigorous agitation, duplicate 100-μL samples were assayed for amylase activity. Amylase release was expressed as the percent of total amylase activity which appeared in the incubation medium during the incubation period.

Triplicate values from the same incubation tube had a coefficient of variation of 4% and values from triplicate incubation tubes had a coefficient of variation of 9%.

Contraction of the Gall Bladder. The method of Ljungberg[27] was used, in which the contractions of the gall bladder are recorded (enlarged) via a strain gauge transducer. For the assay, the peptides were first dissolved to a concentration of 10 μg/mL in physiological saline to which 1 mg of $NaHCO_3$ and 1 mg of L-cysteine had been added per milliliter. The necessary dilutions were made with physiological saline. The results are shown in Figure 3.

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Synthesis and Antiinflammatory Activity of 6,11-Dihydro-11-oxodibenzo[*b,e*]thiepinalkanoic Acids and Related Compounds[1]

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Acetic acid derivatives of tricyclic systems, such as 6,11-dihydro-11-oxodibenzo[*b,e*]thiepin, 4,10-dihydro-4-oxothieno[2,3-*c*][1]benzothiepin, dibenzo[*b,f*]thiepin, dibenz[*b,f*]oxepin, etc., were synthesized and assayed for antiinflammatory activity. One of the compounds, 6,11-dihydro-11-oxodibenzo[*b,e*]thiepin-3-acetic acid (**52**), was chosen for evaluation in man on the basis of high antiinflammatory activity in both short- and long-term animal assays and a low gastric irritation liability in rats and dogs.

During the past 15 years a very large number of nonsteroidal antiinflmmatory agents, principally aryl- and heteroarylacetic and -propionic acids, have been synthesized[2] with the aim of finding substances with fewer side effects (especially gastric irritation) than phenylbutazone and indomethacin. Of the numerous compounds synthesized, only a few, including ibuprofen,[3] ketoprofen,[4] naproxen,[5] and tolmetin,[6] have met most of the above

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