dihydro-3*H*-benz[*e*]indole (34). A mixture of **42** (1.3 g, 3.3 mmol), Ac_2O (1 ml, ~10 mmol), HOAc (10 ml), and *p*-toluenesulfonic acid (100 mg) was stirred at room temperature under N_2 for 6 days. The resulting solid (900 mg) was triturated with MeOH (50 ml) and filtered. Evaporation of the solvent in vacuo followed by crystallization of the residue from 50% EtOH afforded 250 mg (18%) of product, mp 223–226° dec.

3-(4-Acetoxy-3-carboxyphenyl)-2-phenyl-4,5-dihydro-3*H*-benz[*e*]indole (48). A solution of **42** (26.2 g, 69.2 mmol) in Ac_2O (204.2 g, 2 mol) was heated at 75° for 4 hr. After cooling to room temperature, water (34 g, 1.9 mol) was added and the mixture stirred overnight. Filtration afforded 26.7 g (91%) of pure product, mp 194–196°.

3-(3-Carboxy-4-hydroxyphenyl)-2-phenyl-3*H*-benz[*e*]indole (49). A solution of **42** (7.6 g, 20 mmol) in xylene (350 ml) was refluxed with 10% Pd/C (9.1 g) under N_2 for 24 hr. The catalyst was filtered and washed with 150 ml of hot xylene. The combined xylene filtrates were concentrated to 135 ml and cooled. Filtration and subsequent recrystallization from xylene afforded 3.6 g (48%) of yellow crystals, mp 241.5–242.5°.

1-(4-Hydroxy-3-methoxycarbonylbenzyl)-2-phenyl-4,5,-6,7-tetrahydroindole (52). This compound was prepared from 4-hydroxy-3-methoxycarbonylbenzylamine[26] and α-(2-oxocyclohexyl)acetophenone[3] using general procedure C in 58% yield, oil (NMR, ir).

1-(3-Carboxy-4-hydroxybenzyl)-2-phenyl-4,5,6,7-tetrahydroindole (53). A mixture of **52** (3.2 g, 8.8 mmol), KOH (2.5 g, 44 mmol), MeOH (35 ml), and water (10 ml) was refluxed for 2.5 hr. Dilution with water and acidification (HCl) yielded a crude solid, which on crystallization from MeOH–hexane afforded 1.9 g (63%) of pure product, mp 152.5–154°. Anal. ($C_{22}H_{21}NO_3$) C, H, N.

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Notes

Synthesis and Some Biological Activities of the Tyrosine-8 Analog of Substance P[1]

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[Tyr[8]]-substance P, an undecapeptide having the structure Arg-Pro-Lys-Pro-Gln-Gln-Phe-Tyr-Gly-Leu-Met-NH_2, has been synthesized by the solid-phase technique on a Beckman automatic peptide synthesizer, appropriately purified and biologically characterized. At twice the dosage, [Tyr[8]]-substance P showed the same biological activity response as synthetic substance P for stimulation of contraction of the isolated guinea pig ileum and for decrease in the systemic blood pressure of dogs. On the dog's blood pressure, no qualitative differences were observed, but on the isolated gut, the Tyr^8 analog gave a more gradual increase in the muscle tone than synthetic substance P. [Tyr[8]]-substance P released, in vitro, the luteinizing and follicle-stimulating hormones at a very high dosage but did not release growth hormone, prolactin, or thyrotropin.

The existence of the undecapeptide substance P, having the structure Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2, has been demonstrated in several mammalian tissues.[2-7] Recently, determinations with a radioimmunoassay technique have confirmed the presence of substance P-like material in tissue extracts from the

Table I. Effect of [Tyr^8]-substance P on LH and FSH Release

No.	Dose of [Tyr^8]-SP, I_3, I_4, ng/ml of medium	LH			FSH		
		Δ ng/ml of medium[a]	SEM	p value vs. 1	Δ ng/ml of medium[a]	SEM	p value vs. 1
1		−11	±27		613	±203	
2	1000	−13	±6	ns	666	±182	ns
3	10000	11	±17	ns	586	±218	ns
4	100000	68	±15	0.02	1333	±183	0.02

[a] Δ = mean of 12.

central nervous system.[8] However, the sensitivity of this radioimmunoassay did not permit detection of substance P at concentrations lower than two nanograms per milliliter. Consequently, in order to develop a more sensitive radioimmunoassay technique, we have synthesized an analog of substance P which has a tyrosyl residue substituted for the phenylalanyl residue at position eight to permit labeling with radioactive iodine. Although [Tyr^8]-substance P is known,[8] its synthesis and biological activities have not been published. Therefore, we now report our solid-phase synthesis and some biological activities of [Tyr^8]-substance P.

