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# Antide B, an antagonist of LHRH with cis-3-(4-pyrazinylcarbonylaminocyclohexyl)alanine in position 5

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**Summary.** Several LHRH antagonists with trans-3-(4-pyrazinylcarbonylaminocyclohexyl)alanine (trans-PzACAla) in the position 5 were synthesized and their antiovulatory activity was compared with the activity of the analogs containing cis-PzACAla in this position. In all cases cis-isomer produced more potent analogs. Introduction of cis-PzACAla in the position 5 of Antide gave Antide B which completely inhibits ovulation at a dose of 0.5 $\mu$g/rat. Antide B releases negligible histamine ($ED_{50}$ = 104 $\mu$g/mL), and has excellent solubility in water. Also, an improved synthesis of cis-PzACAla is reported, involving the hydrogenation of 4-aminophenylalanine on a rhodium catalyst to give the desired cis-isomer with a 53% yield.

**Keywords:** Amino acids – LHRH-antagonists – Unnatural amino acids – Antiovulatory activity – Histamine release – Solid phase peptide synthesis

**Abbreviations:** Cpa – 3-(4-chlorophenyl)alanine; ILys – $N^{\varepsilon}$-isopropyllysine; Nal – 3-(2-naphthyl)alanine; NicLys – $N^{\varepsilon}$-nicotinoyllysine; Pal – 3-(3-pyridyl)alanine; PicLys – $N^{\varepsilon}$-picolinoyllysine; PzACAla – 3-(4-pyrazinylcarbonylaminocyclohexyl)alanine; Qal – 3-(3-quinolyl)alanine.

## Introduction

There is a great interest in designing highly potent and reversible antagonists of the luteinizing hormone releasing hormone (LHRH), pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly$NH_2$, to inhibit the release of pituitary gonadotropins for the regulation of reproduction, as reviewed by Karten and Rivier (1986), and by Karten (1992). In the design of antagonists, unnatural amino acids were introduced with success in positions 1, 2, 3, 5, 6, and 8. In the most potent antagonists,

the first three positions are occupied by the sequence NAcDNal[1], DCpa[2], DPal[3] which is very hard to substitute (Theobald et al., 1991; Nestor et al., 1992; Suzuki et al., 1992; Rivier et al., 1992; Zhang et al., 1993; Janecka et al., 1994). On the contrary, many quite different in size, shape, and electron properties substituents can occupy position 5 and 6 to produce very potent analogs (Theobald et al., 1991; Nestor et al., 1992; Suzuki et al., 1992; Rivier et al., 1992; Zhang et al., 1993; Janecka et al., 1994).

In our earlier papers (Ljungqvist et al., 1988, 1990, 1991; Janecka et al., 1991), we reported on a new unnatural amino acid, L- or D-3-(4-pyrazinylcarbonyl-aminocyclohexyl)alanine (**3**) (PzACAla) which was very successfully used in position 5 or 6, respectively, of LHRH antagonists. Both cis- and trans-**3** were used in position 6, and cis-**3** usually gave more potent analogs (Ljungqvist et al., 1988). However, only cis-**3** was used in position 5.

We now report on the synthesis of LHRH antagonists with trans-**3** in position 5 and their comparison to the antagonists containing cis-**3** in this position. We also describe synthesis and bioassay of a new, potent antagonist [cis-PzACAla[5]]Antide and improved, stereoselective synthesis of cis-**3**.

## Materials and methods

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and were uncorrected. $^1$H NMR spectra were recorded on a Nicolet NT-360 instrument with TMS as an internal standard. Optical rotations were measured with a Perkin-Elmer 141 Polarimeter. Mass spectra were taken on a 5995 Hewlett-Packard instrument. Amino acid analyses were carried out on a Beckman 118CL Amino Acid Analyzer. All α-amino functions of the amino acids were protected by the Boc-group. The unnatural amino acids: Boc-DNal, Boc-DCpa, Boc-DPal, Boc-DNicLys, and Boc-ILys(Z) were synthesized at the Southwest Foundation for Biomedical Research (under contract No1-HD-1-3137 with NIH), and were made available by the Contraceptive Development Branch, Center for Population Research, NICHD. 4-Nitrophenyl 2-pyrazinecarboxylate was prepared according to Ljungqvist and Folkers (1988).

