(16) F. B. Zienty, B. D. Vineyard, and A. A. Schleppnik, *J. Org. Chem.*, **27**, 3142 (1962).
(17) N. J. Leonard and W. C. Wildman, *J. Am. Chem. Soc.*, 71, 3092 (1949).
(18) C. F. Copp and F. H. Hodson, U.S. Patent 3474134 (1969); *Chem. Abstr.*, **72**, P31478c (1970).
(19) V. C. Barry, L. O'Rourke, and D. Twomey, *Proc. R. Ir. Acad., Sect. B*, 51, 223 (1947); *Chem. Abstr.*, **42**, 4134 (1948).

Synthesis and Some Pharmacological Properties of the 23-Peptide 15-Lysine-secretin-(5–27). Special Role of the Residue in Position 15 in Biological Activity of the Vasoactive Intestinal Polypeptide

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The 23-peptide 15-lysine-secretin-(5–27) [S(5–27)] was synthesized on an insoluble support. The residue in position 15 of secretin, aspartic acid, was replaced by lysine, which occupies that position in the vasoactive intestinal polypeptide (VIP), a member of the secretin family. The resulting analogue showed increased VIP-like activity on smooth muscle preparations and unaltered secretin-like activity on pancreatic juice secretion in the rat. The affinity of the new analogue for high-affinity secretin receptors in acinar cells from guinea pig pancreas was less than that of S(5–27) but was higher than that of S(5–27) for high-affinity VIP receptors in the same cells.

The discovery of a series of gastrointestinal peptide hormones, followed by the elucidation of their amino acid sequences, uncovered patterns of structural similarities between them. Obvious sequence homologies exist between gastrin, cholecystokinin-pancreozymin, and caerulein and between secretin, vasoactive intestinal peptide (VIP), glucagon, and gastric inhibitory peptide (GIP). These homologies favor the concept of hormone families.[1] The similarities between the individual members of these families have received considerable attention. Less attention has been devoted to the study of structural *differences* between related peptides. The differences are no less significant for they seem to be responsible for differences in the biological activities of these peptides. In this paper, we present the results of a study designed to elucidate the role of the amino acid residue which occupies position 15 in secretin and VIP.

An analysis of the homologies in the sequences of the members of the secretin family (Figure 1) reveals a conspicuous difference between secretin and VIP with respect to position 15. This position is occupied by an acidic aspartyl residue in secretin[2] and by lysine, a basic moiety, in VIP.[3] Ion pairs, it might be assumed, would result from the interaction of secretin and its putative receptor. Yet, in secretin the 15-aspartyl residue participates in an intramolecular ion pair,[4] so its similar involvement with a cation-forming site in the receptor seemed unlikely. This did not exclude a role in the hormone-receptor interaction for the corresponding residue in VIP. To find evidence for such a role for the 15-lysine of VIP, we synthesized a secretin analogue with lysine replacing aspartic acid in position 15 of secretin. Because of some complications in the synthetic procedure, which will be discussed below, the C-terminal 23-peptide part of secretin rather than the entire sequence of the hormone was modified. This portion of the molecule, S(5–27), has intrinsic activity[5] and, therefore, its 15-lysine analogue seemed to be suitable for comparisons with the unaltered sequence with respect to binding to receptors. The VIP-like effect of C-terminal sequences of VIP was demonstrated earlier.[6]

The 23-peptide, 15-lysine-S(5–27), was synthesized on an insoluble support.[7] The latter, an aminobenzhydryl resin,[8] was acylated with the *o*-nitrophenyl ester of *tert*-butyloxycarbonyl-L-valine[9] and—after deprotection—the chain was lengthened in a stepwise[10] fashion with *tert*-butyloxycarbonylamino acid active esters. Nitro-L-arginine residues were introduced by activation of the *tert*-butyloxycarbonyl derivative with dicyclohexylcarbodiimide[11] in the presence of 1-hydroxybenzotriazole.[12] While in most cases *o*-nitrophenyl esters which were found suitable in solid-phase synthesis were used,[13] threonine was incorporated as the *N-tert*-butyloxycarbonyl-*O*-benzyl-L-threonine 2,4,5-trichlorophenyl ester.[14] The active ester reactions were catalyzed[15] with 1-hydroxybenzotriazole and were followed by the similarly catalyzed acetylation of any unreacted amine with *p*-nitrophenyl acetate. In the introduction of the first few residues, the recently recommended[16] medium, toluene, was used but in the subsequent steps dimethylformamide seemed to be more advantageous.

