by GLC analysis was 65% product ($t_R$ 5.58 min). Impurity peaks were present at 4.34 min (13.4%), 7.13 min (11.0%), and 7.47 min (9.68%). The oil was chromatographed over silica gel (0.04–0.063 mm) in a medium-sized Michel Miller column (MPLC) by eluting with 1% MeOH/99% $CHCl_3$ containing 1 mL of saturated $NH_4OH$/L of solution. Following a 500-mL forerun, 20-mL fractions were collected. Fractions 51–60 contained 0.185 g (10.5%) of product (**3c**). An attempt to crystallize this material as a *p*-TsOH salt failed. Fractions 61–90 contained 0.1463 g (8.31%) of product oil, which was submitted for analysis and testing. The oil had a retention time of 5.58 min on a 2-ft SE 30 column, 100 °C/1 min, programmed 20 °C/min to 250 °C. Byproducts had $t_R$ of 4.34, 5.02, 7.13, and 7.47 min. By TLC (silica gel, 10% MeOH/90% $CHCl_3$ containing 1 drop of $NH_4OH$) the $R_f$ value was 0.46 separable from a side product with $R_f$ 0.55. **3c**: IR (Nujol) 2933, 1730 (C=O) 1647 $cm^{-1}$ (C=N); NMR ($CDCl_3$ $\delta$ 7.16 (1 H, s, N=CH), 6.90 (2 H, s, aromatic CH), 4.10 (2 H, q, $J$ = 7.1 Hz, $OCH_2$), 3.57 (1 H, q, $J$ = 7.1 Hz, $CHCO_2Et$), 2.97 (6 H, s, $N(CH_3)_2$), 2.10 (6 H, s, aromatic $CH_3$), 1.45 (3 H, d, $J$ = 7.1 Hz, $CH_3$), 1.19 (3 H, t, $J$ = 7.1 Hz, ethyl $CH_3$); high-resolution MS, molecular ion peak at $m/e$ 276.1833, calcd for $C_{16}H_{14}N_2O_2$ 276.1838, with fragment ions at $m/e$ 261 ($M^+ - CH_3$), 203 (base peak $M^+ - CO_2Et$), and 158 ($M^+ - (CO_2Et$ and $HNMe_2)$). The yield of product was higher in several smaller scale reactions.

**Attempted Preparation of 4-[[(Dimethylamino)-methylene]amino]-3,5-dimethylbenzeneacetic Acid Ethyl Ester (3i).** Starting material (0.255 g, 1.00 mmol) **2g** was dissolved in 3.0 mL of THF in a flame-dried 25-mL round-bottom flask with a side arm fitted with a rubber stopper. The solution was stirred with a magnetic stirrer bar and kept in a dry ice/acetone bath at −78 °C. A freshly titrated solution of 0.75 mL of *n*-BuLi (1.6 M, 1.20 mmol) was added. The reactants were stirred for 5 min, at which time an aliquot (A) was removed and quenched in aqueous NaOH, extracted with $CHCl_3$ dried over $Na_2SO_4$, and concentrated in vacuo. GLC analysis (see method C) showed 86% **2a**, 4.8% **2g**, and 5.7% of uncharacterized material at a retention time of 4.71 min. To the remaining solution of anion was added 0.183 g (1.20 mmol) of methyl bromoacetate dissolved in 2.0 mL of THF. The reagents were stirred for 1 min at −78 °C at which time the dry ice bath was removed. The solution was stirred until the temperature of the flask approached room temperature. The reaction mixture was then worked up as before. GLC analyses showed 39.8% (**2a**) and 60.2% (**2g**), indicating metal–halogen exchange occurred. None of the desired product **3i** was detected.

**Hypotensive Activity in Conscious Rat.** The blood pressure of restrained female Sprague-Dawley rats was measured directly from chronic indwelling aortic cannulas exteriorized from the nape of the neck. In order to obtain a high level of sympathetic tone, rats were restrained in a towel during the period of blood pressure measurement with a Statham transducer (P23G) and a Grass Model of 5 polygraph. Measurements were made at 4 and 24 h after the oral administration of each compound. Test compounds were suspended in a (carboxymethyl)cellulose vehicle at 10 mL/kg. Blood pressure values of two animals were averaged at each of the three measurement times.[1] An average change of at least 5 mmHg was required posttreatment for statistical significance ($p < 0.05$).

**Acknowledgment.** We are indebted to Michael G. Wendling and John E. Rogers, who collected the hypotensive data in the conscious rat; to Lubomir Baczynskyj and James R. Boal, who ran the mass spectra; to Upjohn's Physical and Analytical Chemistry Department for elemental analyses and IR spectroscopy; to Vic Rizzo and Dave Graber for preparing compounds **1a** and **1e–g**, respectively; and Kathy Wagner, who typed this manuscript.

