Brief Articles

Novel Heterobivalent Tacrine Derivatives as Cholinesterase Inhibitors with Notable Selectivity Toward Butyrylcholinesterase

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Received June 21, 2006

Two series of novel heterobivalent tacrine derivatives were synthesized. A trimethoxy substituted benzene was linked to the tacrine moiety by a hydrazide-based linker. The compounds were evaluated as cholinesterase inhibitors, and trimethoxybenzoic acid derivatives with 11- or 12-atom spacers were the most potent inhibitors of human acetylcholinesterase. The inhibitors showed a surprising selectivity toward human butyrylcholinesterase, where several trimethoxyphenylpropionic acid derivatives had IC_{50} values less than 250 pM.

Introduction

Acetylcholinesterase is the target of drugs that inhibit the hydrolysis of acetylcholine and alleviate the cholinergic deficit associated with Alzheimer's disease.[1] Recently butyrylcholinesterase was considered a potential target because it also regulates acetylcholine levels. The concurrent inhibition of both cholinesterases was shown to provide additional benefits in Alzheimer's disease.[2]

The peripheral binding site (PAS[a]) of acetylcholinesterase located at the entrance of the active-site gorge was shown to be involved in the formation of amyloid-β fibrils.[3] The PAS was recently reported to facilitate the gorge penetration of ligands by π−cation interactions.[4] Tacrine (9-amino-1,2,3,4-tetrahydroacridine), a potent inhibitor for both acetylcholinesterase and butyrylcholinesterase, was the first approved drug for the symptomatic treatment of Alzheimer's disease. Bivalent tacrine-based inhibitors with increased potency were developed that address both the active and peripheral site of acetylcholinesterase. Conceptually, such compounds might simultaneously inhibit acetylcholine hydrolysis and retard the assembly of amyloid-β fibrils. Moreover, π−π or π−cation interactions with the peripheral binding site, particularly with the aromatic moiety of Trp279, might provide selectivity toward acetylcholinesterase because Trp279 is replaced by alanine in butyrylcholinesterase (amino acid numbering throughout this article refers to acetylcholinesterase from *Torpedo californica*).

Part of the work on bivalent inhibitors connected two 1,2,3,4-tetrahydroacridine moieties by an alkyl tether, where a heptylene linker was found to be the optimal spacer.[5] Other studies focused on peripheral π−cation interactions of tacrine heterodimers.[6] Looking for further interaction sites along the active-site gorge, Tyr121 was found to form a hydrogen bond with a protonated nitrogen inserted into the tether.[7] The utilization of π−π interactions at the peripheral binding site was studied for donepezil and related inhibitors.[8] For natural compounds of the arisugacin or territrem family, containing electron-rich aromatics, docking studies proposed a π−π stacking between these moieties and Trp279.[9] The hypothesis that electron-rich aromatics will bind to the peripheral binding site was further established when comparing aromatics of differing electron density.[10]

Following this hypothesis, we decided to explore the potential of simple electron-rich aromatics and to design inhibitors of acetylcholinesterase with tacrine as the active-site binding part. A trimethoxy substituted benzene moiety was chosen as the electron-rich aromatic unit, and ω-aminocarboxylic acids were used to construct the linker. Here, we report a new class of tacrine heterodimers that show the expected activity against acetylcholinesterase but were additionally found to be very potent inhibitors of butyrylcholinesterase.

Chemistry

As the alkylation of 9-amino-1,2,3,4-tetrahydroacridine is impaired by a lack of regioselectivity and as the nucleophilic substitution of 9-chloro-1,2,3,4-tetrahydroacridine by diaminoalkanes often resulted in low yields, we considered using the hydrazine moiety to combine the tetrahydroacridine ring with the linker. Our experiments revealed that the reaction of 9-hydrazino-1,2,3,4-tetrahydroacridine with carboxylic esters failed (data not shown), but we succeeded with the opposite approach reacting 9-chloro-1,2,3,4-tetrahydroacridine with carboxylic hydrazides. Thus, novel heterobivalent derivatives were designed in which the tacrine moiety, with retained basicity, was connected to the trimethoxybenzene unit through a linker of varying length. One carboxamide and one hydrazide functionality were placed in the linker, which might allow for hydrogen bonds with mid-gorge amino acid residues, e.g., with Tyr121.

