Potent Gonadotropin Releasing Hormone Antagonists with Low Histamine-Releasing Activity[†,1]

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Received April 20, 1992

The incorporation of Arg residues into position 6 of gonadotropin releasing hormone antagonists had resulted in compounds with increased in vivo potency but also made these analogues potent mast cell degranulators. We have focused on the substitution of position 8 by hArg(R)$_2$ (N^G,$N^{G'}$-dialkylhomoarginine) substitutions, based on the hypotheses that the Arg-Pro sequence is of major importance for this side effect and that shielding of the charge may be an effective way to block degranulation. Analogues in four series were evaluated: (A) [*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^{3,6},Arg^5,hArg(R)$_2$^{8},D-Ala^{10}]GnRH, (B) [*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^{3,6},hArg(R)$_2$^{5,8},D-Ala^{10}]-GnRH, (C) [*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^{3,6},hArg(R)$_2$^{8},D-Ala^{10}]GnRH, (D) [*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^3,D-hArg(R)$_2$^{6},hArg(R)$_2$^{8},D-Ala^{10}]GnRH. Although substitution by hArg(Et)$_2$, hArg(Bu), hArg(CH$_2$)$_3$, and hArg(CH$_2$CF$_3$)$_2$ was tested, in each series the hArg(Et)$_2$ residue was superior. Two compounds were considered for clinical evaluation: [*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^{3,6},hArg(Et)$_2$^{8},D-Ala^{10}]GnRH and [*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^3,D-hArg(Et)$_2$^{6},hArg-(Et)$_2$^{8},D-Ala^{10}]GnRH (ganirelix acetate). These compounds had high potency for ovulation suppression and low histamine-releasing potency in vitro (ED_{50} = 0.6, 0.29 μg/rat and EC_{50} = 196, 13 μg/mL, respectively). Ganirelix is currently in Phase II clinical trials and appears to be the most potent GnRH antagonist tested in humans (based upon ED_{50} for 24-h suppression of testosterone levels).

Structure–activity studies with gonadotropin releasing hormone (pGlu^1-His^2-Trp^3-Ser^4-Tyr^5-Gly^6-Leu^7-Arg^8-Pro^9-Gly^{10}-NH$_2$, LHRH, GnRH) antagonists led to a class of highly potent and extremely hydrophobic analogues[2-4] (e.g. [*N*-Ac-Pro^1,D-*p*F-Phe^2,D-Nal(2)^{3,6}]GnRH,[4] [*N*-Ac-D-*p*Cl-Phe^1,D-*p*Cl-Phe^2,D-Trp^3,D-*p*Cl-Phe^6]GnRH[3]). These compounds had very low water solubility and were administered in corn oil or propylene glycol/saline mixtures. A study of analogues containing polar substitutions in order to increase aqueous solubility led to [*N*-Ac-D-pCl-Phe^1,D-pCl-Phe^2,D-Trp^3,D-Arg^6,D-Ala^{10}]GnRH,[5] in which D-Arg replaced the usual hydrophobic, aromatic residue in position 6. This compound was very potent[5] and surprisingly long-acting compared to previous hydrophobic analogues.[4,5] Synthesis of further members of this "D-Arg^6" class resulted in even more potent analogues[6-11] [e.g. [*N*-Ac-D-Nal(2)^1,D-*p*F-Phe^2,D-Trp^3,D-Arg^6]GnRH (1)].

We hypothesized that the very long duration of action of the "D-Arg^6" class of analogues was due to hydrophilic depoting in the body through electrostatic interaction between the positively charged guanidine function on Arg and the negatively charged head groups of the cell membrane phospholipids.[10] We prepared a class of amino acids, the N^G,$N^{G'}$-dialkylhomoarginines [hArg(R)$_2$], which was designed to stabilize this interaction and increase the hydrophilic depoting effect.[12,13] GnRH antagonists con-

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† This paper is dedicated to Professor Ralph Hirschmann on the occasion of his 70th birthday.

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(1) Contribution No. 352 from the Institute of Bio-Organic Chemistry, Syntex Research. Some of these data were presented in a preliminary form at the 20th European Peptide Symposium, Tübingen, Germany, September 1988. The abbreviations for natural amino acids and protecting groups followed the recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* 1984, *138*, 9). The N^G,$N^{G'}$-dialkylhomoarginines are abbreviated hArg(R)$_2$ for the convenience of the readers, with the appropriate alkyl residue inserted. Other abbreviations used are the following: Nal(2), 3-(2-naphthyl)alanine; Pal(3), 3-(3-pyridyl)alanine; *p*X-Phe, *p*-halophenylalanine.

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0022-2623/92/1835-3942$03.00/0

taining this substitution were of high potency and had a very prolonged duration of action[13-15] (e.g. detirelix, **3**, $t_{1/2} \approx 48$ h in man[16,17]).

Schmidt, et al.,[18] noted a facial and paw edema in rats treated with 1.25 mg/kg of **1**, and further studies with **1** or other "D-Arg^6" antagonists showed that they were potent degranulators of rat peritoneal mast cells in vitro.[9,19-21] Studies in animal models[22-24] suggest a rough correlation between the in vitro degranulation potencies and in vivo effects (hypotension, facial and paw edema, rodent tail blueing). Human clinical trials have demonstrated a rapid and profound suppression of gonadotrophin secretion but, in addition, occasional mild, disseminated flushing suggestive of histamine release due to mast cell degranulation (MCD) was noted.[17] The propensity of the "D-Arg^6" class of GnRH antagonists to cause MCD has prevented them from being actively pursued for pharmaceutical development.

