Conformational Preference for the Binding of Biaryl Substrates and Inhibitors to the Active Site of Phenylethanolamine *N*-Methyltransferase[1a,b]

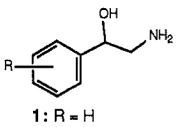
Gary L. Grunewald,* Anne E. Carter,[1c] Daniel J. Sall,[1d] and James A. Monn[1d]

Department of Medicinal Chemistry, University of Kansas, Lawrence, Kansas 66045. *Received June 18, 1987*

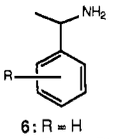
We have previously described regions of steric bulk tolerance in the aromatic-ring binding site of phenylethanolamine *N*-methyltransferase (PNMT, EC 2.1.1.28) for phenylethanolamine substrates and α-methylbenzylamine inhibitors. For bound substrates, this region is located in the vicinity of the para position of the aromatic ring, while for bound α-methylbenzylamine inhibitors, it is located in the region complementary to the meta position. In the present study, we sought to determine the preferred conformation of the biaryl portion of (*m*-phenylphenyl)- and (*p*-phenylphenyl)ethanolamine (**4** and **5**, respectively) as well as for *m*-phenyl- and *p*-phenyl-α-methylbenzylamine (**7** and **8**, respectively) for PNMT active site interactions. Planar derivatives of **4**, **5**, **7**, and **8** were obtained through the synthesis of 2-(1-fluorenyl)-2-hydroxyethylamine (**9**), 2-(2-fluorenyl)-2-hydroxyethylamine (**10**), 1-(1-fluorenyl)ethylamine (**11**), and 1-(2-fluorenyl)ethylamine (**12**). The four fluorene derivatives were examined for in vitro activity as substrates and inhibitors of the PNMT-catalyzed reaction. As in the case of **4**, **5**, **7**, and **8**, we have observed a positional preference for the alkylamine side chain with respect to the biphenyl skeleton present in **9**–**12**. Thus, fluorenylethanolamine **10** ("*p*-biphenyl") displays a Michaelis constant (K_m = 26 μM) that is approximately 10 times lower than that for **9** ("*m*-biphenyl", K_m = 297 μM); in the α-methylbenzylamine inhibitors, fluorenyl derivative **11** ("*m*-biphenyl", K_i = 4.14 μM) is approximately 40 times better than **12** ("*p*-biphenyl", K_i = 185 μM) for in vitro inhibition of PNMT. In each case, conformational restriction of the biaryl system present in **4**, **5**, **7**, and **8**, such that the aromatic rings are coplanar, resulted in enhanced affinity for the PNMT active site. Thus, conformational restriction of ethanolamine **5** (K_m = 82 μM) as in **10** (K_m = 26 μM) and α-methylbenzylamine **7** (K_i = 89 μM) as in **11** (K_i = 4.14 μM) leads, in each case, to a stronger enzyme–ligand dissociable complex. These results, in conjunction with others from these laboratories, indicate that the PNMT active site beyond the zone that interacts with the central aromatic ring portion of phenylethanolamine substrates and α-methylbenzylamine inhibitors is essentially a flat, hydrophobic pocket.

During the course of our studies directed toward the development of a selective inhibitor of phenylethanolamine *N*-methyltransferase (PNMT, EC 2.1.1.28), we became interested in the region of the active site that accommodates the aromatic ring portion of the natural substrate (norepinephrine) and other phenylethanolamine substrates. We have found that a wide variety of straight chain alkyl[2] and alicyclic[3] ethanolamines are methylated in the PNMT-catalyzed reaction, oftentimes more effectively than phenylethanolamine (**1**) itself; we have, therefore, postulated that this region of the PNMT active site is a rather long, flat, hydrophobic region to which either alkyl- or arylethanolamine derivatives can bind during catalysis.

The work of Fuller has supported this hypothesis[4] and furthermore has suggested that there might exist an optimal orientation for steric bulk beyond the central aromatic ring in **1** for binding to the enzyme surface (i.e. 2-(2-phenanthryl)-2-hydroxyethylamine (**2**) binds much more effectively to the PNMT active site than does the 3-phenanthryl derivative **3**). The directional nature of steric-bulk tolerance for bound phenylethanolamines was later confirmed in our laboratories through the synthesis and in vitro evaluation of *m*- and *p*-phenyl-substituted derivatives of phenylethanolamine, **4** and **5**, respectively.[5] In particular, we found (consistent with the phenanthryl data) that substitution at the para position of **1** with a bulky phenyl group (as in **5**) results in an enhancement in activity as a substrate for PNMT (K_m = 82 μM, V_{max} = 4.3, 100 × (V_{max}/K_m) = 5.2) relative to **1** itself (K_m = 70 μM, V_{max} = 2.2, 100 × (V_{max}/K_m) = 3.1), whereas similar substitution in the meta position as in **4** results in a diminished ability to bind to the active site surface (K_m = 653 μM) and a significantly reduced turnover rate (V_{max} = 0.46, 100 × (V_{max}/K_m) = 0.07).



1: R = H
4: R = m-Ph
5: R = p-Ph



6: R = H
7: R = m-Ph
8: R = p-Ph

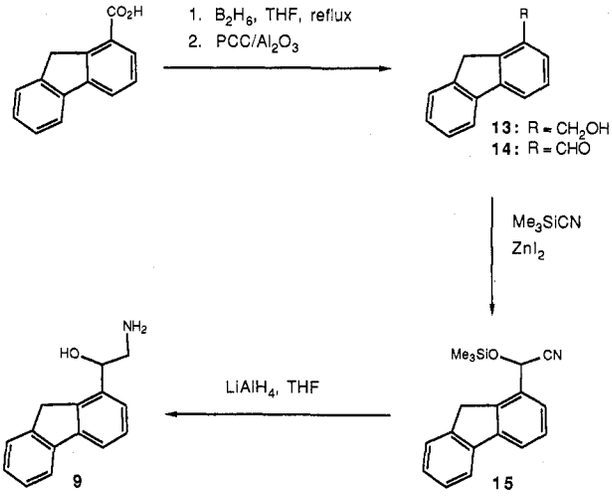
We have also examined the directional nature of steric bulk on the binding of inhibitors of the benzylamine class to PNMT.[5] In this case, however, we found that phenyl substitution in the meta position of α-methylbenzylamine (**6**) results in a dramatic enhancement in potency for **7** (K_i = 89 μM) with respect to **6** (K_i = 460 μM), whereas activity as a PNMT inhibitor is diminished in the *p*-phenyl analogue **8** (K_i = 737 μM).