**Registry No.** **1a**, 51550-40-4; **1a** (free base), 33089-74-6; **1b**, 114886-08-7; **1b** (free base), 75211-09-5; **1c**, 114886-09-8; **1c** (free base), 86538-00-3; **1d**, 114886-10-1; **1d** (free base), 67791-83-7; **1e**, 75211-12-0; **1f**, 76662-31-2; **1g**, 67346-15-0; **1g'**, 114886-11-2; **1h**, 51062-42-1; **2a**, 114886-12-3; **2a** (free base), 24053-84-7; **2b**, 2350-56-3; **2b** (free base), 15851-79-3; **2c**, 1934-03-8; **2c** (free base), 2103-44-8; **2d**, 114886-13-4; **2d** (free base), 15851-80-6; **2e**, 36192-46-8; **2e** (free base), 36192-18-4; **2f**, 114886-14-5; **2f** (free base), 36172-55-1; **2g**, 114886-15-6; **2h**, 69618-84-4; **2h** (free base), 50884-21-4; **3a**, 114886-17-8; **3a** (free base), 114886-16-7; **3b**, 114886-18-9; **3c**, 114886-19-0; **3d**, 114886-20-3; **3e**, 114886-22-5; **3f**, 114886-23-6; **3g**, 114886-24-7; **3h**, 114886-25-8; **4**, 114886-26-9; **5**, 114886-27-0; **6**, 114886-28-1; $(EtO)_3CH$, 122-51-0; 2,4-$Me_2$-1-$NH_2C_6H_3$, 95-68-1; 2,5-$Cl_2$-1-$NH_2C_6H_3$, 95-82-9; 3,4-$Cl_2$-1-$NH_2C_6H_3$, 95-76-1; 2,3-$Cl_2$-1-$NH_2C_6H_3$, 608-27-5; 2,4-$Cl_2$-1-$NH_2C_6H_3$, 554-00-7; 2,6-$Me_2$-1-$NH_2C_6H_3$, 87-62-7; clonidine, 4205-90-7; 2,6-dichloroaniline, 608-31-1; *N*-methylformamide, 123-39-7; methylamine hydrochloride, 593-51-1; 2-chloroaniline, 95-51-2; 2,4,6-trimethylaniline, 88-05-1; *N,N*-dimethylformamide dimethyl acetal, 4637-24-5; 2,6-dimethyl-4-bromoaniline, 24596-19-8; 2-amino-5-methylpyridine, 1603-41-4.

# Effect of the $CH_2NH$ and $CH_2NAc$ Peptide Bond Isosteres on the Antagonistic and Histamine Releasing Activities of a Luteinizing Hormone-Releasing Hormone Analogue[1]

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The effect of the $CH_2NH$ and $CH_2NAc$ peptide bond isosteres on the antagonistic and histamine releasing activities of the LH-RH antagonist [*N*-Ac-D-Nal[1],D-Phe[2,3],D-Arg[6],Phe[7],D-Ala[10]]LH-RH was investigated. The moieties were introduced by facile, racemization-free solid-phase synthesis in an attempt to reduce the histamine releasing activity inherent to the most potent analogues while retaining high antiovulatory activity. The $\psi[CH_2NH]$ isostere was incorporated at each CONH site with the exception of 8–9, which involves Pro, by reductive alkylation with a protected amino acid aldehyde in the presence of $NaBH_3CN$ during conventional solid-phase peptide synthesis. The $\psi[CH_2NH]$ group was extremely resistant to derivatization and could only be partially acetylated to give $\psi[CH_2NAc]$. The analogues were cleaved from the resin with simultaneous deprotection by anhydrous hydrogen fluoride and purified to homogeneity in two stages: gel permeation followed by preparative reversed-phase liquid chromatography. The analogues were assayed in standard rat antiovulatory and in vitro histamine release assays. The isosteres caused a loss of the antiovulatory activity of the antagonist at the 50-$\mu$g dose when incorporated at the positions 1–2, 2–3, 3–4, and 7–8. Incorporation at the other positions resulted in a less marked reduction in activity relative to the unmodified parent analogue. No significant effect was noted on the potent histamine releasing activity of the analogues.

The current interest in antagonists of the luteinizing hormone-releasing hormone, Glp-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-$NH_2$ (LH-RH),[2] for the potential control of fertility and hormone-dependent tumors has been tem-

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Table I. Peptide Chromatographic and Purity Data