The juxtaposition of multiple positive charges is thought to be important in causing MCD.[21,25,26] The "D-Arg^6" analogues have positively charged guanidine groups on the side chain at position 6 in addition to that on the Arg at position 8 of the native hormone. Therefore, initial attempts to reduce histamine release involved increasing the distance between these residues.[25,26] Thus [*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Trp^3,Arg^5,D-Tyr^6,D-Ala^{10}]GnRH was less potent in releasing histamine from rat peritoneal mast cells in vitro,[25] and had surprisingly high GnRH antagonistic potency in view of the unusual Arg-for-Tyr switch in positions 5 and 6. Similarly, [*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Trp^3,Arg^5,D-Glu(AA)^6,D-Ala^{10}]GnRH (**2**) had 10-fold less MCD potency compared to **1**.[27] Later, in a study of the effect of substitutions of ϵ-isopropyllysine [Lys(iPr)] in GnRH antagonists, it was found that incorporation of this residue resulted in compounds with reduced MCD potency.[26] Thus, [*N*-Ac-D-Nal(2)^1,D-C-α-Me-*p*Cl-Phe^2,D-Pal(3)^3,D-Arg^6,Lys(iPr)^8,D-Ala^{10}]GnRH had high antiovulatory potency (ED_{100} = 1 μg/rat, noon proestrus) but a reduced potency for MCD.[26] Similarly, [*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^3,Ile^5,D-Lys(iPr)^6,Lys(iPr)^8,D-Ala^{10}]GnRH had only 1% of the MCD potency of **1** but an antiovulatory $ED_{100} < 1.0$ μg/rat.[26]

A number of peptides cause mast cell degranulation, and a common feature shared by many of them is the sequence Arg-Pro.[28] This suggested that the Arg^6-Pro^9 sequence of GnRH is pivotal for the expression of this side effect. Accordingly, we have studied systematically[29] the effect of replacement of the Arg^6 residue by a series of *N*,*N*′-dialkylhomoarginine residues, which resulted in prolonged duration of action and increased potency for ovulation inhibition when incorporated into position 6. These analogues were designed to test the hypothesis that steric hindrance of the guanidino function [e.g. hArg(Et)$_2$, hArg(CH$_2$)$_3$, hArg(Bu)] and reduced basicity [e.g. hArg-(CH$_2$CF$_3$)$_2$] may diminish the MCD potency of the analogues.

Chemistry. The hArg(R)$_2$ analogues were prepared as described[13] or, using an improved method, directly from unprotected Lys. This new method relies on selective reaction of the ϵ-amino function with *S*-methyl-*N*,*N*′-diethylisothiourea and removal of any unreacted Lys as Boc-Lys(Boc)-OH after the protection step. All peptides were synthesized by the Merrifield solid-phase method[30] using (benzhydrylamino)-polystyrene–1%-divinylbenzene resin.[31] N^{α}-*t*-butyloxycarbonyl (BOC) protection was used on all amino acids. The side-chain protection of amino acids was as follows: Arg(Tos), Tyr(2,6-Cl$_2$Bzl),[32] Ser(Bzl). The side chains of the hArg(R)$_2$ analogues were protected only by protonation. The decreased basicity of the hArg-(CH$_2$CF$_3$)$_2$ side chain (pK_a 9.5) made synthesis using an unprotected side chain more problematic, however, and more complex mixtures resulted. Anhydrous liquid HF was used for the final deprotection and cleavage from the

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resin.[33] The crude peptides were purified by preparative high-performance liquid chromatography (prep-HPLC) as described.[13] Other unnatural amino acids were prepared and resolved as described.[13]

Bioassay. The analogues were tested in a standard rat antiovulatory assay[34] using 50:50 propylene glycol/saline as vehicle. In vitro mast cell degranulation potency was assayed using rat peritoneal mast cells by quantitation of histamine released into the medium following incubation with the analogues.[35]

Biological Results and Discussion. Previous discussion of factors responsible for the histamine-releasing potency of GnRH antagonists focused on the key hydrophobic N-terminus and the presence of two positive charges at the C-terminus.[21,25,26] A decrease in the hydrophobicity of the N-terminus by substitution with *p*F-Phe at position 2 or Pal(3)[36] at position 3 decreased the MCD potency of the compounds. However, the global hydrophobicity of the molecule is probably an important factor since **4**, which has decreased N-terminal [D-Pal(3) for D-Trp[3]]—but increased C-terminal (Phe for Leu[7])—hydrophobicities, has the highest MCD potencies reported for a GnRH antagonist. The more recent report[28] that the Arg-Pro sequence is a common structural factor in neuropeptides which cause MCD and Roeske et al.'s demonstration that Lys(iPr)[8] analogues have reduced MCD potency[26] caused us to focus on position 8. Following the hypothesis that steric hindrance on the positive charge in position 8 would decrease MCD potency, we studied the replacement of Arg[8] with several sterically hindered hArg(R)$_2$ analogues.[29] These substitutions were coupled with other modifications (e.g. Pal(3)[3], D-*p*F-Phe[2]) that decreased N-terminal hydrophobicity relative to detirelix, increased distance between the positive charges (e.g. Arg[5]), increased steric hindrance on both positive charges [e.g. hArg(R)$_2$[5,8] or hArg(R)$_2$[6,8]], or decreased the number of positive charges in the molecule [hArg(R)$_2$[8] only]. The majority of the analogues therefore fall into four generic structures with either Trp or Pal(3) at positions 3 and 6, for example (A) [*N*-Ac-D-Nal(2)[1],D-*p*Cl-Phe[2],D-Pal(3)[3,6],Arg[5],hArg(R)$_2$[8],D-Ala[10]]GnRH, (B) [*N*-Ac-D-Nal(2)[1],D-*p*Cl-Phe[2],D-Pal(3)[3,6],-hArg(R)$_2$[5,8],D-Ala[10]]GnRH, (C) [*N*-Ac-D-Nal(2)[1],D-*p*Cl-Phe[2],D-Pal(3)[3,6],hArg(R)$_2$[8],D-Ala[10]]GnRH, (D) [*N*-Ac-D-Nal(2)[1],D-*p*Cl-Phe[2],D-Pal(3)[3],D-hArg(R)$_2$[6],hArg(R)$_2$[8],D-Ala[10]]GnRH.

The new amino acid analogues hArg(CH$_2$)$_3$ and hArg(Bu) were chosen as substitutions likely to be even more sterically hindered (e.g. compare MCD EC$_{50}$'s of **5** vs detirelix) than the previously reported hArg(Et)$_2$. It should be noted that Roeske et al. have incorporated hArg(Et)$_2$ into a GnRH antagonist and found a decrease in MCD potency ([*N*-Ac-D-Nal(2)[1],D-α-Me,*p*Cl-Phe[2],D-Pal(3)[3],D-Arg[6],hArg(Et)$_2$[8],D-Ala[10]]GnRH, EC$_{50}$ = 4.9 ± 0.33 μg/mL for in vitro histamine release).[26] In addition, we have incorporated hArg(CH$_2$CF$_3$)$_2$, an amino acid analogue with significantly reduced basicity compared to Arg, into position 8.