En route to the truncated compounds **15a** and **16a** (Scheme 1, see also Supporting Information), 3,4,5-trimethoxybenzoic acid (**1**) and 3-(3,4,5-trimethoxyphenyl)propionic acid (**2**) were converted to methyl esters **3** and **4** using oxalyl chloride and sodium methylate. Treatment with hydrazine hydrate yielded hydrazides **5** and **6**. The subsequent reaction with 9-chloro-1,2,3,4-tetrahydroacridine (**8**)[11] required thorough optimization and was found to proceed smoothly in ethanol at 140 °C in a sealed glass reactor. The resulting hydrochlorides were subjected

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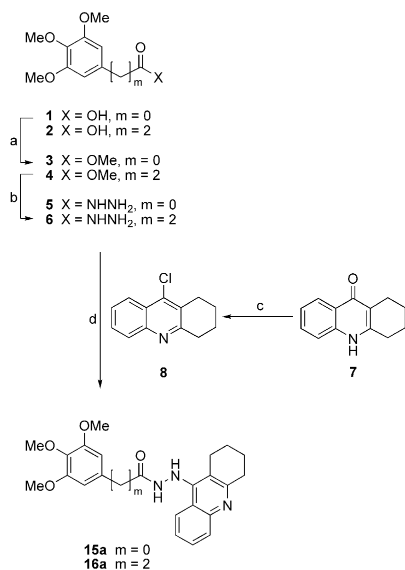
[a] Abbreviations: EeAChE, acetylcholinesterase from *Electrophorus electricus*; hAChE, human acetylcholinesterase; hBChE, human butyrylcholinesterase; PAS, peripheral anionic site; TcAChE, acetylcholinesterase from *Torpedo californica*.

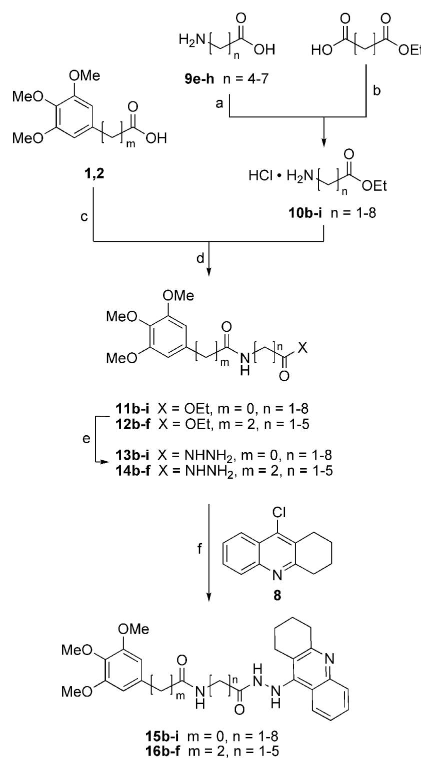
10.1021/jm060742o CCC: $33.50 © 2006 American Chemical Society
Published on Web 11/10/2006

Scheme 1. Synthesis of the Truncated Analogues **15a** and **16a**[a]



[a] (a) (1) $(COCl)_2$, CH_2Cl_2, room temp, 2 h; (2) NaOMe, MeOH, 5 h; (b) N_2H_4(aq), EtOH, reflux, 24 h; (c) (1) $POCl_3$, 130 °C, 3 h; (2) NaOH(aq); (d) (1) EtOH, 140 °C (autoclave), 24 h; (2) NaOH(aq).

