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Conformational Requirements for Norepinephrine Uptake Inhibition by Phenethylamines in Brain Synaptosomes. Effects of α-Alkyl Substitution

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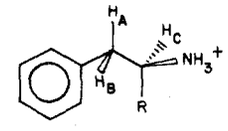
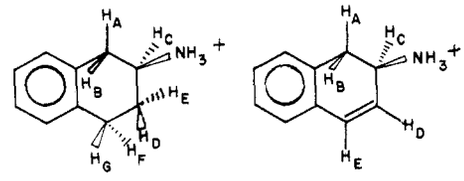
Amphetamine is a strong competitive antagonist of brain synaptosomal [^{3}H]norephinephrine ([^{3}H]NE) uptake. Its α-ethyl analogue is much less active, while 2-aminotetralin and 1,2-dihydro-2-aminonaphthalene, in which the α-ethyl group is tied to the aromatic ring, possess about the same inhibitory potency as amphetamine. The conformational properties of these compounds in solution were studied by ^{1}H and ^{13}C NMR methods. Only small differences between amphetamine and α-ethylphenethylamine hydrochlorides were observed in the relative rotamer populations due to rotation around the C_α–C_β bond of the side chain. In D_2O the gauche conformation is slightly favored, while in $CDCl_3$ the trans conformation is the predominant one. Conformational analysis of the α-ethyl group in α-ethylphenethylamine showed that this group exists in two equally populated conformations in both solvents. It is suggested that these conformations hinder the approach of α-ethylphenethylamine to the brain synaptosomal NE uptake sites.

Several phenethylamine analogues have been shown to inhibit the uptake of [^{3}H]norepinephrine (NE) by either brain synaptosomes[1,2] or heart tissue.[3] Amphetamine (**1**)



1 R = CH_3
2 R = C_2H_5



3 **4**

is known to be a strong competitive antagonist of synaptosomal [^{3}H]NE uptake. This inhibitory ability can vary drastically with relatively small structural modifications and is governed by strict stereoelectronic requirements.[1-7] Evidence from studies with rigid analogues suggests that the pharmacophoric conformation for interaction with the synaptosomal NE uptake sites is one in which the protonated amino group of **1** exists in a trans position with respect to the phenyl ring.[4-7]

In the present study we have examined the effect of α-alkyl substitution on the ability of the drug to inhibit [^{3}H]NE uptake in brain synaptosomes. We have investigated the conformations of α-ethylphenethylamine (**2**) 2-aminotetraline (**3**), and 1,2-dihydro-2-aminonaphthalene (**4**) (Figure 1) using ^{1}H NMR spectroscopy and have compared the results with already published data on the conformation of **1**.[8] The molecular flexibility of these compounds in D_2O was also examined by ^{13}C spin–lattice relaxation time (T_1) measurements.

Results and Discussion

Conformational Analysis. Amphetamine and α-Ethylphenethylamine. The three possible perfectly staggered conformers arising from rotation around the C_α–C_β bond for **1** and **2** are shown in Figure 1. An important ambiguity in the conformational analysis of α-substituted phenethylamines was the uncertainty in the assignment of the diastereotopic benzylic H_A and H_B protons in the ^{1}H NMR spectra. This problem was recently resolved for compound **1** through the stereospecific substitution of each of the two benzylic protons with a deuterium atom.[8] The ^{1}H NMR spectra of the hydrochlorides of the two β-*d* diastereomers in $CDCl_3$ showed that the H_A proton was more downfield than H_B. This was

