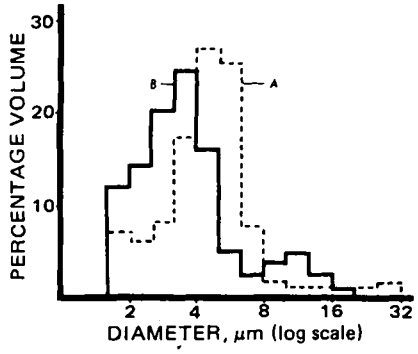

Figure 5—*Crystal size distribution of calcium oxalate dihydrate (A) and calcium oxalate monohydrate (B) used for the dissolution rate studies.*

[determined by automated particle counter[12] (Fig. 5)]. Table II summarizes the structure-dependent properties of calcium oxalate monohydrate and dihydrate. The dihydrate has a higher solubility and dissolution rate and a lower zeta potential than the monohydrate. The dihydrate crystals are stable in air at 4° for 2 weeks; however, when these crystals are kept in normal saline at 37°, they are stable for only 24 hr. After 24 hr, gradual conversion to monohydrate occurs.

The observation that calcium oxalate dihydrate has a higher solubility, higher dissolution rate, and undergoes gradual dissolution–recrystallization to calcium oxalate monohydrate is consistent with recent results reported on the crystallization of calcium oxalate in the rat kidney (8). It offers the most probable explanation for the gradual transformation of calcium oxalate dihydrate to calcium oxalate monohydrate in rat kidney following the administration of 4-hydroxy-L-proline. Detailed study of the difference in the zeta potential between the monohydrate and the dihydrate could lead to a better understanding of the role of crystal aggregation in stone formation.

[12] Coulter Counter model TA II.

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ACKNOWLEDGMENTS

Presented to the APhA Academy of Pharmaceutical Sciences, Washington, D.C., April 1980.

Supported by the Medical Research Council of Canada.

The authors thank Mr. M. Cousineau for his technical assistance.

Potential Thyroliberin Affinity Labels II: Chloroacetyl Substituted Phenylalanyl Prolineamides

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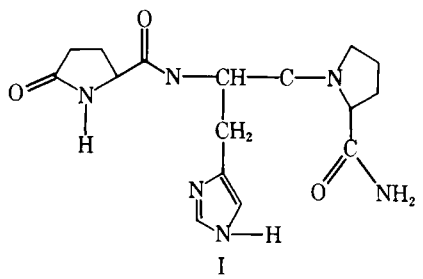
Received August 3, 1981, from the **Department of Medicinal Chemistry, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60680*, and the ‡*Department of Medicine, School of Medicine, Tulane University, New Orleans, LA 70112.* Accepted for publication December 9, 1981.

Abstract □ Three analogs of thyroliberin (I) were prepared. These compounds, *N-m*-chloroacetylbenzoyl-phenylalanyl-prolineamide (VI*a*), *N-p*-chloroacetylbenzoyl-phenylalanyl-prolineamide (VI*b*) and *N*-chloroacetyl-alanyl-phenylalanyl-prolineamide (IX), were designed as potential I antagonist affinity labels. However, no significant antagonist activity was observed. Compounds VI*a* and IX were found to have weak agonist activity. Cyclo (Phe-Pro) an analog of the I metabolite, cyclo (His-Pro), was found, however, to have significant I antagonist activity, but no agonist activity.

Keyphrases □ Thyroliberin—potential affinity labels, chloroacetyl substituted phenylalanyl prolineamides □ Hormones—peptide, thyroliberin affinity labels, chloroacetyl substituted phenylalanyl prolineamides □ Receptor-hormone interactions—affinity label analogs, characterization, thyroliberin

Thyroliberin (I) is a peptide hormone from the hypothalamus that can release thyrotropin (1) and prolactin (2) from the anterior pituitary both *in vivo* and *in vitro*. Affinity label analogs of I that irreversibly bind to the I receptor could aid in the characterization of the I receptor and the study of receptor–hormone interactions. The use of a naltrexamine affinity label to isolate a complex with an opiate receptor, which was recently reported (3) while these studies were in progress, is a similar approach.