Scheme 2. Synthesis of **15b−i** and **16b−f**[a]



[a] (a) $SOCl_2$, EtOH, room temp, 12 h; (b) (1) DPPA, PhMe, 80 °C, 2 h; (2) *t*-BuOH, PhMe, reflux, 24 h; (3) HCl, EtOAc, room temp, 12 h; (c) $(COCl)_2$, CH_2Cl_2, room temp, 2 h; (d) DIEA, CH_2Cl_2, room temp, 1 h; (e) N_2H_4(aq), EtOH, reflux, 24 h; (f) (1) EtOH, 140 °C (autoclave), 24 h; (2) NaOH(aq).

to aqueous sodium hydroxide to release the free bases **15a** and **16a**.

The skeleton of various ethyl ω-aminocarboxylates **10b−i** ($n = 1-8$) was introduced between the trimethoxybenzene and tacrine moieties of **15a** and **16a** to receive heterodimeric products with varying tether length. To synthesize ethyl ω-aminocarboxylates **10e−h** ($n = 4-7$), ω-aminocarboxylic acids **9e−h** were reacted with diethyl sulfite[12] (Scheme 2; see also Supporting Information). The route to ethyl 9-aminononanoate **10i** ($n = 8$) included the transformation of ethyl hydrogen sebacate to its monoisocyanate using diphenylphosphorylazide, the reaction with *tert*-butanol to give the Boc-protected intermediate,[13] and deprotection to give **10i**.

Ethyl ω-aminocarboxylates **10b−f** ($n = 1-5$) were then treated with the acyl chlorides of **1** and **2** to yield the amides **11b−f** and **12b−f**, which were converted to hydrazides **13b−f** and **14b−f**, respectively. These were then reacted with **8** to give hydrochlorides from which the desired **15b−f** and **16b−f** were liberated. First kinetic investigations showed **15e**, **16e** ($n = 4$) and **15f**, **16f** ($n = 5$) to inhibit acetylcholinesterase from *Electrophorus electricus* in the lower nanomolar range. Since the trimethoxybenzoyl derivatives (**15e**,**f**) were slightly more potent, it was decided to extend this series even further. Keeping to the established procedure, hydrazides **13g−i** were prepared, which upon reaction with **8** gave the final compounds **15g−i** ($n = 6-8$).

Results and Discussion

Kinetic studies[14,15] on acetylcholinesterase and butyrylcholinesterase from different organisms (*Electrophorus electricus*, *Torpedo californica*, *Homo sapiens*) were carried out to determine IC_{50} values for **15a−i** and **16a−f** (Table 1). Acetylcholinesterases from *Electrophorus electricus* (EeAChE) and *Torpedo californica* (TcAChE) used in this study were soluble G4 tetramers, and the recombinant enzyme from *Homo sapiens* (hAChE) was a mixture of dimers and tetramers.[16] Butyrylcholinesterase from human plasma (hBChE) was used, which is predominantly the water-soluble G4 form.[17] A Lineweaver−Burk plot (Figure 1) illustrates a representative compound, **15h**, to be an almost noncompetitive inhibitor of acetylcholinesterase from *Electrophorus electricus*, and the binding mode was further examined using secondary plots to obtain $K_{ic} = 3.23$ nM and $\alpha = 0.97$ (see Supporting Information). Tacrine shows a pronounced competitive mode of inhibition as can be concluded from $\alpha > 1$.[15] A value $\alpha \approx 1$ for **15h** implicates the potential binding of the inhibitor to the enzyme−substrate complex. Thus, the electron-rich aromatic moiety may also promote an interaction with the PAS when the tacrine moiety cannot occupy the substrate binding site. To reinforce the assumption that compounds **15a−i** and **16a−f** bind simultaneously to the active and peripheral site, we also evaluated the precursors **11h**, **13h**, and 9-hydrazino-1,2,3,4-tetrahydroacridine. The last two hydrazine-containing compounds exhibited a weak inhibitory activity ($IC_{50} = 18$ and 13 μM, respectively), whereas the carboxylic ester **11h** had no effect ($IC_{50} > 250$ μM).

