Synthesis, Characterization, and Anorectic Testing of the Four Stereoisomers of Cyclo(histidylproline)

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All four possible stereoisomers of cyclo(histidylproline) were individually synthesized, purified, and characterized. They were each tested for anoretic activity in rats with a free feeding paradigm over 24 h. Contrary to literature reports, none significantly reduced food intake at any time over the test period.

In recent years there has been an increasing awareness of the role of peptide transmitters and regulators in biological systems. One such compound is thyrotropin releasing hormone (TRH). Originally isolated from the hypothalamus,[1,2] it was later found in other extrahypothalamic tissues and is involved in several diverse biological processes.[3,4] The mechanism by which TRH produces its broad range of activities is unknown. It might involve the action of TRH at multiple recognition sites, or TRH might serve as a precursor for other compounds that are biologically active. Metabolic studies of TRH have yielded two major materials, pyroglutamylhistidylproline (1) and a cyclic dipeptide, cyclo(histidylproline) (2).[5,6] Although no biological effects have been attributed to the former, cyclo(His-Pro) (2) has been shown to have a number of actions[7] including inhibition of prolactin release from the pituitary,[8] antagonism of ethanol sedation,[6] inhibition of abstinence syndrome in opiate-dependent mice,[9] and production of hypothermia.[10]

Our interest in 2 was aroused with a recent report that cyclo(His-Pro) decreases food intake.[11] An intracerebroventricular (icv) dose of 10^{-8} mol was effective in suppression of stress-induced eating, starvation-induced eating, and spontaneous eating over a 10-h period.[12]

We considered making compounds analogous to 2 as potential antiobesity agents but chose to first verify its reported activity. A sample of **2a** was obtained commercially[13] and tested for its ability to inhibit the free-feeding behavior of rats over a 24-h period. No statistically significant decrease in food intake was discerned vs. saline controls at any hourly interval following icv doses of up to 140 nmol (see Biology).

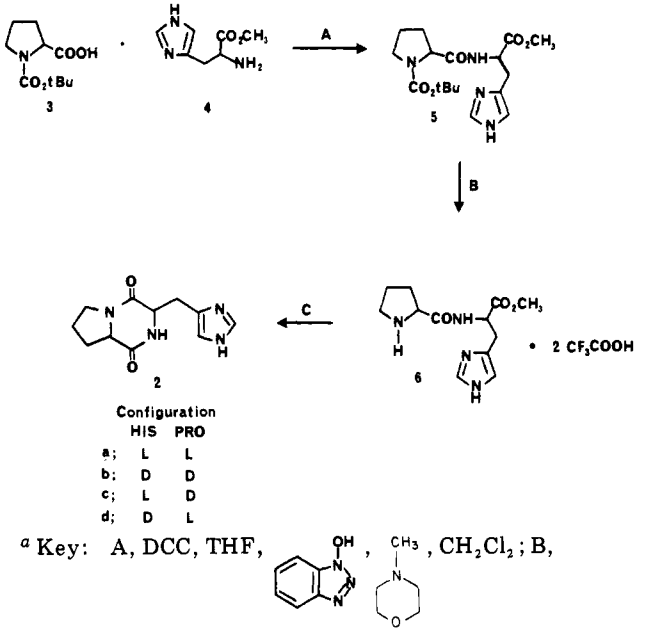
Although there have been numerous references to 2 in the literature since its isolation,[5] only vague details have been reported about its synthesis and chemical characterization.[6,8,14,15] Furthermore, because the diketopiperazine 2 has two optical centers, a pair of diastereomers or four distinct optical isomers are possible. Consequently, when we were unable to reproduce the reported anorectic activity of 2 in a spontaneous feeding paradigm, we could not identify any possible chemical difference between synthetic material reported in the literature and that which we obtained for testing.

Thus, we have synthesized and fully characterized each of the four possible isomers (**2a–d**) of cyclo(His-Pro) and subsequently tested each for anoretic activity (Biology section).