A comparison of the MCD EC$_{50}$'s within the series A analogues (Table I) suggests that hArg(Et)$_2$ is the best substitution at position 8. For the series containing only position 8 substitution (**6**, **7**, **9**), hArg(Et)$_2$[8] has caused a 100-fold decrease in the MCD potency while hArg(CH$_2$)$_3$ and hArg(CH$_2$CF$_3$)$_2$ have caused 10- and 5-fold decreases, respectively. All three analogues have very potent antiovulatory activity, making **6** a significant advance over detirelix. Analogues in this series are structurally related to a literature standard,[27] **2** (in clinical trials), since it also has the pattern of hydrophobic substitution at 3,6 with Arg at position 5. Comparison of **2** with **6** shows the importance of the hArg(Et)$_2$[8] substitution since **6** is 10-fold lower in MCD potency but significantly more potent as an antiovulatory compound.

Within the same structural series, a comparison of **7** and **8** shows that the effect of lowering N-terminal hydrophobicity (*p*Cl-Phe to *p*F-Phe) is to decrease MCD potency (3-fold increase in EC$_{50}$) accompanied by a negative effect on antiovulatory potency. Incorporation of the more hydrophobic Trp[3] (**7** vs **10**, and **9** vs **11**) increases the MCD potency, but has a very adverse effect on antiovulatory potency. An unexpected finding in this series was the relatively high MCD potency of the hArg(CH$_2$CF$_3$)$_2$ (pK_a = 9.5) modification since it should be essentially isosteric with hArg(Et)$_2$ (pK_a > 12.5) but has significantly reduced basicity. Apparently the decreased but still basic character of this residue is not sufficient to remove its potential to contribute to the MCD activity. This substitution is more hydrophobic than hArg(Et)$_2$ and, in certain substitution patterns, may lead to increased MCD potency. The pattern of effects on MCD potency with this residue is not consistent, however. In some analogues a very substantial beneficial effect is seen.

Compounds **12**–**14** (series B) test the effect of steric hindrance at both positions 5 and 8. There are some small structural differences within the series which make direct comparisons less clear-cut. In general the series is disappointing since both MCD and antiovulatory potency are decreased relative to detirelix. The hArg(CH$_2$CF$_3$)$_2$ substitution here (**14**) results in a significant reduction in MCD.

Substitution with a positively charged residue only at position 8 was tested with series C (**15**–**17**). The hArg(Et)$_2$ substitution at position 8 was again superior to hArg(CH$_2$)$_3$ and hArg(Bu), both for decreasing MCD and increasing antiovulatory potency. Compound **15** represents an important advance over previous GnRH antagonists with a >500-fold decrease in MCD activity but very high antiovulatory potency. Compound **18** incorporates an additional modification (Pal(3)[5]) into the series but shows how effective the hArg(CH$_2$CF$_3$)[8] substitution can be. This compound has the lowest MCD potency that we have measured for a GnRH antagonist (>5000-fold less than detirelix and >3-fold less than GnRH itself). The antiovulatory potency is significantly decreased, however, so it is not a desirable development candidate.

When series C is modified to incorporate the more hydrophobic Trp[3,6] instead of Pal(3)[3,6] (**19**–**22**), there is again a negative impact on the resulting analogues. Increased MCD potency (**15** vs **19**) and decreased antiovulatory activity are seen. The 100-fold decrease in MCD