From the data retrieved for acetycholinesterase from *Electrophorus electricus*, a comparison of the corresponding derivatives of both series revealed that in most cases compounds **15** were superior to compounds **16** when containing the same ω-aminocarboxylic acid (e.g., **15e** vs **16e**) and also when considering the overall spacer length (e.g., **15h** vs **16f**). The most potent heterodimeric inhibitors contain an 11- or 12-atom spacer (**15h**, **15i**), which also holds true for the other series, with **16f**, with a total spacer length of 11 atoms, as the best inhibitor. In these series of heterodimers, a heptylene-analogous linker did not provide the optimal distance, suggesting that the type of heterodimer influences the optimal spacer length.

The trend of an increase in activity with increasing linker length, found for acetylcholinesterase from *Electrophorus electricus*, was not observed for *Torpedo californica*. Ranking the potency of **15**, the results with acetylcholinesterase from *Electrophorus electricus* (**a**, **b**, **c**, **d**, **e**, **f**, **g**, **h**, **i**) were closer to

Table 1. Inhibition of Acetylcholinesterase from *Electrophorus electricus* (EeAChE) and *Torpedo californica* (TcAChE), Human Acetylcholinesterase (hAChE), and Human Butyrylcholinesterase (hBChE) by Compounds **15a**−**i** and **16a**-**f**

compd	*n*	IC_{50} [a] ± SEM (nM)				selectivity, IC_{50}(hAChE)/IC_{50}(hBChE)
		EeAChE	TcAChE	hAChE	hBChE	
15a		2260 ± 142	21.1 ± 09	386 ± 24	1.70 ± 0.18	227
15b	1	104 ± 4	163 ± 13	51.6 ± 2.1	27.6 ± 1.0	1.87
15c	2	100 ± 3	49.5 ± 2.0	89.2 ± 6.2	12.8 ± 1.1	6.97
15d	3	60.5 ± 4.8	26.3 ± 2.0	241 ± 8	7.13 ± 0.46	33.8
15e	4	20.2 ± 1.1	18.1 ± 2.1	17.0 ± 1.4	1.68 ± 0.15	10.1
15f	5	20.1 ± 1.4	55.7 ± 4.3	18.2 ± 0.6	0.969 ± 0.098	18.8
15g	6	9.76 ± 0.44	25.2 ± 0.8	5.65 ± 0.31	0.523 ± 0.018	10.8
15h	7	3.25 ± 0.40	17.2 ± 0.3	5.24 ± 0.20	0.293 ± 0.014	17.9
15i	8	2.73 ± 0.25	5.99 ± 0.44	5.08 ± 0.22	1.38 ± 0.04	3.68
16a		294 ± 28	40.1 ± 2.1	365 ± 21	12.1 ± 0.3	30.2
16b	1	122 ± 8	40.1 ± 4.3	40.7 ± 3.5	24.0 ± 0.6	1.70
16c	2	102 ± 10	68.9 ± 4.4	135 ± 5	6.03 ± 0.33	22.4
16d	3	52.1 ± 5.4	158 ± 1	72.1 ± 1.6	0.226 ± 0.018	319
16e	4	54.7 ± 3.4	102 ± 4	59.00 ± 0.52	0.139 ± 0.011	424
16f	5	20.2 ± 2.0	39.8 ± 1.0	8.39 ± 0.14	0.141 ± 0.004	59.5
AS-1397		ni[b]	34700 ± 2600	ni[b]	3370 ± 340	12000[c]

[a] IC_{50} values were determined in duplicate on a minimum of at least four different inhibitor concentrations. [b] ni = no inhibition at 100 μM. [c] Taken from ref 19.