Chemistry

All of the isomers **2a–d** were individually synthesized by the three-step sequence pictured in Scheme I, beginning with the appropriate enantiomers of *N*-(*tert*-butyloxycarbonyl)proline (3) and histidine methyl ester (4). Coupling was accomplished with dicyclohexylcarbodiimide in the presence of *N*-hydroxybenzotriazole in good yields (see Table I). The products 5 were subjected to treatment with

Scheme I[a]



[a] Key: A, DCC, THF, , CH_2Cl_2; B, CF_3COOH; C, $KHCO_3$ (2 equiv), CH_3OH, reflux.

Table I. Reaction Yields and Characteristics of 2

product	reactn yields,[a] % A	B	C[b]	$[\alpha]_D$, deg (CH_3OH)	mp, °C
2a[c]	81	100	26	−119.10 (*c* 1.0)[d,e]	162–165
2b	88	100	36	+123.2 (*c* 0.96)[e]	158–162.5
2c[f]	71[g]	100	44	+58.7 (*c* 0.94)	194–196.5
2d[h]	68[g]	100	13[i]	−53.8 (*c* 0.31)	184–187.5

[a] See Scheme I for reactants and reagents. [b] Yields of purified products. All were flash chromatographed on silica gel with a 5:1 $CHCl_3$–CH_3OH elution system. Subsequently, **2a** and **2b** crystallized on standing; whereas, **2c** and **2d** crystallized when triturated with a trace amount of CH_3OH. [c] See ref 6 and 15. [d] Elemental analysis ($C_{11}H_{14}N_4O_2 \cdot H_2O$) C, H, N indicated that this material was a monohydrate, and the IR spectrum verified the presence of water. [e] **2a** was reported[14] to have an $[\alpha]_D$ −64.6 (*c* 0.5, H_2O). We determined **2b** under comparable conditions for comparison, $[\alpha]_D$ +92.7 (*c* 1.02, H_2O). [f] See ref 16. Anal. ($C_{11}H_{14}N_4O_2$) C, H, N. [g] Required chromatography; others were homogeneous by TLC. [h] See ref 16 and 17. [i] Crude yield was comparable, but material was lost finding a method for purification.

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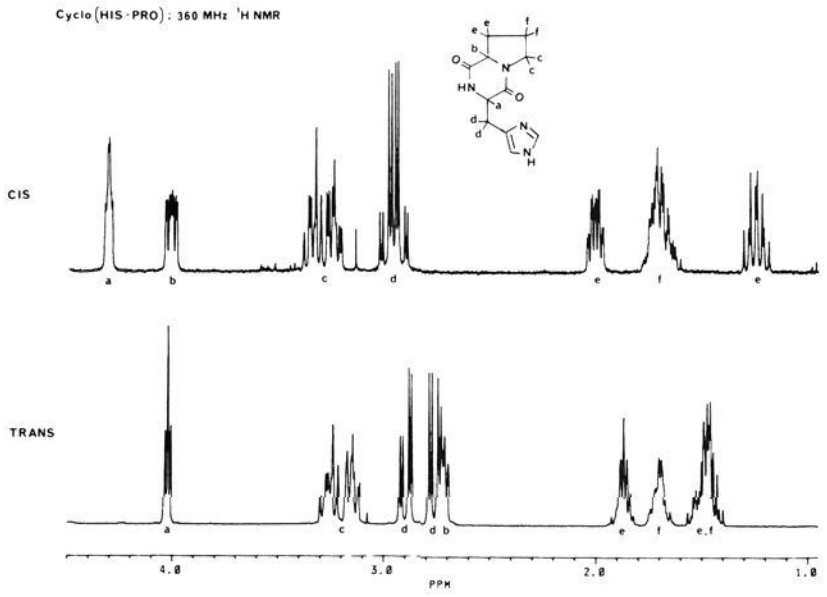

Figure 1.

excess trifluoroacetic acid to remove the *tert*-butyloxycarbonyl protecting group. Concentration of the reaction mixtures gave deprotected amines **6** that were presumed to be the bis(trifluoroacetate) salts. Treatment with 2 equiv of potassium bicarbonate ($KHCO_3$) in refluxing methanol freed the proline amine for subsequent cyclization to diketopiperazines **2**. The rates of these reactions were extremely variable, and they required 6–14 days for completion. We think that the amount of base ($KHCO_3$) used in the reaction is critical and the rate differences reflect incomplete neutralization of the residual trifluoroacetic acid. If an amount in slight excess of that needed to neutralize the acid addition salt was used, the diketopiperazine not only formed rapidly (<12 h) but racemized under the reaction conditions and gave a mixture of diastereomers.