| peptide | HPLC $t_R$, min | HPLC purity, % | TLC $R_f$ 1 | TLC $R_f$ 2 | TLC $R_f$ 3 | TLC $R_f$ 4 | TLC $R_f$ 5 | FAB-MS (M − H$^+$) |
|---|---|---|---|---|---|---|---|---|
| I | 17.8 | 93.6 | 0.39 | 0.79 | 0.68 | 0.27 | 0.55 | 1414 |
| II | 17.9 | 91.7 | 0.42 | 0.81 | 0.66 | 0.21 | 0.48 | 1442 |
| III | 19.2 | 89.8 | 0.43 | 0.82 | 0.69 | 0.29 | 0.59 | 1415 |
| IV | 19.6 | 98.4 | 0.42 | 0.82 | 0.68 | 0.29 | 0.58 | 1414 |
| V | 21.4 | 98.5 | 0.41 | 0.82 | 0.68 | 0.28 | 0.56 | 1414 |
| VI | 19.9 | 94.7 | 0.42 | 0.81 | 0.70 | 0.28 | 0.59 | 1414 |
| VII | 19.6 | 92.5 | 0.41 | 0.81 | 0.70 | 0.28 | 0.57 | 1414 |
| VIII | 20.2 | 93.6 | 0.34 | 0.79 | 0.63 | 0.25 | 0.49 | 1386 |
| IX | 20.3 | 92.9 | 0.34 | 0.78 | 0.65 | 0.26 | 0.47 | 1414 |
| X | 19.6 | 97.0 | 0.23 | 0.65 | 0.58 | 0.13 | 0.36 | 1414 |
| XI | 17.4 | 98.3 | 0.38 | 0.76 | 0.79 | 0.29 | 0.59 | 1456 |
| XII | 17.3 | 98.0 | 0.35 | 0.75 | 0.74 | 0.25 | 0.56 | 1428 |
| XIII | 17.4 | 96.5 | 0.38 | 0.77 | 0.77 | 0.34 | 0.63 | 1456 |
| XIV | 16.4 | 97.9 | 0.33 | 0.75 | 0.75 | 0.28 | 0.55 | 1456 |

pered by observations of adverse effects on histamine release.[3] Presently, the most promising analogues of which [*N*-Ac-D-Nal[1],D-Phe[2,3],D-Arg[6],Phe[7],D-Ala[10]]LH-RH[4] is a typical example contain distinct hydrophobic and hydrophilic regions and include positively charged residues in close proximity. However, when injected subcutaneously into rats at 50–100 times the effective antiovulatory dose, these antagonists have been shown to cause transient edema of the face and extremities[3] and are able to induce a cutaneous anaphylactoid-like response causing a dose-related wheal reaction.[5] Other peptides are also known to elicit the release of histamine including somatostatin, substance P, and neurotensin, and the phenomenon is clearly linked to the presence of highly charged Lys and Arg residues.[6-8]

In an attempt to separate the desired antagonistic properties from the histamine releasing side effects, we reported recently on the effect of modifying the side-chain basicity and hydrophobicity of the positively charged residues in positions 6 and 8 of the former antagonist. The analogues were synthesized by in situ reductive alkylation during the solid-phase peptide synthesis and we were able to show some differential effects on antagonism and histamine release.[9,10] In this paper we report the use of an analogous technique[11,12] for the facile synthesis of analogues containing the $\psi$[$CH_2NH$] peptide bond isostere to investigate the role of the peptide backbone in antagonism and histamine release. This group has recently proven useful in probing peptide backbone involvement in biological signal expression in somatostatin octapeptides[12] and bombesin.[13] In the latter study, one analogue was actually shown to be a potent bombesin antagonist.

## Results and Discussion

**Chemistry.** The sequential replacement of the peptide amide bonds with the $\psi$[$CH_2NH$] isostere proceeded smoothly for most of the analogues. The deprotected resin-bound peptides were reductively alkylated by a protected amino acid aldehyde in the presence of $NaCNBH_3$ in acidified dimethylformamide (DMF). The reactions proceeded normally with few exceptions: Boc-D-Arg(Tos)-CHO could not be formed by using the standard methodology, so the analogue D-Lys[6]$\psi$[$CH_2NH$]Phe[7] was prepared since the substitution of D-Lys for D-Arg in this series of analogues does not have a profound effect upon biological activity. Ac-D-Nal-CHO was particularly impure, as judged by thin-layer chromatography (TLC), and the peptide (III) gave positive (blue colored) Kaiser tests[14] after several reductions. Only a small quantity of the desired Ac-D-Nal[1]$\psi$[$CH_2NH$]Phe[2] analogue was produced. The remainder of the peptides gave rose colored Kaiser tests, typical of the secondary amine product, after one reductive alkylation. Peptide I was prepared by reductive alkylation in the presence of acetaldehyde[9] and gave, unexpectedly, peptide II, the diethylated analogue, as the major product from the synthesis. The N-terminal amino acid D-Nal was coupled as Ac-D-Nal in the presence of HOBt to avoid the possible acetylation of the secondary amino site during the $N^{\alpha}$-acetylation with acetic anhydride. However it was later discovered that the secondary $\psi$[$CH_2NH$]amino group is exceedingly resistant to further reaction. The synthesis of the $\psi$[$CH_2NAc$] analogues produced mixtures of the desired secondary acetylated product along with considerable quantities of the unchanged $\psi$[$CH_2NH$] material. Indeed, acetylation for 24 h with acetic anhydride in the presence of 4-(dimethylamino)pyridine (DMAP) or an excess of acetyl chloride did not ensure a quantitative yield of $\psi$[$CH_2NAc$]. Consequently, only four $\psi$[$CH_2NAc$] analogues were synthesized for this preliminary study.