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Table I

no.	GnRH analog[a]	$[\alpha]^{25}_D$ (c, HOAc), deg	HPLC[b] k'	TLC[c] BAW	TLC[c] BAEW	histamine release[d] EC_{50}, μg/mL	antiovulatory[e] ED_{50}, μg
1	[*N*-Ac-D-Nal(2)^1,D-*p*F-Phe^2,D-Trp^3,D-Arg^6]GnRH[f]	−31.1 (0.8)	0.26	0.37	0.67	0.51	2.4
2	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^3,Arg^5,D-Glu(AA)^6,D-Ala^{10}]GnRH[g]					1.6	<1.5
3	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Trp^3,D-hArg(Et)$_2^6$,D-Ala^{10}]GnRH[h] (detirelix)[i]	−21.6 (0.3)	0.94	0.32	0.61	0.18	~0.6
4	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^3,D-hArg(Et)$_2^6$,Phe^7,D-Ala^{10}]GnRH	−12.8 (0.4)	0.59	0.28	0.58	<0.01	0.5
5	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Trp^3,D-hArg(CH$_2$)$_3^6$,D-Ala^{10}]GnRH	−28.2 (0.3)	0.77	0.33	0.62	0.41	0.56
6	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^{3,6},Arg^5,hArg(Et$_2$)^8,D-Ala^{10}]GnRH	−17.7 (0.5)	0.53	0.22	0.24	23.6	0.425
7	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^{3,6},Arg^5,hArg(CH$_2$)$_3^8$,D-Ala^{10}]GnRH	−22.2 (0.3)	0.44	0.22	0.22	1.5	0.295
8	[*N*-Ac-D-Nal(2)^1,D-*p*F-Phe^2,D-Pal(3)^{3,6},Arg^5,hArg(CH$_2$)$_3^8$,D-Ala^{10}]GnRH	−19.3 (0.3)	0.36	0.22	0.17	6.2	0.5
9	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^{3,6},Arg^5,hArg(CH$_2$CF$_3$)$_2^8$,D-Ala^{10}]GnRH	−14.9 (0.7)	1.01	0.33	0.57	0.95	0.595
10	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Trp^{3,6},Arg^5,hArg(CH$_2$)$_3^8$,D-Ala^{10}]GnRH	−22.9 (0.3)	1.76	0.37	0.65	0.81	>2.0
11	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Trp^{3,6},Arg^5,hArg(CH$_2$CF$_3$)$_2^8$,D-Ala^{10}]GnRH	−14.6 (0.5)	4.53	0.46	0.79	0.56	>2
12	[*N*-Ac-D-Nal(2)^1,D-*p*F-Phe^2,D-Pal(3)^{3,6},hArg(Et)$_2^{5,8}$,D-Ala^{10}]GnRH	−24.3 (0.7)	0.94	0.20	0.39	26.2	–
13	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^{3,6},hArg(Bu)^{5,8},D-Ala^{10}]GnRH	−16.8 (0.4)	1.64	0.36	0.66	9.3	1.5
14	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^3,D-Tyr^6,hArg(CH$_2$CF$_3$)$_2^{5,8}$,D-Ala^{10}]GnRH	−17.9 (0.4)	1.87	0.40	0.71	53	1.15
15	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^{3,6},hArg(Et)$_2^8$,D-Ala^{10}]GnRH	−20.7 (0.3)	0.79	0.35	0.64	196	0.6
16	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^{3,6},hArg(CH$_2$)$_3^8$,D-Ala^{10}]GnRH	−23.5 (0.4)	0.46	0.27	0.45	20	0.68
17	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^{3,6},hArg(Bu)^8,D-Ala^{10}]GnRH	−22.0 (0.4)	0.73	0.38	0.64	15.7	1.2
18	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^{3,6},Pal(3)^5,hArg(CH$_2$CF$_3$)$_2^8$,D-Ala^{10}]GnRH	−12.6 (0.1)	0.75	0.31	0.57	>1000	>1 (4/6)
19	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Trp^{3,6},hArg(Et)$_2^8$,D-Ala^{10}]GnRH	−34.1 (0.3)	2.46	0.55	0.76	16.0	~0.9
20	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Trp^{3,6},hArg(CH$_2$)$_3^8$,D-Ala^{10}]GnRH	−28.7 (0.4)	1.91	0.49	0.73	8.5	>1
21	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Trp^{3,6},hArg(Bu)^8,D-Ala^{10}]GnRH	−32.5 (0.3)	3.01	0.68	0.84	1.6	~1.3
22	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Trp^{3,6},hArg(CH$_2$CF$_3$)$_2^8$,D-Ala^{10}]GnRH	−23.7 (0.03)	3.35	0.71	0.88	>100	>2
23	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^3,D-hArg(Et)$_2^6$,hArg(Et$_2$)^8,D-Ala^{10}]GnRH (ganirelix)[i]	−15.7 (0.2)	1.08	0.28	0.47	13	0.285
24	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^3,D-hArg(CH$_2$)$_3^6$,hArg(CH$_2$)$_3^8$,D-Ala^{10}]GnRH	−22.7 (0.2)	0.68	0.23	0.34	1.33	~0.6
25	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Pal(3)^3,D-hArg(Bu)^6,hArg(Bu)$_1^8$,D-Ala^{10}]GnRH	−22.4 (0.1)	1.45	0.38	0.66	0.64	~0.9
26	[*N*-Ac-D-Nal(2)^1,D-*p*F-Phe^2,D-Pal(3)^3,D-hArg(Et)$_2^6$,hArg(Et)$_2^8$,D-Ala^{10}]GnRH	−21.7 (0.3)	0.85	0.27	0.44	8.0	1.0
27	[*N*-Ac-D-Nal(2)^1,D-*p*F-Phe^2,D-Pal(3)^3,D-hArg(Bu)^6,hArg(CH$_2$CF$_3$)$_2^8$,D-Ala^{10}]GnRH	−17.2 (0.1)	1.33	0.39	0.69	59	1.0
28	[*N*-Ac-D-Nal(2)^1,D-*p*F-Phe^2,D-Pal(3)^3,D-hArg(CH$_2$CF$_3$)$_2^6$,hArg(Et)$_2^8$,D-Ala^{10}]GnRH	−23.1 (0.2)	1.03	0.32	0.61	9.3	–
29	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Trp^3,D-hArg(Et)$_2^6$,hArg(Et)$_2^8$,D-Ala^{10}]GnRH	−23.3 (0.4)	1.92	0.33	0.67	0.1	0.5
30	[*N*-Ac-D-Nal(2)^1,D-*p*Cl-Phe^2,D-Trp^3,D-hArg(CH$_2$)$_3^6$,hArg(CH$_2$)$_3^8$,D-Ala^{10}]GnRH	−23.9 (0.2)	1.25	0.30	0.62	2.5	0.7

[a] Unnatural amino acid abbreviations are given in ref 1. Acceptable amino acid analyses were obtained for all GnRH analogs (see Experimental Section). hArg(CH$_2$CF$_3$)$_2$-containing analogs routinely showed a small amount of Lys in their amino acid analysis profile. [b] k' = (retention volume − void volume)/void volume; conditions in Experimental Section. [c] BAW = 1-BuOH/HOAc/H$_2$O, 4:1:5 (upper phase); BEAW = 1-BuOH/EtOAc/HOAc/H$_2$O, 1:1:1:1. [d] Based on 10 animals per dose group. [e] Compound was administered at noon on proestrus (PE). [f] Reference 10. [g] Reference 27. [h] Reference 12. [i] US Adopted Name (USAN) Council designation.

for 19 relative to detirelix is somewhat offset by its decreased antiovulatory potency, and it is not suitable for clinical development.

In series D, steric hindrance is incorporated into substitutions at positions 6 and 8 (23–28). Compound 23 has almost 100-fold lower MCD activity than detirelix and is the most potent GnRH antagonist that we have ever tested in the rat antiovulatory model. Its increased antagonistic potency relative to detirelix is demonstrated even more clearly in dogs (G. I. McRae and B. H. Vickery, unpublished). This combination of increased antiovulatory potency and decreased potency for release of mast cell mediators suggested that 23 should have a very favorable therapeutic ratio relative to detirelix and makes it an excellent candidate for clinical development.

Compounds 24 and 25 again show that hArg$(CH_2)_3$ and hArg(Bu) are inferior substitutions to hArg$(Et)_2$. Both the MCD potencies and the antiovulatory ED_{50}'s continue the trend seen earlier in series A–C. The *p*F-Phe^2 substitution again gives decreased antiovulatory potency (26 vs 23).