Conformation–activity relationship studies involving the alkylamine portion of amphetamine[6] and benzylamine[7] inhibitors as well as of phenylethanolamine substrates[8] have been performed in our laboratories. The results of these investigations have clarified the conformational and steric parameters that influence the binding of this portion

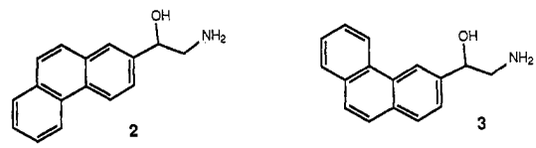
(1) (a) Paper 11 in our series *Conformationally Defined Adrenergic Agents*; for paper 10, see: Grunewald, G. L.; Markovich, K.; Sall, D. J. *J. Med. Chem.*, in press. (b) Taken, in part, from the Dissertation submitted by J.A.M. to the Graduate School of the University of Kansas for the degree Doctor of Philosophy, June, 1987. (c) Summer undergraduate research participant in Medicinal Chemistry, 1984. (d) NIH Predoctoral Trainee (Grant GM 07775) and recipient of the Robert Irsay–Norman Dahle Award in Medicinal Chemistry at the University of Kansas (J.A.M., 1985; D.J.S., 1986).
(2) Vincek, W. C.; Aldrich, C. S.; Borchardt, R. T.; Grunewald, G. L. *J. Med. Chem.* **1981**, *24*, 7.
(3) Davis, D. P.; Borchardt, R. T.; Grunewald, G. L. *J. Med. Chem.* **1981**, *24*, 12.
(4) Fuller, R. W.; Rousch, B. W. *Res. Commun. Chem. Pathol. Pharmacol.* **1977**, *17*, 727.
(5) Rafferty, M. F.; Borchardt, R. T.; Grunewald, G. L. *J. Med. Chem.* **1982**, *25*, 1204.
(6) Grunewald, G. L.; Borchardt, R. T.; Rafferty, M. F.; Krass, P. *Mol. Pharmacol.* **1981**, *20*, 377.
(7) Grunewald, G. L.; Sall, D. J.; Monn, J. A. *J. Med. Chem.*, in press.
(8) Grunewald, G. L.; Ye, Q.; Kieffer, L.; Monn, J. A. *J. Med. Chem.*, in press.

0022-2623/88/1831-0060$01.50/0

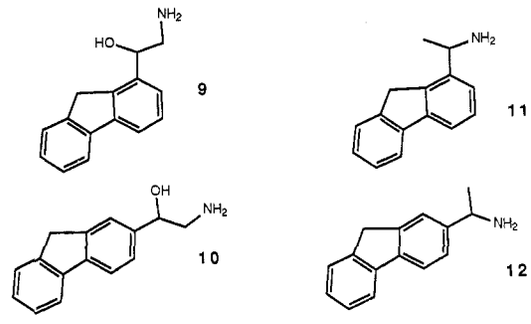
Scheme I



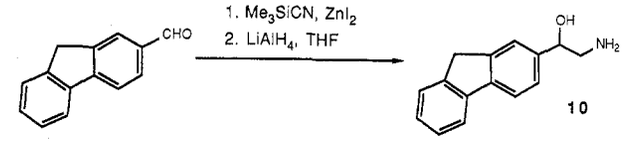
of these different ligands at the PNMT active site. The conformation in which the biphenyl ring system in analogues **4**, **5**, **7**, and **8** interacts with the PNMT active site surface intrigued us. On one hand, the data for phenanthryl derivatives **2** and **3** suggested that a planar arrangement of the biphenyl system is at least capable of binding to the active site surface, while on the other, a coplanar arrangement of the aromatic rings present in **4**, **5**, **7**, and **8** represents a highly unlikely conformation due to van der Waals interactions of the eclipsed ortho hydrogens.



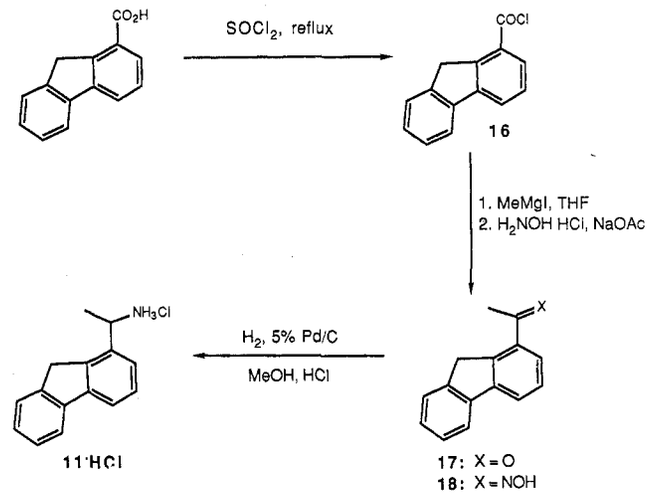
A direct comparison of the kinetic values of ethanolamines **2** and **3** with those of **4** and **5** might clarify this point; however, the data for the phenanthryl analogues are reported only as individual velocities at a particular substrate concentration (i.e. K_m and V_{max} values are not available). In this account, we describe the synthesis and in vitro activity at the active site of PNMT of ethanolamines **9** and **10**, as well as α-methylbenzylamines **11** and **12**, in which the biphenyl system for each analogue is held in a conformationally defined (planar) arrangement through incorporation into the fluorenyl nucleus. Comparison of the in vitro activities as substrates or inhibitors toward PNMT for **9–12** with their flexible counterparts **4**, **5**, **7**, and **8** will lend insight as to the preferred conformation of biphenyl alkylamines when interacting at the PNMT active site surface. This information can then be assimilated with that for the binding of the alkylamine part of these ligands, so as to more fully define the PNMT active site topography, with the ultimate goal of using this information in the design of a PNMT-selective inhibitor.



