1,6-Aminosuberic Acid Analogs of Lysine- and Arginine-Vasopressin and -Vasotocin. Synthesis and Biological Properties[1,2]

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Abstract: Analogs of lysine-vasopressin, lysine-vasotocin, arginine-vasopressin, and arginine-vasotocin in which the terminal amino group and the disulfide group have been replaced, respectively, by a hydrogen atom and an ethylene bridge have been synthesized and tested for their biological activities. These 1,6-aminosuberic acid analogs were prepared by the stepwise method of peptide synthesis. The ω-carboxyl group of the α-aminosuberic acid moiety of each of the acyclic octapeptide intermediates, possessing the general formula H-Tyr-X-Gln-Asn-Asu(ONp)-Pro-Y-Gly-NH_2 (X = Ile or Phe; Y = Lys (Pht) or Arg (Tos)), was condensed by the active ester method with the amino group of the tyrosine moiety to yield the protected cyclic peptides, which were subsequently converted to the free hormone analogs. Upon bioassay, [1,6-aminosuberic acid]-lysine-vasopressin was found to possess 5.3 $\pm$ 0.1 units/mg of rat oxytocic activity, 2.4 $\pm$ 0.1 units/mg of avian vasodepressor activity, 3.2 $\pm$ 0.3 units/mg of rabbit milk ejecting activity, 6.2 $\pm$ 0.2 units/mg of rat pressor activity, and 208 $\pm$ 21.0 units/mg of rat antidiuretic activity. In addition, this analog elicits a half-maximal hydroosmotic response in the *in vitro* toad urinary bladder at a concentration of $(1.57 \pm 0.19) \times 10^{-7}$ *M*. The corresponding values for [1,6-aminosuberic acid]-lysine-vasotocin were 63.6 $\pm$ 2.4, 20.5 $\pm$ 1.2, 36.3 $\pm$ 0.9, 9.6 $\pm$ 0.6, and 34.8 $\pm$ 3.0 units/mg, and $(2.49 \pm 0.87) \times 10^{-9}$ *M*, for [1,6-aminosuberic acid]-arginine-vasopressin, 15.4 $\pm$ 0.5, 12.8 $\pm$ 0.4, 13.7 $\pm$ 1.0, 37.7 $\pm$ 2.8, and 1274 $\pm$ 67 units/mg, and $(4.74 \pm 0.65) \times 10^{-9}$ *M*, and for [1,6-aminosuberic acid]-arginine-vasotocin, 135 $\pm$ 4, 64.2 $\pm$ 1.6, 36.8 $\pm$ 1.6, 27.0 $\pm$ 1.7, and 313 $\pm$ 32 units/mg, and $(2.63 \pm 0.35) \times 10^{-10}$ *M*. While the substitution of the primary amino group by a hydrogen atom affects the biological activities of neurohypophyseal hormones selectively, a subsequent replacement of the disulfide bridge by an ethylene moiety is associated with a general and approximately constant reduction of potency—irrespective of the potency of its disulfide-containing deamino congener. It is tentatively suggested that the substitution of the amino group perturbs the peptide topology locally, whereas the replacement of the disulfide bridge by the ethylene group introduces in first approximation an invariant conformational factor which affects the peptide as a whole. In addition, the results of this study support the contention that a thiol–disulfide interchange does not play a direct role in the hormone–receptor interactions.

A series of studies recently culminated in the proposal of a preferred conformation of oxytocin[4a] and vasopressin[4b] each of which possesses two β turns (or β folds) as its backbone structure, one located in the 20-membered ring and the other in the acyclic peptide component. In ensuing communications we considered the body of information stemming from extensive investigations of the biological activities of neurohypophyseal hormones and synthetic analogs[5] from the vantage point of their three-dimensional structures.[4b,6] A unique relationship between structure and function of these membrane-active peptides is evident, and three predominant categories of conformation variants emerged which determine the activity profile of neurohypophyseal peptides: (a) those which perturb the spatial relationship among all the constituent amino acids by affecting the backbone conformation of the hormone; such structural changes are predicted to either increase or decrease the capacity of the peptide to bind to its specific target organ receptor (affinity) and concomitantly to enhance or diminish the capability of the peptide to generate a response once associated with the receptor (intrinsic activity[7]); (b) those which, while retaining the stability of the backbone conformation, alter the steric environment and charge distribution of limited surface areas in such a manner as to affect affinity and intrinsic activity differentially; and (c) those which do not perturb the peptide backbone but which change the steric and electronic requirements of moieties comprising the active surface of the neurohypophyseal peptide affecting intrinsic activity without altering affinity.

Neurohypophyseal hormone analogs fulfilling the criterion of the first category are deamino-1-seleno-oxytocin and deamino-6-seleno-oxytocin—analogs of deamino-oxytocin in which either of the sulfur atoms is specifically replaced by selenium. All of the biological activities are enhanced to the same degree in the case of

(1) If not otherwise noted, abbreviations follow the rules of the IUPAC–IUB Commission on Biochemical Nomenclature given in *Biochemistry*, **5**, 1445, 2485 (1966); **6**, 362 (1967); *J. Biol. Chem.*, **241**, 2491 (1966).

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(3) (a) Osaka University; (b) Mount Sinai Medical and Graduate Schools of the City University of New York.

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(6) R. Walter, I. L. Schwartz, J. H. Darnell, and D. W. Urry, *Proc. Nat. Acad. Sci. U. S.*, **68**, 1355 (1971).

(7) E. J. Ariëns, "Molecular Pharmacology," Vol. 1, Academic Press, New York, N. Y., 1967; R. F. Furchgott, *Annu. Rev. Pharmacol.*, **4**, 21 (1964); D. R. Waud, *Pharm. Rev.*, **20**, 49 (1968).

crystalline deamino-1-seleno-oxytocin as compared with crystalline deamino-oxytocin, while those of crystalline deamino-6-seleno-oxytocin are uniformly reduced.[6,8] It was concluded that the covalent linkage between residues 1 and 6 in neurohypophyseal peptides is not only the "steric" foundation for the stability of the β turn in the 20-membered ring, but also acts "functionally" as an amplifier—capable of changing the relative spatial relationship of virtually every atom to every other atom in the peptide *via* small changes in the dihedral angle of the bridge component (leaving aside for the moment effects evoked by changes in the intramolecular distance between positions 1 and 6).

A comparison between the biological potencies of a set of neurohypophyseal hormones and a set of analogs, all derived from their parent hormone by an identical structural alteration involving the disulfide bridge, should shed additional light on the "systematic conformational contribution" of this region to the overall topography of these hormonal peptides. For this purpose we turned our attention to lysine-vasopressin, lysine-vasotocin, arginine-vasopressin, arginine-vasotocin, and oxytocin, in which the disulfide bond has been formally substituted by an ethylene bridge and the terminal amino group by a hydrogen atom, resulting in a set of 1,6-aminosuberic acid analogs (Figure 1). The synthesis of [1,6-aminosuberic acid]-8-lysine-vasopressin (Asu-LVP), [1,6-aminosuberic acid]-8-lysine-vasotocin (Asu-LVT), [1,6-aminosuberic acid]-8-arginine-vasopressin (Asu-AVP), and [1,6-aminosuberic acid]-8-arginine-vasotocin (Asu-AVT) followed the general approach developed by Kobayashi, Hase, Kiyoi, and Sakakibara[9] in the case of [1,6-aminosuberic acid]-oxytocin (Asu-OXY). Preliminary reports on the syntheses of Asu-LVP[10] and Asu-AVP[11] have already been published, as has the detailed preparation of crystalline [1,6-aminosuberic acid]-oxytocin.[12]

Each of the 1,6-aminosuberic acid analogs was prepared by the stepwise method of peptide synthesis,[13] elongating the peptide chain with an activated ester of the respective, appropriately protected amino acid. The key intermediates in the preparation of the 1,6-aminosuberic acid analogs are the acyclic octapeptides which are fully protected with the exception of the ω-carboxyl group of the aminosuberic acid residue. In the case of the synthesis of Asu-OXY, this ω-carboxyl group was subsequently converted to the trichlorophenyl ester,[14] which is not affected by catalytic hydrogenation; ring formation was promoted *in situ* in dimethylformamide by catalytic hydrogenation, liberating the terminal amino group which then, *via* nucleophilic attack, displaced the trichlorophenyl group to yield the cyclic amide.[9] The application of an identical synthetic scheme to the preparation of Asu-LVP was impractical since the solubility of the protected intermediates rapidly declined as the peptide chain grew;

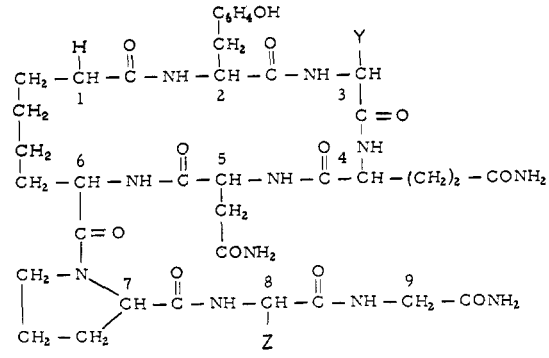

Figure 1. Structures of 1,6-aminosuberic acid analogs of lysine-vasopressin [Y = $CH_2C_6H_5$; Z = $(CH_2)_4NH_2$], lysine-vasotocin [Y = $CH(CH_3)CH_2CH_3$; Z = $(CH_2)_4NH_2$], arginine-vasopressin [Y = $CH_2C_6H_5$; Z = $(CH_2)_3NHC(=NH)NH_2$], arginine-vasotocin [Y = $CH(CH_3)CH_2CH_3$; Z = $(CH_2)_3NHC(=NH)NH_2$], and oxytocin [Y = $CH(CH_3)CH_2CH_3$; Z = $CH_2CH(CH_3)_2$].

moreover, the rate of removal of the N-terminal carbobenzoxy group was slow, particularly in the absence of an equivalent amount of hydrochloric acid. Therefore, the synthetic route of the Asu-vasopressins was modified to the extent that higher molecular weight intermediates were favorably N-protected with the *tert*-amyloxycarbonyl group introduced into peptide chemistry by Sakakibara, *et al.*[15] Following esterification of the ω-carboxyl group of the aminosuberic acid residue of each of the acyclic octapeptide intermediates, the N-terminal protecting group was removed by mild acidolysis and cyclization was achieved in pyridine under high-dilution conditions according to the general procedure of Schwyzer and Sieber.[16] The superiority of the cyclization reaction *via* an active ester rather than the Woodward reagent,[17] azide,[18] or the mixed anhydride method[19] was recently confirmed by Jošt in the case of [1,6-aminosuberic acid]-oxytocin[20] and related analogs.[21]

Several specific points should be made in connection with the syntheses of Asu-LVP, shown in Figure 2, and Asu-LVT, shown in Figure 3. N^{α}-Benzyloxycarbonyl-N^{ϵ}-phthalyl-L-lysine was secured in highly purified form *via* its dicyclohexylammonium salt. The amino acid derivative was then converted to its *p*-nitrophenyl ester, which was allowed to react with a slight excess of glycinamide. Generally, during the synthesis of Asu-LVP and Asu-LVT, the use of *p*-nitrophenyl esters of the respective N-protected amino acid was restricted to a minimum.

