

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

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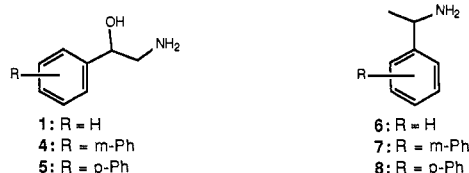
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We have previously described regions of steric bulk tolerance in the aromatic-ring binding site of phenylethanolamine *N*-methyltransferase (PNMT, EC 2.1.1.28) for phenylethanolamine substrates and α -methylbenzylamine inhibitors. For bound substrates, 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, fluorenyl ethanolamine 10 ("*p*-biphenyl") displays a Michaelis constant ($K_m = 26 \mu\text{M}$) that is approximately 10 times lower than that for 9 ("*m*-biphenyl", $K_m = 297 \mu\text{M}$); in the α -methylbenzylamine inhibitors, fluorenyl derivative 11 ("*m*-biphenyl", $K_i = 4.14 \mu\text{M}$) is approximately 40 times better than 12 ("*p*-biphenyl", $K_i = 185 \mu\text{M}$) 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 ($K_m = 82 \mu\text{M}$) as in 10 ($K_m = 26 \mu\text{M}$) and α -methylbenzylamine 7 ($K_i = 89 \mu\text{M}$) as in 11 ($K_i = 4.14 \mu\text{M}$) 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 alkyl² and alicyclic³ 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 hypothesis⁴ 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 ($K_m = 82 \mu\text{M}$, $V_{\text{max}} = 4.3$, $100 \times (V_{\text{max}}/K_m) = 5.2$) relative to 1 itself ($K_m = 70 \mu\text{M}$, $V_{\text{max}} = 2.2$, $100 \times (V_{\text{max}}/K_m) = 3.1$), whereas similar substitution in the meta position as in 4 results in a diminished ability to bind to the active site surface ($K_m = 653 \mu\text{M}$) and a significantly reduced turnover rate ($V_{\text{max}} = 0.46$, $100 \times (V_{\text{max}}/K_m) = 0.07$).



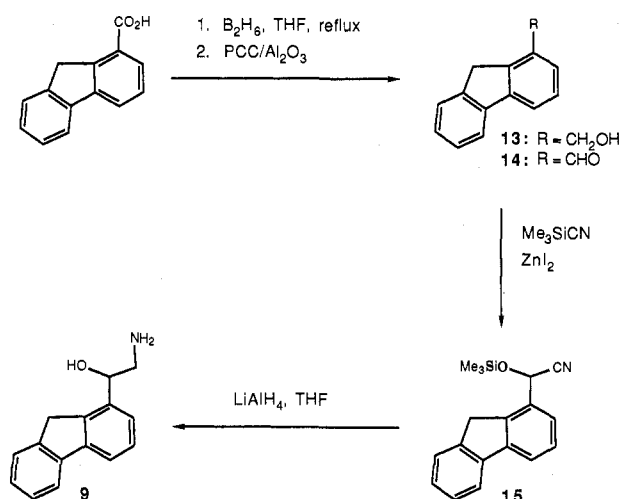
We have also examined the directional nature of steric bulk on the binding of inhibitors of the benzylamine class to PNMT.⁵ In this case, however, we found that phenyl substitution in the meta position of α -methylbenzylamine (6) results in a dramatic enhancement in potency for 7 ($K_i = 89 \mu\text{M}$)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

Conformation-activity relationship studies involving the alkylamine portion of amphetamine⁶ and benzylamine⁷ inhibitors as well as of phenylethanolamine substrates⁸ 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

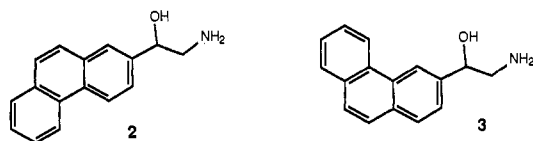
- (1) (a) Paper 11 in our series *Conformationally Defined Adrenergic Agents*; for paper 10, see: Grunewald, G. L.; Markovich, K.; Sall, D. J. *J. Med. Chem.*, in press. (b) Taken, in part, from the Dissertation submitted by J.A.M. to the Graduate School of the University of Kansas for the degree Doctor 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.; Borchartt, R. T.; Grunewald, G. L. *J. Med. Chem.* 1981, 24, 7.
- (3) Davis, D. P.; Borchartt, 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.; Borchartt, R. T.; Grunewald, G. L. *J. Med. Chem.* 1982, 25, 1204.
- (6) Grunewald, G. L.; Borchartt, 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.