Synthesis. The tyrosine-8 analog of substance P was synthesized by the solid-phase technique[9,10] on a Beckman Model 990 peptide synthesizer. The benzhydrylamine (BHA) type resin,[11] originally described by Pietta and Marshall,[12] was used for the synthesis since this resin avoids difficulties with alkylation[13] encountered in esterifying methionine to chloromethylated resins.

N^{α}-*tert*-Butyloxycarbonyl (Boc) protection was used throughout the synthesis. Side-chain protecting groups were carbobenzoxy (Z) for lysine, tosyl (Tos) for arginine, and 2,6-dichlorobenzyl[14] (2,6-Cl_2-Bzl) for tyrosine. Deprotection of the amino-protected intermediates on the resin was accomplished with 50% trifluoroacetic acid (TFA) in methylene chloride, with neutralization of the resulting TFA salt by 10% triethylamine in methylene chloride to give the free amino group. A 2.5-fold excess of each *tert*-butyloxycarbonylamino acid was coupled in dicyclohexylcarbodiimide (DCC)–methylene chloride with an average coupling time of 6 hr. Boc-Gln, however, was incorporated as a fivefold excess of its ONP active ester in dimethylformamide with an 11-hr coupling time in the absence of DCC. Completeness of coupling was monitored by the ninhydrin color test procedure of Kaiser et al.,[15] and when necessary a double coupling was done (particularly in the case of Pro).

Cleavage of the peptide from the resin, with simultaneous removal of the protecting groups and formation of the carboxyl terminal amide, was effected with anhydrous, liquid hydrogen fluoride (HF) in the presence of 10% anisole for 1 hr at 0°.[16,17] The crude, deprotected peptide was purified by gel filtration and partition chromatography. Purity of the peptide was evaluated by amino acid analysis, thin-layer chromatography, and electrophoresis.

Biological Activity. [Tyr^8]-substance P was found to be very active on both dog's blood pressure and guinea pig's isolated intestine. In these studies, the activity of the Tyr^8 analog was compared with that of our original preparation of synthetic substance P.[18] [Tyr^8]-substance P stimulated contraction of the isolated guinea pig ileum in concentrations of tenths of a nanogram of peptide per milliliter of bath fluid. The potency, however, was half of that obtained with the original synthetic substance P, i.e., 2 ng of [Tyr^8]-substance P were equivalent to 1 ng of substance P (Figure 1).

Intravenous injection of [Tyr^8]-substance P in doses higher than 5 ng/kg of body weight elicited a transient decrease in the systemic arterial blood pressure of the dog lasting from 2 to 20 min depending on the dose. Again the potency of [Tyr^8]-substance P was about half that of the original synthetic substance P; i.e., in the dog, 10 ng/kg of body weight of [Tyr^8]-substance P gave an identical decrease in blood pressure as 5 ng/kg of body weight of substance P, e.g., from 140 to 110 mmHg. On the dog's blood pressure, no qualitative differences were observed, but on the isolated gut, [Tyr^8]-substance P gave a more gradual increase in the muscle tone than synthetic substance P.

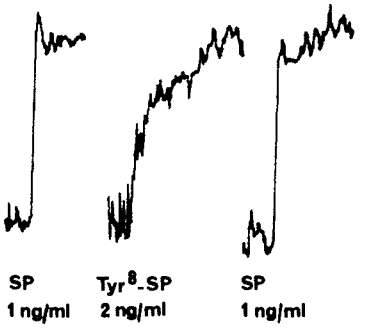

Figure 1. Effect of synthetic substance P (SP) and [Tyr^8]-substance P ([Tyr^8]-SP) on the isolated guinea pig ileum suspended in a 3-ml bath of oxygenated Tyrode's solution at 38°.

As shown in Table I, [Tyr^8]-substance P released LH and FSH in vitro, but only at a very high dosage, and had no antagonist activity on LH or FSH release. Large doses of the Tyr^8 analog (100 μg/ml) have no growth hormone, prolactin, or thyrotropin hormonal-releasing activities. These results are in agreement with those obtained for our original sample of synthetic substance P.