After the incorporation of 23 amino acids, the synthesis was continued to the completion of the 27-membered chain of the secretin analogue only with an aliquot of the peptidyl-resin. An attempt to remove the secretin analogue from the resin resulted in a material which, according to the data of amino acid analysis after enzymic degra-

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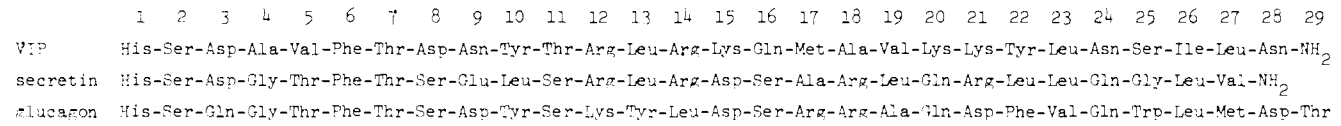

Figure 1. The secretin family [another related peptide, the gastric inhibitory peptide (GIP), is not shown].

dation,[17] contained the aminosuccinylglycine sequence rather than the aspartylglycine sequence near the N terminus (cf. Figure 1). It seems that the acidolytic cleavage with trifluoromethanesulfonic acid[18] resulted in ring closure at this delicate position.[19] Therefore, the studies described below were carried out on the C-terminal 23-peptide that was obtained from the corresponding peptidyl-resin by similar acidolysis, followed by chromatography on Sephadex LH 20 in methanol. After conversion to the acetate salt, the product was further purified by chromatography on a second Sephadex LH 20 column in 0.005 N HCl.[20] The 23-peptide, 15-lysine-S(5–27), appeared as a single spot on thin-layer chromatograms and gave satisfactory amino acid analyses after hydrolysis both with 6 N HCl and with aminopeptidase M.

In the rat, intravenous injection of the new analogue produced a significant increase in pancreatic secretory flow (12.9 ± 1.9 μL per 10 min per gram wet weight of pancreas). The efficacy (20%, when expressed as a fraction of the maximal response to secretin) of 15-lysine-S(5–27) was similar to that of the unaltered C-terminal tricosapeptide of secretin and to that of the 15-asparagine analogue.[4,5] Thus, while sensitive to these compounds, rat exocrine pancreatic secretion did not discriminate among them.

On isolated, superfused smooth muscle preparations,[21] particularly on strips of rat gastric fundus, guinea pig lung, and guinea pig trachea, 15-lysine-S(5–27) caused relaxations similar to those observed with VIP.[6] The relaxant potency of the new analogue was three to four times that of S(5–27) (i.e., doses one-fourth to one-third as large were equipotent) and similar to that of 15-asparagine-S(5–27), which was found earlier[22] to exhibit an enhanced VIP-like activity relative to S(5–27).

Acinar cells from guinea pig pancreas possess two classes of receptors for VIP and secretin. One class has a high affinity for VIP and a low affinity for secretin; the other has a low affinity for VIP and a high affinity for secretin. The new 23-peptide analogue had significantly higher affinity for the VIP-preferring receptors than did the parent S(5–27) or native secretin (Table I). The affinity of 15-lysine-S(5–27) for these receptors was identical with that of 15-asparagine-S(5–27) reported previously[23] (Table I). The affinity of the 15-lysine analogue for the secretin-preferring receptors was significantly less than that for S(5–27) or 15-asparagine-S(5–27) (Table I). Thus, both classes of receptors could clearly distinguish among the residues that occupy position 15 in the two peptides. These observations suggest that the residue in position 15 indeed plays a major role in the determination of the characteristic activities of VIP. Yet, for the enhancement of VIP-like potency the presence of a basic residue in this position might be less important than the *absence* of an acidic function. The question whether the different behavior of S(5–27) and 15-lysine-S(5–27) in these biological systems is due to the difference in the charge of the side chains or the concomitant alteration of the conformation of the chain cannot be answered at this time. The only firm conclusion that can be drawn from our experimental results is that the nature of the residue in position 15 is a significant determinant of the biological activities of VIP.