This was examined further by subjecting pure samples of **2b** (cis) and **2c** (trans) to $KHCO_3$ (1 equiv) in refluxing methanol. Within minutes isomerization could be detected by thin-layer chromatography (TLC). We allowed these isomerizations to continue for 4 h even though no further change in the isomer ratio could be detected by TLC after 1 h. The final ratio of *cis*- to *trans*-**2** was determined to be approximately 1:3.5 by high-pressure liquid chromatography (HPLC)[17] for both reactions. Furthermore, we were interested in the path of this transformation. Reports have indicated that base-promoted cis–trans isomerization of diketopiperazines containing proline proceeds exclusively via inversion of configuration at the proline α-carbon.[18,19] Since we were starting with optically pure samples (**2b** and **2c**), the products would also be optically pure if the isomerization occurred specifically at the proline α-position. However, both product mixtures displayed no optical rotation, suggesting complete racemization. We must assume, therefore, that epimerization takes place at both optical centers in this molecule under our reaction conditions. This is in contrast to the earlier report, and we offer no explanation other than to point out differences between conditions that might account for the discrepancy. We used $KHCO_3$ as the base in refluxing methanol whereas the earlier study used 0.1 M NaOH at 26 °C for 10–30 min.[12,18]

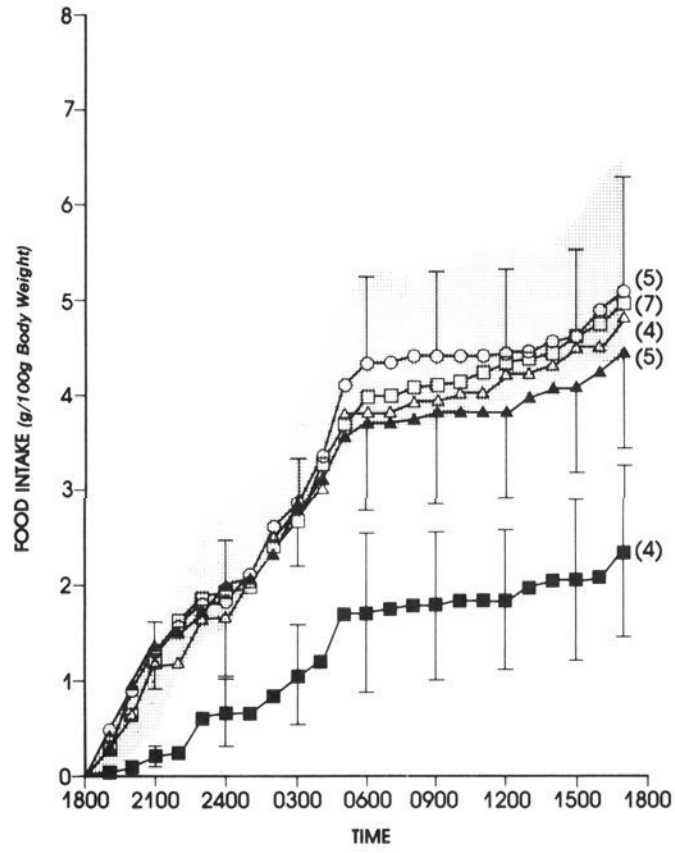

Figure 2. Effect of icv-administered cyclo(His-Pro) stereoisomers (100 nmol) and bombesin (1 μg/0.62 nmol) on cumulative food intake of freely feeding rats: ○, L-His-D-Pro, **2c**; △, D-His-L-Pro, **2d**; □, L-His-L-Pro, **2a**; ▲, D-His-D-Pro, **2b**, ■, bombesin. Mean values are plotted. Bars indicate SEM, and the number of rats per group is given in parentheses. Stippled area indicates mean ±1 standard deviation for 16 saline-injected rats.