### *cis- and trans-N$^{\alpha}$-Boc-4-aminocyclohexyl-L-alanine (cis-**2**) and (trans-**2**)*

A suspension of N$^{\alpha}$-Boc-4-aminophenyl-L-alanine **1** (4.0 g, 143 mmol) and rhodium on alumina powder (2.0 g, rhodium content 5%) in ethanol (250 mL) was hydrogenated at 20 psi for 60–80 h, while the temperature was maintained between 70 and 80°C. The progress of the reaction was monitored by TLC. The catalyst was filtered and the filtrate was concentrated under reduced pressure to give a crude mixture of cis- and trans-**2**. HPLC on an analytical silica column with $CHCl_3$ : MeOH : $H_2O$ (60 : 30 : 5) mixture as the eluting solvent showed cis-**2**/trans-**2** ratio to be 80 : 20. The mixture was separated into the isomers and purified by column chromatography on silica gel 230–400 mesh (eluant; $CHCl_3$ : MeOH : $H_2O$ 60 : 30 : 5). Elution yielded pure cis-**2** (2.16 g; 53%) followed by trans-**2** (0.52 g; 13%).

cis-**2**: m.p. 229–234°C (dec.), $[\alpha]_D^{24} = -11.4°$ (c = 1.4, 25% aq. acetic acid); lit. (Rao et al., 1991) for D-isomer $[\alpha]_D^{26} = +10.6°$ (c = 2.46, 25% aq. acetic acid); $^1$H NMR identical to that described (Rao et al., 1991) for the D-isomer.

trans-**2**: m.p. 252–255°C (dec.), $[\alpha]_D^{24} = -12.2°$ (c = 0.65, 25% aq. acetic acid); lit. (Rao et al., 1991) for D-isomer $[\alpha]_D^{26} = +10.6°$ (c = 2.07, 25% aq. acetic acid); $^1$H NMR identical to that described (Rao et al., 1991) for the D-isomer.

*cis-N$^{\alpha}$-Boc-3-(4-pyrazinylcarbonylaminocyclohexyl)-L-alanine (cis-3) (cis-PzACAla)*

A solution of cis-**2** (2.0 g, 7.0 mmol) and 4-nitrophenyl 2-pyrazinecarboxylate (1.72 g, 7.0 mmol) in DMF (50 mL) was stirred for three days at room temperature. DMF was evaporated with high vacuum, the residue was dissolved in EtOAc (50 mL) and extracted with saturated sodium bicarbonate solution (2 × 15 mL). The combined aqueous fractions were acidified with 10% HCl to pH 3, and extracted with EtOAc (3 × 30 mL). The organic fractions were washed with water (2 × 15 mL) and brine (15 mL), dried and evaporated to give a crude product which was purified by column chromatography on silica gel. Elution with chloroform : methanol 8 : 2 gave pure cis-**3** (1.9 g; 69%); m.p. 69–71°C, $[\alpha]_D^{24} = -9.2°$ (c = 1.0, MeOH), $^1$H NMR($CDCl_3$) $\delta$ 1.25–1.90 (m, 11H), 1.42 (s, 9H), 4.19–4.27 (m, 1H), 4.31–4.40 (m, 1H), 5.00 (bd, J = 8.0 Hz, 1H), 7.96 (bd, J = 8.0 Hz, 1H), 8.52 (dd, J = 2.5, 1.5 Hz, 1H), 8.73 (d, J = 2.5 Hz, 1H), 9.37 (d, J = 1.5 Hz, 1H). Anal. Calcd for $C_{19}H_{28}N_4O_5$: C, 58.15; H, 7.19; N, 14.28. Found: C, 58.01; H, 7.22; N, 14.35.