N-Hydroxysuccinimide esters[22] were used generally for the individual elongation steps. Several new *N*-hydroxysuccinimide esters were prepared in the course of this work, *viz.* those of benzyloxycarbonyl-L-asparagine (II), *tert*-amyloxycarbonyl-L-phenylalanine (III), *N*-*tert*-amyloxycarbonyl-*O*-*tert*-butyl-L-tyrosine (IV), and *tert*-amyloxycarbonyl-L-isoleucine (V). Although *N*-hydroxysuccinimide esters of N-protected glutamine

(8) R. Walter, W. Gordon, I. L. Schwartz, F. Quadrifoglio, and D. W. Urry, *Proc. 9th Eur. Peptide Symp., 1968*, 50 (1968).
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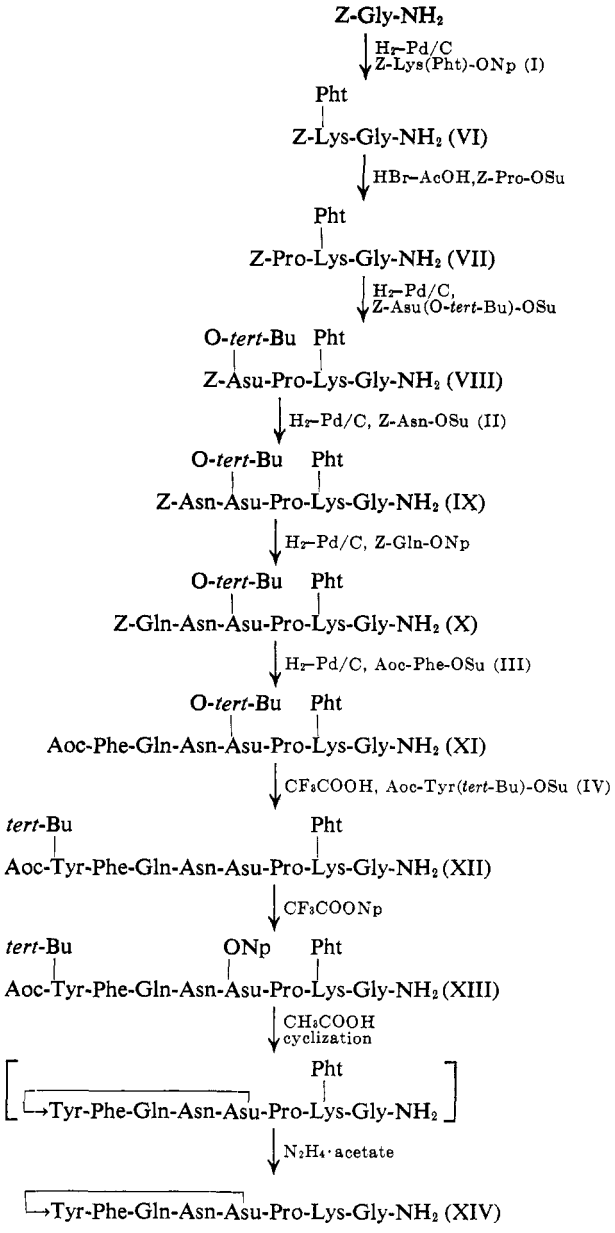

Figure 2. Scheme for the synthesis of [1,6-aminosuberic acid]-8-lysine-vasopressin.

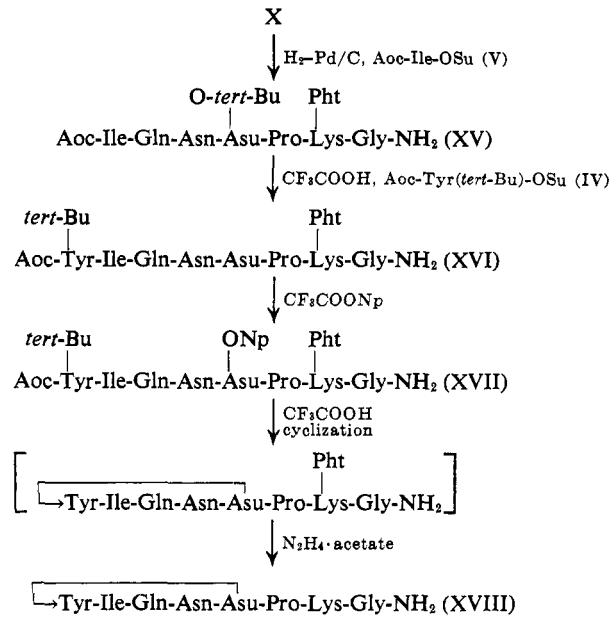

Figure 3. Partial scheme for the synthesis of [1,6-aminosuberic acid]-8-lysine-vasotocin.

have been prepared,[23,24] the formation of L-α-aminoglutarimide derivatives in the course of preparing these esters by the mixed anhydride method[19] or dicyclohexylcarbodiimide method (DCCI)[25] has been encountered repeatedly.[24,26] Therefore, in the present study the benzyloxycarbonyl-L-glutamine was activated by the *p*-nitrophenyl ester method.[13] Since the α-*N*-hydroxysuccinimide ester of benzyloxycarbonyl-L-α-aminosuberic acid ω-*tert*-butyl ester was obtained as an oily compound by the DCCI method, in subsequent syntheses the trifluoroacetate method[27] was employed, which was found to be more practical in this case. The *tert*-amyloxycarbonyl group of the N-terminal phenylalanyl residue of the heptapeptide XI was removed by treatment with trifluoroacetic acid, which also deesterified the ω-*tert*-butyl ester of the α-aminosuberic acid residue; the liberated N-terminal amino group of the heptapeptide was then acylated by treating with *tert*-amyloxycarbonyl-*O*-*tert*-butyl-L-tyrosine *N*-hydroxysuccinimide ester (IV). The ω-carboxyl group of the α-aminosuberic acid residue of the octapeptide XII was then esterified with *p*-nitrophenyl trifluoroacetate to yield XIII. Following acidolytic removal of N- and O-protecting groups of the tyrosyl residue, cyclization of the peptide was achieved by intramolecular peptide bond formation between the amino terminal of the acyclic peptide and the ω-carboxyl group of the aminosuberic acid residue. A small amount of uncyclized compound was removed with CM-Sephadex. The N^{ϵ}-phthalyl group of the lysine residue was subsequently removed with hydrazine acetate[28] and the resulting hormone analog XIV was purified by ion-exchange chromatography and gel filtration. The Asu-LVT was prepared analogously except that the *N*-hydroxysuccinimide ester of *tert*-amyloxycarbonyl-L-isoleucine was used instead of the phenylalanine analog. The homogeneities of both Asu-LVP (XIV) and Asu-LVT (XVIII) were ascertained by high-voltage paper electrophoresis, paper chromatography, amino acid analysis, and elemental analysis.

Turning to Asu-AVP and Asu-AVT, their syntheses followed the pattern just described for XIV and XVIII (Figures 4 and 5, respectively). The guanidino group of the arginine residue was protected throughout the synthesis by a tosyl group, which is notably unaffected at room temperature by acidolysis, by saponification, by hydrazinolysis, and by catalytic hydrogenation.[29] However, Mazur and Plume[30] reported recently that a

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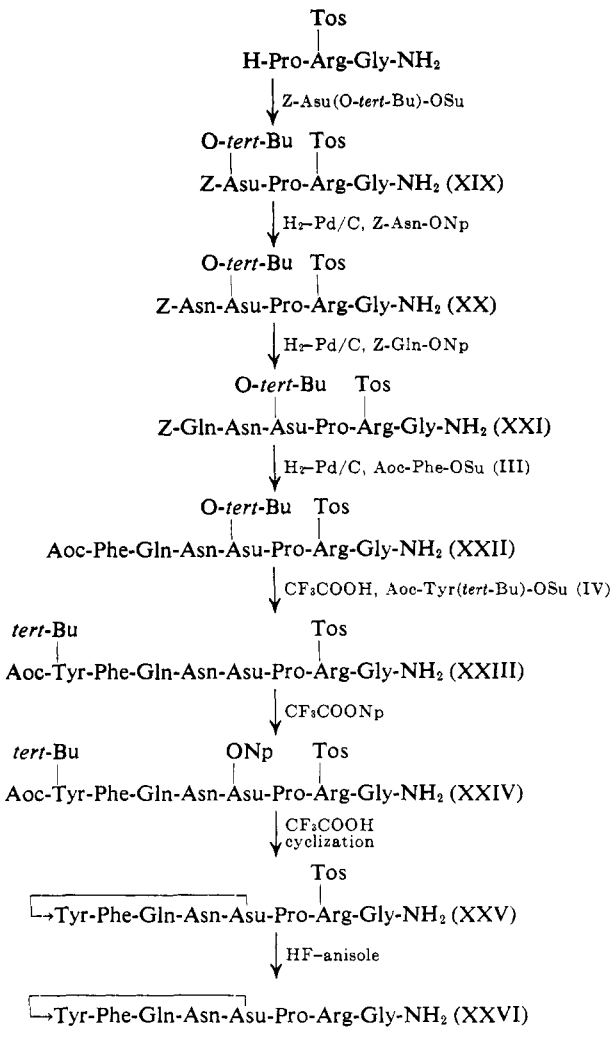

Figure 4. Scheme for the synthesis of [1,6-aminosuberic acid]-8-arginine-vasopressin.

tosyl group attached to the guanidino group is removed safely by treatment with anhydrous hydrogen fluoride[31] in the presence of anisole. Thus, starting with the known L-prolyl-N^G-tosyl-L-arginylglycinamide,[32] the peptide chains were elongated to give XXIII and XXVIII. These acyclic intermediates were converted to the partially protected cyclic peptides XXV and XXX, respectively, as described. The N^G-tosyl group was then removed by treatment with hydrogen fluoride and final product purification was achieved by gel filtration on Sephadex.