Figure 1 shows the 360-MHz ^{1}H NMR spectra in D_2O for cyclo(D-His-D-Pro) (**2b** (cis)) and cyclo(D-His-L-Pro) (**2d** (trans)) except for the downfield imidazole ring protons that appear as singlets at 6.70 and 7.46 ppm for both isomers. The enantiomers of each (**2a** and **2c**, respectively) have spectra that are identical with those pictured. The proton assignments were made from decoupling studies. The significant upfield shift (ca. 1.25 ppm) of the proton labeled b in the trans isomer is consistent with previous reports of arylmethyl side chain substituted diketopiperazines.[20–22] The preferred conformation of this type of substituent is one in which the aromatic ring lies over the diketopiperazine ring that exists in a boat conformation. Consequently, the bridgehead proton experiences the shielding effect of the π cloud of the aromatic imidazole.

Biology

Food intake was measured in free-feeding rats following intracerebroventricular (icv) injections of compounds or saline.

No significant alterations in hourly food intake were observed, through 24-h post-dosing, when commercial **2a**

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was administered at 15, 72, or 140 nmol/rat, icv (n = 5 per dose group, n = 12 for saline controls).

None of the four synthesized stereoisomers of cyclo(His-Pro) (**2a–d**) significantly affected food intake at any hour, through 24-h post-dosing, when administered at 100 nmol/rat, icv (Figure 2). Bombesin, injected at 1 μg/rat (0.62 nmol), was run as a positive control[23] and significantly reduced food intake through 10-h post-dosing. Although food intake was still decreased by 60% at 24 h after bombesin administration, this effect was not statistically significant.

Discussion

Potent anorectic activity has been ascribed to cyclo(L-His-L-Pro),[11,12] particularly with respect to effects on spontaneous eating. We were unable to demonstrate this activity in our laboratories with commercial or synthetic **2a**, which presumably represents the naturally occurring stereoisomer. However, the lack of detailed characterization of any of the isomers **2a–d** in the literature, as well as the apparent instability to base-induced isomerization, led us to question whether another of the isomers **2b–d** might actually be an anorectic molecule. In this respect, both **2a** and **2c** have been reported to produce hypothermia in cold-exposed rats, with a more severe effect observed following **2a** injection.[24] Stereospecific binding of cyclo(His-Pro) isomers to adrenal particulate fraction has also been demonstrated, with **2c** and **2d** being approximately 10 times less potent than **2a** in displacing labeled peptide.[16] As shown in Figure 2, however, none of the compounds **2a–d** had any effect in a free-feeding paradigm, which readily identified bombesin as an anoretic agent. At this point we have no explanation for the discrepancy between our findings and those of Morley et al.[12] The possibility remains either that the compound they tested was something other than an isomer of cyclo(His-Pro) or that their material was impure and contained an unidentified active impurity.

Since cyclo(His-Pro) is purported to have numerous other biological activities, this report should serve as a comparison to ensure the character and purity of materials used in future testing.

Experimental Section

Melting points were determined on a Thomas-Hoover Unimelt capillary melting point apparatus and are uncorrected. ^{1}H NMR were recorded on a Bruker AM-360 instrument with D_2O as solvent and internal standard set at 4.9 ppm. Elemental analyses were carried out by Atalntic, Microlab, Inc., Atlanta, GA. Optical rotations were determined on a Perkin-Elmer 241 using a 10-cm cell. Bombesin and synthetic angiotensin II were purchased from Peninsula Laboratories and Calbiochem-Behring, respectively. All TLC's were done on Whatman MK6F silica gel (1 × 3 in., 200 μm) plates that were developed with $CHCl_3$–CH_3OH–H_2O (65:25:4) and visualized with iodine vapor.