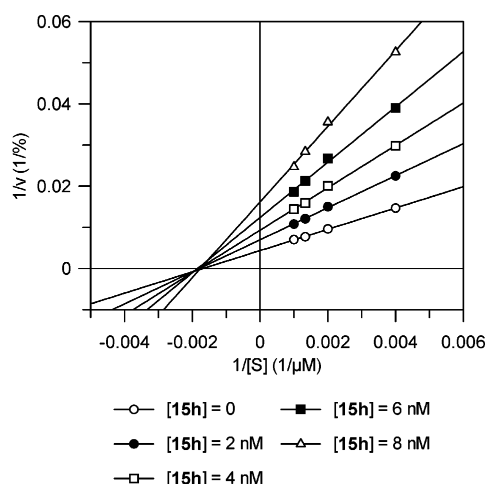


Figure 1. Lineweaver−Burk plot for the inhibition of acetylcholinesterase from *Electrophorus electricus* by **15h** at different substrate concentrations.

those from the human enzyme (**a**, **d**, **c**, **b**, **f**, **e**, **g**, **h**, **i**) than those from *Torpedo californica*. The compound with the most extended linker (**15i**), which was synthesized on the basis of the initial data for the *Electrophorus electricus* enzyme, turned out to be the most potent inhibitor of all three acetylcholinesterases investigated.

Against our expectations all compounds had IC_{50} values in the lower nanomolar or even picomolar range toward human butyrylcholinesterase and thus proved to be more or less selective (Table 1). Particularly compounds **15a**, **16d**, and **16e** showed a considerable preference toward butyrylcholinesterase and may become valuable tools for selective cholinesterase assays.[18] To compare this data with established inhibitors, AS-1397 (an ethopropazine analogue with even higher affinity) was included.[19] While there was no inhibition observed for acetylcholinesterase from *Electrophorus electricus* or the human enzyme, butyrylcholinesterase inhibition was found in the lower micromolar range. The selectivity value reported is about 50 times higher than those found for compounds **15a**, **16d**, and **16e**. Nevertheless, compared with other potent tacrine-derived cholinesterase inhibitors,[7] this selectivity is notable. Whereas the trimethoxybenzoic acid derivatives **15** were superior to trimethoxyphenylpropionic acid derivatives **16** regarding acetylcholinesterase inhibition, the opposite trend was observed for butyrylcholinesterase, where **16d**−**f** had IC_{50} values less than 250 pM.

In conclusion, a concise synthetic entry to novel inhibitors of acetylcholinesterase and butyrylcholinesterase is reported. A new type of a hydrazine-based linker was developed that allows for the introduction of a variety of electron-rich aromatic units linked to a tacrine moiety. Such heterodimers will provide further insights into interactions along the gorge between the active and peripheral binding site of both cholinesterases and might meet the requirements for an improved pharmacological profile as potential drug candidates for the therapy of Alzheimer's disease.

Experimental Section

Ethyl 9-Aminononanoate Hydrochloride (10i). Ethyl hydrogen sebacate (5.54 g, 24.0 mmol) was dissolved in toluene (30 mL), and diphenylphosphorylazide (5.6 mL, 26.0 mmol) and triethyl amine (3.6 mL, 26.0 mmol) were added. The solution was heated to 80 °C for 2 h to allow isocyanate formation. Subsequently, anhydrous *tert*-butanol (20 mL) was added, and the reaction mixture was refluxed for 12 h. After the mixture was cooled to room temperature, the solvent was evaporated in vacuo and the remaining residue was taken up in diethyl ether (100 mL). Filtering through silica gel, washing with diethyl ether (100 mL), and evaporation in vacuo yielded the Boc-protected ethyl 9-aminononanoate as a colorless oil. For deprotection, this oil was dissolved in ethyl acetate (20 mL) and 4 M hydrochloric acid in ethyl acetate (20 mL) was added. **10i** (4.40 g, 77%) was recovered by suction filtration as a white precipitate, mp 129−132 °C, lit.[12] 125 °C. ^{1}H NMR (DMSO-d_6) δ 1.16 (t, 3 H, J = 7.1 Hz), 1.25 (bs, 8 H), 1.45−1.59 (m, 4 H), 2.25 (t, 2 H, J = 7.4 Hz), 2.71 (app t, 2 H, J = 7.7 Hz), 4.03 (q, 2 H, J = 7.2 Hz), 8.03 (s, 3 H); ^{13}C NMR (DMSO-d_6) δ 14.26, 24.54, 25.90, 27.01, 28.44, 28.46, 28.57, 33.63, 38.80, 59.74, 172.98.