Asu-LVP, Asu-LVT, Asu-AVP, and Asu-AVT were assayed for the biological activities characteristic of posterior pituitary hormones, *viz.*, the reduction of avian blood pressure, the contractile response of the uterus, the stimulation of milk ejection from the mammary gland, the increase of blood pressure in mammals, and the alteration of the permeability to water of the mammalian distal nephron and the amphibian urinary bladder. The potencies of Asu-LVP, Asu-LVT, Asu-AVP, and Asu-AVT along with those of crystalline Asu-OXY are listed in Table I. For an analysis of

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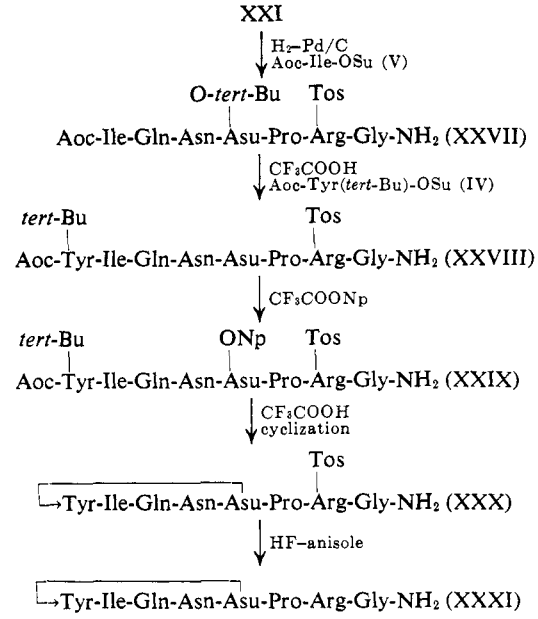

Figure 5. Partial scheme for the synthesis of [1,6-aminosuberic acid]-8-arginine-vasotocin.

the biological data contained in Table I it is advantageous to consider independently, in consecutive steps, the effects of replacement of the terminal amino group by hydrogen (deamino analogs) in neurohypophyseal hormones and the substitution of the disulfide group by an ethylene bridge. As can be seen in Table II, the ratio of the specific biological activity of a deamino analog to that of its parent hormone is *greater than one* in the oxytocic, milk-ejecting, vasodepressor and antidiuretic assays, but *less than one* in the pressor assay. This finding of *selective effects* with respect to individual biological activities—*i.e.*, some activities are enhanced while others are decreased compared to those of the parent molecule—is in line with earlier considerations that the replacement of the amino group will exert local rather than general perturbations in the peptide topology (see category b) (for more detailed discussion of this point see ref 4b and 6).

Turning now to an analogous comparison of the potency ratios between the individual biological activities of the deamino analogs and their corresponding Asu analogs, it can be seen that the Asu analogs are *consistently less active* than their disulfide-containing counterparts (Table III, read horizontally). Moreover, a comparison of the potency for a particular biological activity of different deamino and Asu analog pairs reveals that the ratio values are rather consistently independent of the absolute potency values (Table III, read vertically). We suggest that the replacement of the disulfide group by an ethylene bridge, while allowing retention of the gross features of the β structure of the 20-membered ring component, introduces in first approximation an invariant conformational factor into the individual parent peptides (deamino analogs), as judged by the consistency of the effect on their specific potency in several biological systems. Thus, it is tentatively concluded that in analogy to the replacements of sulfur by selenium,[6] the replacement of sulfur by an ethylene bridge perturbs the backbone conformation of the peptide sufficiently to alter the spatial relationship among all constituent amino acids (category a).

Table I. Biological Potencies[a,b] of 1,6-Aminosuberic Acid Analogs of Lysine-vasopressin, Lysine-vasotocin, Arginine-vasopressin, Arginine-vasotocin, and Oxytocin

Compd	Depressor (fowl)	Oxytocic (rat)	Milk-ejecting (rabbit)	Pressor (rat)	Antidiuretic (rat)	Water flux[c] (toad)
Asu-LVP	2.4 ± 0.1	5.3 ± 0.1	3.2 ± 0.3	6.2 ± 0.2	208 ± 21.0	$(1.57 \pm 0.19) \times 10^{-7}$
Asu-LVT	20.5 ± 1.2	63.6 ± 2.4	36.3 ± 0.9	9.6 ± 0.6	34.8 ± 3.0	$(2.49 \pm 0.87) \times 10^{-9}$
Asu-AVP	12.8 ± 0.4	15.4 ± 0.5	13.7 ± 1.0	37.7 ± 2.8	1274 ± 67	$(4.74 \pm 0.65) \times 10^{-9}$
Asu-AVT	64.2 ± 1.6	135 ± 4	36.8 ± 1.6	27.0 ± 1.7	313 ± 32	$(2.63 \pm 0.35) \times 10^{-10}$
Asu-OXY[d]	46.9 ± 1.8	160 ± 4.7	148 ± 8	0.11 ± 0.01	4.9 ± 0.3	$(3.80 \pm 0.74) \times 10^{-8}$

[a] Expressed in USP units/mg ± SEM, unless otherwise noted. [b] Values reported are those calculated for the anhydrous peptides. [c] Molar concentration ± SEM required for obtaining half-maximal responses; reference standards were the respective hormone congeners. Values were then normalized to the value of crystalline deamino-oxytocin as described by Eggena, *et al.*[34] [d] Values reported by Yamanaka, *et al.*,[12] however, recalculated for the *anhydrous* crystalline peptide.

Table II. Comparison of Biological Potencies of Selected Neurohypophyseal Hormones and Their Deamino Analogs[a]

Compounds	Depressor (fowl)	Oxytocic (rat)	Milk-ejecting (rabbit)	Antidiuretic (rat)	Pressor (rat)
LVP[b]:De-LVP[b]	48:61 (1:1.3)	4.8:12 (1:2.5)	31:32 (1:1)	203:301 (1:1.5)	300[h]:126 (1:0.4)
AVP[c]:De-AVP[d]	100:150 (1:1.5)	12:27 (1:2.2)	~70:80 (1:1)	503:1300 (1:2.6)	487:370 (1:0.8)
OXY[e]:De-OXY[f]	507:975 (1:1.9)	546[g]:803 (1:1.5)	410:541 (1:1.3)	2.7:19 (1:7)	3.1:1.4 (1:0.4)

[a] Expressed in USP units/milligram. Numbers in parentheses are ratios of potencies of neurohypophyseal hormones and their corresponding deamino analogs. [b] R. D. Kimbrough, Jr., W. D. Cash, L. A. Branda, W. Y. Chan, and V. du Vigneaud, *J. Biol. Chem.*, **238,** 1411 (1963). [c] J. Meienhofer, A. Trzeciak, R. T. Havran, and R. Walter, *J. Amer. Chem. Soc.*, **92,** 7199 (1970). [d] R. L. Huguenin and R. A. Boissonnas, *Helv. Chim. Acta*, **49,** 695 (1966). [e] W. Y. Chan and V. du Vigneaud, *Endocrinology*, **71,** 977 (1962). [f] B. M. Ferrier, D. Jarvis, and V. du Vigneaud, *J. Biol. Chem.*, **240,** 4264 (1965). [g] W. Y. Chan, M. O'Connell, and S. R. Pomeroy, *Endocrinology*, **72,** 279 (1963). [h] J. Meienhofer and Y. Sano, *J. Amer. Chem. Soc.*, **90,** 2996 (1968).

Table III. Potency Ratios[a] of Deamino Analogs and 1,6-Aminosuberic Acid Analogs of Neurohypophyseal Hormones

Compounds	Depressor (fowl)	Oxytocic (rat)	Milk-ejecting (rabbit)	Antidiuretic (rat)	Pressor (rat)
De-LVP:Asu-LVP	25:1	2.2:1	10:1	1.4:1	20:1
De-AVP:Asu-AVP	12:1	1.7:1	5.8:1	1.0:1	9.8:1
De-OXY:Asu-OXY	21:1	4.8:1	3.6:1	3.9:1	12.7:1

[a] Potencies for deamino analogs are those from Table II and for Asu analogs are those from Table I.

Dose–response studies comparing each of the Asu analogs with its corresponding hormone on two levels of biological complexity—the sac preparation of the toad urinary bladder according to the method of Bentley[33] as modified by Eggena, *et al.*,[34] and the adenylate cyclase assay of a broken cell preparation of toad bladder epithelium—are in line with the original finding that a direct chemical interaction of the disulfide group of neurohypophyseal hormones with the receptor is not required for initiating a physiological response.[35,36] Moreover, our results support the contention that the replacement of the disulfide group by an ethylene bridge is associated with a "consistent" effect on peptide topography and consequently potency. As can be seen from the upper panels of Figure 6, in the intact bladder all peptides give a similar maximal hydroosmotic response, although these peptides differ strikingly in the relative concentrations required to elicit a half-maximal hydroosmotic response (E_{50}). The E_{50} values found for the neurohypophyseal hormones confirm those values which have been reported earlier.[34] Most significantly, the E_{50} values of the Asu analogs are found to differ by a fairly constant factor when compared to their corresponding hormonal congeners. A comparison of cyclic 3′,5′-AMP production (picomoles of cyclic 3′,5′-AMP/mg per min) in response to LVP, LVT, AVP, AVT, or OXY reveals that these hormones stimulate adenylate cyclase of toad bladder epithelium to a different degree at optimal concentrations (Figure 6, lower panel). Asu analogs, tested under identical conditions, evoke a similar response pattern; however, in all instances they stimulate the adenylate cyclase to a lesser degree than their naturally occurring principles. A strong correlation exists between the rate of cyclic 3′,5′-AMP production in the adenylate cyclase system and the concentration of the agonistic peptides required to elicit a hydroosmotic response in the intact bladder.[37]

Asu analogs are valuable peptidyl probes for various chemical, physicochemical, and biological studies of their corresponding hormones, and are potentially useful therapeutic agents. In contrast to the disulfide-containing oxytocin, and particularly to its diseleno analog,[39] crystalline Asu-OXY is chemically completely stable during concentration procedures and lyophilization.[12] In the present study we found this also to be the case with Asu-LVP, Asu-LVT, Asu-AVP, and Asu-AVT (Table IV). Moreover, boiling aqueous solutions of these peptides for at least 3 hr, as well as repeated freez-

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Table IV. Stability of 1,6-Aminosuberic Acid Analogs of Neurohypophyseal Hormones during Lyophilization and Heat Treatment[a]

Compd	Boiled in water/non-boiled	Lyophilized (H_2O)/non-lyophilized	Lyophilized aq $N(C_2H_5)_3$/non-lyophilized
Asu-LVP	1.01	1.04	1.02
Asu-LVT	0.96	1.00	0.97
Asu-AVP	1.01	0.99	0.96
Asu-AVT	1.03	1.01	0.99
Asu-OXY	0.98	1.02[b]	1.03[b]

[a] Numbers in the table are activity ratios. Asu-LVP and Asu-AVP were assayed for rat pressor activity; Asu-LVT, Asu-AVT, and Asu-OXY for avian vasodepressor activity. [b] Values reported by Yamanaka, *et al.*, ref 12.

ing and thawing, is without effect on their biological potency—findings which indicate that the disulfide bond is the locus which initiates the sequence of irreversible chemical events resulting in the denaturation of neurohypophyseal hormones.

The complete resistance of Asu analogs to certain enzymes, such as aminopeptidases or enzymes acting *via* a reduction or oxidation of the disulfide group, renders these molecules useful for preliminary screening of various animal tissues for the presence of enzymes capable of inactivating neurohypophyseal hormones by mechanisms other than those involving the cystine residue.[40-42] Over and above their value as screening agents, they may serve as markers in studies aimed at the purification of one or more enzymic principles.[43] It is anticipated that detailed comparisons of Asu analogs with their disulfide congeners now in progress will resolve current controversies concerning the quantitative significance of neurohypophyseal hormone inactivation by disulfide reductases.[41,44,45] In fact, in accord with an earlier prediction,[42] we found recently (unpublished) that [7-glycine]-Asu-OXY[46] exhibits a prolonged rat uterotonic activity. Moreover, preliminary results indicate that the Asu analogs themselves evoke a prolonged response when compared to their natural congeners (unpublished).