*trans-N$^{\alpha}$-Boc-3-(4-pyrazinylcarbonylaminocyclohexyl)-L-alanine (trans-3) (trans-PzACAla)*

This compound was synthesized as described above starting from trans-**2** (1.0 g, 3.5 mmol) and 4-nitrophenyl 2-pyrazinecarboxylate (0.86 g, 3.5 mmol). Purification by column chromatography as above gave trans-**3** (0.92 g; 67%), m.p. 188–190°C, $[\alpha]_D^{24} = -6.3$ (c = 1.1, MeOH), $^1$H NMR($CDCl_3$) $\delta$ 0.97–2.03 (m, 11H), 1.32 (s, 9H), 3.71–3.84 (m, 1H), 4.11–4.20 (m, 1H), 5.15 (bd, J = 8.0 Hz, 1H), 8.05 (bd, J = 8.0 Hz, 1H), 8.45 (dd, J = 2.4, 1.1 Hz, 1H), 8.62 (d, J = 2.4 Hz, 1H), 9.21 (d, J = 1.1 Hz, 1H). Anal. Calcd for $C_{19}H_{28}N_4O_5$: C, 58.15; H, 7.19; N, 14.28. Found: C, 58.20; H, 7.15; N, 14.38.

*Peptide synthesis*

Peptide synthesis was performed by the solid phase method on a BHA-resin, as described (Humphries et al., 1979). Cleavage of the peptide from the resin with concomitant deprotection of all side chain protective groups was achieved by the treatment with doubly distilled HF at 0°C in the presence of 10% p-cresol for 1 h. The HF was then evaporated, first by the water aspirator, and then at high vacuum overnight. The residue from the HF step was extracted with ethyl ether to remove non-peptide side products. The peptides were then extracted with 20% acetic acid and the extract was lyophilized. Purification was achieved by gel filtration on Sephadex G-25 with 6% acetic acid as an eluant, followed by chromatography on Sephadex LH-20. The solvent system was $H_2O$ : n-BuOH : HOAc : MeOH, 90 : 10 : 10 : 8. The purified peptides showed single spots on TLC in four solvent systems. The purity was further checked by analytical HPLC using a Waters instrument with 660 solvent programmer and a Vydac $C_{18}$ peptide column, 25 × 3.6 mm i.d. The purity of the peptides was estimated 97–99%. Calculated values for protonated molecular ions were always in agreement with data obtained using FAB technique. Amino acid analyses were carried out after hydrolysis in constant boiling HCl for 24 h using standard procedures. The unnatural amino acids were qualitatively determined with the exception of Pal which was quantified. All results were in agreement with theory within the limits of experimental error. The analytical data are in Table 1.

*Biological assays*

The AOA was determined in rats as described (Corbin et al., 1975), by counting, on estrus, the number of ova by 4-day cycling rats after a single sc injection of the LHRH analog in corn oil was administered between 12 and 12:30 p.m. on proestrus. The in vitro histamine release test in rat mast cells was performed as described (Hook et al., 1985; Karten et al., 1987) and the results are reported as $ED_{50}$ value which is the concentration of the analog in $\mu$g/mL that releases 50% of total releasable histamine.

**Table 1.** Physicochemical characteristics of new LHRH antagonists

| Analog No. | FAB-MS $MH^+$ | TLC[a] | | | | | HPLC[b] |
|---|---|---|---|---|---|---|---|
| | | $R_fA$ | $R_fB$ | $R_fC$ | $R_fD$ | $R_fE$ | $R_t$ (min.) |
| **5** | 1634 | 0.78 | 0.36 | 0.49 | 0.67 | | 7.6 |
| **7** | 1633 | 0.74 | 0.40 | | 0.69 | 0.92 | 9.1 |
| **9** | 1620 | 0.72 | 0.39 | | 0.55 | 0.87 | 8.2 |
| **11** | 1619 | 0.74 | 0.43 | | 0.69 | 0.91 | 8.8 |
| **12** | 1633 | 0.58 | 0.39 | | 0.55 | 0.71 | 7.6 |

[a] TLC solvent systems: A, n-Bu : py : HOAc : $H_2O$, 4 : 1 : 1 : 2; B, n-Bu : HOAc : $H_2O$, 4 : 1 : 2; C, n-Bu : py : HOAc : $H_2O$, 40 : 1 : 10 : 20; D, n-Bu : py : HOAc : $H_2O$, 30 : 10 : 3 : 12; E, EtOAc : py : HOAc : $H_2O$, 5 : 5 : 1 : 3

[b] HPLC conditions: Solvent A was 0.01 M $KH_2PO_4$ adjusted to pH = 3 with $H_3PO_4$, and solvent B was 80% acetonitrile and 20% A. A linear gradient of 0–80% B in 20 min. was used to elute the peptides. The flow rate was 1.5 mL/min. and the absorbance was measured at 210 nm

## Results and discussion

Four new analogs of LHRH with trans-PzACAla in position 5 were synthesized (compounds **5**, **7**, **9**, and **11**) and their activity was compared to the activity of analogs containing cis-PzACAla in this position (Table 2). In all cases cis-PzACAla produced more potent peptides.