Ethyl 9-((3,4,5-Trimethoxybenzoyl)amino)nonanoate (11i). 3,4,5-Trimethoxybenzoic acid (**1**) (2.12 g, 10.0 mmol) was dissolved in anhydrous dichloromethane (20 mL) with catalytic amounts of *N*,*N*-dimethylformamide. Oxalyl chloride (1.0 mL, 11.6 mmol) was added while stirring, and once the gas evolution had ceased, it was evaporated to dryness. The residue was again dissolved in dichloromethane (10 mL), and ethyl 9-aminononanoate hydrochloride (**10i**) (2.38 g, 10.0 mmol) was suspended in the solution. After dropwise addition of *N*-ethyl-*N*,*N*-diisopropylamine (3.5 mL, 20.0 mmol) over 1 h, washing with water and a saturated solution of sodium hydrogen carbonate, drying with anhydrous sodium sulfate, and evaporating in vacuo yielded a crude product that was further purified by recrystallization from ethyl acetate/*n*-hexane to give

11i (3.24 g, 82%) as white crystals, mp 80−83 °C. ^{1}H NMR (DMSO-d_6) δ 1.15 (t, 3 H, J = 7.3 Hz), 1.22−1.32 (m, 8 H), 1.46−1.55 (m, 4 H), 2.25 (t, 2 H, J = 7.4 Hz), 3.23 (app q, 2 H, J = 6.6 Hz), 3.69 (s, 3 H), 3.81 (s, 6 H), 4.03 (q, 2 H, J = 7.1 Hz), 7.15 (s, 2 H), 8.33 (t, 1 H, J = 5.7 Hz); ^{13}C NMR (DMSO-d_6) δ 14.24, 24.57, 26.56, 28.51, 28.73, 28.74, 29.29, 33.64, 39.40, 56.13, 59.72, 60.17, 104.90, 130.04, 139.97, 152.65, 165.52, 172.99. Anal. ($C_{21}H_{33}NO_6$) C, H, N.

***N*-(9-Hydrazino-9-oxononyl)-3,4,5-trimethoxybenzamide (13i).** Ethyl 9-((3,4,5-trimethoxybenzoyl)amino)nonanoate **11i** (1.98 g, 5.0 mmol) was added to a mixture of hydrazine hydrate (100%, 2.5 mL, 51.4 mmol) and absolute ethanol (20 mL), and the solution was refluxed for about 24 h. The course of the reaction was followed by TLC (toluene/acetone/methanol, 7:2:1). Once no more ester could be detected, the solvent and excess hydrazine were removed by evaporation. The crude product was recrystallized from ethanol to obtain **13i** (1.46 g, 77%) as white crystals, mp 133−136 °C. ^{1}H NMR (DMSO-d_6) δ 1.17−1.33 (m, 8 H), 1.42−1.54 (m, 4 H), 1.98 (t, 2 H, J = 7.4 Hz), 3.22 (app q, 2 H, J = 6.6 Hz), 3.68 (s, 3 H), 3.81 (s, 6 H), 4.12 (bs, 2 H), 7.15 (s, 2 H), 8.36 (t, 1 H, J = 5.5 Hz), 8.88 (s, 1 H); ^{13}C NMR (DMSO-d_6) δ 25.38, 26.67, 28.80, 28.87, 28.88, 29.38, 33.58, 39.46, 56.15, 60.22, 104.86, 130.06, 139.93, 152.69, 165.56, 171.76. Anal. ($C_{19}H_{31}N_3O_5$) C, H. N: calcd 11.02, found 10.39.