In addition, the chemically stable, biologically active Asu analogs are valuable in conformational investigations. For example, studies of the circular dichroic spectra of a series of Asu analogs not only lend support to our earlier assignment of the absolute configuration of the disulfide bridge in oxytocin[8,47] but, moreover, allow a quantitative assignment of various transitions to individual chromophoric moieties.[48]

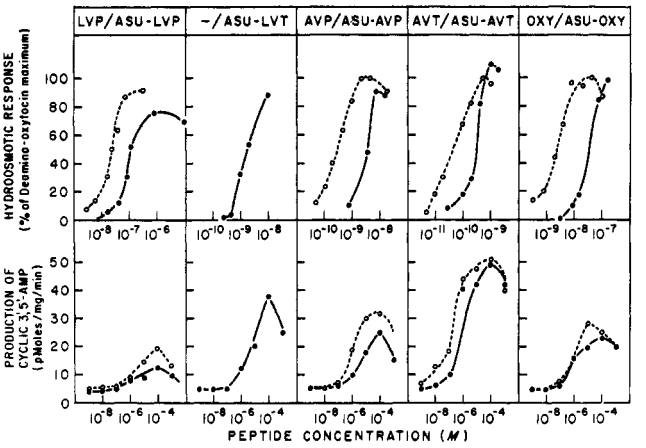

Figure 6. Comparison of dose–response curves of 1,6-aminosuberic acid neurohypophyseal hormone analogs (●—●) with their respective hormones (○----○) in eliciting a hydroosmotic response in the isolated, intact toad urinary bladder (upper panel) and in stimulating cyclic 3′,5′-AMP production in an adenylate cyclase preparation of bladder epithelium (lower panel). The hydroosmotic response is expressed as the per cent of the maximum response obtained with the corresponding natural hormone. All values were then normalized to E_{50} values for deaminooxytocin as reported in ref 34. Each curve represents an average of data obtained from a minimum of nine paired hemibladders. The production of cyclic 3′,5′-AMP is expressed in pmol/mg per min. All curves of agonistic peptides were obtained by direct comparison with deamino-oxytocin and normalized. Each curve represents an average of three or four independent experiments.

Experimental Section

Melting points (uncorrected) were determined by the capillary method. Each reaction step was checked for completion by thin-layer chromatography using silica gel (Kiesel-gel G, Merck) with either chloroform–methanol–acetic acid (95:5:3, v/v/v) (S_1) as solvent system and/or 1-butanol–acetic acid–water (4:1:1, v/v/v) (S_2). Material was stained with ninhydrin and/or with iodine vapor. If necessary, the chromatogram was sprayed with hydrobromic acid, dried on a heating plate, and then tested with ninhydrin solution. For paper chromatography on Toyo filter paper no. 51 the following solvent systems were used: S_2, 1-butanol–pyridine–water (4:1:1, v/v/v) (S_3), and pyridine–acetic acid–water (50:35:15, v/v/v) (S_4). Paper electrophoresis was performed on Toyo filter paper no. 514 using 0.2 *M* pyridine–acetic acid buffer solution (pH 4.8) and the peptides were located with ninhydrin and/or Pauly spray.[49] Coupling reactions as well as deprotection reactions by acidolysis or catalytic hydrogenolysis (5% palladium/charcoal at atmospheric pressure) were carried out at room temperature unless otherwise stated. Solvents were dried over anhydrous sodium sulfate and were removed under reduced pressure. Solid material was collected by filtration. Column effluents were monitored by uv absorption at 280 nm. All products were dried over phosphorus pentoxide *in vacuo* at room temperature unless otherwise noted. Samples used for elementary analysis and optical rotation determinations were dried a second time under the conditions specified.

Toads (*Bufo marinus*, originating in the Dominican Republic) were obtained from National Reagents, Inc., Bridgeport, Conn., Sprague-Dawley rats (200–300 g) and female New Zealand White rabbits from Marland Farms, Wayne, N. J., and male Leghorn chickens from Shamrock Farm, Wayne, N. J. A Grass polygraph in conjunction with Statham transducers P23AC was used for blood pressure determinations in rat, chicken, and rabbit and with transducers P23BC (high sensitivity) for intramammary pressure measurements in rabbit. A heart-smooth muscle lever transducer (Harvard Instruments) connected *via* an "adaptor" panel to a Grass polygraph was used for measuring isotonic contractions by the four-point method. Procaine–HCl (1%) was purchased from Spencer-Mead, Inc., Valley Stream, N. Y., reserpine from J. T. Baker Chemical Co., Phillipsburg, N. J., atropine sulfate monohydrate from Cal-

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biochem, Los Angeles, Calif., dibenzyline (phenoxybenzamine HCl) from Smith, Kline & French, Philadelphia, Pa., Inactin from Chem Fabrik Promonta, Hamburg, Germany, urethane (ethyl carbamate) from Fisher Scientific, Fairlawn, N. J., and heparin from Nutritional Biochemical Corp., Cleveland, Ohio.

For the adenylate cyclase assay of toad bladder epithelium the following reagents were used: 2-phosphoenolpyruvic acid, tricyclohexylammonium salt, and pyruvate kinase from rabbit muscle (178 eu/mg of protein) obtained from Calbiochem, Los Angeles, Calif.; myokinase from rabbit muscle (approximately 600–1000 units/mg of protein) from Sigma Chemical Co., St Louis, Mo.; adenosine 5′-triphosphate, disodium salt (ATP) from Schwarz/Mann, Orangeburg, N. Y.; adenosine 3′,5′-cyclic phosphate, free acid (cyclic AMP) from Nutritional Biochemicals, Cleveland, Ohio. Adenosine 5′-triphosphate-α-^{32}P (specific activity 2.3–6.9 Ci/mmol) was obtained from International Chemical and Nuclear Corp., Irvine, Calif.

N^{α}-Benzyloxycarbonyl-N^{ϵ}-phthalyl-L-lysine Dicyclohexylammonium Salt. N^{ϵ}-Phthalyl-L-lysine hydrochloride[50] (68.8 g, 0.22 mol) was dissolved in water (1 l.) and an aqueous solution (1.7 l.) of sodium bicarbonate (59 g, 0.70 mol) was added. The mixture was treated with benzyloxycarbonyl chloride (35.8 g, 0.25 mol) under vigorous stirring for 30 min at 0–5° and for an additional 2 hr at room temperature. Excess benzyloxycarbonyl chloride was extracted with ether, and the aqueous layer was acidified to pH 2 with 6 *N* hydrochloric acid. An oily precipitate appeared which was extracted twice with a total volume of 2 l. of ethyl acetate. The organic extract was washed with water, dried, and concentrated. The residual oil (89 g) was dissolved in a mixture of ethyl acetate (100 ml) and ether (100 ml), and a solution of dicyclohexylamine (40 ml, 0.20 mol) in ether (400 ml) was added to obtain crystals of the dicyclohexylammonium salt of the lysine derivative: 101 g; mp 150–153°. The crude product was dissolved in methanol (500 ml) and stored overnight at room temperature. The precipitate which appeared was filtered and the mother liquor was concentrated. The solid residue was crystallized from a mixture of methanol (300 ml) and ether (500 ml): 82 g; mp 156–157.5°. From the mother liquor additional product was obtained: 8 g; mp 155–157°. These products were combined and recrystallized from a mixture of methanol (200 ml) and ether (400 ml): 57.7 g (44.4%); mp 158–159°; $[\alpha]^{25}D$ +6.1° (*c* 2.0, methanol).

Anal. Calcd for $C_{34}H_{45}O_6N_3$: C, 69.0; H, 7.67; N, 7.10. Found: C, 68.9; H, 7.65; N, 7.05.

N^{α}-Benzyloxycarbonyl-N^{ϵ}-phthalyl-L-lysine. The dicyclohexylammonium salt (53.3 g, 0.09 mol) was shaken with a mixture of ethyl acetate (300 ml) and 1 *N* sulfuric acid (150 ml); the ethyl acetate layer was washed several times with water and dried. Removal of the solvent gave a crystalline product, which was collected by filtration with the aid of a small amount of ethyl acetate: 31.7 g (86%); mp 129–130.5°. A sample was dried at 60° for 8 hr: $[\alpha]^{25}D$ −1.8° (*c* 3.0, methanol).

Anal. Calcd for $C_{22}H_{22}O_6N_2$: C, 64.4; H, 5.40; N, 6.83. Found: C, 64.2; H, 5.35; N, 6.77.

N^{α}-Benzyloxycarbonyl-N^{ϵ}-phthalyl-L-lysine *p*-Nitrophenyl Ester (I). N^{α}-Benzyloxycarbonyl-N^{ϵ}-phthalyl-L-lysine obtained above (28.7 g, 0.07 mol) was dissolved in ethyl acetate (200 ml) together with *p*-nitrophenol (11.7 g, 0.084 mol). A solution of dicyclohexylcarbodiimide (15.9 g, 0.077 mol) in ethyl acetate (100 ml) was added with stirring at 0°. After 1 hr the cooling bath was removed while stirring was continued for an additional 3 hr at room temperature. A few drops of acetic acid were added to the reaction mixture, and the dicyclohexylurea formed was filtered after 30 min. The filtrate was concentrated and the resulting residue was induced to crystallization by trituration with ethanol. The crude product was collected and recrystallized from ethanol: 31.3 g (84.1%); mp 99–100°. A sample was dried at 60° for 8 hr: $[\alpha]^{22}D$ −16.0° (*c* 4.2, dimethylformamide).

Anal. Calcd for $C_{28}H_{25}O_8N_3$: C, 63.3; H, 4.74; N, 7.91. Found: C, 63.3; H, 4.56; N, 7.84.

Benzyloxycarbonyl-L-asparagine *N*-Hydroxysuccinimide Ester (II). A solution of dicyclohexylcarbodiimide (21.7 g, 0.105 mol) in ethyl acetate (100 ml) was stirred into a solution of benzyloxycarbonyl-L-asparagine (26.6 g, 0.1 mol) and *N*-hydroxysuccinimide (12.6 g, 0.11 mol) in dimethylformamide (100 ml) at 0°. After 3 hr a small amount of acetic acid was added. The dicyclohexylurea was filtered off after 30 min, and ethyl acetate was removed by evaporation. The remaining dimethylformamide solution was treated with a large volume of *n*-hexane. The oily product which separated was collected by decantation, and washed with *n*-hexane and then with ether. The product, crystallized from isopropyl alcohol, was collected and washed with ether. The crude material was recrystallized rapidly at room temperature from dimethylformamide by addition of isopropyl alcohol: 16.4 g (45.2%); mp 129–130°. A sample was dried at 60° for 10 hr: $[\alpha]^{24}D$ −27.5° (*c* 2.0, dimethylformamide).

Anal. Calcd for $C_{18}H_{17}O_7N_3$: C, 52.9; H, 4.72; N, 11.6. Found: C, 52.8; H, 4.73; N, 11.5.