The alkylated peptides were cleaved from the support, with simultaneous side-chain deprotection, by acidolysis

(1) Abbreviations used in this paper for amino acids, protecting groups, and peptides follow the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature and Symbols as described in *Eur. J. Biochem.* **1972**, *27*, 201 and *J. Biol. Chem.* **1975**, *250*, 3215; Glp, pyroglutamic acid; D-Nal, 3-(2-naphthyl)-D-alanine; $\psi$[$CH_2NH$], peptide bond replaced by a [$CH_2NH$] bond.

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Table II. Amino Acid Analyses

| peptide | Ser | Pro | Ala | Tyr | Phe | Nal | Arg | X[a] | $t_R$[b] |
|---|---|---|---|---|---|---|---|---|---|
| I | 0.93 | 0.99 | 1.00 | 0.97 | 2.34 | | 1.95 | | |
| II | 0.91 | 1.00 | 1.00 | 1.02 | 2.25[c] | | 2.10 | | |
| III | 0.91 | 1.04 | 1.00 | 1.03 | 1.70[c] | | 1.84 | | 63.7[d] |
| IV | 0.83[c] | 0.90 | 1.00 | 0.98 | 1.00 | 0.91[c] | 1.92 | | |
| V | | 1.04 | 1.00 | 0.97 | 1.85[c] | 1.08 | 1.94 | | |
| VI | | 1.09 | 1.00 | | 3.13 | 0.90 | 1.62 | 0.86 | 57.8 |
| VII | 0.70[c] | 0.94 | 1.00 | | 2.23[c] | 0.99 | 1.08 | | |
| VIII | 0.99 | 1.08 | 1.00 | 1.04 | 2.09 | 0.99 | 1.51[e] | | 66.9 |
| IX | 1.09 | 1.13 | 1.08 | 1.00 | 1.97 | 0.96 | 0.83[c] | | |
| X | 1.09 | | | 1.00 | 2.85 | 0.81 | 1.49[c] | 0.65 | 58.7 |
| XI | 0.78 | 0.98 | 1.00 | | 3.03 | 1.02 | 0.92 | | |
| XII | 0.89 | 1.10 | 1.00 | 1.17 | 2.10 | 1.05 | 1.58[e] | | |
| XIII | 0.93 | | 1.00 | | 2.86 | 0.91 | 1.65[c] | | |
| XIV | 0.97 | | | 1.01 | 3.00 | 1.00 | 1.98 | 0.63 | 58.7 |

[a] $\psi[CH_2NH]$ dipeptide calculated as Leu. [b] Retention time of $\psi[CH_2NH]$ dipeptide in minutes. [c] Amino acid ratio reduced by proximity of dipeptide. [d] Ammonia and D-Nal$\psi[CH_2NH]$D-Phe coeluted. [e] Arg and D-Lys$\psi[CH_2NH]$Phe coeluted.

in anhydrous hydrogen fluoride. The crude materials were then desalted by gel filtration. All peptides were purified to homogeneity, as judged by TLC and the purities greater than 90% by analytical reversed-phase high-performance liquid chromatography (RP-HPLC) (see Table I), by a single elution from a preparative $C_{18}$, reversed-phase column. The correct structures were confirmed by fast atom bombardment mass spectrometric analysis (FAB-MS) and amino acid analysis and the data are given in Tables I and II, respectively. However, it was noted that peptide III, which was the least pure of the analogues, also gave an $(M - H^+)$ value of 1415 by FAB-MS (calculated value 1414). Since this analogue did not produce any interesting biological data, no attempt was made to purify it further.

**Biology.** The antagonist [*N*-Ac-D-Nal[1],D-Phe[2,3],D-Arg[6],Phe[7],D-Ala[10]]LH-RH was chosen for this study of the effect of the peptide backbone on histamine releasing activity and antiovulatory activity (AOA). This analogue had previously been used by this group in similar investigations of the role of side-chain hydrophobicity and hydrophilicity in positions 6 and 8,[9,10] had high antiovulatory activity (56% blockade at 0.5 μg, $ED_{50}$ = 0.11 μg $mL^{-1}$), and could be produced in good yield.[4]

The minimum-energy conformations of native LH-RH and some analogues have been investigated by molecular dynamics techniques.[15-17] These investigations lead to the description of a cyclic conformation for the antagonists which contains a β-bend around D-Arg[6]–Phe[7] stabilized by transannular hydrogen bonds: Tyr[5](CO)···Arg[8](NH), Tyr[5](NH)···Arg[8](CO), D-Phe[3](CO)···D-Ala[10](NH), and D-Phe[3](NH)···D-Ala[10](CO). This conformation is illustrated in Figure 1. One may expect the loss of the ability to hydrogen bond to affect the biological activity of the molecule either by the loss of structural stability conferred by the transannular bonding or by the inability of the backbone to hydrogen bond to the receptor. Additionally, the increased flexibility of the molecule about the reduced bond compared with the rigidity of the normal peptide amide bond is expected to alter the conformational integrity of the molecule and thus its biological activity.