Table I. Apparent Affinities[a] of S(5–27) and Its Analogues for Receptors on Guinea Pig Acinar Cells

	concn, M, required to occupy 50% of receptors		
receptor	S(5–27)	Asn^{15}-S(5–27)	Lys^{15}-S(5–27)
VIP preferring	46 (±18) × 10^{-7}	8 (±4) × 10^{-7}	10 (±4) × 10^{-7}
secretin preferring	2.3 (±0.9) × 10^{-7}	1.9 (±0.8) × 10^{-7}	12 (±5) × 10^{-7}

[a] The apparent affinities of the secretin fragments for the VIP-preferring receptor were calculated from their abilities to inhibit the increase in cAMP caused by 10^{-9} M VIP.[24] The apparent affinities of the secretin fragments for the secretin-preferring receptor were calculated from their abilities to inhibit the increase in cAMP caused by 10^{-9} M secretin.[24] Values given are means (±1 SD) from three separate experiments.

Experimental Section

Synthesis. Benzhydrylamine hydrochloride resin (polystyrene, 1% cross-linked with divinylbenzene) was purchased from Beckman Instruments, Palo Alto, Calif. Completion of the incorporation of the amino acid residues was monitored with a ninhydrin test. Amino acid analyses were performed on a Beckman Spinco 120C analyzer on samples obtained by hydrolysis of the peptidyl-resin in propionic acid–HCl at 130 °C for 16 h. After removal of the peptide from the resin, samples were hydrolyzed in 6 N HCl at 110 °C for 16 h. In digestions with aminopeptidase M, the procedure of Hofmann et al.[17] was followed, but with a digestion time of 72 h. Thin-layer chromatography was performed on Analabs prescored anasil CF cellulose plates (5 × 20 cm) and on Brinkman precoated silica gel (60-F 254) plates. Spots were detected with ninhydrin or fluorescamine or by charring with ammonium hydrogen sulfate. The following solvent system was used: 1-butanol–pyridine–acetic acid–water (60:24:6:20). Reagent grade solvents were dried over a 4Å molecular sieve (Davison).

Peptidyl-resin. The synthesis was carried out on 5 g of aminobenzhydryl resin with a total capacity of 2 mmol. The following cycle of operations was applied: (1) 15% triethylamine (TEA) for 3 min; (2) 15% TEA for 10 min; (3) CH_2Cl_2, three times, 20 min; (4) DMF, three times, 10 min; (5) active ester, hydroxybenzotriazole (HOBt), and diisopropylethylamine (DIEA) in equivalent amounts in DMF; (6) DMF, 3 × 10 min; (7) *p*-nitrophenyl acetate, HOBt, and DIEA in DMF; (8) DMF, six times, 20 min; (9) CH_2Cl_2, three times, 10 min; (10) 40% F_3Ac in CH_2Cl_2, 5 min; (11) 50% F_3Ac in CH_2Cl_2, 30 min; (12) CH_2Cl_2, six times, 20 min.

The reaction time was between 16 and 24 h except in the coupling of N^{α}-*tert*-butyloxycarbonyl-N^{ϵ}-2-chlorobenzyloxycarbonyl-L-lysine *o*-nitrophenyl ester and *N*-*tert*-butyloxycarbonyl-*O*-benzyl-L-serine *o*-nitrophenyl ester where 48 h was allowed for the completion of acylation.