Compounds 27 and 28 combine the most effective position 8 substitutions for decreased MCD potency [hArg$(Et)_2$ from 6, 15, 23; hArg$(CH_2CF_3)_2$ from 14, 18, 22] with effective position 6 substitutions. While substantial decreases in MCD potency were obtained, the antiovulatory results were not favorable enough for them to be development candidates.

In our early studies on GnRH agonists and antagonists, we hypothesized that greatly increased duration of action could be imparted to peptides by making use of "hydrophobic depoting" in the body.[10] Thus hydrophobic amino acid substitutions were visualized as causing the peptide to partition out of the plasma and into hydrophobic domains (e.g. cell membranes and hydrophobic binding sites on plasma proteins such as serum albumin[37]). This was visualized as forming a depot from which the drug could be released minutes or hours later. With the observation that basic amino acid substitutions could also lead to a long $t_{1/2}$, we modified our design hypothesis to encompass a mixed hydrophobic/hydrophilic depoting, focusing on the mixed polar/nonpolar phospholipid head group region of the cellular plasma membrane as the binding site.[10,13] The novel class of amino acids which we designed to optimize this interaction, hArg$(R)_2$, now has been found to contribute to the minimization of the potential for causing the histamine release side reaction.

More recently a series of analogues containing structurally similar, ω-substituted Arg residues was reported.[38,39] These amino acid substitutions (e.g. hArg(Bu,CN) and N^{ϵ}-[5′-(3′-amino-1′H-1′,2′,4′-triazolyl)]lysine) were designed to have much reduced basicity ($pK_a \ll 7$) compared to the Arg and hArg$(R)_2$ class ($pK_a > 12$). While incorporation of these residues into position 6 can lead to moderately potent analogues, it is clear that they cannot replace basic Arg, hArg$(R)_2$, or Lys(R) residues in position 8. These new residues appear to form an amphiphilic class of amino acids with hydrophilic and hydrophobic character. Whether this will be sufficient to impart the long duration of action required for effective GnRH antagonism will await further data.

Throughout these studies the D-Pal(3) substitution[36] proved superior to D-Trp both to decrease MCD potency and to increase antiovulatory potency. We suggest that this may be due to the increased solubility imparted by this substitution, which also can duplicate the electron-rich aromatic character of Trp. The increased solubility may allow a higher initial bolus concentration in the plasma, which may allow greater receptor saturation. In view of the very slow receptor off-rates of GnRH analogues,[40] such high initial loading may offset the decreased duration of action that might be expected for such a more soluble compound.

The goal of these studies was the synthesis of compounds which had not only decreased MCD potency but also increased antiovulatory potency. This dual goal was necessitated by the fact that previous antagonists required doses which are substantially higher (5–20 mg/day) than those for GnRH agonists (≤1 mg/day) in their paradoxical, antifertility applications. Two compounds from this study (15 and 23) admirably fulfilled our goals and underwent advanced pharmacology studies. Compound 23 (USAN designation ganirelix) has recently entered Phase II clinical trials and appears to be the most potent GnRH antagonist tested in humans (based on ED_{50} for 24-h suppression of testosterone levels).[41]

Experimental Section

General Methods. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter in a 1-dm microcell at 25 °C at the concentration indicated (w/v%). Thin-layer chromatography (TLC) was performed in a solvent-vapor-saturated chamber on 5 × 20 cm glass plates coated with a 250-μm layer of silica gel GF (Analtech). The plates were visualized by UV absorption followed by chlorination (Cl_2) and 1% KI/starch spray. Reagents *n*-butyl isothiocyanate, 3,4,5,6-tetrahydro-2-pyrimidinethiol, 1,3-diethylthiourea, and 2,2,2-trifluoroethylamine hydrochloride were supplied by Aldrich.

Analytical HPLC was performed under isocratic conditions on a Spectra-Physics Model 8700 equipped with a 20-μL loop injector (Rheodyne) and an Altex Ultrasphere 5-μm C-18 reversed-phase column (4.6 × 250 mm). The eluent contained 60% CH_3CN (Burdick and Jackson, UV) and was 0.03 M in NH_4OAc (Fluka, puriss) at pH 7. The column effluent was monitored at 215 nm with a Spectra-Physics Model 8400 detector.

Amino Acid Analysis. Amino acid analyses were performed on a Beckman 119CL analyzer in the single-column mode after 18–24-h hydrolysis in 4 N $MeSO_3H$/0.2% 3-(2-aminoethyl)indole reagent[42] (Pierce). The difficult resolution of *p*F-Phe, Trp, Arg, and hArg$(Et)_2$ made the analyses less precise than usual (Table II), but convincing evidence for the presence of these amino acids

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Table II. Amino Acid Analyses