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Table I. ^{1}H NMR Chemical Shifts (δ), Coupling Constants (J, Hz), and Rotamer Distribution (P)[a]

compd	solvent	δ_{H_A}	δ_{H_B}	δ_{H_C}	δ_{H_D}	δ_{H_E}	J_{AC}	J_{BC}	J_{CD}	J_{CE}	P_I	P_{II}	P_{III}	P_{IV}	P_V	P_{VI}
1·HCl[b]	D_2O	2.92	2.94	3.63			7.5	6.9			0.45	0.53	0.02			
	$CDCl_3$	3.22	2.84	3.57			5.1	9.2			0.76	0.21	0.03			
2·HCl	D_2O	2.88	3.09	3.51	1.69	1.75	8.0	6.3	7.2	7.2	0.37	0.60	0.03	0.49	0.49	0.02
	$CDCl_3$	3.21	2.94	3.38	1.72	1.74	5.6	8.8	7.1	7.1	0.71	0.28	0.01	0.48	0.48	0.04
3·HCl	D_2O	3.17	2.91	3.69	1.89	2.25	5.0	9.8	9.7	3.6	1				1	
			(H_F, H_G = 2.97)													
4·HCl	D_2O	3.27	3.10	3.79	6.08	6.90	6.3	6.3	4.8		1				1	
								(J_{DE} = 9.6)								

[a] Conformer ratios were calculated assuming J_t = 11.0 Hz and J_g = 3.5 Hz, which are values obtained from the model compound γ-1,2,5-trimethyl-4-phenyl-4-piperidinol.[21] Values for conformer distribution should be considered as approximate with a possible error of about 10%. The uncertainties involved in these calculations are described in ref 15. [b] From ref 8.

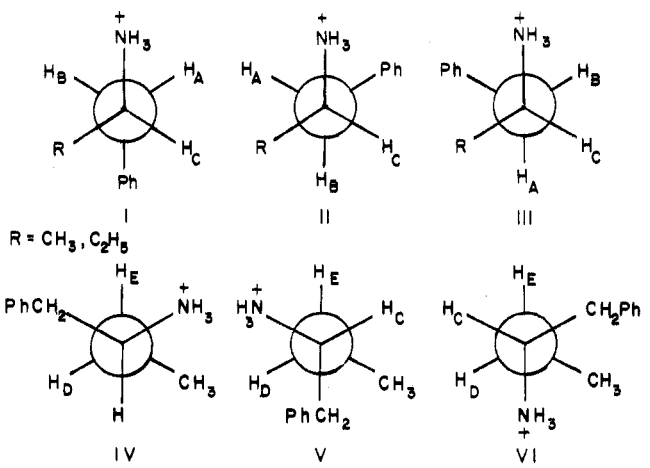

Figure 1. Newman projections of the perfectly staggered conformers around the C_α–C_β bond of **1** and **2** (I–III) and the α side chain CH–CH_2CH_3 bond of **2** (IV–VI).

attributed to a deshielding effect by the $NH_3^+\cdots Cl^-$ ion pair on the gauche vicinal proton. Using a similar argument, we assigned the H_A and H_B benzylic protons in the ^{1}H NMR spectrum of **2** in $CDCl_3$.

H_A and H_B assignment for the hydrochloride salts in D_2O was less straightforward because of the relatively small chemical shift difference between these protons. A more involved method was therefore used for this assignment. We had already observed that in D_2O solutions of the amphetamines β-*d* diastereomeric hydrochlorides the chemical shifts of the H_A and H_B benzylic protons were affected differently by temperature.[13] This was explained as follows: In aqueous solutions the deshielding effect of the NH_3^+ group is reduced by the shielding effect of water molecules hydrogen bonded to the ammonium group. An increase in temperature leads to a reduction in hydrogen bonding, resulting in a more effective deshielding by the NH_3^+ group. Since a trans vicinal proton is considerably further away from the NH_3^+ group than a gauche proton, this effect is much less pronounced on the trans proton. An increase in the temperature of the sample therefore leads to a more marked downfield shift for the gauche proton. The phenomenon of differential chemical-shift changes with temperature enabled us to assign the H_A and H_B benzylic protons in the ^{1}H NMR spectrum of the hydrochloride of **2** in D_2O.