Discussion of Biological Activities and Mechanism of Action. It is expected that [Tyr^8]-substance P should have essentially the same biological activities as substance P since one aromatic amino acid, tyrosine, is substituted for another, phenylalanine. The powerful vasodilating action is probably due to a direct effect on the cells of smooth muscle in the vessel wall, and the effective nanogram dosage is impressive. Picogram levels of the synthetic [Tyr^8]-substance P were sufficient to stimulate contraction of the isolated guinea pig ileum. For each of these activities, however, the [Tyr^8]-substance P was only half as potent as synthetic substance P.

Of the eight different amino acids in the undecapeptide sequence of substance P, Arg^1, Lys^3, Phe^7, and Phe^8 appear to be the four crucial amino acids, based on current interpretations of sequence–conformation–activity relationships[19-22] of the two hypothalamic releasing hormones TRH and LH-RH. The Arg^1 and Lys^3 moieties, in protonated form, could be very important for activity and potency because they contribute an ionic function to the molecule. Since the [Tyr^8]-substance P analog retains these moieties similar biological activity to that of sub-

stance P would be expected.

The Phe^7-Phe^8 moiety probably contributes unique structural specificity to the entire molecule because of possible π–π bond interactions with a receptor site. In the Tyr^8 analog, the phenylalanine at position 8 is replaced by tyrosine, an aromatic amino acid which contains a *p*-hydroxy group on the phenyl ring of the side chain. The electronic and inductive influences of the *p*-hydroxy group of tyrosine could possibly influence the π–π bond interaction of the peptide with a receptor site, and/or it may slightly alter the conformation of the peptide molecule, thus accounting for the lower potency of [Tyr^8]-substance P compared with that of substance P.

The fact that [Tyr^8]-substance P shows low activities to release LH and FSH is surprising but consistent with the activity found for synthetic substance P.[18] The Arg^1 moiety, the aromatic Phe^7-Phe^8 moiety, and possibly some conformational aspects of this undecapeptide might give it slight acceptability at the receptor site for the decapeptide, LH-RH, when LH is released.

The successful use of the Tyr^8 analog of substance P in a radioimmunoassay technique for detection of substance P will be reported in a subsequent paper.[23]

Experimental Section

Synthesis. Benzhydrylamine resin hydrochloride (2.0 g, 0.53 mequiv of NH_2/g, from Beckman) was neutralized by stirring for 10 min with 50 ml of 25% Et_3N–CH_2Cl_2. The resin was filtered, washed with CH_2Cl_2, and dried. The resin was then placed in the reaction vessel of the 990 synthesizer, swelled with CH_2Cl_2, and coupled with a 2.5-fold excess of Boc-Met in DCC–CH_2Cl_2 for 10 hr. The remaining unreacted amino groups on the resin were acetylated with a mixture of Ac_2O and Et_3N in DMF. Amino acid analysis of the resin, after hydrolysis in 6 *N* HCl–propionic acid (1:1) overnight at 110°, gave a value of 0.31 m*M*/g for Met.

The remaining ten amino acids were coupled to the Boc-Met-BHA resin according to the following procedure: (1) three washings with CH_2Cl_2; (2) prewash with 50% TFA–CH_2Cl_2, followed by a 30-min treatment with the same TFA solution; (3) six washings with CH_2Cl_2; (4) two prewashes with 10% Et_3N–CH_2Cl_2, followed by a 15-min treatment with the same Et_3N solution; (5) five washes with CH_2Cl_2; (6) delivery of the *tert*-butyloxycarbonylamino acid in CH_2Cl_2 (DMF for Gln and Arg), followed by a brief stirring period; (7) delivery of a 2.5-fold excess of 10% DCC–CH_2Cl_2 solution, followed by stirring of the reaction mixture for the duration of the coupling time; (8) two washes with CH_2Cl_2; (9) four washes with DMF; (10) four washes with CH_2Cl_2. In the case of coupling Boc-Gln-ONP active esters, step 7 was eliminated. After carrying out ten successive deprotection, neutralization, and coupling cycles with the remaining amino acids, the final protected peptide-resin weighed 3.38 g.

