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SYNTHESES AND ANTICHOLINESTERASE ACTIVITIES OF NOVEL 3-AMINOCARBONYLMETHYLENE-3-METHYL-2,3- DIHYDROBENZOFURAN-5-YL CARBAMATES

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Abstract - Novel carbamates **4** and **5** were synthesized from starting material 5-hydroxy-3-methyl-3*H*-benzofuran-2-one, **17**. The acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities of **4** and **5** were determined against fresh human enzyme, are in the realm of clinically valuable compounds and are discussed. In addition, the reductive properties of the 2-carbonyl group of 3*H*-benzofuran-2-one, possessing an unsaturated substituted group in its 3 position (**9**, **11** and **14**), were studied.

Carbamates based on physostigmine series **1a-1c**, on physovenine series **2a-2c** and on tetrahydrofuro-benzofuran series **3a-3c** have been proven to be potent inhibitors of the enzymes acetylcholinesterase (AChE) or butyrylcholinesterase (BChE), with specific compounds exhibiting remarkable selectivity for one form of the enzyme over the other. These enzymes are validated targets in the treatment of Alzheimer's disease (AD), and their characterization is leading to the development of new and better tolerated drug candidates with actions that may impact disease course. There are numerous reports in the literature describing the synthesis of agents from each of these series.¹⁻¹⁷ Herein, we report the chemical synthesis and anticholinesterase properties of the novel and unexpected carbamates, **4** and **5**.

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These were generated during the planned synthesis of novel carbamate **6**, which possesses an ether linkage in ring-B of physostigmine instead of a N-Me moiety (**Figure 1**).

We, additionally, found that the carbonyl group of the lactone moiety in intermediates **9**, **11** and **14** could be more readily reduced to a methylene group or methine than either a cyano or an ester group in the side chain of these compounds could be reduced (**Scheme 1**). The anticholinesterase activities of carbamates **4** and **5** were considerably less than that of corresponding tricyclic compounds, **1-3** (**Table 1**).

Table 1. Anticholinesterase Actions of Novel versus Known Carbamates: Comparison of the IC₅₀ Values* (±SEM) of Agents Against Human Erythrocyte AChE and Plasma BChE^a

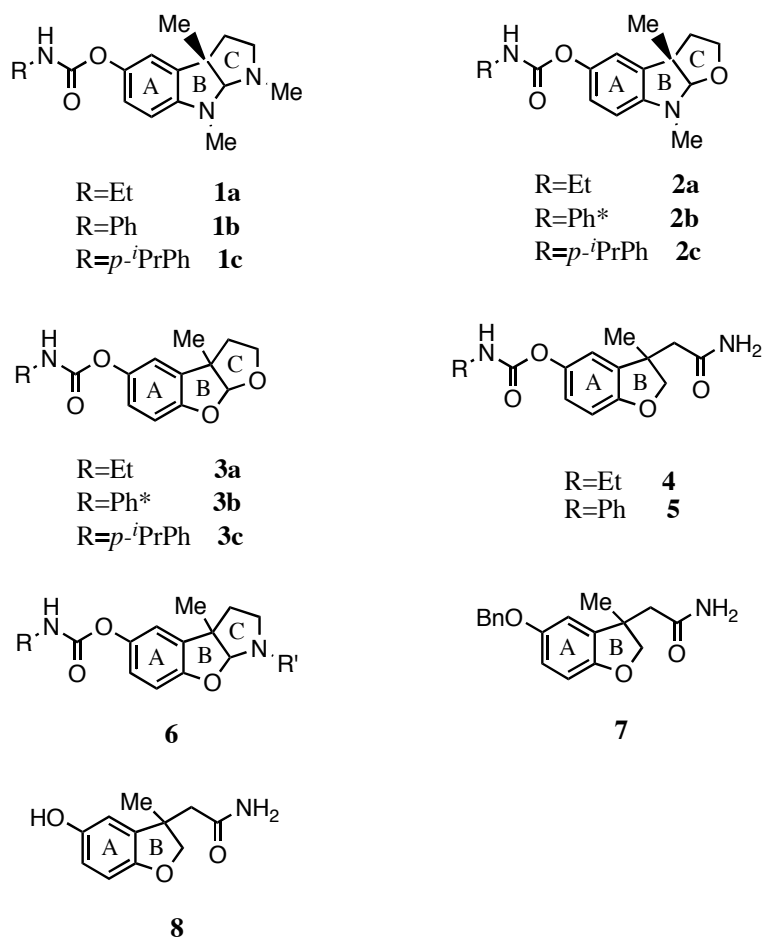
No.	<u>IC₅₀ ± SEM (nM)</u>		Selectivity
	<u>AChE</u>	<u>BChE</u>	
1a	94 ± 12	4 ± 0	23-fold BChE
1b	22 ± 1	1560 ± 45	70-fold AChE
1c	760 ± 20	50 ± 1	15-fold BChE
2a	82 ± 4	2 ± 0	40-fold BChE
2b	13 ± 1	1560 ± 120	120-fold AChE
2c	3860 ± 1000	17 ± 2	225-fold BChE
3a	360 ± 22	6 ± 2	60-fold BChE
3b	23 ± 4	> 30,000	>1300-fold AChE
3c	2650 ± 400	27 ± 4	100-fold BChE
4	5355 ± 359	72 ± 19	74-fold BChE
5	360 ± 7	>30,000	>83-fold AChE
7	>30,000	>30,000	-
8	>30,000	12,000 ± 650	-

