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The Vasoactive Intestinal Peptide (VIP)
VI. The 17-Norleucine Analog of the Sequence 14–28[1]

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A pentadecapeptide amide with the C-terminal sequence (14–28) of the vasoactive intestinal peptide (VIP), but with the methionine residue in position 17 replaced by L-norleucine, was synthesized. The synthesis was carried out through stepwise chain lengthening, by the *in situ* technique. The norleucine-containing pentadecapeptide, L-arginyl-L-lysyl-L-glutaminyl-L-norleucyl-L-alanyl-L-valyl-L-lysyl-L-lysyl-L-tyrosyl-L-leucyl-L-asparaginyl-L-seryl-L-isoleucyl-L-leucyl-L-asparaginamide, was as active in relaxing different smooth-muscle preparations as the methionine-containing parent sequence.

Discovery (*1*), isolation (*2*), structure elucidation (*3*), and synthesis (*4*) of the vasoactive intestinal peptide (VIP) were described earlier. The interesting observation that some C-terminal sequences of VIP exhibit the characteristic pharmacological properties of the entire molecule was also reported (*4*). The relaxation of different smooth-muscle organs (*5*, *6*), e.g., guinea pig trachea, suggests potential usefulness of VIP in medicine. Therefore, it was of interest to explore the possible replacement of the methionine residue in position 17 with a residue that can be introduced without the complications in synthesis caused by methionine. Norleucine, which is isosteric with methionine, was able to replace the sulfur-containing amino acid in several peptide hormones, e.g., in corticotropin (*7*), without loss of activity. In the case of VIP, it seemed possible that the synthesis of the entire chain with 28 amino acid residues might be unnecessary since the C-terminal pentadecapeptide, VIP_{14-28}, showed, albeit at higher dose levels (*4*), the characteristic biological activities of VIP. Therefore, we decided to prepare and examine the 17-L-norleucine analog of VIP_{14-28} (Fig. 1).

Arg-Lys-Gln-Nle-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2
14 15 16 17 18 19 20 21 22 23 24 25 26 27 28

FIG. 1. The 17-norleucine analog of the C-terminal pentadecapeptide of VIP_{14-28}

For the synthesis of 17-L-norleucine VIP_{14-28}, the heptapeptide derivative, *N*-benzyloxycarbonyl-*O*-benzyl-L-tyrosyl-L-leucyl-L-asparaginyl-L-seryl-L-isoleucyl-L-leucyl-L-asparaginamide (*8*), was deprotected by catalytic hydrogenation and the resulting amine was acylated with N^{α}-*t*-butyloxycarbonyl-N^{ε}-2,6-dichlorobenzyloxycarbonyl-

[1] For previous paper in this series, cf. Y. S. Klausner and M. Bodanszky, *Bioorg. Chem.* **2**, 354 (1973).

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L-lysine *o*-nitrophenyl ester (*9*). From here on, the synthesis was carried out by the *in situ* technique (*9*, *10*), meaning that the operations were performed in a single vessel, a modified centrifuge tube (*9*) from which the intermediates were not removed throughout the chain-lengthening procedure. For acylation, *p*-nitrophenyl (*11*) and *o*-nitrophenyl (*8*, *9*) esters of protected amino acids were both used in about 100% excess. Completion of the acylation reactions was monitored with ninhydrin tests. The protecting tert-butyloxycarbonyl groups were removed with trifluoroacetic acid. The N-terminal residue, arginine, was incorporated through the 2,4-dinitrophenyl ester of benzyloxycarbonyl-nitro-L-arginine (*12*). The intermediates were not characterized. The protected pentadecapeptide derivative, N^{α}-benzyloxycarbonyl-nitro-L-arginyl-N^{ε}-2,6-dichlorobenzyloxycarbonyl-L-lysyl-L-glutaminyl-L-norleucyl-L-alanyl-L-valyl-N^{ε}-2,6-dichlorobenzyloxycarbonyl-L-lysyl-N^{ε}-2,6-dichlorbenzyloxycarbonyl-L-lysyl-L-tyrosyl-L-leucyl-L-asparaginyl-L-seryl-L-isoleucyl-L-leucyl-L-asparaginamide, was hydrogenated in the presence of a palladium catalyst and the resulting free pentadeca-peptide (Fig. 1) purified by ion-exchange chromatography on a carboxymethylcellulose column. A product, homogeneous on paper chromatograms and with satisfactory amino acid analysis, was secured.

On bioassay on isolated smooth-muscle organs, the norleucine analog of VIP_{14-28} was approximately as potent as the methionine-containing parent peptide, with about 2% of the potency of VIP (Table 1). Thus, the replacement of methionine with norleucine did not affect the biological activity of the C-terminal pentadecapeptide part of VIP. This relationship might be true for the entire molecule of VIP as well.