***tert*-Amyloxycarbonyl-L-phenylalanine *N*-Hydroxysuccinimide Ester (III).** *tert*-Amyloxycarbonyl-L-phenylalanine, which was isolated from the dicyclohexylammonium salt[51] (9.21 g, 0.02 mol), was dissolved in a mixture of dioxane (20 ml) and ethyl acetate (20 ml) together with *N*-hydroxysuccinimide (2.53 g, 0.022 mol). The mixture was treated with a solution of dicyclohexylcarbodiimide (4.33 g, 0.021 mol) in ethyl acetate (30 ml) at 0°. The reaction mixture was stirred for 15 hr at 4°. A small amount of acetic acid was added to the reaction mixture, dicyclohexylurea was removed by filtration, and the filtrate was concentrated. The residue was triturated with *n*-hexane; the precipitate was filtered, washed with *n*-hexane, and dried. The product was crystallized from isopropyl alcohol, and recrystallized to constant melting point: 6.64 g (88.2%); mp 130–131°. A sample was dried at 60° for 6 hr: $[\alpha]^{18}D$ −52.0° (*c* 2.0, dimethylformamide).

Anal. Calcd for $C_{19}H_{24}O_6N_2$: C, 60.6; H, 6.43; N, 7.44. Found: C, 60.7; H, 6.53; N, 7.62.

***N*-*tert*-Amyloxycarbonyl-*O*-*tert*-butyl-L-tryrosine Dicyclohexylammonium Salt.** A mixture of *O*-*tert*-butyl-L-tyrosine[52] (8.87 g, 0.035 mol), 1 *N* sodium hydroxide (35 ml), triethylamine (9.8 ml, 0.07 mol), and *tert*-amyloxycarbonyl azide[51] (11.0 g, 0.07 mol) dissolved in dioxane (35 ml) was stirred at 36° for 20 hr. Then, dioxane was removed by evaporation and excess azide was extracted with ether. The aqueous layer was carefully acidified to pH 2 with 2 *N* hydrochloric acid, and the oily precipitate was extracted with ethyl acetate. The extract was washed with water, dried, and concentrated. The oily residue was dissolved in *n*-hexane, and crystallized by the addition of dicyclohexylamine; the product was recrystallized twice from *n*-hexane: 16.0 g (85.%); mp 114–116°. A sample was dried at 60° for 3 hr: $[\alpha]^{18}D$ +35.1° (*c* 2.0, dimethylformamide).

Anal. Calcd for $C_{31}H_{52}O_5N_2 \cdot 0.25H_2O$: C, 69.4; H, 9.85; N, 5.22. Found: C, 69.5; H, 10.1; N, 5.06.

***N*-*tert*-Amyloxycarbonyl-*O*-*tert*-butyl-L-tyrosine.** This material was isolated from the dicyclohexylammonium salt (5.33 g, 10 mmol) in the usual manner, and crystallized from *n*-hexane: 3.26 g (92.9%); mp 108–109.5°. A sample was dried at 60° for 3 hr: $[\alpha]^{24}D$ −15.2° (*c* 3.0, dimethylformamide).

Anal. Calcd for $C_{19}H_{29}O_5N$: C, 64.9; H, 8.32; N, 3.99. Found: C, 64.8; H, 8.47; N, 3.99.

***N*-*tert*-Amyloxycarbonyl-*O*-*tert*-butyl-L-tyrosine *N*-Hydroxysuccinimide Ester (IV).** The above compound (2.81 g, 8 mmol) was converted to the *N*-hydroxysuccinimide ester as described for III, but the reaction time was shortened to 1.5 hr. The crystalline product was isolated from *n*-hexane, and recrystallized from the same solvent: 2.82 g (78.5%); mp 112–113°; $[\alpha]^{23}D$ −42.8° (*c* 2.0, dimethylformamide).

Anal. Calcd for $C_{23}H_{32}O_7N_2$: C, 61.6; H, 7.19; N, 6.25. Found: C, 61.3; H, 7.34; N, 6.22.

***tert*-Amyloxycarbonyl-L-isoleucine *N*-Hydroxysuccinimide Ester (V).** *tert*-Amyloxycarbonyl-L-isoleucine isolated from its dicyclohexylammonium salt[51] (8.53 g, 0.02 mol) was mixed with *N*-hydroxysuccinimide (2.42 g, 0.021 mol), and these materials were allowed to react with dicyclohexylcarbodiimide (4.33 g, 0.021 mol) in a mixture of dioxane and ethyl acetate for 2 hr at 0°. The product was isolated as described previously, and recrystallized from ether–petroleum ether: 4.33 g (63.2%); mp 82–83°; $[\alpha]^{23}D$ −24.3° (*c* 4.1, dimethylformamide).

Anal. Calcd for $C_{16}H_{26}O_6N_2$: C, 56.1; H, 7.66; N, 8.18. Found: C, 56.3; H, 7.69; N, 8.18.

N^{α}-Benzyloxycarbonyl-N^{ϵ}-phthalyl-L-lysylglycinamide (VI). Glycinamide which was obtained from benzyloxycarbonylglycinamide[53] (12.5 g, 0.06 mol) by catalytic hydrogenolysis for 8 hr in

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methanol (300 ml) using 0.7 g of catalyst was acylated with compound I (26.6 g, 0.05 mol) in dimethylformamide (100 ml). The product began to precipitate in a few minutes. After 2 hr, water (100 ml) was added, and the precipitated product was collected and recrystallized from dimethylformamide (400 ml) by addition of water (200 ml): 22.2 g (95.2%); mp 215–217°. A sample was dried at 80° for 10 hr: $[\alpha]^{22}D$ −2.2° (*c* 2.1, dimethylformamide).

Anal. Calcd for $C_{24}H_{26}O_6N_4$: C, 61.9; H, 5.62; N, 12.0. Found: C, 61.5; H, 5.52; N, 12.0.

Benzyloxycarbonyl-L-prolyl-N^{ϵ}-phthalyl-L-lysylglycinamide (VII). The compound VI (18.7 g, 0.04 mol) was treated with 25% hydrogen bromide dissolved in glacial acetic acid (40 ml). After 45 min a large volume of ether was added and the resulting precipitate was washed by decantation with ether (three 400-ml portions) and dried in a desiccator over sodium hydroxide. The product was dissolved in dimethylformamide (100 ml) and the solution was treated with benzyloxycarbonyl-L-proline *N*-hydroxysuccinimide ester[22] (15.2 g, 0.044 mol) in the presence of triethylamine (5.6 ml, 0.04 mol) and *N*-ethylmorpholine (5.12 ml, 0.04 mol). After 3 hr the oily product was precipitated by the addition of 1% aqueous acetic acid (400 ml). After standing overnight at 0°, the solid material was collected, washed with water, and crystallized as needles from warm ethyl acetate (300 ml): 20.7 g (92.0%); mp 168–171°. Recrystallization from dimethylformamide (60 ml)–ethyl acetate (100 ml) gave: 17.4 g (77.2%); mp 173–175°; $[\alpha]^{22}D$ −36.4° (*c* 2.1, dimethylformamide). A second crop of 1.2 g (5.3%), mp 171–174°, was recovered from the mother liquor of the recrystallization. A sample was dried at 80° for 8 hr.

Anal. Calcd for $C_{29}H_{33}O_7N_5$: C, 61.8; H, 5.90; N, 12.4. Found: C, 61.7; H, 5.66; N, 12.2.

Benzyloxycarbonyl-ω-*tert*-butyl-L-α-aminosuberyl-L-prolyl-N^{ϵ}-phthalyl-L-lysylglycinamide (VIII). Benzyloxycarbonyl-L-α-aminosuberic acid ω-*tert*-butyl ester (15 mmol), which was isolated from the piperazine salt[9] (5.34 g), was dissolved in dry pyridine (15 ml). *N*-Hydroxysuccinimide trifluoroacetate[27] (6.3 g, 30 mmol) was added to this solution. After 30 min water (100 ml) was added and the resultant oily product was extracted with ethyl acetate. The extract, washed successively with 1 *N* hydrochloric acid and water and dried, was concentrated to give oily α-*N*-hydroxysuccinimide ester of benzyloxycarbonyl-L-α-aminosuberic acid ω-*tert*-butyl ester. This oil was dissolved in dimethylformamide (20 ml) and added to a solution of dimethylformamide (30 ml) containing triethylamine (2.1 ml, 15 mmol) and L-prolyl-N^{ϵ}-phthalyl-L-lysylglycinamide hydrochloride derived from compound VII (8.45 g, 15 mmol) by catalytic hydrogenolysis for 14 hr in a mixture of ethanol (300 ml), water (75 ml), and 1 *N* hydrochloric acid (15 ml) as solvent in the presence of 3 g of catalyst. The reaction mixture was allowed to stand for 2 days. Aqueous acetic acid (1%, 100 ml) was added to the reaction mixture and the precipitated oil was extracted with ethyl acetate (100 ml). The extract was washed with water, dried, and concentrated. The residue was crystallized from a small amount of ethanol containing ether; the crystals were collected and recrystallized twice from ethanol: 8.73 g (73.6%); mp 112–115°. A sample was dried at 70° for 6 hr: $[\alpha]^{18}D$ −37.5° (*c* 2.0, dimethylformamide).

Anal. Calcd for $C_{41}H_{54}O_{10}N_6$: C, 62.3; H, 6.88; N, 10.6. Found: C, 62.3; H, 7.04; N, 10.5.

Benzyloxycarbonyl-L-asparaginyl-ω-*tert*-butyl-L-α-aminosuberyl-L-prolyl-N^{ϵ}-phthalyl-L-lysylglycinamide (IX). Compound VIII (11.86 g, 15 mmol) was subjected to catalytic hydrogenolysis for 10 hr in a mixture of ethanol (300 ml) and 1 *N* hydrochloric acid (15 ml) as described above. The material obtained after removal of catalyst and solvent was dissolved in dimethylformamide (100 ml) and compound II (6.18 g, 17 mmol) was added with stirring, along with triethylamine (2.1 ml, 15 mmol). After 80 min the product was precipitated with ether (500 ml), collected, and washed successively with ether, ethyl acetate, 1% acetic acid, and water. The crude product was recrystallized from 90% ethanol: 11.05 g (81.3%); mp 188–190°. A sample was dried at 80° for 10 hr: $[\alpha]^{19}D$ −38.0° (*c* 2.0, dimethylformamide).

Anal. Calcd for $C_{45}H_{60}O_{12}N_8$: C, 59.7; H, 6.68; N, 12.4. Found: C, 59.5; H, 6.96; N, 12.6.

Benzyloxycarbonyl-L-glutaminyl-L-asparaginyl-ω-*tert*-butyl-L-α-aminosuberyl-L-prolyl-N^{ϵ}-phthalyl-L-lysylglycinamide (X). The benzyloxycarbonyl group of compound IX (10.86 g, 12 mmol) was removed by catalytic hydrogenolysis for 6 hr at 35–40° in a mixture of ethanol (500 ml), water (30 ml), and 1 *N* hydrochloric acid (15 ml) in the presence of 3 g of catalyst. The resulting material was allowed to react with benzyloxycarbonyl-L-glutamine *p*-nitrophenyl ester[13] (6.02 g, 15 mmol) in dimethylformamide (60 ml) in the presence of triethylamine (2.1 ml, 15 mmol). After 24 hr acetic acid (2 ml) and ethyl acetate (300 ml) were added to the reaction mixture; the precipitate formed was collected, washed with ethyl acetate and water, and dried. Reprecipitation from 90% ethanol gave 10.95 g of the product: 88.3%; mp 189–191° dec. A sample was dried at 80° for 10 hr: $[\alpha]^{19}D$ −36.9° (*c* 1.6, dimethylformamide).