no.	1 D-Nal(2)	2 A	3 B	4 Ser	5 C	6 D	7 Leu	8 E	9 Pro	10 Ala	NH_3
4	0.87	0.96[a]	1.19[b]	0.88	0.91[c]	0.86[d]	0.99[e]	1.02[f]	1.00	1.08	1.87
5	0.99	0.99[a]	0.93[g]	0.92	0.97[c]	1.15[h]	0.99	0.98[f]	0.96	1.05	1.12
6	0.96	1.06[a]	1.01[b]	0.88	1.00[f]	1.01[b]	0.96	0.91[d]	1.08	1.04	1.38
7	1.06	1.16[a]	1.00[b]	0.84	1.06[f]	1.00[b]	0.96	1.25[h]	1.07	0.98	0.98
8	0.95	1.01[i]	1.02[b]	0.88	1.02[f]	1.02[b]	0.96	0.82[h]	0.93	1.02	0.99
9	1.11	1.14[a]	1.03[b]	1.03	1.11[f]	1.03[b]	0.94	0.76[j]	1.05	0.96	1.54
10	1.03	1.01[a]	1.05[g]	0.96	0.98[f]	1.05[g]	0.97	1.37[h]	1.03	1.05	0.95
11	0.83	1.12[a]	0.93[g]	1.05	1.08[f]	0.93[g]	0.95	0.83[j]	0.96	0.96	1.46
12	1.06	0.95[i]	0.95[b]	0.89	0.99[d]	0.95[b]	0.99	0.99[d]	1.04	1.01	1.15
13	1.09	0.94[a]	1.01[b]	0.83	0.95[k]	1.01[b]	0.96	0.95[k]	1.12	1.04	1.31
14	0.96	0.97[a]	1.11[b]	0.87	1.14[j]	1.01[b]	1.01	1.14[j]	0.96	0.98	1.16
15	1.08	1.05[a]	1.02[b]	0.93	1.01[c]	1.02[b]	0.96	1.02[d]	1.11	1.03	1.10
16	1.05	1.01[a]	1.11[b]	0.92	1.02[c]	1.11[b]	0.97	1.15[h]	0.93	1.00	1.03
17	1.01	1.04[a]	1.10[b]	0.94	0.99[c]	1.10[b]	0.98	1.02[k]	1.02	1.03	1.16
18	0.90	1.04[a]	0.95[b]	0.96	0.95[b]	0.95[b]	0.97	1.16[j]	1.08	1.03	1.19
19	0.96	1.03[a]	1.12[g]	0.94	1.06[c]	1.12[g]	0.94	1.01[d]	1.06	1.00	1.02
20	0.98	1.08[a]	1.15[g]	0.93	1.08[c]	1.15[g]	0.98	1.17[h]	0.90	0.93	0.98
21	1.02	1.01[a]	1.10[g]	0.93	1.07[c]	1.10[g]	0.92	0.91[k]	1.05	1.02	1.07
22	1.09	1.00[a]	1.06[g]	0.96	1.07[c]	1.06[g]	0.95	0.89[j]	0.96	0.98	1.82
23	0.80	0.84[a]	0.94[b]	0.90	0.94[c]	0.93[d]	0.99	0.93[d]	1.01	1.07	0.97
24	1.02	1.03[a]	1.16[b]	0.99	0.99[c]	1.33[h]	0.97	1.33[h]	1.14	1.04	0.97
25	1.07	1.03[a]	1.23[b]	0.96	1.06	0.93[k]	0.98	0.93[k]	1.00	0.97	0.96
26	0.81	0.98[i]	0.98[b]	0.90	0.97[c]	1.03[d]	0.97	1.03[d]	1.16	1.06	0.97
27	1.02	0.95[i]	0.95[b]	0.82	1.02[c]	1.01[k]	1.02	0.96[j]	1.06	1.06	1.35
28	1.00	1.25[i]	1.25[b]	0.83	1.05[c]	0.92[j]	1.03	0.92[d]	1.00	0.97	1.04
29	1.04	0.95[a]	0.96[g]	0.90	0.93[c]	0.91[d]	1.00	0.91[d]	1.03	1.07	0.93
30	0.91	0.91[a]	0.90[g]	0.80	0.93[c]	10.7[h]	0.88	1.07[h]	1.05	1.19	1.07

[a] pCl-Phe. [b] Pal(3). [c] Tyr. [d] hArg(Et)$_2$. [e] Phe. [f] Arg [g] Trp. [h] hArg(CH$_2$)$_3$. [i] pF-Phe. [j] hArg(CH$_2$CF$_3$)$_2$. [k] hArg(Bu).

was obtained in each case. The buffer sequence pH 3.25 (50 min), pH 4.12 (67 min), pH 6.25 (100 min) was used. Satisfactory amino acid analyses (±10%) were obtained for compounds **4–30**.

Peptide Synthesis. Protected peptides were prepared on a Beckman 900 synthesizer using a standard program, employing 50% CF_3CO_2H/CH_2Cl_2 and 10% Et_3N/CH_2Cl_2 as deprotection and neutralization reagents, respectively. Final deprotection/cleavage was performed with anhydrous (CoF_3), redistilled liquid HF containing 10% anisole as scavenger for 1 h at 0 °C. The crude product was converted to the AcO form by passage through a column of the weakly basic anion exchanger AG 3 (AcO form; Bio-Rad) in H_2O and was lyophilized. This material was purified by reversed-phase preparative HPLC as described,[13] using a 2.5 × 100 cm column (Altex) packed with 25–40 μm Lichroprep RP-18 (E. Merck) or 30 μm Vydac C18 TP. The eluent contained various proportions of CH_3CN (~60%) and H_2O, depending on the hydrophobicity of the compound (k'), but in each case the eluent was 0.03 M in NH_4OAc (pH 7). The fractions containing the product (analytical HPLC) were pooled and concentrated to dryness. The bulk of the NH_4OAc was sublimed under vacuum from the flask into a Kjeldahl head by use of a 40 °C water bath. The residual traces were removed by lyophilization three times from H_2O to yield the pure product as a fluffy white powder.

***N*$^{\alpha}$-(*tert*-Butoxycarbonyl)-*N*^{G},*N*$^{G'}$-diethyl-D-homoarginine (31) [Boc-D-hArg(Et)$_2$-OH].** **A. *S*-Methyl-*N*,*N'*-diethylisothiouronium Hydriodide (32).** Methyl iodide (83 mL, 1.32 mol) was added dropwise to a chilled (0–5 °C) solution of *N*,*N'*-diethylthiourea (155 g, 1.17 mol) in methanol (600 mL). After completion of addition, the solution was heated at 80 °C for 2 h. The solution was concentrated to ~100 mL, and 200 mL of Et_2O was added. The solution was cooled and the resulting solid was filtered, washed with ether, and dried under vacuum to yield the product as 290 g (91% yield) of white solid of mp 72–73 °C. Anal. Calcd for $C_6H_{15}N_2SI$ (274.10): C, 26.29; H, 5.52; N, 10.22. Found: C, 26.20; H, 5.78; N, 10.23.

B. A solution of D-lysine hydrochloride (27.4 g, 0.15 mol) in 150 mL of 2 N NaOH was adjusted to pH 10.65 ± 0.05 with 2 N NaOH. A warm solution (60 °C) of the salt **32** (41.1 g, 0.15 mol) in 4 N NaOH was extracted with 200 mL of CH_2Cl_2 and the resulting CH_2Cl_2 solution was added to the warm (60 °C), vigorously stirred solution of D-Lys. Positive N_2 pressure was maintained in order to sweep the evolved MeSH to a 200-mL bleach (NaOCl) trap. The pH was maintained at 10.65 ± 0.05 by the addition of 4 N NaOH as needed. After the completion of the addition, the solution was stirred at 60 °C overnight. The reaction was 60% complete (amino acid analysis), so an additional batch of **32** (20 g, 0.075 mol) was converted to the free base and added as a solution in CH_2Cl_2 as above. The reaction was maintained at pH 10.65 and 60 °C for another 24-h period, by which time conversion was 80%. The reaction mixture was extracted with EtOAc to remove *N*,*N'*-diethylurea.