***N*-(*tert*-Butoxycarbonyl)-L-prolyl-L-histidine Methyl Ester (5).** 1,3-Dicyclohexylcarbodiimide (2.58 g, 12.5 mmol) in CH_2Cl_2 (40 mL) was added to a stirred, heterogeneous mixture of *N*-*tert*-butoxycarbonyl)-L-proline (**3**; 3.12 g, 12.5 mmol), 1-hydroxybenzotriazole hydrate (1.77 g, 13 mmol), L-histidine methyl ester dihydrochloride, **4** (3.02 g, 12.5 mmol), and *N*-methylmorpholine (2.25 mL, 25 mmol) in THF (150 mL) at 0 °C under N_2. After 2 h, the ice bath was removed and the reaction stirred at room temperature for 2 days. The reaction mixture was then cooled to 0 °C and stirred for 30 min. The solid urea was filtered off and the filtrate concentrated under reduced pressure. The crude residue was dissolved in $CHCl_3$ and washed with brine, then saturated $NaHCO_3$, and brine once again. The organic phase was dried over $MgSO_4$, filtered, and concentrated to give 3.71 g (81%) of a yellowish oil. The oil showed one spot by TLC (R_f 0.75).

L-Prolyl-L-histidine Methyl Ester Bis(trifluoroacetate) (6). *N*-(*tert*-Butoxycarbonyl)-L-prolyl-L-histidine methyl ester (**5**; 2.20 g, 6.0 mmol) was dissolved in cold (0 °C) trifluoroacetic acid (60 mL) for 1 h and at room temperature for 2 h. The trifluoroacetic acid was then removed at reduced pressure to give a gummy oil that was dissolved and reevaporated from methanol thrice. The crude product was used in the next step without further purification. It gave a streaky spot by TLC (R_f 0.15).

Cyclo(L-histidyl-L-proline) (2a). Potassium bicarbonate (1.20 g, 12 mmol) was added to a solution of L-prolyl-L-histidine methyl ester bis(trifluoroacetate) (**6**; 6 mmol; based on 100% yield in previous step) in 200 mL methanol. The reaction was refluxed under N_2 and monitored by TLC (R_f 0.53 for **2a** and **2b**, 0.46 for **2c** and **2d**). After 6 days the solvent was removed under vacuum and the product purified by column chromatography, giving 0.36 g (26%) of an oil that crystallized upon standing.

Anorexia Test. Male, Sprague–Dawley rats (Charles River CD strain, 340–515 g) were habituated to individual metabolism cages, which were equipped with automated feeding monitors (Coulbourn Instruments), until body weights and food intake stabilized.

Food was provided as 45-mg pellets (Bio-Serv), which were singly delivered into a feeding trough. A photodetector sensed removal of the pellet and triggered delivery of another pellet. A 10-channel printing counter was used to record pellets delivered.

Animals were kept on a 12-h light–12-h dark cycle, with food and water continuously available. Rats were then implanted with 21-gauge lateral ventricular cannulae, which were affixed to the skull (AP = −1.0, Lat = −1.5 from Bregma) such that the tip of the guide cannula was 1 mm above the roof of the lateral ventricle. A minimum of 5 days post-surgery was allowed before studies were performed. Each compound was dissolved in 0.9% saline and the appropriate dose administered intracerebroventriculary in a volume of 10 μL. Injections were given just prior to the onset of the dark period (1800 h) by a Hamilton syringe and a 26-gauge needle that was passed through the guide cannula into the lateral ventricle. Food intake was recorded at hourly intervals. Statistical comparisons to saline-injected controls were performed by the Student's t-test. After anorexia tests were completed, cannula patency was assessed by the drinking response to an intracerebroventricular injection of 500 ng of angiotensin II. Cannula placement was verified post-mortem by histological examination after cresyl violet injection.

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Registry No. **2a**, 53109-32-3; **2b**, 98049-14-0; **2c**, 86195-58-6; **2d**, 75685-88-0; L-**3**, 15761-39-4; D-**3**, 37784-17-1; L-**4**, 7389-87-9; D-**4**, 4467-54-3; L,L-**5**, 66024-29-1; D,D-**5**, 97997-77-8; L,D-**5**, 97997-78-9; D,L-**5**, 97997-79-0; L,L-**6**, 97997-80-3; D,D-**6**, 97997-82-5; L,D-**6**, 97997-84-7; D,L-**6**, 97997-86-9.

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