Anal. Calcd for $C_{50}H_{68}O_{14}N_{10}$: C, 58.1; H, 6.63; N, 13.6. Found: C, 57.9; H, 6.63; N, 13.5.

***tert*-Amyloxycarbonyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-ω-*tert*-butyl-L-α-aminosuberyl-L-prolyl-N^{ϵ}-phthalyl-L-lysylglycinamide (XI).** Compound X (5.17 g, 5 mmol) was subjected to catalytic hydrogenolysis for 13 hr at 35–40° in a mixture of dimethylformamide (50 ml), ethanol (50 ml), water (30 ml), and 1 *N* hydrochloric acid (6 ml) as described above. The catalyst was filtered off and volatile solvent was evaporated. Water was removed from the dimethylformamide azeotropically with toluene under reduced pressure. Compound III (2.26 g, 6 mmol) and triethylamine (0.84 ml, 6 mmol) were added to the resulting solution. After 60 min, acetic acid (1 ml) and ethyl acetate (250 ml) were added to the reaction mixture which was kept overnight at 0°. The precipitate formed was filtered, washed successively with ethyl acetate, ethanol, and water, and dried. The crude material was reprecipitated from 90% ethanol: 4.41 g (76.0%); mp 188–190° dec. A sample was dried at 80° for 10 hr: $[\alpha]^{22}D$ −35.4° (*c* 1.5, dimethylformamide).

Anal. Calcd for $C_{57}H_{81}O_{15}N_{11}$: C, 59.0; H, 7.04; N, 13.3. Found: C, 58.9; H, 7.29; N, 13.4.

***N*-*tert*-Amyloxycarbonyl-*O*-*tert*-butyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-L-α-aminosuberyl-L-prolyl-N^{ϵ}-phthalyl-L-lysylglycinamide (XII).** Compound XI (2.09 g, 1.8 mmol) was dissolved and kept in trifluoroacetic acid (9 ml) for 2 hr. Removal of solvent gave an oily residue which was triturated with ether and dried over sodium hydroxide. The powder thus obtained was dissolved in dimethylformamide (10 ml) and treated with compound IV (0.99 g, 2.2 mmol) in the presence of *N*-ethylmorpholine (0.46 ml, 3.6 mmol) for 40 hr. Ethyl acetate (100 ml) was added to precipitate the product, which was collected. The crude material was suspended in 90% ethanol (80 ml), refluxed for a few minutes, and then cooled to room temperature. The precipitated product was collected, washed with 90% ethanol, then with absolute ethanol, and dried: 2.08 g (86.3%); mp 230–232° dec. A sample was dried at 80° for 10 hr: $[\alpha]^{25}D$ −37.0° (*c* 1.0, dimethylformamide).

Anal. Calcd for $C_{66}H_{90}O_{17}N_{12}$: C, 59.9; H, 6.85; N, 12.7. Found: C, 59.6; H, 6.79; N, 12.8.

***N*-*tert*-Amyloxycarbonyl-*O*-*tert*-butyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-ω-*p*-nitrophenyl-L-α-aminosuberyl-L-prolyl-N^{ϵ}-phthalyl-L-lysylglycinamide (XIII).** *p*-Nitrophenyl trifluoroacetate[9,27] (1.3 g, 5:5 mmol) was stirred into a suspension of compound XII (1.46 g, 1.1 mmol) in a mixture of dimethylformamide (16 ml) and pyridine (8 ml). The suspension changed to a clear solution within a few minutes while stirring was continued at 50°. A large excess of ether was added after 3 hr. The precipitated product was collected and reprecipitated twice from dimethylformamide–ether: 1.20 g (75.5%); mp 217–220° dec. A sample was dried at 60° for 8 hr: $[\alpha]^{25}D$ −29.2° (*c* 1.0, dimethylformamide).

Anal. Calcd for $C_{72}H_{93}O_{19}N_{13}$: C, 59.9; H, 6.49; N, 12.6. Found: C, 60.0; H, 6.49; N, 12.6.

[1,6-Aminosuberic acid]-8-lysine-vasopressin (XIV). Compound XIII (1.15 g, 0.8 mmol) dissolved in trifluoroacetic acid (4 ml) was kept for 1 hr. The oily residue secured after removal of solvent was solidified by trituration with ether; the precipitate was collected, washed with ether, and dried over sodium hydroxide. The dried material (1.20 g) was dissolved in dimethylformamide (8 ml) and this solution was added under stirring over a period of 4 hr to pyridine (800 ml) kept at a temperature of 50°. After 1 hr of additional stirring at 50° the reaction mixture was concentrated to a small volume and, by addition of ether, the product precipitated. The precipitate was collected, washed with ether, and dried, 0.95 g. This material was dissolved in 100 ml of a mixture of methanol–water (2:1, v/v) and the solution was passed through a column (1.2 × 12 cm) of CM-Sephadex C-25 (H^+ form). The column was eluted with the same solvent and effluent and washings were combined and concentrated. The resulting residue was dissolved in acetic acid and lyophilized, 0.84 g. The lyophilizate was dissolved in methanol (2 ml) and a small amount (0.15 g) of precipitate formed overnight at room temperature was removed. Subsequently, a large volume of ether was added to the filtrate to pre-

cipitate the phthalylated cyclic peptide (0.61 g, *ca.* 0.54 mmol) which was then dissolved in a freshly prepared 2 *M* solution of hydrazine acetate in dimethylacetamide (5.4 ml) and kept overnight. Then, the reaction mixture was diluted with water (25 ml) and the solution was adjusted with acetic acid to pH 3.5–4. Overnight at 4° crystals appeared (phthalyl hydrazide) which were filtered off. The filtrate was passed through a column (3 × 14 cm) of CM-Sephadex C-25 (H^+ form), which was washed thoroughly with water until the uv absorption of the washings became negligible. The product was eluted from the column with a mixture of pyridine–acetic acid–water (30:4:66, v/v/v).[54] The effluent was concentrated almost to dryness, water was added, and the solution was lyophilized. The lyophilizate was dissolved in 3 ml of 0.5 *M* ammonium acetate solution (pH 6.3) and placed on a column of CM-Sephadex C-25 (2 × 50 cm) which had been previously equilibrated with the same solution. The column was eluted with 0.5 *M* ammonium acetate solution (pH 6.3) at the rate of 12 ml/hr; the effluent, monitored for uv absorption, was collected in 5-g fractions. The content of tubes 32–43 was pooled and lyophilized three times from water to give a fluffy powder (380 mg). This powder was then dissolved in 0.1 *N* acetic acid (2 ml) and subjected to gel filtration on a Sephadex G-25 column (fine, 2 × 135 cm) with 0.1 *N* acetic acid as solvent. Fractions 54–62 were pooled, lyophilized, and dried, 370 mg. The yield was 40%, based on XII. This material was found to be homogeneous as judged by paper electrophoresis and paper chromatography: R_f 0.23 (S_2) and 0.13 (S_3). A sample was dried at 100° for 24 hr: $[\alpha]^{23}D$ −79.6° (*c* 0.56, 1 *N* acetic acid). Loss of weight upon drying was 7.2%.

Anal. Calcd for $C_{48}H_{68}O_{12}N_{12}\cdot 3H_2O$: C, 54.4; H, 7.04; N, 15.9. Found: C, 54.3; H, 6.81; N, 15.9.

Amino acid analysis[55] (6 *N* hydrochloric acid, 105°, 39 hr in presence of a small amount of phenol) gave the following molar ratios: Tyr, 1.00; Phe, 1.00; Glu, 1.02; Asp, 0.99; Asu, 1.00; Pro, 1.05; Lys, 1.07; Gly, 1.00.

***tert*-Amyloxycarbonyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-ω-*tert*-butyl-L-α-aminosuberyl-L-prolyl-N^ϵ-phthalyl-L-lysylglycinamide (XV).** Compound X (2.58 g, 2.5 mmol) was subjected to catalytic hydrogenolysis for 13 hr in the presence of 1.5 g of catalyst in a mixture of dimethylformamide (20 ml), ethanol (20 ml), water (10 ml), and 1 *N* hydrochloric acid (2.7 ml) at 35–40°. The decarbobenzoxylated product was isolated as described for XI. Compound V (1.03 g, 3 mmol) and triethylamine (0.36 ml, 2.7 mmol) were added to the dimethylformamide solution. Since the reaction was found to proceed very slowly, additional active ester V (0.34 g, 1 mmol) and *N*-ethylmorpholine (0.13 ml, 1 mmol) were added 4 days later. After an additional day the product was precipitated by addition of a large amount of an ether–ethyl acetate mixture (1:1, v/v); the precipitate was filtered, washed with ether, and dried. Reprecipitation from 90% ethanol gave: 2.02 g (71.9%); mp 192–194° dec. A sample was dried at 100° for 19 hr: $[\alpha]^{23}D$ −36.5° (*c* 2.1, dimethylformamide).

Anal. Calcd for $C_{54}H_{83}O_{15}N_{11}\cdot H_2O$: C, 56.7; H, 7.49; N, 13.5. Found: C, 56.3; H, 7.39; N, 13.8.

***N*-*tert*-Amyloxycarbonyl-*O*-*tert*-butyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-L-α-aminosuberyl-L-prolyl-N^ϵ-phthalyl-L-lysylglycinamide (XVI).** Partial deprotection of XV (1.46 g, 1.3 mmol) was achieved as described for XI. The dried powder thus obtained was treated with compound IV and worked up as described for XII, giving: 1.51 g (89.8%); mp 234–236° dec. A sample was dried at 100° for 18 hr: $[\alpha]^{24}D$ −39.9° (*c* 1.0, acetic acid).

Anal. Calcd for $C_{68}H_{92}O_{17}N_{12}$: C, 58.7; H, 7.19; N, 13.0. Found: C, 58.6; H, 7.12; N, 13.1.

***N*-*tert*-Amyloxycarbonyl-*O*-*tert*-butyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-ω-*p*-nitrophenyl-L-α-aminosuberyl-L-prolyl-N^ϵ-phthalyl-L-lysylglycinamide (XVII).** Compound XVI (635 mg, 0.49 mmol) suspended in a mixture of dimethylformamide (8 ml) and pyridine (8 ml) was esterified with *p*-nitrophenyl trifluoroacetate (1.06 g, 4.5 mmol) as described for XIII: 591 mg (83.5%); mp 229–233° dec. A sample was dried at 60° for 10 hr: $[\alpha]^{26}D$ −26.4° (*c* 1.1, dimethylformamide).

Anal. Calcd for $C_{69}H_{95}O_{19}N_{13}\cdot 2H_2O$: C, 57.3; H, 6.90; N, 12.6. Found: C, 57.4; H, 6.74; N, 12.7.