The aqueous solution was cooled to 0 °C, diluted with 300 mL of dioxane/H_2O (1:1), and treated with MgO (6.0 g, 0.15 mol). The heterogeneous mixture was treated with di-*tert*-butyl dicarbonate (32.7 g, 0.15 mol) in 50 mL of dioxane (dropwise) and maintained at room temperature and pH 10 by the addition of 2 N NaOH for ~20 h.

The mixture was filtered and the filtrate was evaporated to one-half volume at reduced pressure to remove dioxane. The aqueous solution was extracted with EtOAc to remove excess di-*tert*-butyl dicarbonate, acidified to pH 6.5 with 1 N HCl at 0 °C, and re-extracted with EtOAc to remove Boc-D-Lys(Boc)-OH. The aqueous layer was concentrated to dryness at reduced pressure and the residue was dissolved in EtOH. The crude product was adsorbed onto silica gel (50 g) in a round-bottom flask by concentration of the EtOH solution to dryness at reduced pressure. The solid was added to the top of a 750-g silica gel column packed in CH_3CN and the product was eluted with a step gradient of 2 L of CH_3CN, 2 L of CH_3CN/H_2O (95:5), and 2 L of CH_3CN/H_2O (9:1). The fractions containing the pure product were pooled, concentrated to an oil at reduced pressure, triturated with Et_2O, and dried under vacuum. Boc-D-hArg(Et)$_2$-OH was obtained as 22.5 g (41% yield) of white solid, mp 65–70 °C, $[\alpha]^{25}_D$ −15.9° (*c* 0.13, MeOH). Anal. Calcd for $C_{16}H_{32}N_4O_4 \cdot H_2O$ (362.48): C, 53.02; H, 9.45; N, 15.45. Found: C, 52.91; H, 9.92; N, 15.08.

***N*$^{\alpha}$-(*tert*-Butoxycarbonyl)-*N*^{G},*N*$^{G'}$-propano-D-homoarginine [Boc-D-hArg(CH$_2$)$_3$-OH].** The preparation proceeded from D-Lys as described above for Boc-D-hArg(Et)$_2$-OH except that 3,4,5,6-tetrahydro-2-pyrimidinethiol was converted to the alkylating species. The protected amino acid was obtained as a white foam (49% yield), mp 58–60 °C, $[\alpha]^{25}_D$ 6.73° (*c* 1.1, HOAc). Anal. Calcd for $C_{15}H_{28}N_4O_4 \cdot 3H_2O$ (382.47): C, 47.10; H, 8.96; N, 14.65. Found: C, 47.38; H, 8.29; N, 14.27.

***N*-*n*-Butylthiourea.** To *n*-butyl isothiocyanate (25 g, 217.4 mmol) was added dropwise concentrated NH_4OH (29%, 52.5 mL, 431 mmol). After completion of the addition, and stirring at room temperature for 0.5 h, the mixture was heated at 100 °C

for 1 h. The reaction mixture was allowed to stand at room temperature overnight and filtered, and the white solid was dissolved in EtOAc. The solution was dried (Na_2SO_4), filtered, and concentrated to dryness, and the product was recrystallized from EtOAc/hexane to yield the product as 26 g (91% yield) of white solid, mp 75–76 °C. Anal. Calcd for $C_5H_{12}N_2S$ (132.23): C, 45.42; H, 9.15; N, 21.19. Found: C, 45.17; H, 9.44; N, 21.48.

N^{α}-(*tert*-Butoxycarbonyl)-N^{G}-*n*-butyl-D-homoarginine [Boc-D-hArg(Bu)-OH]. The preparation proceeded from D-Lys as described above for Boc-D-hArg(Et)$_2$-OH except that *N*-*n*-butylthiourea was converted to the alkylating species. The protected amino acid was obtained as a white foam (37% yield), mp 101–105 °C, $[\alpha]^{25}_D$ 5.1° (*c* 0.4, HOAc). Anal. Calcd for $C_{16}H_{32}N_4O_4 \cdot 0.5H_2O$ (353.47): C, 54.36; H, 9.41; N, 15.85. Found: C, 54.59; H, 9.48; N, 15.63.

N^{α}-(*tert*-Butoxycarbonyl)-N^{G},$N^{G'}$-bis(2,2,2-trifluoroethyl)-D-homoarginine Hydrochloride [Boc-D-hArg(CH$_2$CF$_3$)$_2$-OH]. **A. *N*,*N*-Bis(2,2,2-trifluoroethyl)thiourea (33).** To a vigorously stirred mixture of sodium bicarbonate (17.5 g, 208 mmol) and thiophosgene (4.0 g, 34.8 mmol) in cold (0–5 °C) CH_2Cl_2 (125 mL) was added a solution of 2,2,2-trifluoroethylamine hydrochloride (9.4 g, 69.53 mmol) in 50 mL of H_2O.[43] The reaction mixture was stirred at 0 °C for 2 h and at room temperature overnight. The solid was filtered, washed with water and ether, and dried under vacuum. Recrystallization of the solid from EtOAc/hexane gave the product as 6.5 g of white solid (78% yield), mp 154–157 °C. Anal. Calcd for $C_5H_6N_2SF_6$ (240.18): C, 25.00; H, 2.52; N, 11.66. Found: C, 25.20; H, 2.53; N, 11.58.