* IC₅₀ Value: concentration required to inhibit 50% of enzyme activity

^a The IC₅₀ data of compounds **1a-1c**, **2a-2c** and **3a-3c** are from Ref. 8.

Table 2. Comparison of the IC₅₀ Values (nM) ($\pm$ SEM) of Anticholinesterases of Clinical Interest

No.	AChE	BChE	Selectivity
Tacrine	190 $\pm$ 40	47 $\pm$ 10	4-fold BChE
Donepezil	22 $\pm$ 8	4150 $\pm$ 1700	188-fold AChE
Galantamine	800 $\pm$ 60	7300 $\pm$ 830	9-fold AChE
Physostigmine	28 $\pm$ 2	16 $\pm$ 3	2-fold BChE

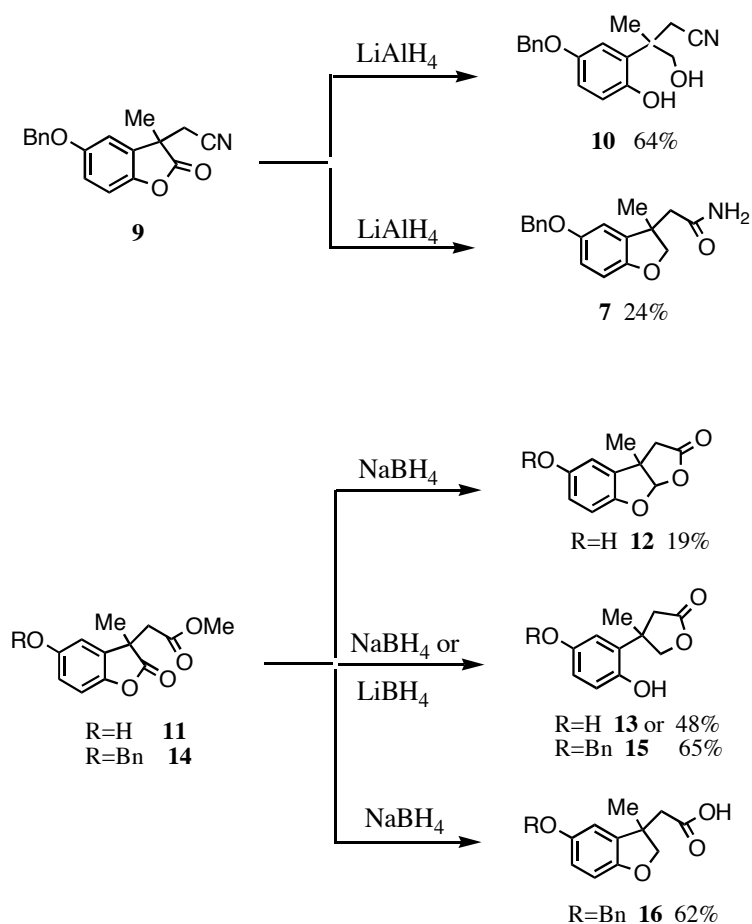
Figure 1. Chemical Structures of Novel Carbamates and Known Analogues

* The phenyl group was substituted by methyl in its own ortho-position.