Scheme II



Scheme III



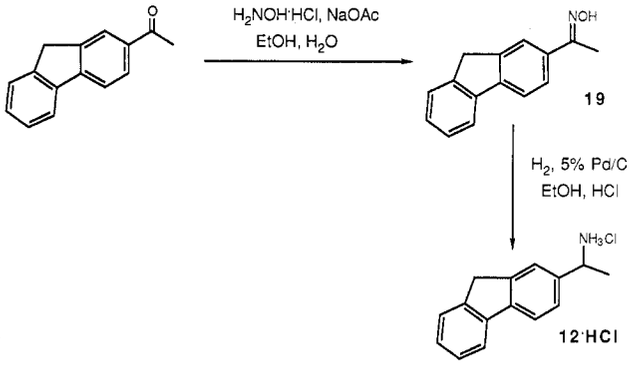
Chemistry

The fluorenylethanolamine **9** was prepared in three steps from commercially available 1-fluorenecarboxylic acid (Scheme I). Reduction of the carboxylic acid with diborane cleanly afforded the corresponding alcohol **13** in high yield (91%). Conversion of **13** to aldehyde **14**[9] was smoothly accomplished by oxidation with pyridinium chlorochromate adsorbed onto neutral alumina.[10] Addition of cyanotrimethylsilane to a solution of **14** in CH_2Cl_2 containing a catalytic amount of ZnI_2 generated the intermediate *O*-(trimethylsilyl)cyanohydrin[11] **15**, which, without purification, was reduced to the desired ethanolamine **9** with lithium aluminum hydride ($LiAlH_4$). The regioisomer **10** was obtained in a similar manner from commercially available 2-fluorenecarboxaldehyde (Scheme II).

The preparation of the α-methylbenzylamine analogue **11** is depicted in Scheme III. Commercially available 1-fluorenecarboxylic acid was converted to the known acid chloride **16**,[9] which was treated (at −70 °C in THF) with methylmagnesium iodide. Solvent choice has been described as crucial in this type of reaction if monoaddition products are desired.[12] In this case, a complex product mixture was generated, and purification of the desired methyl ketone **17** was not readily accomplished; however, chromatographic purification of the corresponding oxime derivative **18** was facile. Thus, the crude reaction mixture resulting from the Grignard addition of MeMgI to **16** was treated with hydroxylamine hydrochloride and sodium acetate in boiling EtOH–H_2O. The mixture of syn- and anti-oximes **18** was then purified by preparative centrifugal TLC. Reduction of **18** by catalytic hydrogenation in the presence of 5% Pd/C and HCl afforded the desired amine hydrochloride **11**·HCl in high yield (95%). A similar route was employed for the preparation of α-methylbenzylamine

(9) Bergmann, E. D.; Ikan, R. *J. Am. Chem. Soc.* **1956**, *78*, 2821.
(10) Cheng, Y.; Liu, W.; Chen, S. *Synthesis* **1980**, 223.
(11) Evans, D. A.; Carroll, G. L.; Truesdale, L. K. *J. Org. Chem.* **1974**, *39*, 914.
(12) Sato, F.; Inoue, M.; Oguro, K.; Sato, M. *Tetrahedron Lett.* **1979**, 4303.

Scheme IV



derivative **12**·HCl via the oxime **19** from commercially available 2-acetylfluorene (Scheme IV).

Biochemistry

Conformationally defined biphenylylalkylamines **9–12**, as well as phenylethanolamine (**1**), α-methylbenzylamine (**6**), and their biaryl derivatives **4**, **5**, **7**, and **8** were evaluated as their hydrochloride salts for activity as both substrates and inhibitors of PNMT. Bovine adrenal PNMT,[13] which had been purified according to the method of Connett and Kirshner through the isoelectric precipitation step,[14] was used. In vitro activity was assessed by use of a standard radiochemical assay that has been previously described for both substrates[15] and inhibitors.[6] For the determination of the kinetic constants for substrates, at least five concentrations of the variable substrate were assayed. Inhibition constants were determined by using at least three different concentrations of the inhibitor with phenylethanolamine as the variable substrate.

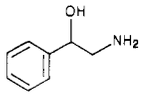
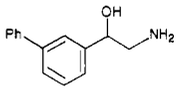
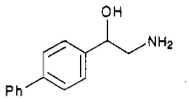
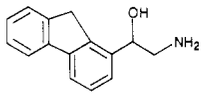
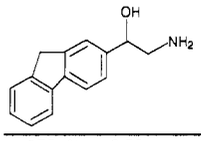
Results and Discussion

As expected, no activity as a substrate was observed for benzylamine analogues **6**, **7**, **8**, **11**, or **12** at concentrations up to 2 mM, while phenylethanolamine (**1**) and its analogues **4**, **5**, **9**, and **10** underwent PNMT catalysis with varying degrees of efficiency (Table I).

Examination of the data in Table I reveals the effect of biphenyl position and conformation on activity as substrates for PNMT. As we have previously reported,[5] introduction of an aromatic ring in the meta position of phenylethanolamine (**1**) results in a severely diminished ability for **4** to interact with the active site (reflected in the large Michaelis constant for **4**; K_m = 653 μM) compared with **1** (K_m = 70 μM). Restriction of the biphenyl segment of **4** by its incorporation into the fluorene nucleus (compound **9**) results in a 2.5-fold enhancement in binding affinity (K_m = 297 μM), suggesting that a flat biphenyl arrangement may be preferred for optimal active-site interactions. This conformational optimization does not, however, overcome the negative influence of the *m*-phenyl substitution.

A similar pattern of activity is evident for analogues **5** and **10**, although in this case, PNMT active-site interactions are optimized (relative to **1**) by a para arrangement between the ethanolamine side chain and phenyl substituent. Thus, para substitution of **1** (K_m = 70 μM, V_{max} = 2.2, 100 × (V_{max}/K_m) = 3.1) with an aromatic ring

Table I. In Vitro Activity of Arylethanolamines as Substrates for PNMT

compound	no.	K_m ± SEM (μM)	V_{max} ± SEM[a]	100 × (V_{max}/K_m)
	1	70 ± 4	2.2 ± 0.07	3.1
	4	653 ± 56	0.46 ± 0.02	0.07
	5	82 ± 6	4.3 ± 0.3	5.2
	9	297 ± 38	0.046 ± 0.003	0.015
	10	26.3 ± 0.95	1.76 ± 0.03	6.7

[a] Units of V_{max} are nanomoles of product formed per milligram of protein per minute.

