Identification of a 3-Hydroxylated Tacrine Metabolite in Rat and Man: Metabolic Profiling Implications and Pharmacology[1]

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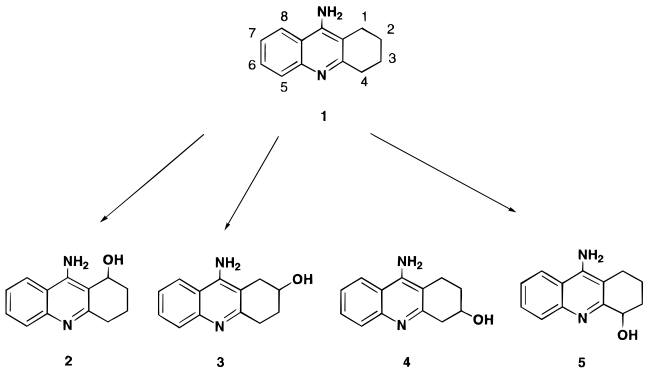
Discrepancies in urinary metabolic profiles in rats administered tacrine (**1**) suggested the presence of an unidentified metabolite of **1**. Chromatographic methods were developed that allowed isolation of a metabolite fraction containing both 1-hydroxytacrine (**2**) and an unknown metabolite from rat urine. Mass spectral analysis indicated this metabolite to be a monohydroxylated derivative, which upon two dimensional COSY NMR analysis could be assigned as 3-hydroxytacrine (**4**). This structural assignment was confirmed by independent synthesis of **4**. Compound 4 was also identified as a human urinary metabolite of **1**. Biologically, **4** was found to have in vitro human red blood cell acetylcholinesterase inhibitory activity similar to that of **2** and 4-hydroxytacrine (**5**) and approximately 8-fold less than that of **1**. These results underscore the need to conduct rigorous structural identification studies, especially in cases where isomeric metabolites are possible, in assessing the accuracy of chromatographic profiling techniques.

Introduction

Tacrine (**1**, Cognex) is a cholinesterase inhibitor with demonstrated efficacy in treating patients with mild to moderate Alzheimer's disease (AD),[1,2] a condition associated with decreased central cholinergic function.[3,4] In addition to gastrointestinal side effects, tacrine administration to AD patients has associated a relatively high incidence, up to 50% of patients treated, of asymptomatic elevations of liver marker enzymes (alanine aminotransferase (ALT)).[5−7] The metabolic fate of **1** in animals and man is characterized by rapid and extensive oxidative metabolism with marked differences in species plasma and urinary metabolic profiles.[8−12] Clearly, identification of these metabolites and assessing their pharmacological properties are necessary to gain insight into the contribution of metabolites to the overall pharmacological profile of **1**. While investigating the urinary metabolic profile of **1** in rat, we observed a difference in relative amounts of 1-, 2-, and 4-hydroxytacrine metabolites (**2**, **3**, and **5**, respectively) (Scheme 1) quantitated using our analytical method compared to results reported by Hsu et al.[12] This observed difference could not be explained by dose, route of administration, or collection period. Hsu et al. found **2** and **3** as major urinary excretion products, while we observed **2** and **5** as major urinary metabolites. To rationalize this apparent discrepancy, we conducted a series of chromatographic and spectral investigations ultimately leading to the identification and characterization of a new tacrine metabolite, namely, 3-hydroxytacrine (**4**). The identification and synthesis of **4** and its cholinesterase inhibition properties are described below.

Scheme 1. Metabolism of **1** in Rat



Chemistry

The synthesis of 3-hydroxytacrine (**4**) is outlined in Scheme 2. The general synthetic strategy utilized by Shutske[13] for the synthesis of dihydroxytacrine derivatives was applied to the synthesis of **7**. The tetrahydroaminoacridine ring system was built with a phenyldimethylsilyl group as a masked form of the desired hydroxy functionality. Attempts to build the ring system with a protected alcohol in the 3-position consistently led to the formation of 1,2-dihydro-9-aminoacridine (an elimination byproduct) as the only isolated product. Briefly, 1,3-dione **7**[14] was condensed with anthranilonitrile under Dean−Stark conditions to give **8** in 47% yield. Cyclization of **8** was accomplished with zinc chloride as catalyst to produce **9** in 80% yield. Reduction of the carbonyl group of **9** was efficiently accomplished with $^{t}BuNH_2-BH_3$ and $AlCl_3$ in CH_2Cl_2 at 0 °C.[15] Conversion of the phenyldimethylsilyl group of **10** into the desired hydroxy group was accomplished by a two-step sequence involving treatment of **10** with $HBF_4 \cdot Et_2O$ to give crude **11** in 60% yield and oxidation of **11** with basic H_2O_2/KF to give the desired compound **4** in 22% yield for the final step.