In the incorporation of Leu^{26} and Gly^{25}, 0.1 M solutions of the active ester in toluene were used without any added catalyst or base. At the completion of the acylation the beads gave negative reaction with ninhydrin, but they turned intensely yellow, probably due to the adsorbed nitrophenol. Washing with toluene did not release nitrophenol; hence, the resin was washed with DMF. The incorporation of Gln^{24} was carried out in DMF. Arginine was introduced as Boc-Arg(NO_2) with DCC with the addition of equimolar amounts of 1-hydroxybenzotriazole (HOBt) and diisopropylethylamine (DIEA). For acetylation after reaction with the active esters of Val^{27} and Leu^{26}, *p*-nitrophenyl acetate was used without the addition of HOBt–DIEA. At the 12-peptide stage the peptidyl resin weighed 5.26 g. The losses were due, in

part, to the removal of small aliquots after each chain lengthening for ninhydrin reaction and for amino acid analyses. From this point on 5.0 g of peptidyl-resin was used for the continuation of the synthesis. The 23-peptidyl resin weighed only 5.78 g. There are obvious losses in the deprotection steps: trifluoroacetic acid removes a not negligible portion of the peptide from the resin.

15-Lysine-secretin-(5–27). A sample of the tricosapeptide resin (400 mg) was treated with 20% trifluoromethanesulfonic acid in trifluoroacetic acid (4 mL) for 2 h. After filtration, the resin was washed with F_3Ac (5 mL). The acids were removed in vacuo and the residue was dried in vacuo over NaOH. After 1 h the crude material was treated with ether and the ether solubles were removed by centrifugation. After overnight storage in a desiccator in vacuo over NaOH, the residue was dissolved in methanol (1 mL) and applied to a column of Sephadex LH-20 poured in methanol (30 g, 2.4 × 45 cm) and eluted with methanol; 1-mL fractions were collected. Fractions 55–70 contained the major component. After removal of the solvents the residue was dissolved on 50% aqueous methanol (1 mL) and passed through a small column (0.7 × 7.5 cm) of Dowex 1-acetate. The column was washed with the same solvent mixture (9 mL). After evaporation the residue was dissolved in 0.005 N HCl (1 mL) and applied to a Sephadex LH-20 column (30 × 2.4 cm) prepared with 0.005 N HCl. Elution was carried out with the same solvent. After 40 mL of eluate 2.5-mL fractions were collected. They were monitored by UV absorption and also examined by TLC (silica gel, fluorescamine, and charring, solvent system A). Fractions 21–34 contained the desired tricosapeptide amide (56 mg). According to the recovery in amino acid analysis, this material contained only 28 mg of peptide corresponding to an overall yield of 13%: R_f (cellulose) 0.58; R_f (silica gel) 0.33. Amino acid analysis after acid hydrolysis gave Lys, 1.1; Arg, 3.6; Thr, 1.6; Ser, 2.6; Glu, 3.1; Gly, 1.0; Ala, 1.1; Val, 1.0; Leu, 6.1; Phe, 0.8. After hydrolysis with aminopeptidase M, amino acid analysis gave Lys, 1.1; Arg, 3.7; Thr, 1.7; Glu, 0.6; Gly, 1.0; Ala, 1.1; Val, 1.0; Leu, 5.9; Phe, 0.8. (Ser and Gln were not determined since their peaks were not separated.[25])

Biological Tests. The experiments with smooth muscle preparations were carried out as described for VIP.[21] The flow of pancreatic juice was determined in rats.[5] The affinity of VIP and secretin receptors in acinar cells from guinea pig pancreas (Table I) was measured according to the procedure described in ref 23.

Attempted Synthesis of 15-Lysine-Secretin. Continuation of the chain lengthening until the 27-peptide was completed, cleavage from the resin with CF_3SO_3H, and purification as described for the 23-peptide gave a product which was homogeneous by TLC and had quite satisfactory amino acid analysis. After digestion with aminopeptidase M, however, the values for Asp and Gly were very low (about 0.2 for Asp and 1.2 for Gly). We had to assume that ring closure took place at residues 3 and 4. The enzymic hydrolysis seems to skip aminosuccinylglycine and to go on with the hydrolysis in the rest of the chain. A similar situation can be discerned in the studies of Ondetti and his associates,[24] who used also aminopeptidase M in the digestion of aminosuccinyl peptides.