I

Initial attempts to develop analogs of I as potential af-

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finity labels were based on previously reported structure–activity relationships and were expected to result in compounds that were antagonists of I. These compounds, with general structure *N*-chloroacetyl [ ]Phe-Pyrr, were found, however, to be only weak agonists (4).

In an effort to improve receptor binding affinity, the prolineamide moiety was included in the present compounds. The resulting analogs were expected to have improved agonist potency based on previously reported structure–activity relationships (5). Therefore, compounds of general structure, *N*-chloroacetyl[ ]Phe-Pro-NH_2, were synthesized. Cyclo (Phe-Pro) was also synthesized as an analog of cyclo (His-Pro), a metabolite of I (6, 7).

EXPERIMENTAL

Melting points[1] reported are uncorrected. All amino acid derivatives used were of the L configuration. Thin-layer chromatography was performed on 250 μm silica gel G plates with fluorescent indicator[2]. The following solvent systems were used: (A) chloroform–methanol (39:1); (B) chloroform–methanol (19:1); (C) chloroform–methanol–concentrated ammonium hydroxide (18:1:1), lower phase; (D) chloroform–methanol–concentrated ammonium hydroxide (17:2:1), lower phase. Methods of detection are indicated with the data. Proton magnetic resonance[3] spectra were recorded at 60 MHz in the solvent indicated for each compound and the data are reported in parts per million (δ) downfield from internal tetramethylsilane. Optical rotations were determined with a digital readout polarimeter[4], and the solvent used is indicated with the data. Elemental analyses are reported for new compounds[5].

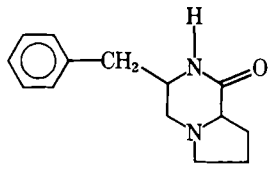
L-Phe-L-Pro-NH_2 Hydrochloride (III)—*N*-Carbobenzoxy-L-Phe (1.5 g, 5.02 mmoles) was dissolved in dry ethyl acetate (60 ml) with stirring and cooled to −40°. *N*-methylmorpholine (0.61 ml, 5.55 mmoles) was added followed immediately with isobutyl chloroformate (8) (0.62 ml, 4.78 mmoles). After 8 min, L-Pro-NH_2 hydrochloride (0.72 g, 4.78 mmoles) was added followed by additional *N*-methylmorpholine (0.53 ml). The reaction mixture was allowed to warm to room temperature and stirred overnight. The mixture was washed with 5% $NaHCO_3$ (2 × 25 ml), 1 *N* HCl (2 × 25 ml), water (2 × 25 ml), dried over anhydrous magnesium sulfate, and evaporated to dryness (3.95 g).

Chromatographic purification on a silica gel column (90 g, 2.5 × 51.5 cm) and elution with chloroform (450 ml) and 5% methanol in chloroform (600 ml) yielded 3.05 g of *N*-carbobenzoxy-L-Phe-L-Pro-NH_2 (II) as a sticky foam from fractions (4 ml) 205 through 230; one spot on TLC, Solvent B, UV and chlorine–tolidine positive, ninhydrin negative; with a confirming PMR spectrum.

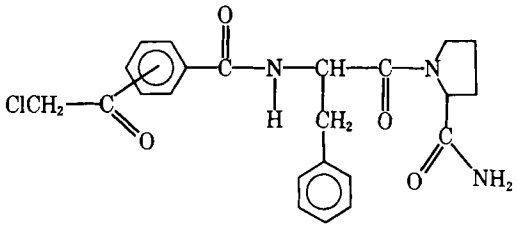
A solution of II (3.05 g) in methanol (55 ml) containing gaseous hydrogen chloride (0.16 g) was hydrogenolyzed for 1 hr at 10 psi and room temperature over 5% palladium on charcoal. Removal of the catalyst and concentration *in vacuo* provided III as a hygroscopic solid which could be used directly in subsequent reactions. Further purification was not possible due to conversion into cyclo (L-Phe-L-Pro) (IV) during crystallization attempts. The PMR spectrum confirmed the assigned structure; one spot on TLC, Solvent D, UV, ninhydrin and chlorine–tolidine positive.