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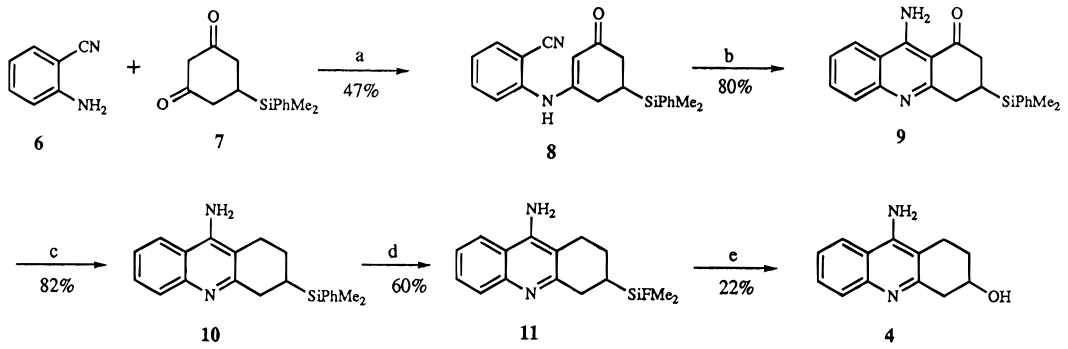
Scheme 2. Synthetic Route to **4**[a]



[a] Reagents: (a) *p*-TsOH·H_2O, benzene, reflux; (b) $ZnCl_2$, toluene, reflux; (c) $AlCl_3$, $^tBuNH_2$·BH_3, CH_2Cl_2, 0 °C; (d) HBF_4·Et_2O, CH_2Cl_2, room temperature; (e) H_2O_2, KF, $NaHCO_3$, 0 °C.

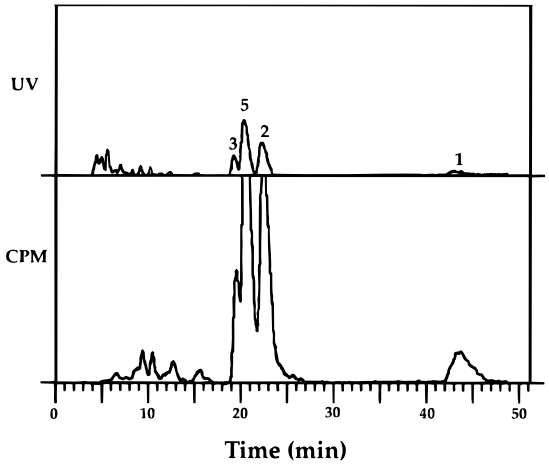


Figure 1. Representative HPLC radioactivity chromatogram of 0−4 h urine from rats administered a single oral 18 mg/kg dose of [^{14}C]**1** using method A.

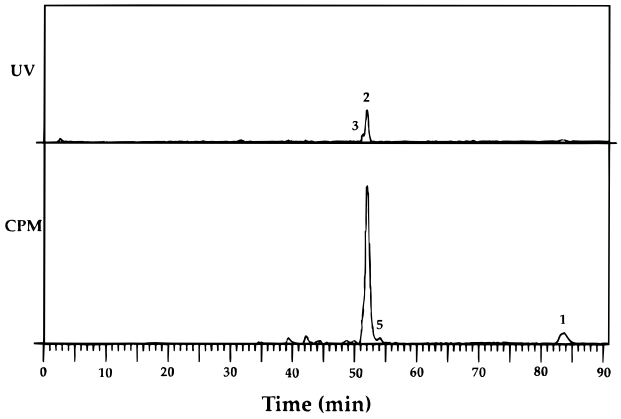


Figure 2. Representative HPLC radioactivity chromatogram of 0−4 h urine from rats administered a single oral 18 mg/kg dose of [^{14}C]**1** using method B.