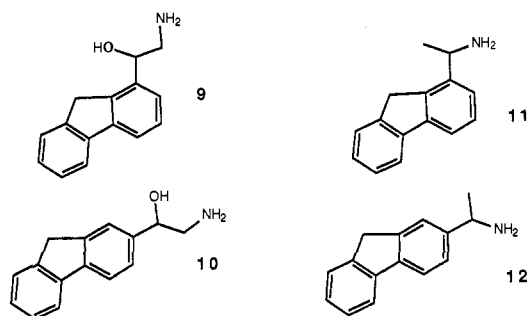
Scheme I



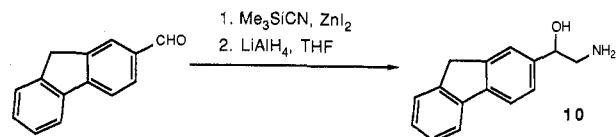
of these different ligands at the PNMT active site. The conformation in which the biphenyl ring system in analogues 4, 5, 7, and 8 interacts with the PNMT active site surface intrigued us. On one hand, the data for phenanthryl derivatives 2 and 3 suggested that a planar arrangement of the biphenyl system 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.



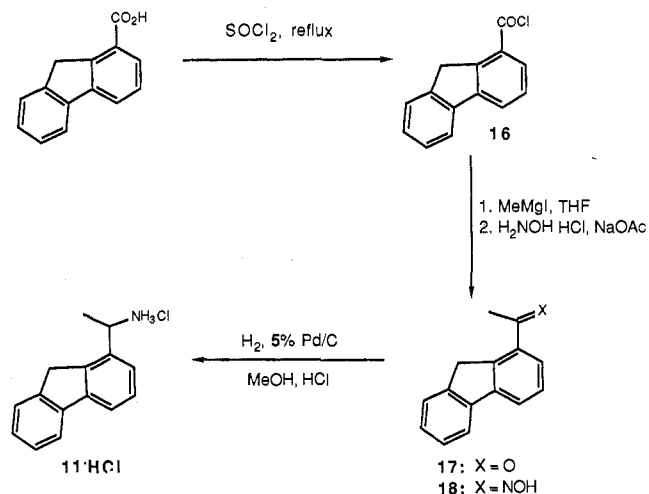
A direct comparison of the kinetic values of ethanolamines 2 and 3 with those of 4 and 5 might clarify this point; however, the data for the phenanthryl analogues are reported only as individual velocities at a particular substrate concentration (i.e. K_m and V_{max} values are not available). In this 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



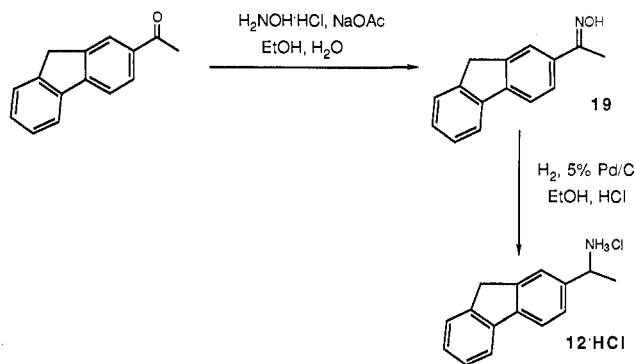
Chemistry

The fluorenylethanolamine 9 was prepared in three steps from commercially available 1-fluorencarboxylic 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 14⁹ 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)cyanohydrin¹¹ 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-fluorencarboxaldehyde (Scheme II).

The preparation of the α -methylbenzylamine analogue 11 is depicted in Scheme III. Commercially available 1-fluorencarboxylic acid was converted to the known acid chloride 16,⁹ which was treated (at $-70^\circ C$ 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 biphenylalkylamines 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 Connert 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 substrates¹⁵ 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; $K_m = 653 \mu\text{M}$) compared with 1 ($K_m = 70 \mu\text{M}$). 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 ($K_m = 297 \mu\text{M}$), 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 ($K_m = 70 \mu\text{M}$, $V_{\max} = 2.2$, $100 \times (V_{\max}/K_m) = 3.1$) with an aromatic ring

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

compound	no.	$K_m \pm \text{SEM}$ (μM)	$V_{\max} \pm \text{SEM}^a$	$100 \times (V_{\max}/K_m)$
	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 ($K_m = 82 \mu\text{M}$) but an enhanced turnover rate ($V_{\max} = 4.3$, $100 \times (V_{\max}/K_m) = 5.2$). Conformational restriction of this biphenyl system (compound 10) results in a two- to threefold decrease in the K_m value ($26 \mu\text{M}$), indicating a slower rate of dissociation of the enzyme-substrate complex, as well as in a decrease in the rate of product formation ($V_{\max} = 1.76$, $100 \times (V_{\max}/K_m) = 6.7$).