Cyclo (L-Phe-L-Pro) (IV)—Hydrogenolysis of II (2.75 g) in methanol (20 ml) and glacial acetic acid (0.5 ml) at 10 psi over 5% palladium on charcoal for 2 hr, removal of the catalyst, and evaporation to dryness gave a residue. This residue was refluxed for 1 hr in benzene (20 ml), filtered hot, and crystals of IV obtained upon addition of petroleum ether, 0.28 g, mp 126–128° [lit. (9) mp 125–127°], one spot on TLC, Solvent A, UV and iodine positive, ninhydrin negative.

3-Chloroacetylbenzoyl-L-Phe-L-Pro-NH_2 (VI*a*)—To a solution of III (0.40 g, 1.34 mmoles), in dry dimethylformamide (4 ml), methylene chloride (6 ml), and *N*-methylmorpholine (0.14 ml), was added 0.21 g of 3-chloroacetylbenzoyl chloride (V*a*) (4) in methylene chloride (4 ml) followed by additional *N*-methylmorpholine (0.11 ml). The reaction mixture was stirred for 3 hr at room temperature.



IV



VI*a*: = *m*-$ClCH_2CO$—
VI*b*: = *p*-$ClCH_2CO$—

Methylene chloride (25 ml) was added, and the mixture was washed with 5% $NaHCO_3$ (35 ml), 1 *N* HCl (25 ml), and water (25 ml). The organic layer was dried over magnesium sulfate, evaporated to dryness, and crystalline VI*a* obtained from ethyl acetate–petroleum ether; yield 0.11 g (25%); mp 152–154°; one spot on TLC, Solvent B, UV and chlorine–tolidine positive, ninhydrin negative; $[\alpha]_D^{28} = -52.4°$ (*C* = 0.867, chloroform). The NMR spectrum supported the assigned structure.

Anal.—Calc. for $C_{23}H_{24}ClN_3O_4$: C, 62.51; H, 5.47; Cl, 8.02; N, 9.51. Found: C, 62.49; H, 5.54; Cl, 8.22; N, 9.44.

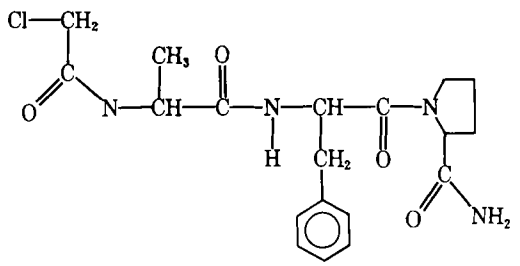
4-Chloroacetylbenzoyl-L-Phe-L-Pro-NH_2 (VI*b*)—A solution of III (0.40 g, 1.34 mmoles), in dimethylformamide (4 ml), methylene chloride (6 ml), and *N*-methylmorpholine (0.14 ml), was treated with 0.25 g of 4-chloroacetylbenzoyl chloride (V*b*) (4) in methylene chloride (4 ml) followed by additional *N*-methylmorpholine (0.11 ml). The reaction was stirred for 3 hr at room temperature. Workup was similar to that for VI*a*.

Crude VI*b* (0.44 g) required column purification on a chloroform packed silica gel column (35 g, 2.5 × 28 cm) eluted with chloroform (200 ml), 30% ethyl acetate in chloroform (200 ml), 60% ethyl acetate in chloroform (200 ml), and ethyl acetate (600 ml). Fractions (4 ml) 235 through 285 contained VI*b* which was crystallized from ethyl acetate–petroleum ether. Purified VI*b* crystallized with 0.5 mole of ethyl acetate, which was confirmed in the PMR spectrum; yield 0.17 g (35%); mp 103–107°; one spot on TLC, Solvent B, UV and chlorine–tolidine positive, ninhydrin negative; $[\alpha]_D^{28} = -32.4°$ (*C* = 1.125, chloroform).