These results convinced us to resynthesize our earlier analog, Antide (Ljungqvist et al., 1988) NAcDNal-DCpa-DPal-Ser-NicLys-DNicLys-Leu-ILys-Pro-DAla$NH_2$, with cis-PzACAla in position 5. Antide was a remarkable antagonist of LHRH which released negligible histamine, and highlightened a new era of safety for such antagonists. Also, Antide caused prolonged duration of action, since a single dose of 10 mg/kg (sc) in male monkeys inhibited the

**Table 2.** Comparison of antiovulatory activity (AOA) for LHRH antagonists with cis- or trans-PzACAla in position 5. General sequence NAc( )[1]-DCpa-DPal-Ser-( )[5]-DPicLys-Leu-( )[8]-Pro-DAla$NH_2$

| Analog No. | Position | | | AOA (rats ovul./total rats) dose in $\mu$g | |
|---|---|---|---|---|---|
| | 1 | 5 | 8 | 0.5 | 0.25 |
| **4**[a] | DQal | cis-PzACAla | ILys | 0/12 | 6/11 |
| **5** | DQal | trans-PzACAla | ILys | 3/6 | |
| **6**[b] | DNal | cis-PzACAla | ILys | 1/9 | 2/6 |
| **7** | DNal | trans-PzACAla | ILys | 2/6 | |
| **8**[c] | DQal | cis-PzACAla | Arg | 0/12 | 1/9 |
| **9** | DQal | trans-PzACAla | Arg | 3/5 | |
| **10**[c] | DNal | cis-PzACAla | Arg | 2/8 | |
| **11** | DNal | trans-PzACAla | Arg | 5/5 | |

[a] Ljungqvist et al., 1991; [b] Ljungqvist et al., 1988; [c] Janecka et al., 1991

**Table 3.** Comparison of Antide B with other potent LHRH antagonists in terms of antiovulatory activity (AOA), histamine release (HR), and solubility in water. General sequence: NAc-( )[1]-DCpa-DPal-Ser-( )[5]-( )[6]-Leu-( )[8]-Pro-DAlaNH$_2$

| Analog No. | Position 1 | 5 | 6 | 8 | AOA rats ovul/total rats dose in $\mu$g 1.0 | 0.5 | 0.25 | HR $ED_{50} \pm SEM$ $\mu$g/mL | Solubility mg/mL |
|---|---|---|---|---|---|---|---|---|---|
| **12** (Antide B) | DNal | cPzACAla | DNicLys | ILys | | 0/12 | 6/12 | 104 | >80 |
| **13**[a] (Antide) | DNal | NicLys | cDPzACAla | ILys | 0/12 | 7/11 | | >300 | 1 |
| **14**[b] | DNal | PicLys | cDPzACAla | ILys | | 0/8 | 2/7 | 28 | >50 |
| **15**[c] | DNal | cPzACAla | DPicLys | ILys | | 1/10 | 2/6 | 49 | >50 |
| **16**[d] (Lystide) | DQal | cPzACAla | DPicLys | ILys | | 0/9 | 4/9 | 171 | >80 |
| **17**[e] (Argtide) | DQal | cPzACAla | DPicLys | Arg | | 0/8 | 1/9 | 31 | >100 |

[a] Ljungqvist et al., 1987; [b] Ljungqvist et al., 1988; [c] Janecka et al., 1993; [d] Ljungqvist et al., 1991; [e] Janecka et al., 1991.