TABLE 1

RELATIVE ACTIVITY OF VIP AND FRAGMENTS ON ISOLATED SMOOTH-MUSCLE ORGANS[a]

Tissue[b]	VIP_{14-28} 10.6 μg/ml	17-Norleucine VIP_{14-28} 10.6 μg/ml	VIP 200 ng/ml
RSS	23	32	23
GPT	15	9	32
GPGB	6 (?)	28	22
CR	9	12	6

[a] Numbers, in arbitrary units, are products of amplitude and duration of response.

[b] RSS, rat stomach strip: GPT, guinea pig trachea; GPGB, guinea pig gallbladder; CR, chick rectum.

EXPERIMENTAL

t-Butyloxycarbonyl-L-norleucine o-nitrophenyl ester. *t*-Butyloxycarbonyl-L-norleucine was prepared by the method of Schnabel (*13*). The product, which was obtained as an oil, gave a single spot with R_f 0.77 on thin-layer plates of silica gel in the system of butanol–acetic acid–water (4:1:1), A sample (2.46 g) was dissolved in pyridine (20 ml),

o-nitrophenol (2.78 g) was added, followed by addition, at 0°C, of dicyclohexylcarbodiimide (2.1 g). After 1 hr, the mixture was allowed to warm up to room temperature. The ir spectrum revealed some unreacted diimide even after 6 hr. Therefore, the reaction was allowed to proceed overnight. The dicyclohexylurea was removed by filtration, and the filtrate and washings were concentrated *in vacuo*. Ether (100 ml) was added and the insoluble precipitate (more urea derivative) filtered off. After removal of the ether *in vacuo*, the residue was taken up in $CHCl_3$ (50 ml) and the solution washed with a 5% solution of citric acid, water, then with 0.1 *N* NaOH until the washings ceased to be orange–red, finally with water. The solution was dried and the chloroform removed *in vacuo*. The crude product (4.3 g) was recrystallized from hot 95% ethanol containing 1% acetic acid. The purified active ester (1.90 g) melted at 90–92°C. From the mother liquor, two additional crops were obtained with the same mp. A total of 2.6 g active ester was secured. On tlc (silica gel) in the solvent system $CHCl_3$–CH_3OH (9:1), a single spot with R_f 0.9 was observed and R_f 0.87 in *n*-BuOH–AcOH–H_2O (4:1:1). For analysis, a sample was recrystallized from ethanol. It was dried *in vacuo* at 35°C for 2 hr; mp 90–92°C; $[\alpha]_D^{25}$ –61.5° (*c* 2, DMF containing 1% AcOH).

Anal. Calcd for $C_{17}H_{24}N_2O_6$ (352.4): C, 57.9; H, 6.9; N, 8.0. Found: C, 57.7; H, 6.7; N, 7.8.

*L-Arginyl-*L*-lysyl-*L*-glutaminyl-*L*-norleucyl-*L*-alanyl-*L*-valyl-*L*-lysyl-*L*-lysyl-*L*-tyrosyl-*L*-asparaginyl-*L*-seryl-*L*-isoleucyl-*L*-leucyl-*L*-asparaginamide.* The protected heptapeptide, *N*-benzyloxycarbonyl-*O*-benzyl-L-tyrosyl-L-leucyl-L-asparaginyl-L-seryl-L-isoleucyl-L-leucyl-L-asparaginamide (8) (700 mg), was dissolved in 80% acetic acid (60 ml) and hydrogenated in the presence of a 10% Pd on charcoal catalyst (250 mg). The resulting partially deprotected heptapeptide acetate (562 mg) was acylated with N^{α}-*t*-butyloxycarbonyl-N^{ϵ}-2,6-dichlorobenzyloxycarbonyl-L-lysine *o*-nitrophenyl ester (*9*) (546 mg) in dimethylformamide (8.5 ml) in the presence of triethylamine (0.09 ml). This reaction and the subsequent operations were carried out in a centrifuge tube (*9*, *10*). The *t*-butyloxycarbonyl group was removed by treatment with trifluoroacetic acid (2.0 ml) at room temperature for 15 min. The partially deprotected octapeptide trifluoroacetate was isolated by concentration of the solution *in vacuo* and trituration of the residue with ether. The product was secured by centrifugation. It was suspended in dimethylformamide (5.4 ml) and acylated in the presence of triethylamine (0.054 ml) with N^{α}-*t*-butyloxycarbonyl-N^{ϵ}-2,6-dichlorobenzyloxycarbonyl-L-lysine *o*-nitrophenyl ester (*9*) (329 mg). The chain was lengthened in the same manner by the *in situ* technique (*9*, *10*) with stepwise incorporation of single amino acid residues as active esters. The *o*-nitrophenyl esters of *t*-butyloxycarbonyl-L-valine (*9*), *t*-butyloxycarbonyl-L-norleucine and *t*-butyloxycarbonyl-L-glutamine (*9*), the *p*-nitrophenyl ester of *t*-butyloxycarbonyl-L-alanine,[3] and finally the 2,4-dinitrophenyl ester of benzyloxycarbonyl-nitro-L-arginine (*12*) were employed as acylating agents. The active esters were applied in excess, about twice the calculated amount. The intermediates were not characterized. The fully protected pentadecapeptide, benzyloxycarbonyl-nitro-L-arginyl-N^{ϵ}-2,6-dichlorobenzyloxycarbonyl-L-lysyl-L-glutaminyl-L-norleucyl-L-alanyl-L-valyl-N^{ϵ}-2, 6-dichlorobenzyloxycarbonyl-L-lysyl-N^{ϵ}-2, 6-dichlorobenzyloxycarbonyl-L-lysyl-L-tyrosyl-L-leucyl-L-asparaginyl-L-seryl-L-isoleucyl-L-leucyl-L-asparaginamide (460 mg), was extracted with methanol (5 ml), centrifuged and reextracted with methanol (5 ml). The