Chemistry As shown in **Scheme 1**, our study indicates the occurrence of a chemoselective reduction with a preference for the carbonyl group associated with the lactone versus the cyano moiety for compound **9**, and similarly a preference for the carbonyl group of the lactone to the ester group of the

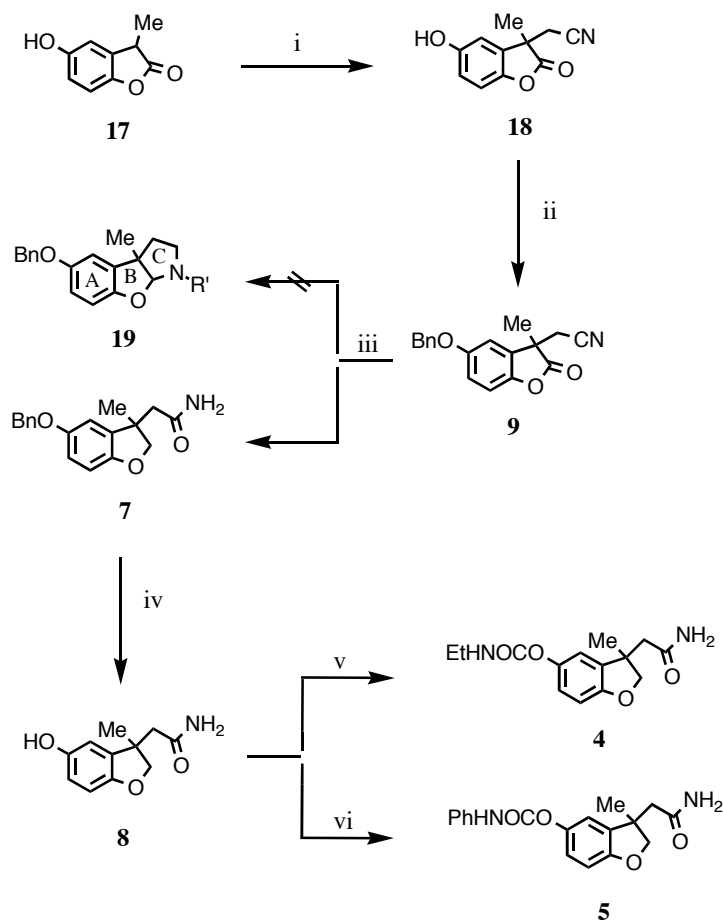
side chain in the case of **11** and **14**. In the latter, there is a regioselectivity for reduction of the carbonyl of the lactones to their side-chain moiety.