(compound **5**) results in a slightly higher Michaelis constant (K_m = 82 μM) but an enhanced turnover rate (V_{max} = 4.3, 100 × (V_{max}/K_m) = 5.2). Conformational restriction of this biphenyl system (compound **10**) results in a two- to threefold decrease in the K_m value (26 μM), indicating a slower rate of dissociation of the enzyme–substrate complex, as well as in a decrease in the rate of product formation (V_{max} = 1.76, 100 × (V_{max}/K_m) = 6.7).

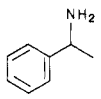
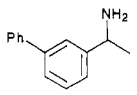
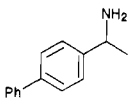
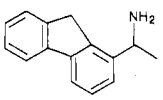
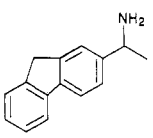
Thus, in both the flexible (**4** and **5**) and conformationally defined (**9** and **10**) biphenylylethanolamine systems, PNMT active site binding (as reflected by the K_m values) is primarily the result of the spatial relationship between the alkylamine side chain and the phenyl substituent. Specifically, a para arrangement is preferred (by approximately 10 times) over the corresponding meta one. Conformational restriction of the biphenyl systems in either case results in a two- to threefold enhancement in binding and a lower turnover rate. The lower K_m values for **9** and **10** compared to **4** and **5** suggest that a planar arrangement of the biphenyl skeleton in substrates of this type is preferred for active-site binding.

The relative activities as inhibitors of PNMT catalysis for α-methylbenzylamine (**6**) and its analogues **7**, **8**, **11**, and **12** (Table II) show a similar trend to that observed for the ethanolamines above. As we have reported,[5] a meta arrangement between the alkylamine side chain and the phenyl substituent is optimal in this type of ligand for PNMT inhibition. Thus, the *m*-phenyl isomer **7** (K_i = 89 μM) is more effective in competing for the PNMT active site with phenylethanolamine than is the unsubstituted derivative **6** (K_i = 460 μM), while the para-substituted regioisomer **8** is effective only at high inhibitor concentrations (K_i = 737 μM).

Conformational restriction of **7** and **8** as in fluorenyl derivatives **11** and **12** results, in each case, in an enhanced ability to bind to the active site (K_i = 4.14 μM for **11** and 185 μM for **12**). Conformational restriction of the biphenyl unit present in the weak inhibitor **8** results in a fourfold enhancement in potency for **12**, completely overcoming the negative influence of the *p*-phenyl substituent. This was not observed in the ethanolamine system (compare ethanolamines **1**, **4**, and **9**, Table I). Finally, holding the spa-

(13) Adrenal PNMT has been found to be similar to the brain enzyme in terms of its susceptibility to inhibitors: Fuller, R. W. *Annu. Rev. Pharmacol. Toxicol.* **1982**, *22*, 31 and references therein.
(14) Connett, R. J.; Kirshner, N. *J. Biol. Chem.* **1970**, *245*, 329.
(15) Grunewald, G. L.; Grindel, J. M.; Vincek, W. C.; Borchardt, R. T. *Mol. Pharmacol.* **1975**, *11*, 694.

Table II. In Vitro PNMT Inhibition by Aryl-α-methylbenzylamines

compound	no.	K_i ± SEM, μM
	6	460 ± 52
	7	89 ± 18
	8	737 ± 31
	11	4.14 ± 0.28
	12	185 ± 11

tially optimized biphenyl skeleton present in **7** in a planar conformation (analogue **11**) results in a dramatic (20-fold) increase in potency as a PNMT inhibitor. This reflects an increase in potency for **11** compared to the unsubstituted α-methylbenzylamine (**6**) by a full 2 orders of magnitude.

In summary, we have examined the effect of biphenyl conformation in PNMT substrates and inhibitors on their ability to bind to the active site. We have determined that (1) the optimal orientation of the biphenyl nucleus (with respect to the amine-containing side chain) when locked into a conformationally defined fluorene skeleton is identical with that observed for flexible biphenyl substrates and inhibitors and (2) a planar arrangement of the biphenyl system present in substrates **4** and **5** as well as in inhibitors **7** and **8** appears to represent the preferred binding conformation of these ligands at the PNMT active site. In each case, a slower rate of dissociation of the enzyme–fluorenylalkylamine complex is observed (compared to the corresponding flexible biaryl ligands), indicative of stronger enzyme–ligand interactions.

These results, together with those obtained for nonaromatic ethanolamine substrates,[2,3] and conformationally defined analogues of amphetamine,[6] benzylamine,[7] and phenylethanolamine,[8] suggest that the PNMT active site is a long, flat, hydrophobic surface, which is at least 11 Å long (from the "end" of the fluorene system to the site of catalysis), 4–5 Å wide, and approximately 2 Å above (or below) the plane defined by the aromatic ring.

Experimental Section

1-Fluorenecarboxylic acid, 2-fluorenecarboxaldehyde, and 2-acetylfluorene were purchased from Aldrich Chemical Co. (Milwaukee, WI). Melting points were determined on a Thomas-Hoover capillary melting point apparatus calibrated with known compounds and are corrected accordingly. Proton nuclear magnetic resonance spectra (^{1}H NMR) and carbon nuclear magnetic resonance spectra (^{13}C NMR) were obtained on either a Varian FT-80A or XL-300 spectrometer with deuteriated chloroform ($CDCl_3$) as the solvent. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (Me_4Si; 0.0 ppm) for ^{1}H NMR and $CDCl_3$ (77.0 ppm) for ^{13}C NMR. Coupling constants (J) are reported in hertz (Hz), and s, d, t, q, and m refer to singlet, doublet, triplet, quartet, and multiplet, respectively. Infrared spectra (IR) were recorded on a Perkin-Elmer IR-727 spectrometer and are calibrated relative to polystyrene (1601 cm^{-1}). Electron-impact mass spectra (EIMS) were obtained on a Varian Atlas CH-5 mass spectrometer. Combustion analyses were performed on a Hewlett-Packard Model 185B CHN Analyzer at the University of Kansas and were within 0.4% of the calculated values. Preparative centrifugal thin-layer chromatography (PCTLC) was performed on a Harrison Model 7924 chromatotron with Merck silica gel 60 PF254 containing $CaSO_4 \cdot 0.5H_2O$ binder. Plate thickness and eluent systems employed are reported in parentheses. Bulb-to-bulb distillations were carried out with a Kugelrohr distillation apparatus (Aldrich), and the boiling range given refers to the internal oven temperature.