Assignment of the H_A, H_B benzylic protons of **2** allowed us to identify the vicinal J_{AC} and J_{AC} coupling constants from which the conformer distribution around the C_β–C_α bond could be calculated (Table I). The vicinal coupling constants (J_{AC}, J_{BC}) were considered as averaged values arising from a mixture of the three staggered conformers I–III (Figure 1). The relative population of each rotamer was calculated from simple equations, which contain J_g and J_t terms for the coupling constants of the perfectly staggered gauche and trans vicinal protons.[14] J_g and J_t values can be determined by means of semiempirical calculations. They can also be estimated from model compounds, where the HCCH angle is fixed. We had already shown that for the analysis of amphetamine analogues the second method provided better internal consistency,[15] and we made use of this approach in the present study. Our analysis showed that the trans conformer I is the principal rotamer for **2** in $CDCl_3$, although its preponderance is somewhat less than in the case of **1**. In D_2O the gauche conformer II predominates. Conformational analysis around the $C_\beta H_2C_\alpha H$–CH_2CH_3 bond was carried out from the J_{CD}, J_{CE} vicinal coupling constants of the two methylene protons of the ethyl group with the methine proton on C_α. H_D and H_E are magnetically nonequivalent and have slightly different chemical shifts. The complicated ^{1}H multiplets due to these two diastereotopic methylene protons could be successfully analyzed only with the help of a very high-field instrument (500 MHz). Calculation of the relative rotamer population in $CDCl_3$ and in D_2O from the J_{CD}, J_{CE} coupling constants revealed that conformers IV and V were equally favored, while conformer VI made only a minor contribution to the total population.

2-Aminotetralin and 1,2-Dihydro-2-aminonaphthalene. The calculation of ^{1}H–^{1}H vicinal coupling constants between protons of the nonaromatic portions of the hydrochlorides of **3** and **4** enabled us to estimate the geometry of the nonaromatic rings. As we did with previous conformational analyses of cyclic systems,[16] we have assumed that here too the two ring systems occur as one preferred conformer.

From the ^{1}H NMR spectrum of **3**, four vicinal coupling constants could be estimated. These include coupling constants of the C_1 and C_3 protons with the C_2 methine protons. The values of the coupling constants (Table I) are consistent with two axial–axial and two axial–equatorial couplings. These coupling constants indicate that the saturated ring of **3** exists as a slightly distorted chair. Using the Karplus equation[17] and following previously described procedures,[15,16] we calculated the following dihedral angles: ϕ ($H_AC_1C_2H_C$) = 54°, ϕ ($H_BC_1C_2H_C$) = 149°, ϕ ($H_CC_2C_3H_D$) = 149°, and ϕ ($H_EC_1C_2H_C$) = 60°.

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Table II. ^{13}C Chemical Shifts (ppm),[a] T_1's (s),[b] and τ_{eff} (ps)

compd		C_1	C_2	C_3	C_4	C_5	C_6	C_β	C_α	CH_2	CH_3
1·HCl	δ	137.2	129.9	130.4	128.3	130.4	129.9	41.0	49.9		18.3
	T_1		2.71	2.76	1.83	2.76	2.71	1.50	2.74		1.66
	τ_{eff}		16.7	16.4	24.8	16.4	16.7	15.1	16.5		9.1
2·HCl	δ	137.0	130.0	130.5	128.4	130.5	130.0	38.7	55.5	25.9	9.9
	T_1		2.28	2.31	1.59	2.31	2.28	1.23	2.23	1.51	3.14
	τ_{eff}		19.8	19.6	28.5	19.6	19.8	18.4	20.3	15.0	4.8
compd		C_9	C_{10}	C_5	C_6	C_7	C_8	C_1	C_2	C_3	C_4
3·HCl	δ	145.0	142.3	129.8	127.8	127.3	130.2	33.8	48.5	27.6	27.3
	T_1			2.19	1.53	1.82	2.28	1.33	2.30	1.34	1.31
	τ_{eff}			20.7	29.6	24.9	19.9	17.0	19.7	16.9	17.3
4·HCl	δ	132.7	131.9	129.9	128.8	129.8	128.3	32.0	46.1	123.1	133.7
	T_1			2.95	2.53	2.95	3.00	1.86	3.37	2.73	3.03
	τ_{eff}			15.3	17.9	15.3	15.1	12.2	13.4	16.6	15.0

[a] Measured from dioxane, which was used as an internal standard (δ 67.4). [b] Measurements were made with 0.75 M solutions of the compounds in D_2O at 37 °C. The results given are the average of three determinations.