Biochemistry

In order to investigate the relative contribution of **2**−**5** to the pharmacological action of **1**, their inhibition of red blood cell acetylcholinesterase from mouse, rat, monkey, and man was evaluated using a modified Ellman assay.[16]

Results and Discussion

Bioanalytical Data. Analysis of synthetic standards of **2**, **3**, and **5** as well as **1**, individually and as a mixture, by an isocratic chromatographic method (method A) resulted in an elution sequence of **3**, **5**, **2**, and **1**. HPLC radioactivity profiling using method A of a directly injected aliquot of 0−4 h urine from a rat administered a single 18 mg/kg oral dose of [^{14}C]**1** showed a series of radiolabeled peaks corresponding to **3**, **5**, and **2** and a peak with identical retention properties as **1** (Figure 1). Metabolites **5** and **2** were present in greatest amounts. These results differed from those reported by Hsu et al.[12] where **3** and **2** were found as major tacrine-derived metabolites in rat urine. Given similarities in the dose, route of administration, and collection periods, we surmised that this discrepancy may have been the result of differences in HPLC methods where Hsu et al.[12] used a phenyl column while we used a cyano column. These differences in the urinary metabolic profiles led us to develop a C-18 reversed-phase gradient HPLC method (method B) where the elution order was **3**, **2**, **5**, and **1**. HPLC radioactivity profile analysis of a directly injected aliquot of 0−4 h rat urine from the same animals revealed **2** as the sole major metabolite with minor amounts of **3** and **5** (Figure 2). Again, unchanged **1** was present in lesser amounts.

The differences in metabolic profile between methods A and B with respect to the amounts of **5** and **2** present in rat urine suggested to us to examine the corresponding isocratic and gradient chromatographic peaks by thermospray (TSI)−LC/MS analysis. The relatively "soft" sample ionization technique of TSI−LC/MS resulted in abundant formation of protonated molecular ions at *m*/*z* 215 corresponding to monohydroxylated derivatives of **1** at retention times consistent with standards of **2** and **5**. There was no evidence for any additional metabolites of **1** in the peaks corresponding to **2** and **5** using either method A or B by TSI−LC/MS. These chromatographic and spectroscopic results suggested that an unknown monohydroxylated derivative of **1** was coeluting with either **5** or **2**, depending on whether method A or B, respectively, was used.

To further elucidate the structure of this unknown metabolite, 0−4 h urine from rats dosed orally with 2 mg/kg (13 μCi) [^{14}C]**1** was extracted and fractionated by semipreparative HPLC (method C). A fraction with similar elution properties as **2** was collected and concentrated. An aliquot of this isolate was chromatographed on the analytical gradient system (method B) and shown to be one peak coeluting with **2**. The isolate fraction was dissolved in deuteriomethanol and subjected to ^{1}H NMR analysis.

The various metabolites resulting from monohydroxylation of the aliphatic ring portion of tacrine are distinguishable by the chemical shifts of the hydroxylated methine proton and the remaining methylene protons. The ^{1}H NMR spectrum of the isolate fraction

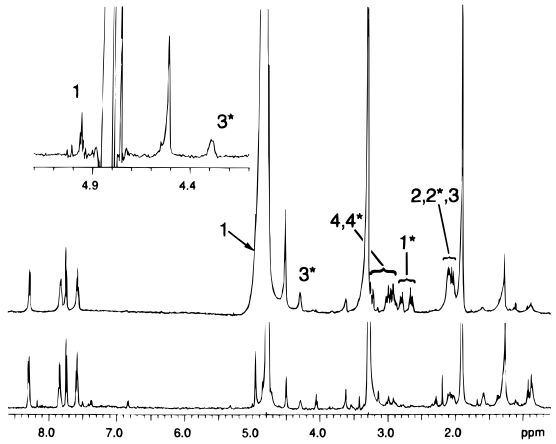


Figure 3. ^{1}H NMR spectra (500 MHz) of major metabolites of **1** from rat and human. Bottom: human urine metabolite fraction. Top: rat urine metabolite fraction. Inset: expansion of the region near water in the rat metabolite spectrum after resolution enhancement and spline base-line correction to resolve the signal at 4.95 ppm. The numbering without and with asterisks refers to **2** and **4**, respectively. Solution and spectral conditions are given in the material and methods section.