S-Adenosyl-L-methionine (AdoMet) was obtained from Sigma Chemical Co. [*methyl*-^{3}H]-*S*-Adenosyl-L-methionine that was used in the radiochemical assays was purchased from New England Nuclear Corp. (Boston, MA). Bovine adrenal glands, required for the purification of the enzyme used in this study, were obtained from Pel-Freez Biologicals (Rogers, AR). Solvents were routinely distilled prior to use; anhydrous tetrahydrofuran (THF) and ether (Et_2O) were distilled from sodium benzophenone ketyl; dry methylene chloride (CH_2Cl_2) was obtained by distillation over phosphorus pentoxide; dry benzene was obtained by distillation from calcium hydride; anhydrous methanol (MeOH) and ethanol (EtOH) were obtained by distillation from magnesium. Unless otherwise stated, all MeOH and EtOH used was anhydrous. Hexanes refers to a mixture of isomeric hexanes (bp 68–70 °C), petroleum ether refers to low-boiling hydrocarbons (primarily pentanes and hexanes, bp 35–60 °C), and brine refers to saturated aqueous NaCl. All reactions requiring inert conditions were performed in oven-dried or flame-dried glassware under a N_2 or Ar atmosphere.

1-(Hydroxymethyl)fluorene (13). A solution of 1-fluorenecarboxylic acid (2.00 g, 9.51 mmol) in THF (200 mL) was treated at room temperature with B_2H_6 in THF (19.0 mL, 19.0 mmol) over a period of 10 min. Upon complete addition, the resulting mixture was warmed under reflux for 29 h. The volatiles were removed under reduced pressure, and the residue was dissolved in EtOAc (100 mL) and washed with 1 N NaOH (3 × 35 mL). The organic layer was dried over $MgSO_4$ and then concentrated under reduced pressure, affording **13** as a tan solid (1.70 g, 8.67 mmol, 91%): mp 144–145 °C. The solid was recrystallized from CH_2Cl_2 as tan needles: mp 147–148.5 °C; ^{13}C NMR (75 MHz) δ 35.2, 63.54, 119.38, 119.95, 125.0, 125.3, 126.7, 126.8, 127.3, 136.82, 141.14, 141.47, 142.07, 143.03; EIMS, m/z (relative intensity) 196 (M^+, 49), 178 (75), 165 (100). Anal. ($C_{14}H_{12}O$) C, H.

1-Fluorenecarboxaldehyde (14).[9] A solution of **13** (1.0 g, 5.1 mmol) in CH_2Cl_2 (50 mL) was added in one portion to a rapidly stirred suspension of pyridinium chlorochromate on neutral alumina[10] (15.3 mmol of PCC) in hexanes (50 mL). The mixture was allowed to stir at room temperature for 8 h, the solid was filtered, and the filtrate was concentrated under reduced pressure to a brown solid, which was purified by PCTLC (4 mm, gradient elution: 100% hexanes to 50% hexanes in ethyl acetate). The homogeneous fractions were combined and concentrated under reduced pressure, affording **14** (0.87 g, 4.5 mmol, 88%): mp 89–90 °C (lit.[9] mp 90 °C); ^{1}H NMR (300 MHz) δ 4.10 (s, 2 H, H9), 7.31–7.88 (m, 7 H, Ar H), 10.14 (s, 1 H, CHO); ^{13}C NMR (75 MHz) δ 36.9, 119.9, 124.9, 125.0, 126.7, 127.3, 127.4, 130.4, 132.4, 139.7, 143.2, 143.6, 143.9, 192.6; EIMS, m/z (relative intensity) 194 (M^+, 100), 165 (79). Anal. ($C_{14}H_{10}O$) C, H.

2-(1-Fluorenyl)-2-hydroxyethylamine (9). A solution of 1-fluorenecarboxaldehyde (**14**; 0.76 g, 3.9 mmol) in CH_2Cl_2 (3 mL) containing ZnI_2 (0.012 g, 0.039 mmol) was treated with cyanotrimethylsilane[11] (0.65 mL, 4.7 mmol) over 5 min. The resulting mixture was allowed to stir for 1 h at room temperature and then was treated with a solution of $LiAlH_4$ in THF (1 M, 7.8 mL, 7.8 mmol). The dark red solution was allowed to stir at room temperature for an additional 3 h, at which time excess $LiAlH_4$ was destroyed by the Fieser method.[16] The liquid was decanted, and the solid was washed with EtOAc (3 × 50 mL). The filtrate was concentrated under reduced pressure, and the residue was partitioned between Et_2O (50 mL) and 1 N HCl (50 mL), the layers were separated, and the organic layer was extracted with 1 N HCl (3 × 50 mL). The combined aqueous pool was washed with Et_2O

(16) Fieser, L. F.; Fieser, M. *Reagents for Organic Synthesis*; Wiley: New York, 1967; p 583.

(100 mL), and then made alkaline with 3 N NaOH (100 mL). The oil that separated was extracted with Et_2O (4 × 100 mL), and the organic pool was dried over K_2CO_3. Concentration under reduced pressure afforded crude **9** as a yellow oil (0.56 g), which crystallized on standing in the air (0.30 g, 1.3 mmol, 34%): mp 166–169 °C; IR (KBr) 3300–2700 (NH_2, OH), 750 cm^{-1}; ^1H NMR (300 MHz) δ 1.20–1.75 (br s, 3 H, exchangeable with D_2O, NH_2, OH), 2.92 (dd, 1 H, J = 13 and 8 Hz, H1), 3.10 (dd, 1 H, J = 13 and 3 Hz, H1), 3.92 (s, 2 H, H9), 4.88–4.94 (m, 1 H, H2), 7.28–7.86 (m, 7 H, Ar H); ^{13}C NMR (75 MHz) δ 35.6, 47.8, 72.6, 119.1, 119.9, 123.8, 124.9, 126.7, 126.8, 127.4, 138.7, 140.1, 141.4, 141.9, 142.9. The HCl salt (**9**·HCl) was formed by passing anhydrous HCl(g) over a rapidly stirred solution of **9** in Et_2O and subsequently recrystallized from MeOH–Et_2O: mp 264 °C dec; EIMS, m/z (relative intensity) 225 (M^+, 8.0), 207 (48), 196 (36), 167 (78), 165 (100). Anal. ($C_{15}H_{16}ClNO$) C, H, N.