[3] Purchased from Bachem, California.

dried material (0.41 g), dec. above 290°C, was hydrogenated in 80% acetic acid (120 ml) in the presence of a 10% Pd on charcoal catalyst (230 mg) for 48 hr. After removal of the catalyst and of the solvent, the residue was dissolved in glacial acetic acid and lyophilized. The crude peptide (280 mg) was purified by chromatography on a carboxymethylcellulose column (2 × 58 cm). A linear gradient of ammonium acetate was used for elution, starting with 0.1 *M* (500 ml) and increasing to 0.55 *M* (500 ml). A flow rate of 24 ml/hr was used and 4-ml fractions were collected. The pentadecapeptide was detected (by uv absorption) in fractions 225–260, but in better purity in fractions 241–260. From the latter fractions, the purified pentadecapeptide (20 mg)[4] was secured by repeated lyophilizations from water. On paper chromatograms in the Watson–Waley system (*14*), the peptide travels as a single but somewhat elongated spot of R_f 0.4. In electrophoresis (Savant flat plate, N AcOH, 29 V/cm) on Whatman No. 1 paper, the peptide moved as a single band 8 cm in 1.5 hr, toward the cathode.

Amino acid analysis: Asp, 2.18; Ser, 1.00; Glu, 1.03; Ala, 1.00; Val, 0.85; Ile, 0.95; Leu, 1.86; Nle, 1.02; Tyr, 0.98; Lys, 2.80; NH_3, 4.06; Arg, 0.93.

A solution of the pentadecapeptide in water revealed a uv absorption spectrum similar to that of tyrosine, $E^{1\%}_{1cm}$ 0.65, corresponding to $\varepsilon = 1356$, calculated for pentaacetate with *M*/ 2086. This is in good agreement with the absorption of the tyrosine residue.

Bioassays were carried out as described in Ref. 5.

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REFERENCES

1. S. I. Said and V. Mutt, *Nature (London)*, **225**, 863 (1970).
2. S. I. Said and V. Mutt, *Eur. J. Biochem.*, **28**, 199 (1972).
3. V. Mutt and S. I. Said, *Eur. J. Biochem.*, **42**, 581 (1974).
4. M. Bodanszky, Y. S. Klausner, and S. I. Said, *Proc. Nat. Acad. Sci., USA*, **70**, 387 (1973).
5. P. J. Piper, S. I. Said, and J. R. Vane, *Nature (London)*, **225**, 1144 (1970).
6. S. I. Said, "Proc. Symposium on Endocrinology (1973)," Heinemann, Ed., Royal Postgrad. Medical School, London, in press.
7. S. Guttmann, J. Pless, and R. A. Boissonnas, *Acta Chim. Hung.*, **44**, 23 (1965).
8. M. Bodanszky, Y. S. Klausner, and V. Mutt, *Bioorg. Chem.*, **2**, 30 (1972).
9. M. Bodanszky, K. W. Funk, and M. L. Fink, *J. Org. Chem.*, **38**, 3564 (1973).
10. M. Bodanszky, M. Kondo, C. Yang Lin, and G. F. Sigler, *J. Org. Chem.*, **39**, 444 (1974).
11. M. Bodanszky, *Nature (London)*, **175**, 685 (1955).
12. M. Bodanszky and M. A. Ondetti, *Chem. Ind.*, 26 (1966).
13. E. Schnabel, *Ann.*, **702**, 188 (1967).
14. S. G. Waley and J. Watson, *Biochem. J.*, **57**, 529 (1954).

[4] About the same amount was present in fractions 225–240. The low recovery is due, at least in part, to the presence of a blocked heptapeptide (an acetyl derivative?) in the crude product.