showed the presence of two multiplet resonances at δ 4.29 and 4.95 in the chemical shift region expected for hydroxy-bearing methines (Figure 3, top trace). The signal resonating at δ 4.95 and the chromatographic retention time of the isolate were consistent with **2**. The signal resonating at δ 4.29 was close to the known chemical shift of the 2-CH in **3** at δ 4.34. The identity of this unknown as **3** was unlikely based on chromatographic differences, and results from a titration experiment where 3 was added to the rat isolate produced a shoulder on the nonresolved multiplet at δ 4.29 as well as an additional set of 1-methylenes. These peaks increased in intensity with increasing amounts of added **3**. The identity of the unknown as **5** was also ruled out based on its chromatographic retention properties and distinguishing chemical shifts, most notably the C4 proton at δ 4.89. Since mass spectral data indicated that the unknown was a monohydroxylated derivative of **1**, it was suspected that the methine at δ 4.29 came from **4**. To further elucidate the structure of the hydroxy isomer, two-dimensional COSY spectra were obtained that allowed correlation of the methine protons with the methylenes on adjacent carbons (Figure 4A). This spectrum showed the expected coupling patterns for **2** and **4**.

HPLC radioactivity profiling of urine obtained from human subjects administered a single oral 40 mg dose of (100 μCi) [^{14}C]**1** using method B showed **1** to be extensively metabolized with no single major metabolite (Figure 5). A 0−4 h aliquot of urine was subjected to the above extraction and purification techniques to isolate the component coeluting with **2**. The isolate was dissolved in deuteriomethanol and subjected to ^{1}H NMR analysis. The ^{1}H NMR spectrum of the isolated human metabolite was similar to that observed in rat and is shown in the bottom trace of Figure 3. Two-dimensional COSY spectra recorded on the human isolate resulted in coupling patterns consistent with the presence of **2** and **4** in the isolate and are shown in Figure 4B. Comparative two-dimensional COSY experiments run on synthetic **2** and **4** confirmed that the coupling patterns were identical with those seen in the isolated metabolite fractions (Figure 4C,D).

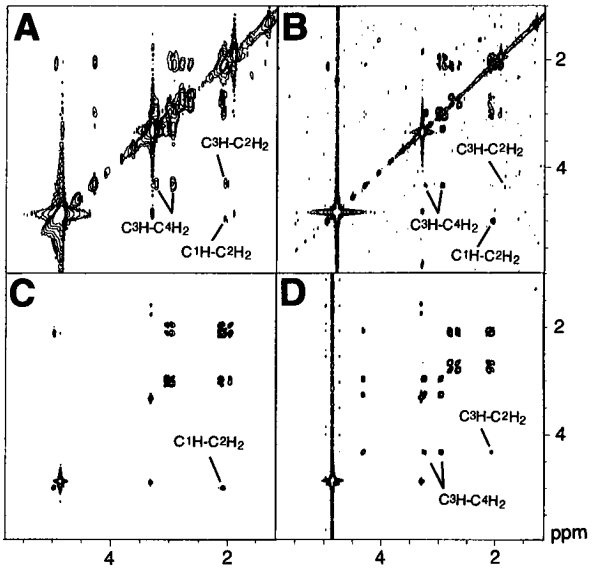


Figure 4. Aliphatic regions of the 500 MHz two-dimensional COSY spectra of **2** and **4**: A, rat urine metabolite fraction; B, human urine metabolite fraction; C, **2**; D, **4**. Cross-peaks critical for assignment of the site of hydroxylation are indicated on the plots. Solution and spectral conditions are given in the material and methods section.