2-(2-Fluorenyl)-2-hydroxyethylamine (10). A solution of 2-fluorenecarboxaldehyde (4.00 g, 20.6 mmol) in CH_2Cl_2 (100 mL) was treated sequentially with ZnI_2 (67 mg, 0.21 mmol) and cyanotrimethylsilane[11] (3.3 mL, 25 mmol). The resulting solution was allowed to stir at room temperature for 1 h, at which time the volatiles were removed under reduced pressure, and the residue was dissolved in THF (50 mL). A solution of $LiAlH_4$ in THF (41.2 mL, 41.2 mmol) was then added dropwise at 0 °C over 10 min. After the addition was complete, the reaction mixture was allowed to warm to room temperature and to remain there for 16 h. Excess $LiAlH_4$ was destroyed by the Fieser method,[16] the liquid was decanted, and the aluminum salts were washed with Et_2O (100 mL total). The filtrate was treated with 1 N HCl (100 mL), the layers were separated, and the organic layer was extracted with 1 N HCl (3 × 50 mL). The aqueous pool was made alkaline by the cautious addition of solid KOH and then extracted with EtOAc (5 × 50 mL). The organic phase was dried over K_2CO_3 and then concentrated under reduced pressure, affording crude **10** as a tan solid, which was purified by PCTLC (4 mm, CH_2Cl_2–CH_3OH–H_4NOH, 250:25:1). Pure **10** was obtained as a white solid (2.50 g, 11.1 mmol, 54%): IR (film) 3500–3000 (NH_2, OH), 1590, 830, 760, 720 cm^{-1}; ^1H NMR (300 MHz) δ 1.6 (br s, 3 H, exchangeable with D_2O, NH_2, OH), 2.85 (dd, 1 H, J = 13 and 10 Hz, H1), 3.20 (dd, 1 H, J = 13 and 5 Hz, H1), 3.87 (s, 2 H, H9), 4.64–4.72 (m, 1 H, H2), 7.26–7.75 (m, 7 H, Ar H); ^{13}C NMR (75 MHz) δ 36.86, 49.42, 74.58, 119.71, 119.83, 122.53, 124.63, 125.02, 126.64, 126.72, 141.19, 141.22, 141.40, 143.31, 143.51. The HCl salt (**10**·HCl) was prepared by passing anhydrous HCl(g) over a rapidly stirred solution of **10** in Et_2O and then recrystallized from MeOH–Et_2O: mp 236 °C dec; EIMS, m/z (relative intensity) 225 (M^+, 8), 195 (100), 167 (67), 165 (97), 152 (30). Anal. ($C_{15}H_{16}ClNO$) C, H, N.

1-Fluorenecarbonyl Chloride (16).[9] A mixture of 1-fluorenecarboxylic acid (6.0 g, 29 mmol) and thionyl chloride (20.8 mL, 285 mmol) was warmed under reflux for 10 h. The excess thionyl chloride was removed by distillation (bp 78–81 °C), leaving **16** as a dark colored oil, which was distilled (bulb-to-bulb, 165–170 °C, 0.8 mm) to a white solid (5.9 g, 26 mmol, 91%); mp 108–109 °C (lit.[9] mp 108 °C); ^1H NMR (300 MHz) δ 4.00 (s, 2 H, H9), 7.25–8.15 (m, 7 H, Ar H); ^{13}C NMR (75 MHz) δ 38.66, 119.95, 124.85, 125.88, 126.85, 127.57, 127.67, 127.77, 129.45, 131.48, 139.32, 143.03, 143.21, 146.20; EIMS, m/z (relative intensity) 228 (M^+, 41), 193 (99), 165 (100), 164 (36), 163 (37).

***syn*- and *anti*-1-Acetylfluorene Oxime (18).** A solution of the preceding acid chloride (**16**; 1.0 g, 4.4 mmol) in THF (50 mL) under an argon atmosphere was cooled to −70 °C. Methylmagnesium iodide (2.2 mL, 4.4 mmol, in Et_2O) was introduced through a syringe in a dropwise fashion over 10 min. Upon complete addition of the Grignard reagent, the reaction mixture was allowed to warm to room temperature over 1 h. Water (50 mL) was added, the layers were separated, and the aqueous layer was extracted with Et_2O (3 × 100 mL). The combined organic extracts were dried ($MgSO_4$) and then concentrated to give a dark oil (0.95 g). A solution of the crude ketone (**17**) in EtOH (10 mL) was then treated with hydroxylamine hydrochloride (0.24 g, 3.5 mmol) and sodium acetate (0.94 g, 12 mmol) in H_2O (10 mL), and the resulting solution was boiled on a steam bath for 2 h. After the mixture cooled to room temperature, Et_2O (50 mL) was added, the layers were separated, and the aqueous layer was extracted with Et_2O (3 × 50 mL). The organic pool was dried ($MgSO_4$) and subsequently concentrated under reduced pressure affording crude **18** as an oily solid (0.48 g), which was purified by PCTLC (2 mm, 50% hexanes in EtOAc) yielding the purified oxime mixture as a white solid (0.39 g, 1.7 mmol, 39% from **16**): IR (KBr) 3300–3100 (OH), 750 cm^{-1}; ^1H NMR (80 MHz, two isomers apparent) δ 2.25 and 2.35 (s, 3 H, CH_3), 3.80 and 4.05 (s, 2 H, H9), 7.08–7.75 (m, 7 H, Ar H), 8.25 (br s, 1 H, exchangeable with D_2O, OH); EIMS, m/z (relative intensity) 223 (M^+, 39), 206 (100), 190 (90), 165 (78). Anal. ($C_{15}H_{13}NO$) C, H, N.