B. Benzyl N^{α}-(Benzyloxycarbonyl)-N^{G},$N^{G'}$-bis(2,2,2-trifluoroethyl)-D-homoargininate Toluenesulfonate (34). Triethylamine (3.3 g, 30 mmol), **33** (3.60 g, 15.0 mmol), and $HgCl_2$ (4.06 g, 15 mmol) were added at room temperature to a solution of benzyl N^{α}-(benzyloxycarbonyl)-D-lysine toluenesulfonate[44] (7.06 g, 13.5 mmol) in CH_3CN (50 mL), and the reaction mixture was refluxed for 12 h to give a thick black mixture. The reaction mixture was cooled, filtered over Celite, and concentrated to dryness. The residue was diluted with water, adjusted to pH 4 with 1 N HCl, and extracted with EtOAc. The EtOAc solution was washed with water and brine and then dried over anhydrous $MgSO_4$. The solution was filtered, concentrated, and passed through a silica gel column eluted with a gradient of CH_2Cl_2/MeOH (9:1 to 4:1). The appropriate fractions were pooled to give **34** as 7.0 g (69% yield) of yellow foam of $[\alpha]^{25}_D$ 10.2° (*c* 1.5, MeOH). Anal. Calcd for $C_{33}H_{38}N_4O_7F_6S$ (748.70): C, 52.94; H, 5.12; N, 7.48. Found: C, 53.29; H, 5.16; N, 7.36.

C. N^{G},$N^{G'}$-Bis(2,2,2-trifluoroethyl)-D-homoarginine Toluenesulfonate (35). A solution of **34** (6 g, 8 mmol) in EtOH (150 mL) was placed in a 500-mL three-necked round-bottom flask fitted with a H_2 inlet (under solution) and magnetic stirrer. The solution was degassed and 10% Pd(C) (1 g) was added, followed by bubbling of H_2 gas over 3 h. The solution was filtered over Celite, washed with EtOH, and concentrated under vacuum to give the product as 4 g (100% yield) of white solid of mp 120–125 °C, $[\alpha]^{25}_D$ −7.76° (*c* 0.4, MeOH). Anal. Calcd for $C_{16}H_{25}N_4F_6SO_3$ (491.49): C, 43.99; H, 5.13; N, 11.40. Found: C, 43.88; H, 5.26; N, 11.20.

N^{α}-(*tert*-Butoxycarbonyl)-N^{G},$N^{G'}$-bis(2,2,2-trifluoroethyl)-D-homoarginine Hydrochloride [Boc-D-hArg(CH$_2$CF$_3$)$_2$-OH]. To a chilled (0 °C) suspension of **35** (1.96 g, 4 mmol) and MgO (0.16 g, 4 mmol) in a mixture of 1 N NaOH (8 mL) and dioxane (8 mL) was added a solution of di-*tert*-butyl dicarbonate (1.05 g, 4.8 mmol) in dioxane (5 mL). The reaction mixture was stirred at 0 °C for 1 h and at ~25 °C for 3 h. The reaction mixture was filtered and concentrated to dryness. The residue was diluted with water and extracted with Et_2O. The aqueous layer was chilled to 0 °C, acidified with 1 N HCl to pH 3.5, and extracted with EtOAc. The EtOAc layer was washed with water and brine and then dried over anhydrous $MgSO_4$. The solution was filtered and concentrated under vacuum to give a foam. The foam was redissolved in MeOH (20 mL) and triturated with AG 3 (Cl) resin. The methanol solution was filtered and concentrated to dryness to give 1.4 g (72% yield) of the product as white foam of mp 122–130 °C and $[\alpha]^{25}_D$ −2.19° (*c* 0.5, MeOH). Anal. Calcd for $C_{16}H_{26}N_4O_4F_6 \cdot HCl$ (488.87): C, 39.31; H, 5.57; N, 11.46. Found: C, 39.41; H, 5.51; N, 11.63.

Rat Antiovulatory Assay.[34] Adult female Simonsen albino rats (Sprague–Dawley strain; >180 g) were acclimatized to laboratory conditions (14:10, light:dark with lights on at 5:00 a.m.) for at least 10 days, after which daily vaginal lavages were taken from each rat between 7:30 and 9:00 a.m. for at least 12 days. Cytology of vaginal lavages was examined microscopically to determine the stage of the estrous cycle. Rats with at least two consecutive normal 4-day cycles preceding the test cycle were selected. The rats were injected subcutaneously with a solution of the analogue in vehicle (50% propylene glycol/0.9% saline) at noon on proestrus.

On the morning of expected estrus, the rats were euthanized, and the oviducts were removed and examined under a dissecting microscope for the presence of freshly ovulated eggs. The eggs were teased out of the oviducts and counted. The percent of females ovulating was plotted against the log dose to calculate the ED_{50} for antiovulatory activity. Usually three or more dose groups (data from a total of 10 animals per dose group) were used to determine the ED_{50}, but occasionally two dose groups were found to be sufficient.

Histamine-Release Assay.[35] Mixed peritoneal cells were recovered from three or four male Sprague–Dawley rats (350 g, Iffa-Credo, France) by intraperitoneal injection of 10 mL of 0.9% NaCl (containing 50 μg/mL heparin). After gentle massage of the abdominal cavity for 1 min, the peritoneal cells were aspirated, pooled, and centrifuged for 5 min at 1500 rpm. After three rinses in Ca^{2+}-free Krebs–Ringer Buffer (KRB) comprised of 141.9 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM Na_2HPO_4, and 0.6 mM KH_2PO_4, the cells were counted under a microscope and diluted in the appropriate volume of Ca^{2+}-free KRB to achieve a cell density of about 2×10^{-6} cells/mL. Aliquots of 0.5 mL were prewarmed with 0.3 mL Ca^{2+}-free KRB for 5 min at 35 °C, and 0.1 mL of the appropriate solution of the drugs tested was added. After 5 min, the reaction was started by the addition of 0.1 mL of KRB containing Ca^{2+} to achieve the required concentration. After 15 min, the reaction was stopped by adding 2.5 mL of ice-cold Ca^{2+}-free KRB and putting the mixture on ice. After centrifugation of the cell suspension, the histamine content of the supernatant was assayed fluorimetrically by the method of Shore,[45] omitting the extraction procedure (excitation 365 nm, emission 450 nm). None of the reagents fluoresced with *o*-phthaldialdehyde at the concentrations used in the experiments. The total histamine content of the cell suspension was determined after sonication (2 min, 5-s pulse frequency). The spontaneous histamine release was subtracted from all the values measured. Histamine release was calculated from the following equation: % histamine release = [(histamine content of supernatant)/(total histamine content of cell suspension)] × 100.

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