In 4 the vicinal coupling constants between the two C_1 protons and the methine proton on C_2 are equal, indicating that the aliphatic ring is considerably flattened. We calculated the following dihedral angles: ϕ ($H_AC_1C_2H_C$) = 48°, ϕ ($H_BC_1C_2H_C$) = 134°, and ϕ ($H_CC_2C_3H_D$) = 55°.

Molecular Flexibility. Information on the molecular dynamics of the compounds included in this study was obtained from the ^{13}C τ_{eff} values of all protonated carbons (Table II). Since the T_1 values for all of the compounds were measured under identical conditions of temperature and concentration, the observed differences in the T_1 values of corresponding carbons in these molecules (Table II) reflect a variation in dynamic behavior of the compounds in solution. In amphetamine hydrochloride, ortho and meta phenyl ring carbons and the α-methine side-chain carbon have approximately similar T_1 and τ_{eff} values, while the β-methylene carbon has a slightly lower τ_{eff}. This reflects flexibility around the C_1–Ph bond. The low T_1 value of the para carbon of 1 can be attributed to the anisotropic motion of the phenyl ring. It is known that monosubstituted phenyl rings rotate around the C_2 symmetry axis, which runs through the C_1 and C_4 carbons and is coincident with the C_4–H bond. This results in more effective relaxation and lower T_1 values for C_4.[18] A similar effect is observed in the phenyl ring of 2. The least-restricted proton in 7 is the side-chain α-methyl group, which has the lowest ^{13}C τ_{eff} value. Nevertheless, this group apparently experiences some degree of restricted rotation, as is revealed when its τ_{eff} is compared to that of the freely rotating CH_2CH_3 methyl group in 2.

In 2, where the α-methyl group of 1 is substituted with an ethyl group, the T_1 values are smaller (longer τ_{eff}) than in 1, indicating overall slower rotation of the entire molecule. The τ_{eff} values of the ethyl carbons are quite revealing. The methylene carbon has a strikingly higher τ_{eff} than the methyl carbon of the ethyl group, reflecting slow rotation around the CH–CH_2 bond. On the other hand, the methyl carbon of the ethyl group experiences very fast rotation with no apparent steric constraints. This last piece of information proved very helpful in the conformational analysis of the α-ethyl group, since it allowed us to eliminate from consideration conformations involving a sterically hindered methyl group.

Except for C_6, all protonated carbons for each of the two cyclic analogues (3 and 4) have similar τ_{eff} values, indicating that these carbons tumble in unison. The low T_1 values for C_6 in both compounds is again a reflection of anisotropic motion in both molecules with the preferred axis of rotation running along the C_6–N and C_2–N bonds. A careful examination of the τ_{eff} values for the other carbons reveals some flexibility for the aliphatic portion in 3, which may be due to conformational ring inversion (breathing) superimposed over the overall tumbling motion of the molecule. This type of motion is less prominent in 4.

Table III. IC_{50} Values of Compounds Studied as [^{3}H]Norepinephrine Uptake Inhibitors[a]

compd	IC_{50}, M
(±)-1·HCl	4.9×10^{-7}
(±)-2·HCl	6.5×10^{-6}
(±)-3·HCl	8.5×10^{-7}
(±)-4·HCl	6.5×10^{-7}

[a] The synaptosomal preparations were incubated for 6 min in a modified Kreb's Ringer solution containing 0.1% ascorbic acid, 10 μM pargyline, radioactive NE (10^7 M), and various concentrations of the inhibitor to be studied. At least seven concentrations of each inhibitor were tested for the IC_{50} estimations. IC_{50} values are the average of two to four duplicate experiments.