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References and Notes

(1) M. Bodanszky in "Endocrinology of the Gut", W. Y. Chey and F. P. Brooks, Ed., C. B. Slack, Inc., Thorofare, N.J., 1974, p 3.
(2) V. Mutt, J. E. Jorpes, and S. Magnusson, *Eur. J. Biochem.*, **15**, 513 (1970).
(3) V. Mutt and S. I. Said, *Eur. J. Biochem*, **42**, 581 (1974).
(4) M. L. Fink and M. Bodanszky, *J. Am. Chem. Soc.*, **98**, 974 (1976).
(5) G. M. Makhlouf, M. Bodanszky, M. L. Fink, and M. Schebalin, *Gastroenterology*, in press.
(6) M. Bodanszky, Y. S. Klausner, and S. I. Said, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 382 (1973).
(7) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).
(8) P. G. Pietta and G. R. Marshall, *J. Chem. Soc. D*, 650 (1970).
(9) M. Bodanszky, K. W. Funk, and M. L. Fink, *J. Org. Chem.*, **38**, 3565 (1973).
(10) M. Bodanszky, *Ann. N.Y. Acad. Sci.*, **88**, 655 (1960); cf. also M. Bodanszky and V. duVigneaud, *Nature (London)*, **183**, 1324 (1959).
(11) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
(12) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
(13) M. Bodanszky and K. W. Funk, *J. Org. Chem.*, **38**, 1296 (1973).
(14) J. Pless and R. A. Boissonnas, *Helv. Chim. Acta*, **46**, 1609 (1963).
(15) W. König and R. Geiger, *Chem. Ber.*, **106**, 3626 (1973).
(16) S. A. Khan and K. M. Sivanandaiah, *Synthesis*, 614 (1976).
(17) K. Hofmann, F. M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966).
(18) H. Yajima, N. Fujii, H. Ogawa, and H. Kawatani, *J. Chem. Soc., Chem. Commun.*, 107 (1974).
(19) C. C. Yang and R. B. Merrifield, *J. Org. Chem.*, 41, 1032 (1976).
(20) W. König, R. Geiger, H. Wissmann, M. Bickel, R. Obermeier, W. Tetz, and R. Uhmann, *Gastroenterology*, **72**, 797 (1977).
(21) S. I. Said, *Am. J. Med.*, **57**, 453 (1974).
(22) S. I. Said, unpublished results.
(23) J. D. Gardner, T. P. Conlon, M. L. Fink, and M. Bodanszky, *Gastroenterology*, 71, 965 (1976).
(24) M. A. Ondetti, A. Deer, J. T. Sheehan, J. Pluscec, and O. Kocy, *Biochemistry*, **7**, 4069 (1968).

Synthesis and β-Adrenoceptor Activity of the Erythro and Threo Isomers of Substituted α-Hydroxytrimetoquinol

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The synthesis and pharmacological activity of erythro and threo isomers of 1-(3′,4′,5′-trimethoxy-α-hydroxybenzyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, **2** and **3**, are reported. The structural assignments of **2** and **3** are based upon NMR spectra of the 6,7-dibenzyl precursors, **6** and **10**, and of the synthetic derivatives of 13α- and 13β-hydroxy-2,3-(dibenzyloxy)-9,10,11-trimethoxytetrahydroprotoberberine, **8** and **12**, respectively. The erythro isomer **2** was a more potent β-adrenoceptor stimulant than the threo isomer **3** in guinea pig atrial, guinea pig tracheal, and rat adipocyte preparations. The differential activity of these compounds on lipolysis was favorably correlated to changes in the stimulation of adenylate cyclase activity and cAMP accumulation in rat adipocytes.

Although 1-benzyltetrahydroisoquinolines have been studied intensively, both chemically and pharmacologically,[1-3] the corresponding α-oxo- and α-hydroxybenzyltetrahydroisoquinolines are less frequently encountered

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