Anal.—Calc. for $C_{23}H_{24}ClN_3O_4 \cdot 0.5\ C_4H_8O_2$: C, 61.79; H, 5.81; Cl, 7.29; N, 8.65. Found: C, 61.77; H, 5.72; Cl, 7.11; N, 8.74.

***N*-Chloroacetyl-L-Ala-L-Phe-L-Pro-NH_2 (IX)**—A solution of *N*-carbobenzoxy-L-Ala (0.95 g, 4.26 mmoles) in dry tetrahydrofuran (80 ml) was cooled to −40°, *N*-methylmorpholine (0.52 ml, 4.73 mmoles) was added, followed by isobutyl chloroformate (0.53 ml, 4.09 mmoles). After 15 sec, III (1.25 g, 4.20 mmoles) was added followed by additional *N*-methylmorpholine (0.46 ml, 4.18 mmoles). The reaction was warmed to room temperature, stirred overnight, and then evaporated to dryness.

The residue was dissolved in methylene chloride (100 ml) and washed with bicarbonate, acid, and water similar to the procedure used for II. The organic layer was dried over magnesium sulfate and taken to dryness. The residue was applied to a chloroform packed silica gel column (35 g, 28 × 2.5 cm) which was eluted with chloroform (250 ml), 1% methanol in chloroform (300 ml), and 1.5% methanol in chloroform (400 ml). The desired product was collected in fractions (4 ml) 167 to 202 that were concentrated to a residue (0.80 g), one spot on TLC, Solvent B, UV and



IX

[1] Determined on a Mel-Temp block; values are uncorrected.
[2] Obtained from E. Merck.
[3] Determined with a Varian Associates T-60A instrument equipped with a Nicolet Instrument Corp. TT-7 Fourier Transform Accessory.
[4] Determined with a Perkin-Elmer model 241.
[5] Performed by Micro-Tech Laboratories, Skokie, Ill.

Table I—Relative Agonist Activity

Peptide	Relative Activity, %
I	100[a]
IV	None[b]
VI*a*	0.0006
VI*b*	None[b]
IX	0.0002

[a] Compound I dose of 0.6 ng. [b] At a dose up to 30 μg.

chlorine–tolidine positive, ninhydrin negative. The PMR spectrum indicated the expected product, *N*-carbobenzoxy-Ala-Phe-Pro-NH_2 (VII) which could be used directly in the next step.

A solution of VII (1.04 g) in methanol (40 ml) containing gaseous hydrogen chloride (0.10 g) and 5% palladium on charcoal (0.35 g) was hydrogenolyzed for 1 hr at 10 psi and room temperature. Removal of the catalyst by filtration, concentration *in vacuo*, and coevaporation with benzene gave Ala-Phe-Pro-NH_2 hydrochloride (VIII) (0.82 g) as a glass which gave the appropriate PMR spectrum, and was one spot on TLC, Solvent C, UV, chlorine–tolidine, and ninhydrin positive.

Chloroacetic anhydride (0.20 g, 1.17 nmoles) was added to a solution of VIII (0.47 g, 1.27 mmoles) in dimethylformamide (3 ml) and methylene chloride (7 ml) followed by *N*-methylmorpholine (0.14 ml, 1.27 mmoles). The reaction was stirred for 6 hr, methylene chloride (10 ml) was added, and the mixture was washed with 5% $NaHCO_3$ (2 × 10 ml), 1 *N* HCl (2 × 10 ml), and water (2 × 10 ml). The organic layer was dried over magnesium sulfate and concentrated to dryness to yield 0.27 g of IX.