Thus, in both the flexible (4 and 5) and conformationally defined (9 and 10) biphenylethanolamine 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 ($K_i = 89 \mu\text{M}$) is more effective in competing for the PNMT active site with phenylethanolamine than is the unsubstituted derivative 6 ($K_i = 460 \mu\text{M}$), while the para-substituted regioisomer 8 is effective only at high inhibitor concentrations ($K_i = 737 \mu\text{M}$).


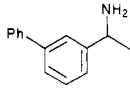
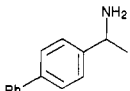
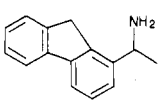
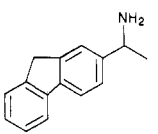
Conformational restriction of 7 and 8 as in fluorenyl derivatives 11 and 12 results, in each case, in an enhanced ability to bind to the active site ($K_i = 4.14 \mu\text{M}$ for 11 and $185 \mu\text{M}$ for 12). Conformational restriction of the biphenyl unit present in the weak inhibitor 8 results 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) Connert, 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.	$K_i \pm \text{SEM}, \mu\text{M}$
	6	460 \pm 52
	7	89 \pm 18
	8	737 \pm 31
	11	4.14 \pm 0.28
	12	185 \pm 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-Fluorenicarboxylic acid, 2-fluorenicarboxaldehyde, 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 (¹H NMR) and carbon nuclear magnetic resonance spectra (¹³C NMR) were obtained on either a Varian FT-80A or XL-300 spectrometer with deuterated chloroform (CDCl₃) as the solvent. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (Me₄Si; 0.0 ppm) for ¹H NMR and CDCl₃ (77.0 ppm) for ¹³C 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 CaSO₄·0.5H₂O 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*-³H]-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 (Et₂O) were distilled from sodium benzophenone ketyl; dry methylene chloride (CH₂Cl₂) 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 N₂ or Ar atmosphere.

1-(Hydroxymethyl)fluorene (13). A solution of 1-fluorenicarboxylic acid (2.00 g, 9.51 mmol) in THF (200 mL) was treated at room temperature with B₂H₆ 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 MgSO₄ 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 CH₂Cl₂ as tan needles; mp 147–148.5 °C; ¹³C 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. (C₁₄H₁₂O) C, H.

1-Fluorenicarboxaldehyde (14).⁹ A solution of 13 (1.0 g, 5.1 mmol) in CH₂Cl₂ (50 mL) was added in one portion to a rapidly stirred suspension of pyridinium chlorochromate on neutral alumina¹⁰ (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); ¹H 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); ¹³C 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. (C₁₄H₁₀O) C, H.

2-(1-Fluorenyl)-2-hydroxyethylamine (9). A solution of 1-fluorenicarboxaldehyde (14; 0.76 g, 3.9 mmol) in CH₂Cl₂ (3 mL) containing ZnI₂ (0.012 g, 0.039 mmol) was treated with cyanotrimethylsilane¹¹ (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 LiAlH₄ 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 LiAlH₄ 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 Et₂O (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 Et₂O

(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 Et₂O (4 × 100 mL), and the organic pool was dried over K₂CO₃. 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 (NH₂, OH), 750 cm⁻¹; ¹H NMR (300 MHz) δ 1.20–1.75 (br s, 3 H, exchangeable with D₂O, NH₂, 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); ¹³C 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 Et₂O and subsequently recrystallized from MeOH–Et₂O: mp 264 °C dec; EIMS, *m/z* (relative intensity) 225 (M⁺, 8.0), 207 (48), 196 (36), 167 (78), 165 (100). Anal. (C₁₅H₁₆ClNO) C, H, N.