biosynthesis of testosterone for periods of 1 week to 60 days. However, the low solubility of Antide in aqueous formulations was a disadvantage (Miller et al., 1990). Introduction of cis-**3** in the position 5 of Antide gave Antide B which inhibits completely ovulation at a dose of 0.5 $\mu$g/rat, releases negligible histamine ($ED_{50}$ = 104 $\mu$g/mL), and has excellent solubility in water. Table 3 provides the comparison of [cis-PzACAla$^5$]Antide **12**, named Antide B and our other potent LHRH antagonists in terms of AOA, histamine release, and solubility in water. Antide B is superior to Antide in AOA assay, inhibiting completely ovulation at 0.5 $\mu$g/rat ($ED_{100}$ for Antide was at 1 $\mu$g). The in vitro histamine release for Antide B is 104 $\mu$g/mL which is a very satisfactory value and compares favorably with the values of other LHRH antagonists (Nestor et al., 1992; Theobald et al., 1991; Rivier et al., 1986; Hocart et al., 1988), though it is three times lower than for Antide. An $ED_{50}$ of 300 $\mu$g/mL for Antide was achieved only for a few other antagonists, but unfortunately none of them was fully active at the dose of 0.5 $\mu$g (Fluoret et al., 1992; Janecka et al., 1993). The only other antagonist in Table 3 better than Antide B is Lystide, which has the same level of antiovulatory activity as Antide B but releases less histamine. The main drawback of Lystide though is the presence of DQal in position 1. This unnatural amino acid is very expensive and difficult to synthesize.

Very good biological activity showed by peptides containing cis-PzACAla prompted us to find an efficient method for the preparation of cis-4-aminocyclohexylalanine (cis-**2**) which after standard acylation with 4-nitrophenyl 2-pyrazinecarboxylate gives cis-PzACAla (cis-**3**). So far cis- and trans-**2** have been prepared by hydrogenation of Boc-4-nitrophenylalanine over $PtO_2$. Purification and separation of the ~1 : 1 mixture of cis- and trans-**2** was performed by preparative HPLC, and finally gave pure cis-**2**, and trans-**2** contaminated with 2.5% of cis-**2**, with 10.6% and 11.2% yield, respectively (Rao et al., 1991).

We found that rhodium-catalyzed low pressure hydrogenation of commercially available Boc-4-aminophenylalanine (**1**) was highly stereoselective and

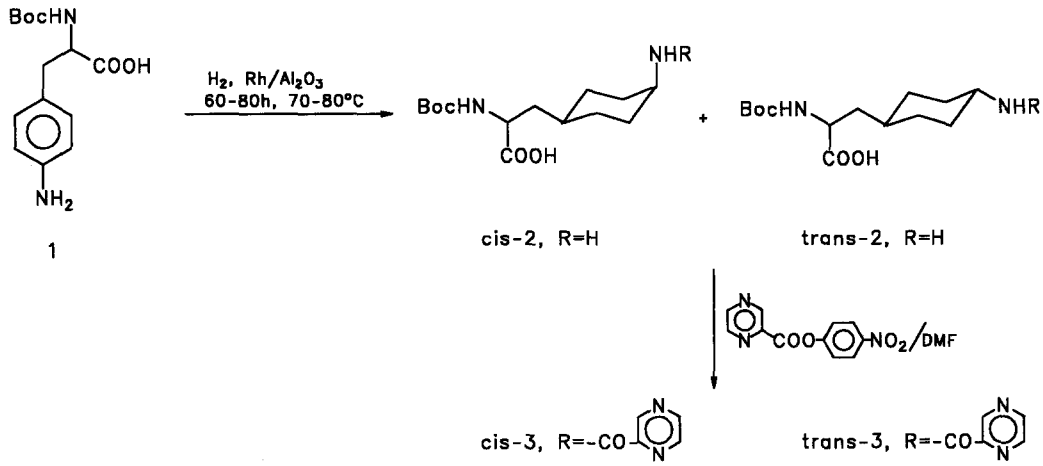


**Fig. 1.** Preparation of cis- and trans-3-(4-pyrazinylcarbonylaminocyclohexyl)alanine (3)

gave a mixture of cis-**2**/trans-**2** in 80 : 20 ratio, as judged from analytical HPLC of the crude product (Fig. 1). Prolonged reaction time (60–80 h) and a moderate temperature (70–80°C) were necessary to complete the reaction. Purification and separation of the crude mixture by column chromatography on silica gel gave pure cis- and trans-**2** with 53% and 13% yield, respectively.

Presented here efficient preparation of cis-**2** makes cis-PzACAla much more available for the synthesis of very active analogs of LHRH such as Antide B.

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