The crude product was applied to a silica gel column (35 g, 28 × 2.5 cm) packed in chloroform and eluted with chloroform (200 ml), 10% acetone in chloroform (200 ml), 20% acetone in chloroform (200 ml), and acetone (200 ml). The desired product was collected in fractions (4 ml) 265 to 280. Pure IX was obtained by crystallization from ethyl acetate–petroleum ether; yield 0.02 g (4%); one spot on TLC, Solvent B, UV and chlorine–tolidine positive, ninhydrin negative; mp 198–202°; $[\alpha]_D^{28} = -68.0°$ (C = 0.883, chloroform); PMR spectrum in deuterochloroform: δ 1.31 [d, $J \simeq$ 7 Hz, 3H(β—CH_3 of Ala)], 1.75 [m, 2H(δ—CH_2 of Pro—NH_2)], 2.06 [m, 4H(β and γ—CH_2 of Pro—NH_2)], 3.00 [m, 2H(β—CH_2 of Phe)], 4.01 [s, 2H (—$COCH_2Cl$)], 4.53–5.25 [m, 3H(overlapping α—CH)], 7.23 [s, 5H (aromatic H of Phe)].

Anal.—Calc. for $C_{19}H_{25}ClN_4O_4$: C, 55.81; H, 6.16; Cl, 8.67; N, 13.71. Found: C, 55.52; H, 6.17; Cl, 8.62; N, 13.43.

Biological Assay—The initial evaluation of the thyroliberin agonist and antagonist activity of these compounds was by the *in vitro* method of Bowers *et al.* (10). Freshly excised whole pituitaries from 20-day-old female rats were incubated in Krebs–Ringer bicarbonate for six 1-hr periods. The incubation medium was replaced after each hour. Test samples were added during the third through the sixth hours (I_3–I_6), and the resultant thyrotropin concentration in the incubation media was measured by radioimmunoassay and compared with the second hour (P_2). Agonist activity was measured by the effects of the analogs alone on the pituitaries. Antagonist activity was measured by the net effect of the analog in combination with synthetic thyroliberin. The bioassay results are summarized in Table I.

RESULTS AND DISCUSSION

As shown in Table I, the agonist potency is weak and is on the same order as that reported previously (4) for the pyrrolidine analogs. The incorporation of prolineamide into thyroliberin analogs has usually resulted in a significant increase in the agonist potency of the resulting peptides relative to the corresponding pyrrolidine derivatives. However, this was not the case for the reported analogs.

The case of IX is striking. Chloroacetylalanyl can be considered to be an open ring analog of pyroglutamyl. Thus, there is only a minor change in structure from the relatively potent analog, *p*-Glu-Phe-Pro-NH_2 (11). Compound IX, however, had only 0.0002% of the agonist potency of thyroliberin. No significant antagonist activity was found for VI*a*, VI*b*, or IX.

The synthesis of IV has been previously reported (9) in conjunction with some spectroscopic studies. The evaluation of the endocrine activity of IV was of interest because of the importance of cyclo (His-Pro) as a metabolite (6, 7) of thyroliberin and the observation that cyclo (His-Pro) could inhibit the thyroliberin-stimulated release of prolactin both *in vitro* (12, 13) and *in vivo* (12). An uncharacterized pineal substance that can inhibit thyroliberin-stimulated thyrotropin release has also been recently reported (14).

Cyclo (Phe-Pro) had no significant agonist activity up to a 30-μg dose; however, antagonist activity was apparent. Thyroliberin (0.6 ng) alone caused the release of 17.3 μg of thyrotropin. In the presence of IV (3 μg and 30 μg), the thyrotropin release was 11.0 μg ($p < 0.01$) and 13.1 μg ($p < 0.05$), respectively. This is of interest because cyclo (His-Pro) has not been reported to have thyroliberin antagonist activity.

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ACKNOWLEDGMENTS

Presented in part at the 180th National Meeting of the American Chemical Society, Las Vegas, Nev., August 24–29, 1980, abstract MEDI 47.

This work forms part of a thesis submitted by R. J. Goebel to the University of Illinois at the Medical Center in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The authors thank Ms. George Ann Reynolds for her expert technical assistance in the performance of the bioassays and Ms. Marion Roberts for her assistance in the preparation of this manuscript.