[1,6-Aminosuberic acid]-8-lysine-vasotocin (XVIII). Compound XVII (1.08 g, 0.75 mmol as dihydrate) dissolved in trifluoroacetic acid (4 ml) was kept for 1 hr. The reaction mixture was then worked up as described for XIV. The dried material (1.04 g) was dissolved in dimethylformamide (7.5 ml) and the solution was added with stirring during a 3-hr period to pyridine (750 ml) kept at 50°. After additional stirring for 2 hr at 50° the crude phthalylated cyclic peptide was isolated as described (0.94 g), then partially purified on CM-Sephadex C-25 (0.69 g, *ca.* 0.63 mmol), dephthalylated, again purified by ion exchange chromatography, and isolated by lyophilization as a fluffy powder (293 mg). This powder was then subjected to gel filtration on a Sephadex G-25 column as described for XIV: 266 mg (overall yield from XVII was 32%). The homogeneity of this material was tested by paper chromatography: R_f 0.25 (S_2) and 0.70 (S_4). A sample was dried at 100° for 24 hr: $[\alpha]^{27}D$ −79.2° (*c* 0.47, 1 *N* acetic acid). Loss of weight upon drying was 7.6%.

Anal. Calcd for $C_{45}H_{70}O_{12}N_{12}\cdot 3H_2O$: C, 52.7; H, 7.47; N, 16.4. Found: C, 53.0; H, 7.12; N, 16.4.

Amino acid analysis (6 *N* hydrochloric acid, 105°, 39 hr in presence of small amount of phenol) gave the following molar ratios: Tyr, 0.95; Ile, 1.05; Glu, 1.04; Asp, 0.97; Asu, 1.02; Pro, 1.02; Lys, 0.95; Gly, 1.01.

Benzyloxycarbonyl-ω-*tert*-butyl-L-α-aminosuberyl-L-prolyl-N^g-tosyl-L-arginylglycinamide (XIX). Benzyloxycarbonyl-L-α-aminosuberic acid ω-*tert*-butyl ester (10 mmol) which was isolated from its piperazine salt (4.23 g) was esterified with *N*-hydroxysuccinimide trifluoroacetate, as described for VIII. The dried, oily *N*-hydroxysuccinimide ester was allowed to react with L-prolyl-N^g-tosyl-L-arginylglycinamide (4.33 g, 9 mmol) in dimethylformamide (30 ml) for 4 days. Water (200 ml) was added to the reaction mixture to give an oily precipitate which was then extracted with ethyl acetate. The organic layer was washed with 1 *N* hydrochloric acid and water, dried, and concentrated to yield a residue which solidified upon trituration with ether: 5.49 g (72.3%); mp 78–100°. A sample was dried at 40° for 5 hr: $[\alpha]^{16}D$ −31.7° (*c* 2.0, dimethylformamide).

Anal. Calcd for $C_{40}H_{58}O_{10}N_8S\cdot 0.5H_2O$: C, 56.4; H, 6.98; N, 13.2; S, 3.76. Found: C, 56.4; H, 7.07; N, 13.1; S, 3.93.

Benzyloxycarbonyl-L-asparaginyl-ω-*tert*-butyl-L-α-aminosuberyl-L-prolyl-N^g-tosyl-L-arginylglycinamide (XX). Compound XIX (5.06 g, 6 mmol as hemihydrate) was subjected to catalytic hydrogenolysis for 12 hr in the presence of 1 g of catalyst in 90% ethanol (55 ml). The free amine, isolated as an oil, was dissolved in dimethylacetamide (20 ml) and allowed to react with benzyloxycarbonyl-L-asparagine *p*-nitrophenyl ester[13] (2.79 g, 7.2 mmol) for 24 hr. The product, precipitated with ethyl acetate, was collected and reprecipitated from ethanol: 3.89 g (67.8%); mp 148–150°. A sample was dried at 100° for 18 hr: $[\alpha]^{18}D$ −30.5° (*c* 2.0, dimethylformamide).

Anal. Calcd for $C_{44}H_{64}O_{12}N_{10}S\cdot H_2O$: C, 54.2; H, 6.82; N, 14.4; S, 3.29. Found: C, 54.2; H, 6.73; N, 14.2; S, 3.20.

Benzyloxycarbonyl-L-glutaminyl-L-asparaginyl-ω-*tert*-butyl-L-α-aminosuberyl-L-prolyl-N^g-tosyl-L-arginylglycinamide (XXI). The benzyloxycarbonyl group of compound XX (4.34 g, 4.5 mmol as monohydrate) was removed by catalytic hydrogenolysis for 5 hr using 1 g of catalyst in a mixture of ethanol (50 ml) and water (10 ml). The decarbobenzoxylated, oily peptide was dissolved in dimethylformamide (10 ml) along with benzyloxycarbonyl-L-glutamine *p*-nitrophenyl ester[13] (2.17 g, 5.4 mmol), and stored for 2 days. The product, precipitated with ethyl acetate, was further purified by reprecipitation from 95% ethanol: 4.14 g (83.5%); mp 168–170°. A sample was dried at 100° for 18 hr: $[\alpha]^{18}D$ −29.2° (*c* 1.9, dimethylformamide).

Anal. Calcd for $C_{49}H_{72}O_{14}N_{12}S\cdot H_2O$: C, 53.3; H, 6.76; N, 15.2; S, 2.91. Found: C, 53.5; H, 6.64; N, 15.0; S, 3.14.

***tert*-Amyloxycarbonyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-ω-*tert*-butyl-L-α-aminosuberyl-L-prolyl-N^g-tosyl-L-arginylglycinamide (XXII).** Compound XXI (2.71 g, 2.5 mmol as monohydrate) was decarbobenzoxylated by catalytic hydrogenolysis using the conditions described in the preceding paragraph. The free base was isolated and dissolved in dimethylformamide (15 ml). Compound III (1.13 g, 3 mmol) was added to the solution. After 3 days the product was precipitated with ethyl acetate, collected, and reprecipitated from 95% ethanol–ethyl acetate: 2.46 g (81.2%); mp 142–146°. A sample was dried at 100° for 18 hr: $[\alpha]^{16}D$ −29.6° (*c* 2.1, dimethylformamide).

Anal. Calcd for $C_{56}H_{85}O_{15}N_{13}S\cdot H_2O$: C, 54.7; H, 7.13; N, 14.8; S, 2.60. Found: C, 54.5; H, 7.23; N, 14.9; S, 2.53.

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N-tert-Amyloxycarbonyl-*O-tert*-butyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-L-α-aminosuberyl-L-prolyl-N^g-tosyl-L-arginylglycinamide (XXIII). Compound XXII (1.85 g, 1.5 mmol as monohydrate) was treated for 1 hr with trifluoroacetic acid (7.5 ml). The trifluoroacetate was isolated as a dry powder which was allowed to react with compound IV (0.81 g, 1.8 mmol) in dimethylacetamide (5 ml) in the presence of *N*-ethylmorpholine (0.4 ml) for 2 days. The precipitate obtained upon addition of ethyl acetate was collected and reprecipitated from 90% ethanol to obtain the product: 1.55 g (74.1%); mp 176–179° dec. A sample was dried at 100° for 18 hr: $[\alpha]^{17}D$ −29.3° (*c* 1.6, dimethylformamide).

Anal. Calcd for $C_{65}H_{94}O_{17}N_4S \cdot H_2O$: C, 56.0; H, 6.94; N, 14.1; S, 2.30. Found: C, 56.1; H, 6.98; N, 14.1; S, 2.24.

N-tert-Amyloxycarbonyl-*O-tert*-butyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-ω-*p*-nitrophenyl-L-α-aminosuberyl-L-prolyl-N^g-tosyl-L-arginylglycinamide (XXIV). Compound XXIII (1.39 g, 1 mmol as monohydrate) was allowed to react with *p*-nitrophenyl trifluoroacetate (1.18 g, 5 mmol) in a mixture of dimethylacetamide (10 ml) and pyridine (10 ml) at 50° for 3 hr. The product was precipitated by adding a large amount of ether, and the precipitate was collected and reprecipitated from dimethylformamide–ether: 1.41 g (92.2%); mp 171–176° dec. A sample was dried at 100° for 19 hr: $[\alpha]^{19}D$ −28.8° (*c* 1.5, dimethylformamide).

Anal. Calcd for $C_{71}H_{97}O_{19}N_{15}S \cdot H_2O$: C, 56.3; H, 6.59; N, 13.9; S, 2.14. Found: C, 56.3; H, 6.55; N, 13.9; S, 2.22.

Cyclic Lactam of L-Tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-L-α-aminosuberyl-L-prolyl-N^g-tosyl-L-arginylglycinamide (XXV). Compound XXIV (1.27 g, 0.85 mmol as monohydrate) was dissolved in trifluoroacetic acid (8 ml) for 1 hr. The reaction mixture was concentrated and triturated with ether to yield a precipitate which was collected, dried, and dissolved in dimethylacetamide (8.5 ml). The solution was added with stirring during a 4-hr period to pyridine (850 ml) and kept at 50°. After an additional hour of stirring at 50°, the pyridine was removed and the product was precipitated from the remaining solution with ether. The precipitate was collected, washed with ether, and dried. The dried powder (1.11 g) was dissolved in 100 ml of a mixture of methanol–water (2:1, v/v), and the solution was passed through columns of Amberlite IR-45 (OH^- form, 1 × 15 cm) and CM-Sephadex C-25 (H^+ form, 1.2 × 14 cm), successively. The columns were washed thoroughly with the same solvent mixture, and combined effluent and washings were concentrated. The remaining water was removed azeotropically with benzene and the resulting solid was collected with the aid of ether. The crude product (0.82 g) was dissolved in a small amount of 90% ethanol and precipitated by the addition of absolute ethanol, 601 mg (58.9%); this compound sintered at 150°. A sample was dried at 100° for 19 hr: $[\alpha]^{16}D$ −35.3° (*c* 1.1, dimethylformamide).

Anal. Calcd for $C_{55}H_{74}O_{14}N_{14}S \cdot 1.5H_2O$: C, 54.4; H, 6.39; N, 16.2; S, 2.64. Found: C, 54.2; H, 6.30; N, 16.2; S, 2.47.

[1,6-Aminosuberic acid]-8-arginine-vasopressin (XXVI). Compound XXV (200 mg, 0.16 mmol as 1.5 hydrate) and anisole (0.20 ml) were kept in anhydrous hydrogen fluoride (10 ml) at 0° for 1 hr. After removal of excess acid the residue was stored over NaOH under reduced pressure for 3 hr. Then the dried material was dissolved in water (20 ml) and the solution was immediately passed through a column of Amberlite IR-45 (acetate form, 1.2 × 17 cm), which was subsequently washed with water. The effluent and washings were combined and lyophilized to yield 188 mg of crude product, which was subjected twice to gel filtration on a Sephadex G-25 column (2 × 138 cm) with 1 *N* acetic acid. Flow rates were about 15 ml/hr. Fractions 55–65 of the second run were pooled and lyophilized to give the final product, 129 mg (63%). Upon paper chromatography this material possessed R_f values of 0.36 (S_2) and 0.19 (S_3) as detected with Pauly and phenanthrenequinone[56] spray reagents. A sample was dried at 100° for 20 hr: $[\alpha]^{27}D$ −66.0° (*c* 0.44, 0.1 *N* acetic acid). Loss of weight upon drying was 7.9%.