3,4,5-Trimethoxy-*N*-(9-oxo-9-(2-(1,2,3,4-tetrahydroacridin-9-yl)hydrazino)nonyl)benzamide (15i). 9-Chloro-1,2,3,4-tetrahydroacridine (**8**) (0.44 g, 2.0 mmol) and *N*-(9-hydrazino-9-oxononyl)-3,4,5-trimethoxybenzamide (**13i**) (0.76 g, 2.0 mmol), dissolved in absolute ethanol (20 mL), were heated to 140 °C for 24 h in a sealed tube. Cooling to room temperature and evaporation yielded a crude product that was taken up in ethanol (5 mL) and diluted with water (20 mL). Addition of 1 M sodium hydroxide solution (2.5 mL) liberated the base as an oily layer. After decantation, the oily residue was suspended in ethyl acetate (25 mL) and shortly refluxed until the final product precipitated. Compound **15i** (0.61 g, 54%) was obtained as a yellow powder, mp 162−165 °C. ^{1}H NMR (DMSO-d_6) δ 1.09−1.33 (m, 8 H), 1.38−1.56 (m, 4 H), 1.73−1.88 (m, 4 H), 2.07 (t, 2 H, J = 7.1 Hz), 2.82 (bs, 2 H), 2.90 (bs, 2 H), 3.22 (app q, 2 H, J = 6.2 Hz), 3.69 (s, 3 H), 3.81 (s, 6 H), 7.16 (s, 2 H), 7.30 (app t, 1 H, J = 7.3 Hz), 7.51 (app t, 1 H, J = 7.4 Hz), 7.64 (s, 1 H), 7.70 (app d, 1 H, J = 8.2 Hz), 8.31 (app d, 1 H, J = 8.5 Hz), 8.34 (t, 1 H, J = 4.7 Hz), 10.02 (s, 1 H); ^{13}C NMR (DMSO-d_6) δ 22.54, 22.71, 24.84, 25.02, 26.61, 28.65, 28.82, 29.34, 33.06, 33.78, 39.42, 56.15, 60.18, 104.91, 115.60, 119.12, 123.21, 123.44, 127.93, 128.41, 130.04, 139.98, 146.82, 148.83, 152.66, 158.13, 165.54, 172.06. Anal. ($C_{32}H_{42}N_4O_5 \cdot 0.5H_2O$) H, N. C: calcd 67.23, found 67.83.

Cholinesterase Inhibition. Acetylcholinesterase from *Electrophorus electricus* (G4, soluble tetramer) was purchased from Fluka (Deisenhofen, Germany). Human acetylcholinesterase (recombinant, expressed in HEK 293 cells, G2/G4) was from Sigma (Steinheim, Germany) and butyrylcholinesterase (human plasma, G4) from Lee Biosolutions (St. Louis, MO). Acetylcholinesterase from *Torpedo californica* (recombinant, expressed in *Pichia pastoris*, G4) was a kind gift from R. Gallitzendörfer, Institute for Physiological Chemistry, University of Bonn, Germany. Cholinesterase inhibition was assayed spectrophotometrically at 412 nm in 100 mM sodium phosphate buffer, 100 mM NaCl, pH 7.3. Acetyl- or butyrylthiocholine (10 mM) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (7 mM) were dissolved in assay buffer. Stock solutions of the inhibitors were prepared in a 1:1 mixture of acetonitrile and 0.1 M HCl. IC_{50} values were determined in duplicate on a minimum of at least four different inhibitor concentrations. Into a cuvette containing 825 μL of assay buffer, were added 50 μL of the DTNB solution, 55 μL of acetonitrile, 10 μL of an inhibitor solution, and 10 μL of a cholinesterase solution (∼3 U/mL), and the mixture was thoroughly mixed. After incubation for 15 min at 25 °C, the reaction was initiated by adding 50 μL of the acetyl- or butyrylthiocholine solution.

Acknowledgment. This work was supported by the Deutsche Forschungsgemeinschaft, Graduiertenkolleg 677.

Supporting Information Available: Detailed experimental and analytical data including spectral assignments for **1**−**8**, **11a**−**h**, **12a**−**f**, **13a**−**h**, **14a**−**f**, **15a**−**h**, **16a**−**f**, and AS-1397; kinetic data for inhibition of acetylcholinesterase from *Electrophorus electricus* by compound **15h**; and elemental analysis results. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM060742O