1-(1-Fluorenyl)ethylamine Hydrochloride (11·HCl). A solution of 1-acetylfluorene oxime (**18**; 0.20 g, 0.90 mmol) in CH_3OH (100 mL) containing concentrated HCl (0.2 mL, 2.4 mmol) was hydrogenated at 3 atm of H_2 over 5% Pd/C (0.02 g) at room temperature for 21 h. The catalyst was removed by filtration, and the volatiles were removed under reduced pressure, affording **11**·HCl as an off-white solid, which was recrystallized from CH_3OH–Et_2O (0.21 g, 0.85 mmol, 95%): mp 264–266 °C dec; EIMS, m/z (relative intensity) 209 (M^+, 6), 194 (38), 193 (21), 192 (100), 191 (39), 167 (22), 166 (12), 165 (47), 152 (9), 139 (6), 97 (21), 44 (29). Anal. ($C_{15}H_{16}ClN$) C, H, N. Approximately 60 mg of the salt was converted to the free base **11**, which was distilled (bulb-to-bulb, 110–112 °C, 0.04 mm) to a colorless oil (52 mg): IR (film) 3410 and 3300 (NH_2), 3100, 3000, 2950, 1590, 760 cm^{-1}; ^1H NMR (300 MHz) δ 1.47 (d, 3 H, J = 6 Hz, CH_3), 1.58 (br s, 2 H, exchangeable with D_2O, NH_2), 3.90 (s, 2 H, H9), 4.41 (q, 1 H, J = 6 Hz, H1), 7.25–7.86 (m, 7 H, Ar H); ^{13}C NMR (75 MHz) δ 24.4, 35.3, 48.6, 118.3, 119.9, 122.6, 124.9, 126.7, 126.8, 127.5, 139.8, 141.7, 141.8, 142.8, 143.9.

1-(2-Fluorenyl)ethylamine Hydrochloride (12·HCl). A solution of 2-acetylfluorene (5.00 g, 24.0 mmol) in EtOH (50 mL) was boiled with a solution of hydroxylamine hydrochloride (2.5 g, 36 mmol) and sodium acetate (9.8 g, 120 mmol) in H_2O (50 mL) for 1 h. The resulting solution was allowed to cool to room temperature, and the product was extracted into EtOAc (5 × 50 mL). The organic pool was dried ($MgSO_4$) and then concentrated under reduced pressure, affording the oxime mixture **19** as a white solid (5.30 g, 23.8 mmol, 99%): mp 192–193.5 °C. A solution of **19** (4.0 g, 18 mmol) in EtOH (200 mL) containing concentrated HCl (2 mL) was hydrogenated (3 atm of H_2) over 5% Pd/C (0.5 g) for 16 h. The catalyst was removed by filtration, and the volatiles were removed under reduced pressure, affording **12**·HCl as a white solid (3.6 g), which was subsequently converted to the free base **12** (2.8 g). Purification by PCTLC (4 mm, CH_2Cl_2–CH_3OH–H_4NOH, 250:25:1) afforded **12** as a white solid (2.30 g, 11.0 mmol, 61%): mp 89–91 °C; ^1H NMR (300 MHz) δ 1.44 (d, 3 H, J = 6 Hz, CH_3), 1.66 (s, 2 H, exchangeable with D_2O, NH_2), 3.88 (s, 2 H, H9), 4.20 (q, 1 H, J = 6 Hz, H1), 7.29–7.78 (m, 7 H; Ar H). A solution of **12** (2.30 g, 11.0 mmol) in Et_2O was treated with HCl(g) to form the salt (**12**·HCl, 2.42 g), which was recrystallized from MeOH–Et_2O as white needles: mp 249–251 °C dec; EIMS, m/z (relative intensity) 209 (M^+, 15), 194 (100), 167 (23), 165 (29). Anal. ($C_{15}H_{16}ClN$) C, H, N.

Radiochemical PNMT Assay. The assay employed in this investigation has been described elsewhere.[6,15] Briefly, a typical assay mixture consisted of 50 μL of 0.5 M phosphate buffer (pH 8.0), 25 μL of a 10 mM solution of unlabeled AdoMet, 5 μL of [*methyl*-^3H]AdoMet, containing approximately 2 × 10^6 dpm (specific activity ca. 15 Ci/mmol), 25 μL of substrate solution, 25 μL of inhibitor solution (if added), 25 μL of the enzyme preparation, and sufficient water to achieve a final volume of 250 μL. After incubation for 30 min at 37 °C, the reaction was terminated by the addition of 250 μL of 0.5 M borate buffer (pH 10) and extracted with 2 mL of toluene–isoamyl alcohol (7:3). The organic layer was removed and transferred to a scintillation vial and diluted with cocktail for counting. The mode of inhibition was ascertained by inspection of the $1/V$ vs $1/S$ plot of the data.

Acknowledgment. We are grateful to Kevin Criscione for his assistance in the biochemical evaluation of some of the compounds in this study. Research grants from the University of Kansas General Research Fund and from the U.S. Public Health Service (Grant HL 34193) made this work possible.

Registry No. **1**, 7568-93-6; **4**, 110826-95-4; **5**, 110826-96-5; **6**, 98-84-0; **7**, 110826-97-6; **8**, 92247-32-0; **9**, 110826-98-7; **9**·HCl, 110827-08-2; **10**, 110826-99-8; **10**·HCl, 110827-09-3; **11**, 110827-00-4;

11·HCl, 110827-06-0; 12, 110827-01-5; 12·HCl, 54398-93-5; 13, 73728-55-9; 14, 95264-32-7; 15, 110827-02-6; 16, 55341-64-5; 17, 36272-09-0; 18 (isomer 1), 110827-04-8; 18 (isomer 2), 110827-05-9; 19, 110827-07-1; PNMT, 9037-68-7; Me_3SiCN, 7677-24-9; $SOCl_2$, 7719-09-7; MeMgI, 917-64-6; H_2NOH·HCl, 5470-11-1; 1-fluorenecarboxylic acid, 6276-03-5; 2-fluorenecarboxaldehyde, 30084-90-3; 2-(2-fluorenyl)-2-[(trimethylsilyl)oxy]acetonitrile, 110827-03-7; 2-acetylfluorene, 781-73-7.