The reduced peptide bond analogues all had much lower antiovulatory activities than the parent antagonist (see Table III), with many having no measurable activity at the 50-μg dose (peptides III, IV, V, and IX). However, the modifications had little significant effect on the in vitro histamine releasing activities of the peptides. Several analogues still maintained significant antiovulatory activity. The removal of the acetyl carbonyl (peptide I) produced an analogue with moderate activity (I, 82% AOA

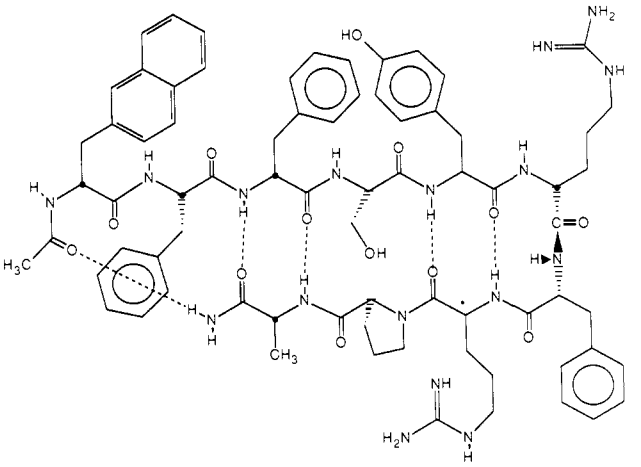

**Figure 1.** Schematic representation of the predicted hydrogen-bonded structure of the antagonist [*N*-Ac-D-Nal[1],D-Phe[2,3],D-Arg[6],Phe[7],D-Ala[10]]LH-RH (• denotes D configuration).

Table III. Antiovulatory and Histamine Releasing Activities of Analogues with the General Formula [*N*-Ac-D-Nal[1],D-Phe[2,3],D-Arg[6],Phe[7],D-Ala[10]]LH-RH Containing the $\psi[CH_2NH]$Isostere

| peptide | reduced peptide bond | antiovulatory activity[a] | in vitro histamine release: $ED_{50}$[b] |
|---|---|---|---|
| | parent analogue | 56 at 0.5 (9) | 0.11 ± 0.04 |
| I | *N*-ethyl-D-Nal[1] | 17 at 6 (6)<br>82 at 12 (11) | 0.34 ± 0.10 |
| II | *N*,*N*-diethyl-D-Nal[1] | 0 at 6 (9) | 0.45 ± 0.04 |
| III | D-Nal[1]$\psi[CH_2NH]$D-Phe[2] | 0 at 50 (9) | 1.50 ± 0.38 |
| IV | D-Phe[2]$\psi[CH_2NH]$D-Phe[3] | 0 at 50 (7) | 0.35 ± 0.09 |
| V | D-Phe[3]$\psi[CH_2NH]$Ser[4] | 0 at 50 (11) | 0.43[c] |
| VI | Ser[4]$\psi[CH_2NH]$Tyr[5] | 63 at 12 (8) | 0.19[c] |
| VII | Tyr[5]$\psi[CH_2NH]$D-Arg[6] | 100 at 12 (8) | 1.50 ± 0.29 |
| VIII | D-Lys[6]$\psi[CH_2NH]$Phe[7] | 60 at 50 (10) | 1.90 ± 0.06 |
| IX | Phe[7]$\psi[CH_2NH]$Arg[8] | 0 at 50 (11) | 0.46 ± 0.03 |
| X | Pro[9]$\psi[CH_2NH]$D-Ala[10] | 44 at 6 (9)<br>92 at 12 (13) | 0.21[c] |

[a] Expressed as the percentage of (*n*) rats blocked at a dose of *x* μg. [b] Expressed as the mean $ED_{50}$ ± standard error in units of μg $mL^{-1}$. [c] Mean of two determinations.

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Table IV. Antiovulatory and Histamine Releasing Activities of Analogues with the General Formula [*N*-Ac-D-Nal[1],D-Phe[2,3],D-Arg[6],Phe[7],D-Ala[10]]LH-RH Containing the $\psi$[$CH_2$NAc]Isostere

| peptide | acetylated reduced peptide bond | antiovulatory activity[a] | in vitro histamine release: $ED_{50}$[b] |
|---|---|---|---|
| XI | Ser[4]$\psi$[$CH_2$NAc]Tyr[5] | 0 at 50 (13) | 0.45[c] |
| XII | Tyr[5]$\psi$[$CH_2$NAc]D-Arg[6] | 0 at 50 (6) | 4.00 ± 1.3 |
| XIII | D-Lys[6]$\psi$[$CH_2$NAc]Phe[7] | 33 at 50 (9) | 1.50[c] |
| XIV | Pro[9]$\psi$[$CH_2$NAc]D-Ala[10] | 75 at 12 (12)<br>100 at 50 (14) | 0.28[c] |