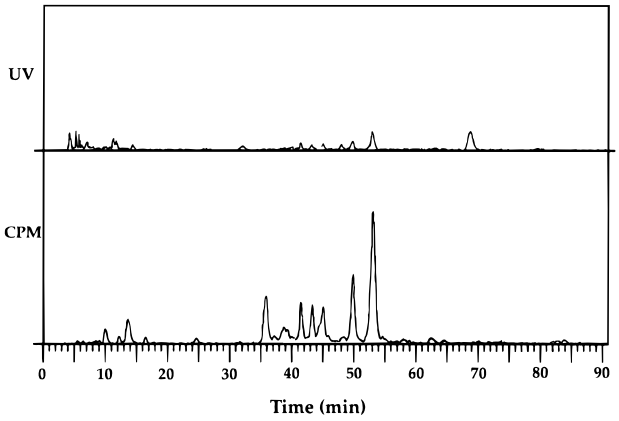


Figure 5. Representative HPLC radioactivity chromatogram of 0−4 h urine from human subjects administered a single oral 40 mg dose of [^{14}C]**1** using method B. HPLC peak eluting at 53 min coelutes with **2**. Compound **5** coelutes with peak eluting at 54.5 min.

Biochemistry. In order to investigate the relative contribution of **2**−**5** to the pharmacological action of **1**, in vitro IC_{50} values for the inhibition of red blood cell acetylcholinesterase were obtained for **1** and the monohydroxylated metabolites in mouse, rat, monkey, and man (Table 1). The IC_{50} for **4** was 8-fold higher than that of **1** in the human preparation and similar in magnitude to that determined for **2** and **5**. Metabolite **3** displayed the weakest inhibitory activity of the hydroxylated regiomers in all preparations examined.

Conclusion

A new monohydroxylated metabolite of **1**, **4**, was identified in urine from rats and humans following oral **1** administration. This metabolite, depending on chromatographic conditions, can coelute with either **2** or **5**. Interestingly, the in vitro AchE inhibition activities of **4** are similar to those of **2** and **5** and differ significantly from that of the less potent regiomer **3**. Whether or not this new metabolite significantly contributes to the central cholinergic effects of **1** in vivo is not known.

Table 1. IC_{50} Values (μM ± SE) for the Inhibition of Red Blood Cell Acetylcholinesterase[a]

	1	2	3	4	5
human	0.078 ± 0.005	0.702 ± 0.054	1.61 ± 0.137	0.623 ± 0.035	0.521 ± 0.017
monkey	0.075 ± 0.004	0.954 ± 0.023	2.30 ± 0.078	0.759 ± 0.036	0.476 ± 0.025
rat	0.046 ± 0.003	0.361 ± 0.023	1.77 ± 0.097	0.483 ± 0.072	0.411 ± 0.012
mouse	0.143 ± 0.010	1.000 ± 0.019	4.82 ± 0.085	1.210 ± 0.164	1.43 ± 0.074

[a] Inhibition assays were performed as described in ref 16.

These results underscore the need to conduct rigorous structural identification studies, especially in cases where isomeric metabolites are possible, in assessing the accuracy of chromatographic profiling techniques.

Experimental Section

General Methods. Air- or moisture-sensitive reactions were carried out in flame-dried glassware under an atmosphere of nitrogen or argon. Organic solutions were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure on a rotary evaporator. Thin-layer chromatography (TLC) was carried out on E. Merck silica gel 60-F254 precoated glass plates (0.25 mm thickness). Medium pressure liquid chromatography (MPLC) was performed with E. Merck silica gel 60, 230−400 mesh ASTM. Melting points were determined on a Thomas Hoover capillary melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian Unityplus-400 NMR spectrometer. Chemical shifts are reported in ppm downfield from tetramethylsilane (internal standard). Mass spectra were recorded on a Finnigan 4500 mass spectrometer or a VG analytical 7070E/HF mass spectrometer; the spectra are described by the molecular peak (M) and its relative intensity as well as the base peak (100%). Elemental analyses were performed on a CEC 240XA elemental analyzer. Where analyses are indicated by the symbols of the elements, the results are within 0.4% of the theoretical values. The syntheses of **2**, **3**, and **5** were accomplished using the procedures detailed by Hsu et al.,[12] while the syntheses of [^{14}C]**1** and [^{14}C]**2** are described by McNally et al.[17]