2-(2-Fluorenyl)-2-hydroxyethylamine (10). A solution of 2-fluorencarboxaldehyde (4.00 g, 20.6 mmol) in CH₂Cl₂ (100 mL) was treated sequentially with ZnI₂ (67 mg, 0.21 mmol) and cyanotrimethylsilane¹¹ (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 LiAlH₄ 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 LiAlH₄ was destroyed by the Fieser method,¹⁶ the liquid was decanted, and the aluminum salts were washed with Et₂O (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 K₂CO₃ and then concentrated under reduced pressure, affording crude **10** as a tan solid, which was purified by PCTLC (4 mm, CH₂Cl₂–CH₃OH–H₄NOH, 250:25:1). Pure **10** was obtained as a white solid (2.50 g, 11.1 mmol, 54%): IR (film) 3500–3000 (NH₂, OH), 1590, 830, 760, 720 cm⁻¹; ¹H NMR (300 MHz) δ 1.6 (br s, 3 H, exchangeable with D₂O, NH₂, 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); ¹³C 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 Et₂O and then recrystallized from MeOH–Et₂O: mp 236 °C dec; EIMS, *m/z* (relative intensity) 225 (M⁺, 8), 195 (100), 167 (67), 165 (97), 152 (30). Anal. (C₁₅H₁₆ClNO) C, H, N.

1-Fluorencarbonyl Chloride (16).⁹ A mixture of 1-fluorencarboxylic 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); ¹³C 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 Et₂O) 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 Et₂O (3 × 100 mL). The combined organic extracts were dried (MgSO₄) 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 H₂O (10 mL), and the resulting solution was boiled on a steam bath for 2 h. After the mixture cooled to room temperature, Et₂O (50 mL) was added, the layers were separated, and the aqueous layer was extracted with Et₂O (3 × 50 mL). The organic pool was dried (MgSO₄) 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, CH₃), 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 D₂O, OH); EIMS, *m/z* (relative intensity) 223 (M⁺, 39), 206 (100), 190 (90), 165 (78). Anal. (C₁₅H₁₃NO) C, H, N.

1-(1-Fluorenyl)ethylamine Hydrochloride (11·HCl). A solution of 1-acetylfluorene oxime (**18**; 0.20 g, 0.90 mmol) in CH₃OH (100 mL) containing concentrated HCl (0.2 mL, 2.4 mmol) was hydrogenated at 3 atm of H₂ 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 CH₃OH–Et₂O (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. (C₁₅H₁₆ClN) 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 (NH₂), 3100, 3000, 2950, 1590, 760 cm⁻¹; ¹H NMR (300 MHz) δ 1.47 (d, 3 H, *J* = 6 Hz, CH₃), 1.58 (br s, 2 H, exchangeable with D₂O, NH₂), 3.90 (s, 2 H, H9), 4.41 (q, 1 H, *J* = 6 Hz, H1), 7.25–7.86 (m, 7 H, Ar H); ¹³C 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 H₂O (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 (MgSO₄) 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 H₂) 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, CH₂Cl₂–CH₃OH–H₄NOH, 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, CH₃), 1.66 (s, 2 H, exchangeable with D₂O, NH₂), 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 Et₂O was treated with HCl(g) to form the salt (**12**-HCl, 2.42 g), which was recrystallized from MeOH–Et₂O as white needles: mp 249–251 °C dec; EIMS, *m/z* (relative intensity) 209 (M⁺, 15), 194 (100), 167 (23), 165 (29). Anal. (C₁₅H₁₆ClN) 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 × 10⁶ 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; Me₃SiCN, 7677-24-9; SOCl₂,

7719-09-7; MeMgI, 917-64-6; H₂NOH·HCl, 5470-11-1; 1-fluorenicarboxylic acid, 6276-03-5; 2-fluorenicarboxaldehyde, 30084-90-3; 2-(2-fluorenyl)-2-[(trimethylsilyloxy)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-homoarginines¹

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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(R₂)-OH; R = Me, Et, Pr, *i*-Pr, Bu, hexyl, cyclohexyl, (Et, Me₂NPr)] 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-Phe²,D-Trp³,D-hArg(Et)⁶,D-Ala¹⁰]LH-RH (detirelix), had an ED₅₀ of 0.7 μg in the rat antioviulatory 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-NH₂>), 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-Trp⁶,Pro⁹-NHET]LH-RH;⁵ 100 × LH-RH potency, rat estrus suppression assay⁷). Unnatural D-amino acids of substantially greater hydrophobicity⁷⁻¹⁰ were then incorporated, and even higher potencies resulted (e.g., [D-Nal(2)⁶]LH-RH; nafarelin; 200 × LH-RH potency, rat estrus suppression assay⁸). 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 binding¹³⁻¹⁵ 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 albumin²⁰ 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 role²¹ in the prolonged biological half-life ($t_{1/2} = 2.4-3.3$ h in women²² 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-

- (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'}*-dialkyl-homoarginines are abbreviated hArg(R₂) 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.
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