[a] Expressed as the percentage of ($n$) rats blocked at a dose of $x$ $\mu$g. [b] Expressed as the mean $ED_{50}$ ± standard error in units of $\mu$g $mL^{-1}$. [c] Mean of two determinations.

at 12 $\mu$g, 17% at 6 $\mu$g, $ED_{50}$ = 0.34), which was reduced by the addition of a second ethyl group at the N-terminus (peptide II, 0% at 6 $\mu$g, $ED_{50}$ = 0.45). The analogue Ser[4]$\psi$[$CH_2$NH]Tyr[5] (peptide VI, 63% at 12 $\mu$g, $ED_{50}$ = 0.19) showed some retention of activity as did peptide VII, Tyr[5]$\psi$[$CH_2$NH]D-Arg[6] (VII, 100% at 12 $\mu$g, $ED_{50}$ = 1.50), peptide X, Pro[9]$\psi$[$CH_2$NH]D-Ala[10] (X, 92% at 12 $\mu$g, 44% at 6 $\mu$g, $ED_{50}$ = 0.21), and to a lesser extent peptide VIII, D-Lys[6]$\psi$[$CH_2$NH]Phe[7] (VIII, 60% at 50 $\mu$g, $ED_{50}$ = 1.90).

According to the calculated structure of LH-RH, the $\beta$-bend is stabilized by transannular hydrogen bonds between Tyr[5] and Arg[6]. Peptide VII, which has no Tyr[5](CO), would be unable to form one of these hydrogen bonds, yet it is the most active of this series of analogues (VII, 100% at 12 $\mu$g, $ED_{50}$ = 1.50). The N-terminus is held in close proximity to the C-terminus and it is possible that hydrogen bonding occurs between Ac(CO) and D-Ala[10]($NH_2$). The antiovulatory activity of peptide I (*N*-ethyl-D-Nal[1], 17% at 6 $\mu$g, $ED_{50}$ = 0.34) is much lower than the parent peptide (56% AOA at 0.5 $\mu$g), which is in accordance with either the loss of an N- to C-terminal hydrogen bond, or the loss of a crucial interaction of the acetyl carbonyl with the receptor. Likewise, the loss of a putative transannular hydrogen bond between D-Phe[3](CO)···D-Ala[10](NH) seems to correspond to the loss of activity with peptide V. Interestingly, peptide X (Pro[9]$\psi$[$CH_2$NH]D-Ala[10], 44% at 6 $\mu$g, $ED_{50}$ = 0.21), peptide VI (Ser[4]$\psi$[$CH_2$NH]Tyr[5], 63% at 12 $\mu$g, $ED_{50}$ = 0.43), and peptide VIII (D-Lys[6]$\psi$-[$CH_2$NH]Phe[7], 60% at 50 $\mu$g, $ED_{50}$ = 1.90) maintained significant activity which is consistent since none are involved in possible transannular hydrogen bonding. Indeed, each modification is situated between residues which are involved in internal hydrogen bonding and therefore the flexibility conferred by the reduced peptide bond is constrained. Replacements in the pendant, hydrophobic N-terminus presumably introduce excessive flexibility and loss of activity.

Acetylation of the reduced peptide bond presumably restricts the flexibility of the isostere and further prevents hydrogen bonding. This modification to the isostere produced some interesting effects (see Table IV). Ser[4]$\psi$[$CH_2$NAc]Tyr[5] (peptide XI) caused a loss of activity relative to the unacetylated analogue (VI) (XI, 0% AOA at 50 $\mu$g, $ED_{50}$ = 0.45 and VI, 63% at 12 $\mu$g, $ED_{50}$ = 0.19). Loss of activity was also observed with the Tyr[5]$\psi$-[$CH_2$NAc]D-Arg[6] analogue (XII) relative to Tyr[5]$\psi$-[$CH_2$NH]D-Arg[6] (VII), together with a slight improvement in the $ED_{50}$ (XII, 0% AOA at 50 $\mu$g, $ED_{50}$ = 4.00 and VII, 100% at 12 $\mu$g, $ED_{50}$ = 1.50). However, D-Lys[6]$\psi$-[$CH_2$NAc]Phe[7] (XIII, 33% at 50 $\mu$g, $ED_{50}$ = 1.50) exhibited only a slight reduction in antiovulatory activity compared with the parent analogue (VIII, 60% at 50 $\mu$g, $ED_{50}$ = 1.90), as did Pro[9]$\psi$[$CH_2$NAc]D-Ala[10] (XIV, 75% AOA at 12 $\mu$g, $ED_{50}$ = 0.28 compared with Pro[3]$\psi$[$CH_2$NH]D-Ala[10]X, 92% at 12 $\mu$g, $ED_{50}$ = 0.21).