Synthetic Methods. *N*-[5-(Dimethylphenylsilyl)-3-oxocyclohexen-1-yl]-2-aminobenzonitrile (8). A solution of 30 g (0.10 mol) of crude 5-(dimethylphenylsilyl)cyclohexane-1,3-dione (**7**),[14] 14.1 g (0.10 mol) of anthranilonitrile (**6**), and 3.0 g of *p*-toluenesulfonic acid monohydrate in 500 mL of benzene was refluxed with a Dean−Stark trap for 2 h. The solution was cooled, concentrated in vacuo, dissolved in EtOAc, and washed with saturated $NaHCO_3$ solution and brine. The EtOAc extract was dried ($MgSO_4$), filtered, concentrated, and chromatographed (MPLC: gradient 50−75% EtOAc in hexane) to give 19.3 g (47%) of **8** as a yellow solid. A portion was recrystallized from EtOAc−hexanes to give a light yellow solid, mp 127−130 °C. ^{1}H NMR (DMSO-d_6): 0.33 (6H, d, J = 6.8 Hz), 1.58−1.67 (1H, m), 2.01−2.11 (2H, m), 2.37−2.48 (2H, m), 4.84 (1H, s), 7.38−7.42 (5H, m), 7.55−7.57 (2H, m), 7.71 (1H, dd, J = 8 Hz), 7.85 (1H, d, J = 8 Hz), 9.03 (1H, s). MS (CI): 347 (M + 1, 100). Anal. ($C_{21}H_{22}N_2OSi$) C, H, N.

(±)-9-Amino-3-(dimethylphenylsilyl)-3,4-dihydroacridin-1(2*H*)-one (9). A suspension of 10.0 g (28.86 mmol) of **8** and 11.80 g (86.59 mmol) of $ZnCl_2$ in 750 mL of toluene was refluxed under N_2 for 12 h, with mechanical stirring. The mixture was concentrated and partitioned between $CHCl_3$ and 10% NH_4OH. The $CHCl_3$ extract was dried ($MgSO_4$), filtered, concentrated, and chromatographed (MPLC: EtOAc:hexane, 1:1) to give 8.03 g (80%) of **9** as a light yellow solid. A portion was recrystallized from EtOAc−hexanes to give a white solid, mp 184−187 °C. ^{1}H NMR (DMSO-d_6): 0.35 (6H, d, J = 3.1 Hz), 1.68−1.76 (1H, m), 2.48−2.54 (2H, m), 2.77−2.90 (2H, m), 7.37−7.44 (4H, m), 7.54−7.59 (2H, m), 7.63−7.70 (2H, m), 8.31 (1H, d, J = 8.2 Hz), 8.37 (1H, broad s), 9.92 (1H, broad s). MS (CI): 347 (M + 1, 100). Anal. ($C_{21}H_{22}N_2OSi\cdot0.25H_2O$) C, H, N.

(±)-9-Amino-3-(dimethylphenylsilyl)-1,2,3,4-tetrahydroacridine (10). The reductive deoxygenation procedure of Lau et al.[15] was followed. To a suspension of 10.34 g (77.55 mmol) of $AlCl_3$ in 200 mL of CH_2Cl_2 was added in one portion 13.50 g (155.23 mmol) of borane−*tert*-butylamine complex, at 0 °C under nitrogen. The mixture was stirred at 0 °C for 30 min until a clear solution resulted. A solution of 4.48 g (12.93 mmol) of **9** in 150 mL of CH_2Cl_2 was added dropwise. The reaction mixture was allowed to slowly warm to room temperature overnight. The fluorescent yellow solution was concentrated, the reaction carefully quenched with dropwise addition of ice-cold water, and the mixture basified with concentrated NH_4OH to pH ∼9−10 and extracted into $CHCl_3$. The $CHCl_3$ extract was dried ($MgSO_4$), filtered, and concentrated, and the crude foamy yellow solid was chromatographed ($CHCl_3$:MeOH:NH_4OH, 90:10:1) to give 3.54 g (82%) of **10** as a light yellow solid, mp 141−150 °C. ^{1}H NMR (DMSO-d_6): 0.33 (6H, d, J = 1.4 Hz), 1.23−1.28 (1H, m), 1.44−1.48 (1H, m), 2.02−2.07 (1H, m), 2.42−2.45 (1H, m), 2.58−2.72 (2H, m), 2.76−2.82 (1H, m), 6.30 (2H, s), 7.26 (1H, dd, J = 8 Hz), 7.38−7.41 (3H, m), 7.46 (1H, dd, J = 8 Hz), 7.55−7.60 (3H, m), 8.12 (1H, d, J = 8 Hz). MS (CI): 333 (M + 1, 100). Anal. ($C_{21}H_{24}N_2Si$) C, H, N.