The protected peptide-resin was treated with 35 ml of CoF_3-dried HF, in the presence of 3.5 ml of anisole, for 60 min at 0°.[16,17] After removal of excess HF in vacuo, the resin was washed with 50 ml of EtOAc and 25 ml of Et_2O to remove anisole, followed by 150 ml of 0.2 *N* HOAc to extract the peptide, and filtered. The crude, lyophilized product (a sticky, yellow semisolid) was subjected to gel filtration on a 102 × 2.5 cm column of Bio-Gel P-2 eluted with 0.2 *N* HOAc, with detection of the peptide by uv at 260 nm. Lyophilization of the fractions corresponding to the major peptide peak gave 591 mg of yellow solid. This material was subjected to partition chromatography on an 85 × 1.5 cm column of Sephadex G-25 eluted with upper phase of the system 0.1% HOAc–*n*-BuOH–Pyr (11:5:3) with detection of the peptide by the Folin–Lowry procedure[24] at 700 nm. Lyophilization of the fractions corresponding to the desired peptide peak gave 149.2 mg (19.9%) of [Tyr^8]-substance P.

Amino acid analysis of the product, after hydrolysis with 6 *N* HCl containing 4% thioglycolic acid overnight at 110°, gave the following ratios: Arg, 0.96; 2Pro, 2.00; Lys, 1.02; 2Glu, 2.02; Phe, 1.02; Tyr, 0.98; Gly, 0.98; Leu, 1.05; Met, 0.97; $3NH_3$, 2.92. The peptide showed a specific optical rotation of $[\alpha]^{24}D$ –53.8° (*c* 0.92, MeOH). TLC methods on silica gel plates showed one spot with the following R_f's in the given systems, detected by uv, ninhydrin, and chlorotolidine: R_f 0.90, EtOAc–Pyr–HOAc–H_2O (5:5:1:3); R_f 0.13, $CHCl_3$–MeOH–concentrated NH_4OH (60:45:20); R_f 0.00, EtOH–H_2O (7:3); R_f 0.09, *n*-BuOH–HOAc–EtOAc–H_2O (1:1:1:1); R_f 0.05, *n*-PrOH–1 *N* HOAc (2:1). The peptide showed one spot moving toward the cathode on electrophoresis on a thin-layer cellulose plate in pH 6.4 buffer of Pyr–HOAc–H_2O (10:0.4:90).

Biological Activities. A section of guinea pig ileum was suspended in a 3-ml bath of the standard oxygenated Tyrode's solution at 38°. The effect of the peptide on the isolated intestinal tissue was measured upon addition of tenths of a nanogram of peptide per milliliter of bath fluid.

Analyses of the circulatory effect of the peptides were performed on dogs anesthetized with pentobarbital. The arterial blood pressure was recorded through a catheter inserted in a carotid artery connected to a Statham pressure transducer.

Pituitary hormonal releasing activities were obtained from in vitro studies on pituitaries of 20-day-old female Sprague–Dawley rats (Charles River Laboratory). Two pituitaries were incubated for a total of 6 hr at 37° in 1 ml of lactated Ringer's solution (Travenol Laboratories) in 10-ml Teflon beakers in a Dubnoff shaker. Medium was removed each hour for radioimmunoassay[25] LH, FSH, GH, PRL, and TSH and then fresh medium was added. After two preincubation periods (P_1, P_2), the synthetic peptide was added to the incubation medium (I_3, I_4, I_5, I_6) and LH-RH was added at I_5 and I_6. Agonist activity was determined from the hormonal release at I_3 and I_4 and antagonist or synergistic activity from I_5 and I_6. The RIA reagents for FSH, PRL, GH, and TSH were distributed by NIAMDD, NIH. The values for these assays are calculated in terms of nanograms of the following standards: LH-LER-1240-2 (0.60 NIH-LH-SI units/mg), FSH (2.1 NIH-FSH-SI units/mg), GH (0.61 units/mg), PRL (111 units/mg), and TSH [0.22 USP (bovine) units/mg].

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Synthetic Metabolites of Neurohypophyseal Hormones. [Des-9-glycinamide]oxytocin and [Des-9-glycinamide,des-8-leucine]oxytocin[1]

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Syntheses and biological properties are reported for two analogs of oxytocin in which the glycinamide and the leucylglycinamide moiety, respectively, have been deleted from the parent hormone. Both [des-9-glycinamide]oxytocin and [des-9-glycinamide,des-8-leucine]oxytocin are weak agonists in the rat uterotonic and antidiuretic assays but possess no detectable rat pressor activity. In addition, [des-9-glycinamide]oxytocin is an inhibitor of the oxytocin-induced vasodepressor response in fowl but is a potent agonist in the hydroosmotic assay of the toad urinary bladder.