Anal. Calcd for $C_{48}H_{67}O_{12}N_{14} \cdot C_2H_4O_2 \cdot 2.5H_2O$: C, 52.8; H, 6.74; N, 17.2. Found: C, 52.9; H, 6.65; N, 17.2.

Amino acid analysis (6 *N* hydrochloric acid, 105°, 48 hr in the presence of a small amount of phenol) gave the following molecular ratios: Tyr, 0.98; Phe, 1.00; Glu, 1.02; Asp, 1.02; Asu, 0.96; Pro, 1.02; Arg, 1.00; Gly, 0.96.

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tert-Amyloxycarbonyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-ω-*tert*-butyl-L-α-aminosuberyl-L-prolyl-N^g-tosyl-L-arginylglycinamide (XXVII). Compound XXI (2.76 g, 2.5 mmol as monohydrate), decarbobenzoxylated by catalytic hydrogenolysis for 8 hr in the presence of 1 g of catalyst in a mixture of ethanol (50 ml) and water (10 ml), was allowed to react with compound V (1.03 g, 3 mmol) in dimethylformamide (7 ml). After 2 days an additional 0.34 g (1 mmol) of ester V and *N*-ethylmorpholine (0.32 ml) were added. Five days later ethyl acetate was added to precipitate the product. The precipitate was collected, dissolved in 50% ethanol, and reprecipitated with water: 1.95 g (66.3%); mp 175–179° dec. A sample was dried at 100° for 18 hr: $[\alpha]^{25}D$ −31.1° (*c* 2.0, dimethylformamide).

Anal. Calcd for $C_{58}H_{87}O_{15}N_{13}S \cdot H_2O$: C, 53.2; H, 7.50; N, 15.2; S, 2.68. Found: C, 52.8; H, 7.53; N, 15.3; S, 2.50.

N-tert-Amyloxycarbonyl-*O-tert*-butyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-L-α-aminosuberyl-L-prolyl-N^g-tosyl-L-arginylglycinamide (XXVIII). Compound XXVII (1.79 g, 1.5 mmol as monohydrate) was treated with trifluoroacetic acid, then allowed to react with IV and worked up as described for XXIII. Reprecipitation from 90% ethanol gave 1.52 g (75.6%) of the product: mp 202–204° dec. A sample was dried at 100° for 18 hr: $[\alpha]^{25}D$ −25.3° (*c* 1.1, dimethylformamide).

Anal. Calcd for $C_{62}H_{96}O_{17}N_{14}S \cdot H_2O$: C, 55.4; H, 7.35; N, 14.6; S, 2.39. Found: C, 55.0; H, 7.37; N, 14.5; S, 2.26.

N-tert-Amyloxycarbonyl-*O-tert*-butyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-ω-*p*-nitrophenyl-L-α-aminosuberyl-L-prolyl-N^g-tosyl-L-arginylglycinamide (XXIX). Compound XXVIII (1.34 g, 1 mmol as monohydrate) was treated as described for XXIV, giving: 1.39 g (93.9%); mp 202–204° dec. A sample was dried at 100° for 19 hr: $[\alpha]^{25}D$ −25.2° (*c* 1.1, dimethylformamide).

Anal. Calcd for $C_{68}H_{99}O_{19}N_{15}S \cdot H_2O$: C, 55.2; H, 6.87; N, 14.2; S, 2.17. Found: C, 55.0; H, 6.81; N, 14.3; S, 1.94.

Cyclic Lactam of L-Tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-L-α-aminosuberyl-L-prolyl-N^g-tosyl-L-arginylglycinamide (XXX). Compound XXIX (1.26 g, 0.86 mmol as monohydrate) was treated with trifluoroacetic acid and then the partially deprotected peptide was cyclized and worked up as described for XXV. The crude peptide (1.09 g) in a mixture of methanol–water (2:1, v/v) was passed successively through columns of Amberlite IR-45 (OH^- form, 1.8 × 6 cm) and of CM-Sephadex C-25 (H^+ form, 1.2 × 12 cm). The columns were washed thoroughly with the same solvent. The effluent and washings were combined and concentrated to give an oily residue which was lyophilized from acetic acid. The lyophilizate (0.73 g) was purified by reprecipitation from 95% ethanol and ether, 0.69 g (68.3%). A sample was dried at 100° for 19 hr: $[\alpha]^{25}D$ −27.4° (*c* 1.1, dimethylformamide). This compound sintered at 160°.

Anal. Calcd for $C_{52}H_{76}O_{14}N_{14}S \cdot H_2O$: C, 53.3; H, 6.71; N, 16.7; S, 2.73. Found: C, 53.3; H, 6.65; N, 16.4; S, 2.38.

[1,6-Aminosuberic acid]-8-arginine-vasotocin (XXXI). Compound XXX (200 mg, 0.17 mmol as monohydrate) and anisole (0.2 ml) were kept in anhydrous hydrogen fluoride (10 ml) at 0° and treated as described for XXVI. The crude product obtained (176 mg) was purified by gel filtration on Sephadex G-25 (2 × 138 cm) with 0.1 *N* acetic acid; the flow rate was 20 ml/hr. Fractions 54–60 were combined and lyophilized: 139 mg (69%). Upon paper chromatography this material possessed R_f values of 0.22 (S_2) and 0.13 (S_3) as detected with Pauly and phenanthrenequinone spray reagents. A sample was dried at 100° for 20 hr: $[\alpha]^{23}D$ −74.5° (*c* 0.47, 1 *N* acetic acid). Loss of weight upon drying was 7.4%.

Anal. Calcd for $C_{45}H_{70}O_{12}N_{14} \cdot C_2H_4O_2 \cdot 2H_2O$: C, 51.5; H, 7.18; N, 17.9. Found: C, 51.3; H, 6.81; N, 17.9.

Amino acid analysis (6 *N* hydrochloric acid, 105°, 48 hr in the presence of a small amount of phenol) gave the following molecular ratios: Tyr, 0.98; Ile plus Asu, 2.11; Glu, 1.00; Asp, 0.98; Pro, 1.02; Arg, 0.95; Gly, 1.00.

Bioassay Methods. For all standard bioassays the four-point design was used in which the neurohypophyseal extract obtained from the U. S. Pharmacopeia was the reference standard; this extract possesses 2.5 units/ml of oxytocic activity and 2.1 units/ml of rat pressor activity. A minimum of three animals was used for the quantification of the oxytocin-like activities and no less than six animals for the determination of the vasopressin-like activities. Determinations for oxytocic activities were performed on isolated uterine horns from rats in natural estrus, determined on the morning of the assay by vaginal smear. The method used was that of Holton[57] as modified by Munsick,[58] utilizing Mg^{2+}-free van Dyke-

Hastings solution as bathing fluid. Milk-ejecting activity was determined on urethane-anesthetized, lactating rabbits following the procedure of van Dyke, *et al.*,[59] as modified by Chan.[60] Avian vasodepressor assays were performed on conscious chickens according to the procedure of Munsick, *et al.*[61] The pressor properties of the polypeptides were determined on atropinized, urethane-anesthetized male rats following the procedures of the U. S. Pharmacopeia.[62] The ability of the polypeptides to enhance water transport (*i.e.*, to induce antidiuresis) was examined in Inactin- and ethanol-anesthetized, hydrated male Sprague-Dawley rats, according to the method of Jeffers, *et al.*,[63] as modified by Sawyer;[64] maximal depression of the rate of urine flow was taken as the effective response. Water transport across the toad urinary bladder was measured according to the method of Bentley[33] as modified by Eggena, *et al.*[34]

Lyophilization, Heat Treatment, Freezing, and Thawing of Asu Analogs. Samples of Asu analogs were prepared for lyophilization experiments in water (pH 6.0) and aqueous triethylamine (pH $\approx$ 9), and for heat treatment in water, as described by Yamanaka, *et al.*[12] After completion of the experiments the stock solutions and experimental samples were diluted identically and assayed for rat pressor activity[62] in the case of Asu-LVP and Asu-AVP and for avian vasodepressor assay[61] in the case of Asu-LVT and Asu-AVT. Another set of aliquots of Asu analogs dissolved in water was frozen in a bath at $-66°$ and thawed three times prior to bioassay; no loss of biological potency was detected. A four-point procedure was used to compare the activities of experimental and control samples. Three low doses of analog (one for each sample) were compared with three high doses at random.

Preparation of Toad Bladder Adenylate Cyclase Extract. Female toads, maintained on moist peat moss until sacrificed, were rapidly pithed and exsanguinated by perfusion through the heart (ventricle) with Ringer's solution (Na^+, 111.1; K^+, 3.5; Cl^-, 116.4; HCO_3^-, 2.4; and Ca^{2+}, 0.89 m*M*; total solute concentration 220 mOsm/kg of H_2O; pH in air 7.8–8.1). Both hemibladders were removed and rinsed with Ringer's solution at room temperature. The epithelial cell layer was scraped off the bladder in a Petri dish maintained on ice with the aid of two glass slides. All subsequent stages of preparation were performed at 0–4°. The cells were collected in 2 ml of 0.225 *M* sucrose containing 0.1 m*M* EGTA and 0.01 *M* Tris-HCl, pH 7.5, and centrifuged at 300 $\times$ *g* for 5 min (supernatant discarded). The packed cells were resuspended and again centrifuged as before. They were then homogenized in 2 ml of the same sucrose medium with a tight-fitting glass homogenizer and Teflon pestle and centrifuged at 600 $\times$ *g* for 10 min. The supernatant was discarded and the pellet (consisting mostly of broken cell membranes as revealed by a phase contrast microscope) was resuspended in 2 ml of the same medium and centrifuged once more at 600 $\times$ *g* for 10 min. The pellet was diluted to a protein concentration[65] of 1–2.5 mg/ml and quick-frozen in an acetone–Dry Ice mixture. The preparation was stored at $-70°$ for up to 4 hr prior to use. A different bladder preparation was used for each independent experiment.

Adenylate Cyclase Assays. Adenylate cyclase activity was assayed according to previously described methods[38,66] with the following modifications: (a) assays were performed at pH 8.0, the optimum pH for neurohypophyseal hormone stimulation;[67,68] (b) incubation periods were 20 min; and (c) the final protein concentration of the toad bladder adenylate cyclase preparation was 0.5–2.0 mg/ml of incubation mixture.

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Secretin. V.[1] Solvent Effects and Conformational Freedom

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Abstract: A comparison of the effects of solvents on the ORD spectra of secretin, glucagon, and lysozyme revealed different types of conformational freedom. In this respect, there is considerable similarity between secretin and several peptides corresponding to longer C-terminal sequences of its chain. The analogy in the solvent-induced conformational change suggests that, in water, the C-terminal part of the hormone is not helical.

Earlier studies of the ORD–CD spectra of the gastrointestinal hormone (porcine) secretin revealed the existence of a preferred conformation in its 27-membered peptide chain. The close similarity of these

His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Asp-Ser-Ala-Arg-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-NH_2
15 16 17 18 19 20 21 22 23 24 25 26 27
porcine secretin[2]

spectra with those of (hen egg white) lysozyme indicated the presence of a helical[3] portion in the molecule.

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