(±)-9-Amino-3-hydroxy-1,2,3,4-tetrahydroacridine (4). The two-step general procedure of Oliver et al.[14] for the replacement of $PhMe_2Si$ by OH was followed. A solution of 2.51 g (7.55 mmol) of **10** in 150 mL of CH_2Cl_2 was treated with 10.0 mL (57.7 mmol) of 85% tetrafluoroboric acid−diethyl ether complex. A solid immediately formed. After 2 h at room temperature, all solids had gone into solution. Another portion of 5.0 mL of 85% tetrafluoroboric acid−diethyl ether complex was added; the solution was stirred for 2 h, concentrated, suspended in ice-cold water, basified with saturated $NaHCO_3$ solution, and extracted into $CHCl_3$. The $CHCl_3$ extract was washed with brine, dried ($MgSO_4$), filtered, and concentrated to give 1.24 g (60%) of crude **11** as a white-yellow solid. This material was used without further purification.

To an ice-cold suspension of 1.07 g (3.9 mmol) of crude **11**, 2.1 g (36.1 mmol) of KF, and 2.6 g (30.9 mmol) of $NaHCO_3$ in 30 mL of MeOH:THF (1:1) was added dropwise 13.5 mL (132.1 mmol) of 30 wt % H_2O_2 solution in water. The mixture was stirred at room temperature for 12 h, filtered through a pad of Celite, concentrated, and partitioned between 10% NH_4OH and $CHCl_3$. The $CHCl_3$ extract was dried ($MgSO_4$), filtered, concentrated, and chromatographed by MPLC ($CHCl_3$:MeOH:NH_4OH, 90:10:1) to give 181 mg (22%) of **4** as a light yellow solid, mp 171−178 °C dec. ^{1}H NMR (DMSO-d_6): 1.69−1.78 (1H, m), 1.98−2.01 (1H, m), 2.46−2.54 (1H, m), 2.66−2.77 (2H, m), 3.02 (1H, dd, J = 3.7, 16.8 Hz), 4.01−4.03 (1H, m), 4.84 (1H, broad s), 6.37 (2H, broad s), 7.28 (1H, dd, J = 8 Hz), 7.48 (1H, dd, J = 8 Hz), 7.63 (1H, d, J = 8 Hz), 8.14 (1H, d, J = 8 Hz). MS (CI): 215 (M + 1, 100). Anal. ($C_{13}H_{14}N_2O\cdot0.25H_2O$) C, H, N.

Analytical and Spectroscopic Methods: HPLC Method A. HPLC isocratic radioactivity analysis was performed with an ALLTECH Econosphere CN analytical column (5 μm, 250 mm × 4.6 i.d.) in series with an Upchurch uptight precolumn packed with C-18 pellicular media (30−40 μm). Radioactivity was monitored online with a Radiomatic IC flow detector with UV detection at 325 nm. The mobile phase consisted of 0.05 M ammonium formate, pH 3.1:acetonitrile (95:5, v/v). Analytes were eluted at 1 mL/min. The flow rate of the scintillant (Flo-Scint III) was set at 3 mL/min.

HPLC Method B. HPLC gradient radioactivity profiling was performed with a Waters μBONDAPAK C-18 column (10 μm, 300 mm × 3.9 i.d.) with the above precolumn, instruments, and conditions. The mobile phase consisted of 0.1 M ammonium acetate, pH 4.0, and acetonitrile. Analytes were eluted at 1 mL/min with a linear gradient starting at 0% acetonitrile for 8 min and increasing to an acetonitrile concentration of 10% over 27 min. The acetonitrile concentration was maintained at 10% for an additional 55 min.

HPLC Method C. Metabolite isolation was accomplished by gradient semipreparative HPLC with a Whatman M-9

ODS-3 column (500 mm × 9.4 i.d.) in series with the above precolumn. The fraction coeluting with **2** was separated from other radiolabeled components with a linear gradient starting at 0% acetonitrile for 10 min and increasing to a final acetonitrile concentration of 10% over 30 min. The acetonitrile concentration was maintained at 10% for an additional 80 min. Analytes were eluted at 5 mL/min with UV monitoring at 325 nm.