Inhibition of Synaptosomal [^{3}H]NE Uptake. (±)-Amphetamine is a strong inhibitor of the uptake of [^{3}H]norephinephrine ([^{3}H]NE) into brain synaptosomes (Table III). An increase in the length of the α side chain by one carbon unit to give 2 results in a decrease of inhibitory potency by over 10-fold. Most of this inhibitory activity is restored when the α-ethyl group is tied back on the aromatic ring to give 3 or 4 (Table III). The stereoselectivity of these compounds is discussed elsewhere.[19]

From [^{3}H]NE uptake inhibition studies with rigid analogues of amphetamine it has been established that the pharmacophoric conformation at the uptake sites involves an antiperiplanar more or less coplanar alignment of the phenyl ring and the protonated amino group (conformation I in Figure 2).[4-7] Our results confirm previous observations. The trans pharmacophoric conformation is less predominant for the active compound 1 in aqueous media. However, this conformation predominates in $CDCl_3$. This is most probably due to the formation of an ion pair between the protonated ammonium group and the chloride anion.[20] Such a condition could very well reflect the

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(20) We have obtained evidence for such ion pair formation with other analogues, when we observed changes in rotamer distribution around the C_β–C_α bond when the anion was varied. Bulkier anions showed increased preference for the trans conformer.

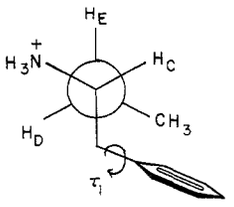

Figure 2. Twisting of the phenyl ring around the Ph–C bond (τ_1) in conformation V of Figure 1, allowing for free rotation of the CH_2CH_3 methyl group in α-ethylphenethylamine.

situation at the active site, where the ammonium group would be expected to first interact with a corresponding anionic site of the receptor to form an analogous ion pair.

Our results indicate that the reduced activity of **2** cannot be attributed to conformational preference around the C_α–C_β bond, since no substantial difference exists between **1** and **2** in this molecular property. However, the results from the conformational analysis of the α-ethyl group offer an alternate explanation. The ^1H vicinal coupling constant data show that the ethyl group in **2** exists in two nearly equally populated conformations (IV and V, Figure 2) in both solvents. In both of these conformations, steric factors would hinder the simultaneous interaction of the phenyl and ammonium groups with a relatively planar surface on the active site. In conformation IV, the steric hindrance is due to the CH_2CH_3 methyl group which protrudes beyond the level of the plane joining the ammonium and phenyl groups. With regard to conformation V, the plane of the phenyl ring is, in all likelihood, at an angle with the side chain ($\tau_1 = 90°$ would be the optimum angle, Figure 2). This out-of-plane phenyl ring conformation would not allow proper alignment of this molecule on the active site. The evidence for the out-of-plane conformation comes from the T_1 data that show free rotation (low τ_{eff}) for the CH_2CH_3 methyl group. A conformation in which the phenyl ring was coplanar ($\tau_1 = 0°$) with the side chain would sterically hinder the methyl group rotation and result in considerably higher τ_{eff} values than those observed.

When the two carbons of the α-ethyl group are tied back on the aromatic ring to give **3** and **4**, inhibitory activity at the synaptosomal active uptake sites is restored. According to our argument, this geometry allows simultaneous access of the ammonium and phenyl groups to the flat receptor surfaces.

Experimental Section

The (±) hydrochloride salts of **1** and **2** were prepared according to literature procedures.[9] The (±) hydrochlorides of **3** and **4** were a generous gift of Dr. D. E. Nichols, Purdue University. ^1H NMR spectra were recorded on Bruker WH-270 and WM-500 spectrometers operating at 270 and 500 MHz, respectively, using 0.01 M solutions at 25 °C. Chemical shifts were measured relative to sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (TSP) and tetramethylsilane, which were used as internal standards for D_2O and $CDCl_3$ solutions, respectively.