Potent, Long-Acting Luteinizing Hormone-Releasing Hormone Antagonists Containing New Synthetic Amino Acids: *N*,*N*′-Dialkyl-D-homoarginines[1]

John J. Nestor, Jr.,*† Ram Tahilramani,† Teresa L. Ho,† Georgia I. McRae,‡ and Brian H. Vickery‡

Institutes of Bio-Organic Chemistry and Biological Sciences, Syntex Research, Palo Alto, California 94304.
Received May 26, 1987

A new series of unnatural amino acids has been prepared and incorporated into antagonistic analogues of luteinizing hormone-releasing hormone (LH-RH), on the basis of the hypothesis that stabilization of a proposed phospholipid membrane interaction might yield analogues with high potency and a prolonged duration of action. Thus a series of N^G,$N^{G'}$-dialkyl-D-homoarginine analogues [H-D-hArg(R_2)-OH; R = Me, Et, Pr, *i*-Pr, Bu, hexyl, cyclohexyl, (Et, Me_2NPr)] was conveniently prepared by semisynthesis from D-Lys using the appropriate dialkylcarbodiimide. A number of the analogues that were prepared by using these new amino acid analogues exhibited very high potency and a prolonged duration of action. One of the most potent members of the series, [N-Ac-D-Nal(2)[1],D-pCl-Phe[2],D-Trp[3],D-hArg(Et_2)[6],D-Ala[10]]LH-RH (detirelix), had an ED_{50} of 0.7 μg in the rat antiovulatory assay when administered at noon on proestrus and only 2.5 μg when administered 24 h earlier, at noon on diestrus II. This antagonist is undergoing detailed biological and clinical evaluation.

During the course of the numerous synthetic studies directed toward the understanding of the relationship between structure and biological activity of analogues of luteinizing hormone-releasing hormone (LH-RH; <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), the importance of hydrophobic substitutions for the Gly residue in position 6 was recognized.[2-4] Thus, substitution of the D form of natural amino acids yielded higher potencies as the hydrophobicity of the side chain increased[5,6] (e.g., [D-Trp[6],Pro[9]-NHEt]LH-RH;[5] 100 × LH-RH potency, rat estrus suppression assay[7]). Unnatural D-amino acids of substantially greater hydrophobicity[7-10] were then incorporated, and even higher potencies resulted (e.g., [D-Nal(2)[6]]LH-RH; nafarelin; 200 × LH-RH potency, rat estrus suppression assay[8]). Quantitative structure–activity relationships (QSAR) were derived[11,12] from earlier data which confirmed that the most potent monosubstituted LH-RH agonists should be those with substitutions substantially more hydrophobic than D-Trp. These potency increases were variously ascribed to increased receptor binding[13-15] and/or protection from proteolysis.[16-19] However, in the case of nafarelin, a significant role is played by this molecule's ability to bind to serum albumin[20] and phospholipid membranes (P. Felgner, unpublished). The efficient association with serum albumin (80% bound by equilibrium dialysis measurements), presumably with the hydrophobic binding site on serum albumin, is thought to play a role[21] in the prolonged biological half-life ($t_{1/2}$ = 2.4–3.3 h in women[22] for a dose of 5 μg, sc) of nafarelin. This complex may serve as a depot for the drug which protects it from proteolysis and clearance through the kidney. Thus, a hydrophobic depot effect may be important for highest potency in the LH-RH agonist series.

Since competitive antagonists of LH-RH must be present at the receptors continuously in order to compete with endogenous pulses of LH-RH, a prolonged biological $t_{1/2}$ is even more critical than for LH-RH agonists. Increased hydrophobicity at position 6 as well as high global hydrophobicity evolved as the formula for highest potency in the antagonist series also.[23-25] For example, [*N*-Ac-

† Institute of Bio-Organic Chemistry.
‡ Institute of Biological Sciences.

(1) Contribution No. 207 from the Institute of Bio-Organic Chemistry, Syntex Research. Some of these data were presented in a preliminary form at the 8th American Peptide Symposium, Tucson, AZ, May 1983. 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; Pal(4), 3-(4-pyridyl)alanine; pX-Phe, *p*-halophenylalanine.
(2) Rivier, J.; Brown, M.; Rivier, C.; Ling, N.; Vale, W. In *Peptides* **1976**; Loffet, A., Ed.; Editions de l'Université de Bruxelles: Brussels, 1976; p 427 and references cited therein.
(3) Schally, A. V.; Coy, D. H.; Meyer, C. A. *Annu. Rev. Biochem.* **1978**, *47*, 89 and references cited therein.
(4) Nestor, J. J., Jr. In *LHRH and Its Analogs—Contraceptive and Therapeutic Applications*; Vickery, B. H., Nestor, J. J., Jr., Hafez, E. S. E., Eds.; MTP: Boston, 1984; p 3.
(5) Vale, W.; Rivier, C.; Brown, M.; Ling, N.; Monahan, M.; Rivier, J. In *Clinical Endocrinology*, 5th Suppl.; McIntyre, I., Ed.; Blackwell Scientific: Oxford, 1976; p 2615.
(6) Coy, D. H.; Vilchez-Martinez, J. A.; Coy, E. J.; Schally, A. V. *J. Med. Chem.* **1976**, *19*, 423.
(7) Nestor, J. J., Jr.; Ho, T. L.; Simpson, R. A.; Horner, B. L.; Jones, G. H.; McRae, G. I.; Vickery, B. H. *J. Med. Chem.* **1982**, *25*, 795.
(8) Nestor, J. J., Jr.; Ho, T. L.; Simpson, R. A.; Horner, B. L.; Jones, G. H.; McRae, G. I.; Vickery, B. H. In *Peptides: Synthesis–Structure–Function*; Rich, D. H., Gross, E., Eds.; Pierce Chemical: Rockford, IL, 1981; p 109.
(9) Nestor, J. J., Jr.; Horner, B. L.; Ho, T. L.; Jones, G. H.; McRae, G. I.; Vickery, B. H. *J. Med. Chem.* **1984**, *27*, 320.
(10) Nestor, J. J., Jr.; Ho, T. L.; Tahilramani, R.; Horner, B. L.; Simpson, R. A.; Jones, G. H.; McRae, G. I.; Vickery, B. H. In *LHRH and Its Analogs—Contraceptive and Therapeutic Applications*; Vickery, B. H., Nestor, J. J., Jr., Hafez, E. S. E., Eds.; MTP: Boston, 1984, p 23.
(11) Nadasdi, L.; Medzihradszky, K. *Biochem. Biophys. Res. Commun.* **1981**, *99*, 451.
(12) Zeelen, F. J. *CHEMTECH* **1983**, 419.
(13) Heber, D.; Odell, W. D. *Biochem. Biophys. Res. Commun.* **1978**, *82*, 67.
(14) Perrin, M. H.; Rivier, J. E.; Vale, W. W. *Endocrinology (Baltimore)* **1980**, *106*, 1289.

0022-2623/88/1831-0065$01.50/0