LC/MS. Rat and human urine aliquots were analyzed by thermospray LC/MS using HPLC conditions described for methods A and B. Mass spectra were recorded on a VG Trio-2 spectrometer operated in the plasmaspray positive ion detection mode, with a source temperature of 250 °C, capillary temperature of 225 °×b0C, and a discharge current of 0 μA. Full scans (600−150 Da) were taken repetitively at a scan rate of 1 s decade^{-1}.

NMR Spectroscopy. Samples for NMR were prepared by dissolving the lyophilized metabolite in 0.5 mL of 99.96% deuteriomethanol and transferring into 5 mm Wilmad 528 NMR tubes. Synthetic reference standards were similarly prepared by dissolving 0.5 mg of compound in 0.5 mL of 99.96% deuteriomethanol. NMR spectra were collected on a Bruker AMX500 spectrometer. One-dimensional ^{1}H NMR spectra were recorded at 25 and 30 °C using a sweep width of 5530 Hz and 8K complex data points. Signal averaging over 32−1024 scans, depending on sample concentration, was used in conjunction with application of an exponential weighting function (LB = 1.0 or 2.0 Hz) to enhance signal-to-noise in the Fourier transformed data. When necessary, frequency domain resolution was enhanced by application of a Lorentzian-to-Gaussian weighting function (GB = 0.07, LB = −5.0 Hz). Two-dimensional correlated spectroscopy (COSY) experiments[18] were acquired at either 25 or 30 °C. The COSY spectra for metabolite fractions were obtained by recording 128 or 256 t_1 blocks of 96−112 transients each (2.0 s relaxation delay) with a time-domain digital resolution of 10.8 Hz in t_2 and 23.5 or 47 Hz in t_1. The intense water signal was minimized by application of a low-power, selective rf pulse during the relaxation delay. COSY spectra for synthetic standards were acquired by recording 256 t_1 blocks of four transients each with a time-domain digital resolution of 7.8 Hz in t_2 and 15.6 Hz in t_1 and a relaxation delay of 1.3 s. In all cases the COSY data were treated with a sine-bell window function and zero-filled once in t_2 and twice in t_1 prior to Fourier transformation and magnitude calculation.

Biochemistry Methods: Acetylcholinesterase Assay. A microplate colorimetric Ellman assay[16] was used for the determination of enzyme activity. The substrate solution contained 500 μM acetylthiocholine and 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) in 50 mM sodium phosphate buffer, pH 8.0. The assays were initiated by adding 100 μL of an appropriate dilution of acetylcholinesterase (AchE) (1:20 for mouse and rat, 1:100 for monkey and human) to 200 μL of substrate solution containing concentrations of inhibitor ranging from 100 μM to 1 nM. Blank (background) values were determined by the addition of buffer with no enzyme to the substrate solution. All assays were performed in triplicate. The microplates were read in the kinetic mode of a Molecular Devices Thermomax microplate reader at room temperature and set for 405 nm wavelength. The dose of each compound producing 50% inhibition of enzyme activity (IC_{50}) was extrapolated from the dose−response curve. Human AchE (type XIII) was obtained from human red blood cell ghosts purchased from Sigma Chemical Co. Red blood cells from monkey, rat, and mouse were collected after washing in isotonic saline and then osmotically lysed in distilled water. The membranes were pelleted at 20000*g* for 30 min at 4 °C. The AchE was extracted from the membranes by homogenizing in 5 vol of 50 mM borate buffer, pH 8.0, containing 1% Triton X-100. The homogenate was centrifuged at 10000*g* for 30 min at 4 °C. The supernatant was removed, aliquoted in 1 mL Eppendorf tubes, and stored at −70 °C until needed. All other assay materials were obtained from Sigma Chemical Co.

Metabolism Studies: Rat Experiments. Fasted male Wistar rats were administered [^{14}C]**1** at either 18 mg/kg (120 μCi) or 2 mg/kg (13 μCi) as single oral solution free base equivalent doses. Urine collected through 24 h was analyzed by HPLC radioactivity profiling.

Human Experiment. As part of the drug development process for **1**, healthy male volunteers received single oral 40 mg free base equivalent (100 μCi) solution doses of [^{14}C]**1**. Urine collected through 24 h was analyzed by HPLC radioactivity profiling.

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