Thus, the antagonist has little tolerance for modifications in the backbone and it is unlikely that other, synthetically difficult, peptide bond isosteres will produce any better results. The present technique offers a relatively rapid method for a preliminary investigation of a peptide backbone before further labor-intensive syntheses are performed.

The results obtained from investigations of the same isostere with bombesin, where two antagonists were produced by single peptide bond replacements,[13] suggest that LH-RH itself should be studied for antagonism resulting from the incorporation of peptide bond replacements. This approach to producing an LH-RH antagonist would be doubly advantageous since the resulting compounds should have very low histamine releasing potencies and contain relatively few synthetic amino acids. This study is currently in progress.

## Experimental Section

**Materials.** 4-Methylbenzhydrylamine hydrochloride resin[18] (ca. 0.7 mequiv $g^{-1}$) was obtained from Vega Biotechnologies Inc. *tert*-Butoxycarbonyl (Boc) protected amino acids were purchased from Bachem Inc., Torrance, CA, or Advanced ChemTech Inc., Louisville, KY. The reactive side chains of the amino acids were masked as follows: Arg, $N^g$-tosyl; Lys, $N^\epsilon$-fluorenylmethoxycarbonyl; Ser, *O*-benzyl; Tyr, *O*-2-bromobenzyloxycarbonyl. 3-(2-Naphthyl)-D-alanine was provided by the Southwest Foundation for Research and Education, San Antonio, TX, through the courtesy of Dr. Marvin Karten, Contraceptive Development Branch, Center for Population Research, National Institutes of Health, Rockville, MD. All reagents and solvents were ACS grade or better and used without further purification.

**Amino Acid Aldehydes.** The protected amino acid aldehydes were prepared in two steps by using a modification of the method of Fehrentz and Castro:[19] the protected amino acids were converted to the corresponding *N,O*-dimethylhydroxamates by reaction with an excess of *N,O*-dimethylhydroxylamine hydrochloride (1.5 equiv) and dicyclohexylcarbodiimide (1.5 equiv) in dichloromethane containing an excess of diisopropylethylamine (4 equiv) at 0 °C. The reaction was allowed to warm up to ambient temperature over 16 h with stirring. The crude, *N,O*-dimethylhydroxamates were isolated as oils after washing with 3 M HCl (3 × 30 mL), saturated $NaHCO_3$ solution (3 × 30 mL), and water (3 × 30 mL), drying over $MgSO_4$, and evaporation to dryness at reduced pressure. The *N,O*-hydroxamates were then reduced with $LiAlH_4$ in ether/tetrahydrofuran at 0 °C. The reaction was followed by TLC and worked up as above to give the crude protected amino acid aldehydes. Although no attempt was made to purify the products further, the optical purity of the resulting reduced peptide bonds has been demonstrated in a model system.[20]

**Peptide Synthesis.** The peptides were assembled on 4-methylbenzhydrylamine functionalized (ca. 0.7 mequiv $g^{-1}$) 1% cross-linked polystyrene resin, on 1-mmol scales utilizing a Vega Model 50 synthesizer by using a modified solid-phase procedure.[21,22] The reduced peptide bonds were formed by the reductive alkylation of the deprotected $N^\alpha$-amino group with the appropriate protected amino acid aldehyde (3.0 equiv) in the

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presence of $NaBH_3CN$ (10 equiv) in DMF (25 mL) containing 1% acetic acid at ambient temperature for 16 h.

**Peptide Cleavage.** The decapeptides were cleaved from the resin support, with simultaneous side-chain deprotection, by acidolysis with anhydrous hydrogen fluoride containing anisole (~15% v/v) and dithiothreitol (~0.3% w/v) as scavengers for 1 h at 0 °C.

**Purification.** The crude peptides were subjected initially to gel permeation chromatography on Sephadex G25 (2.5 × 100 cm) with 50% acetic acid eluent. Final purification was effected by preparative RP-HPLC on $C_{18}$ bonded silica gel (Vydac $C_{18}$, 10-15 μm, 1.0 × 45 cm) eluted with a linear acetonitrile gradient with a constant concentration of trifluoroacetic acid (0.1% v/v). The linear gradient was generated with a Chromat-a-Trol Model II (Eldex Laboratories Inc.) gradient maker. The separations were monitored at 280 nm, by TLC on silica gel plates (Merck F60) and by analytical RP-HPLC. The fractions containing the product were pooled, concentrated in vacuo, and subjected to filtration. Each peptide was obtained as a fluffy white powder of constant weight by lyophilization from aqueous acetic acid. The purity of the final peptides was assessed by RP-HPLC and TLC in five solvent systems, and the results are given in Table I. Analytical RP-HPLCs were recorded with a Vydac $C_{18}$ support (4.6 × 250 mm, 5 μm, 300-Å pore size, Liquid Separations Group). A linear gradient from 30% to 60% acetonitrile over 30 min with a constant concentration of trifluoroacetic acid (0.1% v/v) was employed for all the analyses at a flow rate of 1.5 mL $min^{-1}$. Column eluent was monitored at 215 nm. The retention time and purity of each peptide was assessed by an Shimadzu C-R3A recording integrator. Each peptide produced only one spot, at a loading of ~10 μg, in each of the following solvent systems when visualized by UV or chlorine/starch–iodide:[23] 1, ethyl acetate–pyridine–acetic acid–water, 10:5:1:3; 2, ethyl acetate–pyridine–acetic acid–water, 5:5:1:3; 3, butan-1-ol–acetic acid–water–ethyl acetate, 1:1:1:1; 4, butan-1-ol–acetic acid–water, 4:1:1; and 5, propan-2-ol–1 M acetic acid, 2:1.