^{13}C spin–lattice relaxation times (T_1) were measured on a Bruker WP-60 spectrometer operating at 15.08 MHz using 0.75 M solutions in D_2O at 37 °C. EDTA (10^{-4} M) was used to suppress effects of possible paramagnetic impurities. All solutions were degassed by four freeze–pump–thaw cycles to remove all oxygen. T_1 values for all carbons directly attached to protons were determined simultaneously with complete ^1H decoupling. The method used was a (180°–t–90°–T) inversion recovery sequence, where t is experimentally varied and T is equal to at least five times the longest T_1 to be measured. The T_1 calculations were performed on a Nicolet BNC-12 minicomputer using the Bruker T_1 program/II, which estimates the T_1 values from peak intensities. Each reported T_1 value is the average of at least three determinations. Assignment of resonances was made by analogy and with the help of off-resonance decoupling, when necessary.

Spectral Analysis. ^1H chemical shifts and vicinal ^1H–^1H coupling constants for all protons in the side chains of **1** and **2** and in the aliphatic portions of **3** and **4** were extracted from the corresponding ^1H spectra. The two benzylic protons in the α-alkylphenethylamines and those of the C_1 carbon in compounds **3** and **4** were analyzed as the AB portion of an ABC spin system, while the CH protons in C_2 of compounds **3** and **4** were analyzed as the M component of ABMXY and ABMX spin systems, respectively. In addition, the methylene protons of the α-ethyl group in **2** and the protons on the C_3 carbon of **3** were considered as the MN portion of $AMNX_3$ and AMNXY systems, respectively. Initial estimates of the spectral parameters were obtained by standard methods[10,11] and then refined by spectral stimulation with the Nicolet ITRCAL program.

Molecular Flexibility. The ^{13}C T_1 values for all protonated carbons were used to calculate the corresponding effective correlation times (τ_{eff}),[12] which measure the period of molecular reorientation of a C–H vector through a given angular displacement. Effective correlation times can thus serve as a measure for the motion of individual ^{13}C atoms and provide a description of the molecule's dynamic behavior in solution. Such measurements allow us to make semiquantitative comparisons on the flexibility of closely related molecules in solution and also give us information about specific molecular interactions that may affect their flexibility.

Synaptosomal Uptake of [^3H]Norepinephrine. Male Sprague–Dawley rats (150–200 g) were killed by decapitation, and the brain cortices were rapidly dissected, weighed, and homogenized in 0.32 M sucrose by a motor-driven Teflon pestle–glass Potter–Elvehjem homogenizer to obtain a 20% homogenate. A crude nuclei fraction was obtained by centrifugation at 1000g for 15 min in a refrigerated Sorvall RC-2B centrifuge (0–4 °C). The supernatant was decanted and subsequently centrifuged at 11000g for 20 min to pellet the synaptosomes. This crude synaptosomal fraction was resuspended in the same volume of a modified Kreb's Ringer solution (20 mM Na_2HPO_4, 1.2 mM NaH_2PO_4, 0.5 mM $CaCl_2 \cdot 2H_2O$, 5 mM KCl, 125 mM NaCl, 1.2 mM $MgSO_4$, 5 mM glucose) containing 0.1% ascorbic acid and 10 μM pargyline. Incubations were carried out in duplicate. Each tube received 150 μL of tissue suspension and 300 μL of various concentrations of drugs in modified Ringer's solution. After a preequilibration of 3 min at 37 °C, 0.05 μCi of [^3H]NE was added in 50-μL aliquots, together with unlabeled NE to provide a final NE concentration of 1×10^{-7} M. Incubations were continued for 6 min at 37 °C and terminated by the addition of 3 mL of ice-cold modified Ringer's solution. The contents of each tube were rapidly centrifuged (0–4 °C) at 11000g for 20 min. After the supernatant was decanted, the pellet was dispersed thoroughly in 200 μL of 50% ethanol using glass beads. The radioactivity of 100 μL of the latter suspension was then counted by liquid scintillation spectrometry in vials containing 8 mL of Biofluor. The counting efficiency was monitored by internal and external standards. Blank values were obtained by measuring the amount of radioactivity due to [^3H]NE taken up by the synaptosomal preparation in the absence of drugs, when the temperature of the incubation mixture was between 0 and 4 °C.

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