It has been suggested that the milk-ejecting hormone, oxytocin,[2a] can serve as precursor or prohormone for the enzymatic generation in vivo of biologically active fragments.[2b-4] There is an ever-increasing number of peptides both synthetic and isolated from natural sources which comprise N-terminal and C-terminal sequences of oxytocin and vasopressin and which exhibit endocrine and extra-endocrine effects clearly distinct from those of the neurohypophyseal hormones.[5] The isolation from natural sources of peptides is compatible with results obtained from studies of the metabolism of neurohypophyseal hormones by proteolytic enzymes present in tissue extracts, body fluids, and intact animals. These studies have shown that the peptide bonds between residues 1 and 2 (Cys-Tyr) and 7 and 8 (Pro-Leu, Arg, or Lys) as well as 8 and 9 (Leu, Arg, or Lys-Gly) are the initial sites for hydrolysis of the hormones (for a recent summary see ref 6).

In light of these results it became important to obtain authentic samples of analogs of oxytocin in which the glycinamide and the leucylglycinamide moieties, respectively, have been deleted. In this paper we describe the synthesis of H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-OH (dG-OXY) and H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-OH (dLG-OXY) along with some of their potencies for those biological activities which are characteristic of oxytocin. For the synthesis of dG-OXY, Z-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys (Bzl)-Pro-Leu-resin (I) was obtained by the general solid-phase procedure of Merrifield[7] as applied to the synthesis of vasopressin[8] with further modifications noted in the Experimental Section. Removal of the peptide from the resin and deblocking of the N-CBz and O-Bzl groups were achieved by acidolysis with HBr–F_3CCO_2H to give the hydrobromide of the S-benzylated octapeptide. Debenzylation with Na in boiling NH_3[9] followed by aqueous $K_3Fe(CN)_6$[10] oxidation of the intermediary dithiol to the disulfide yielded crude dG-OXY, which was purified by partition chromatography.[11]

Similarly, dLG-OXY was prepared in a stepwise manner through the protected heptapeptide resin, Z-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro-resin (II). Treatment of II with HBr–F_3CCO_2H produced the dibenzyl heptapeptide hydrobromide which upon conversion to the disulfide and partition chromatography gave homogenous dLG-OXY.

Table I. Comparison of Some Biological Activities of [Des-Gly-NH_2[9]]oxytocin and [Des-Gly-NH_2[9],des-Leu[8]]oxytocin with Those of Oxytocin[a]

	Uterotonic (rat)	Vasodepressor (fowl)	Pressor (rat)	Antidiuretic (rat)	Water flux[b] (toad)
Oxytocin[c]	546[d]	507	3.1	2.1	2.3×10^{-9}
dG-OXY	2.4 ± 0.07	Inhibition	Negl	0.08 ± 0.003	4.0×10^{-9}
dLG-OXY	2.7 ± 0.5	0.10 ± 0.03	Negl	0.06 ± 0.006	

[a] Expressed in USP units/mg ± SEM unless otherwise noted. [b] Molar concentration required for obtaining half-maximal response; data were normalized to the value of crystalline deamino-oxytocin, which also served as standard as described by Eggena et al.[29] [c] Values reported by W. Y. Chan and V. du Vigneaud, *Endocrinology*, 71, 977 (1962). [d] Value reported by R. L. Huguenin and R. A. Boissonnas, *Helv. Chim. Acta*, 49, 695 (1966).

The two analogs of oxytocin have been assayed for biological activities characteristic of neurohypophyseal hormones and some of these data are summarized in Table I. Both dG-OXY and dLG-OXY are weak agonists in the in vitro rat uterotonic assay and the rat antidiuretic assay, and they show no activity either as agonists or antagonists in the rat pressor assay at single doses of as high as 1 mg. In contrast, much of the hydroosmotic potency determined in the toad urinary bladder assay is retained upon deletion of the glycinamide moiety from oxytocin. The fact that dG-OXY retains such a high degree of the activity which oxytocin exhibits in this amphibian system is reminiscent of the high natriferic activity in the frog skin[12] retained by [des-Gly[9]]oxytocin (an analog of oxytocin in which the glycine residue has been omitted, but the terminal carboxamide group was kept).[13] The above results are in line