**Amino Acid Analysis.** The peptides were hydrolyzed in vacuo (110 °C, 20 h) in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole[24] (Pierce). Amino acid analyses were performed on the hydrolysates with an LKB 4150 analyzer, equipped with an Ultropac 11 column (6 × 215 mm) and a Shimadzu C-R3A recording integrator with in-house software. The buffer sequence pH 3.20 (13.5 min), pH 4.25 (27 min), pH 10.00 (borate; 33 min) and temperature sequence 50 °C (5 min), 55 °C (5 min), 58 °C (30.5 min), 65 °C (7 min), 80 °C (26 min) were used. Standard retention times were as follows: His, 54.9; Nal, 59.3; Lys, 60.3; $NH_3$, 63.5; Arg, 66.0 min, respectively. The results are given in Table II.

**Mass Spectrometry.** FAB-MS was conducted by Oneida Research Services, Inc., Whitesboro, NY, with krypton at 8 kV with a current of 5 μA while scanning from 700 to 1600 amu at 2.0 $scan^{-1}$. The results are given in Table I.

**Biological Assays.** The antiovulatory activity of each analogue was determined in Sprague-Dawley rats in a standard assay[25] using a 40% propane-1,2-diol/0.9% saline vehicle. The results (given in Table III) are expressed as the percentage of ($n$) rats which did not ovulate at a dose of $x$ micrograms of analogue. The in vitro histamine releasing activity of each analogue was determined with peritoneal mast cells from male Sprague-Dawley rats in a standard assay[26] and the results are given as the $ED_{50}$ values expressed in μg $mL^{-1}$. (Standard compound 48/80 has an $ED_{50}$ = 0.58 in this assay system.)

**Acknowledgment.** We gratefully acknowledge the technical assistance of Ellen Shaw for conducting the antiovulatory bioassays and Dr. Marven Karten of the Contraceptive Development Branch, Center for Population Research, National Institutes of Health, Rockville, MD, for arranging the in vitro histamine release bioassays. The in vitro histamine release assays were conducted by Drs. William A. Hook and Reuben P. Siraganian of the Clinical Immunology Section, Laboratory of Microbiology and Immunology, National Institute of Dental Research, National Institutes of Health, Bethesda, MD. This work was supported by NIH Contract NO1-HD-4-2832 to D.H.C.

**Registry No.** I, 115186-25-9; II, 115186-26-0; III, 115204-12-1; IV, 115204-13-2; V, 115204-14-3; VI, 115204-15-4; VII, 115204-16-5; VIII, 115204-17-6; IX, 115204-18-7; X, 115204-19-8; XI, 115186-27-1; XII, 115186-28-2; XIII, 115186-29-3; XIV, 115186-30-6; [N-Ac-D-$Nal^1$,D-$Phe^{2,3}$,D-$Arg^6$,$Phe^7$,D-$Ala^{10}$]LH-RH, 106881-55-4; BOC-D-Nal-OH, 76985-10-9; BOC-D-Phe-OH, 18942-49-9; BOC-Ser($CH_2$Ph)-OH, 23680-31-1; BOC-Tyr[(2-Br)Z]-OH, 47689-67-8; BOC-D-Lys(FMOC)-OH, 115186-31-7; BOC-Pro-OH, 15761-39-4; BOC-Phe-OH, 13734-34-4; BOC-Arg(Tos)-OH, 13836-37-8; BOC-D-Nal-NMeOMe, 115186-32-8; BOC-D-Phe-NMeOMe, 115186-33-9; BOC-Ser($CH_2$Ph)-NMeOMe, 115186-34-0; BOC-Tyr[[2-Br)Z]-NMeOMe, 115186-35-1; BOC-D-Lys(FMOC)-NMeOMe, 115186-36-2; BOC-Pro-NMeOMe, 115186-37-3; BOC-Phe-NMeOMe, 87694-53-9; BOC-D-Nal-H, 115186-38-4; BOC-D-Phe-H, 77119-85-8; BOC-Ser($CH_2$Ph)-H, 79069-54-8; BOC-Tyr-[(2-Br)Z]-H, 108104-78-5; BOC-D-Lys(FMOC)-H, 115186-39-5; BOC-Pro-H, 69610-41-9; BOC-Phe-H, 72155-45-4; MeN-HOMe·HCl, 1117-97-1.

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