Scheme 1. Selective Carbonyl Reduction of Lactone in **9**, **11** and **14**^a

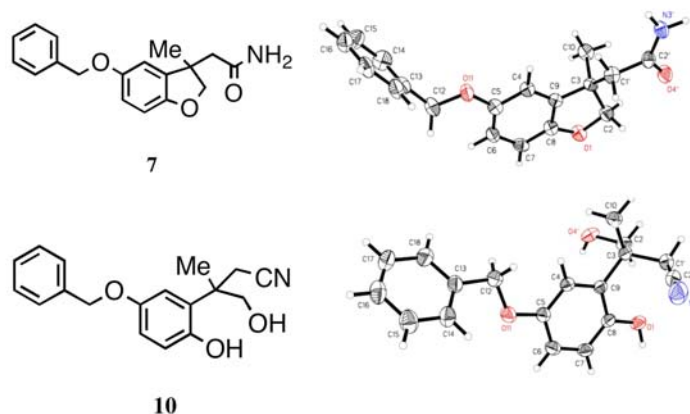


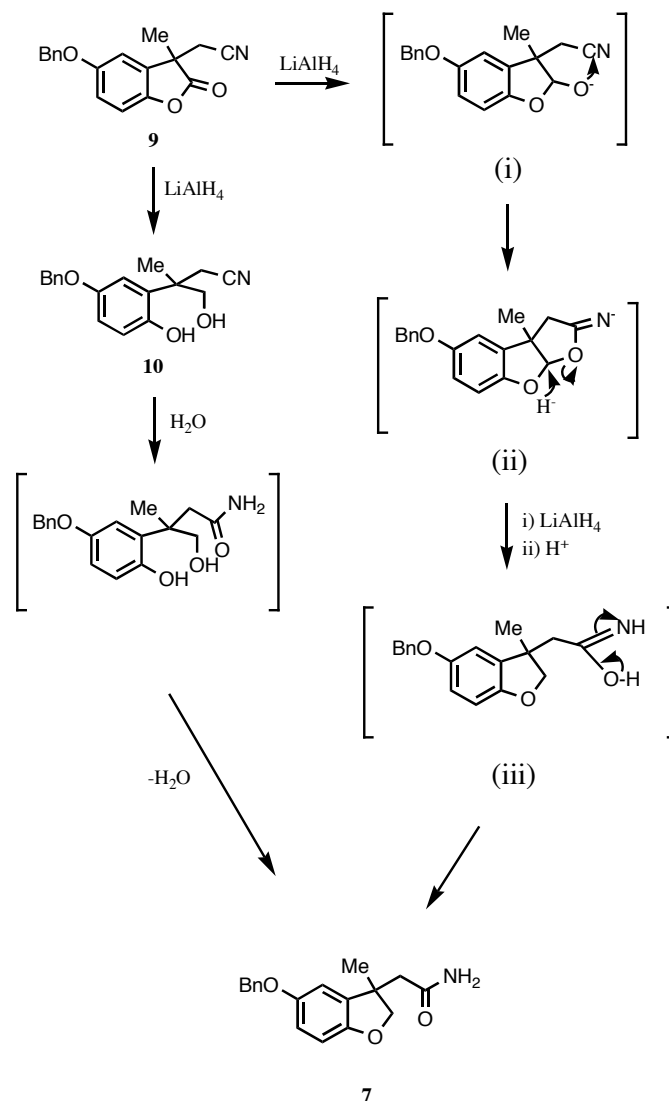
^a Compound **11** to **12**, **13** and compound **14** to **15**, **16** come from ref. 9.

We have previously reported the synthesis of physostigmine, physovenine and related analogues from their oxindoles **9a** and **9b**^{10,12,14-17} (see **Table 3 re: 9a** and **9b**). As a consequence, we planned to construct the ring-C of compound **19** using a similar strategy (**Scheme 2**). However, in practice this generated amide **7** and nitrile **10**, whose structures were confirmed by X-ray crystallographic analyses (**Figure 2**). The likely mechanism is shown in **Scheme 3**.

Scheme 2. Syntheses of Amide **7** and Carbamates **4** and **5**^a

^a Reagents and conditions: i) $\text{ClCH}_2\text{CN}/\text{DMF}$, NaH , $0^\circ\text{C}\sim\text{rt}$, 68.0h, 63%; ii) $\text{BrCH}_2\text{Ph}/\text{MeCN}$, K_2CO_3 , rt, 66.5 h, 72%; iii) $\text{LiAlH}_4/\text{THF}$, $0^\circ\text{C}\sim\text{rt}$, 80min, 24%; iv) 20% $\text{Pd}(\text{OH})_2/\text{C}$, MeOH , rt, 3h, 83%; v) Na , $\text{EtNCO}/\text{Et}_2\text{O}$, rt, 15 min, 60%; vi) Na , $\text{PhNCO}/\text{Et}_2\text{O}$, EtOAc , rt, 16 min, 27%.

Figure 2. X-Ray Crystallographic Pictures and Corresponding Chemical Structures of Compounds **7** and **10**.

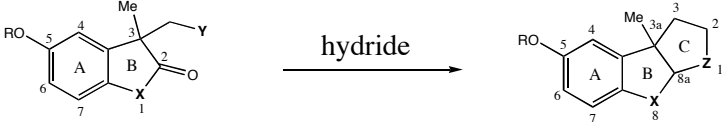
Scheme 3. Likely Mechanism for the Formation of Amide 7

In **Scheme 3**, the carbonyl group of lactone **9** was reduced or reduced and then dehydrated to yield a methylene group prior to the reduction or hydrolysis of the cyano group in **9**. In support of this premise, compound **10** was isolated and characterized. This chemoselective reduction is noteworthy since the reducing agent, lithium aluminum hydride, which was reacted with the substrates possessing the skeleton of 3*H*-benzofuran-2-one, has not previously been reported to provide selectivity between cyano and ester groups.¹⁸

In contrast, as shown in **Table 3**, reduction of the 2-carbonyl group in oxindoles **9a-9c** (i.e., where X is N-Me) is more difficult than in 3*H*-benzofuran-2-ones **9**, **9d** and **9e**. Hence, the corresponding yields of generated ring-C formation from starting materials **9a** to **9d** are decreased from 98% to 10%. In the case of an oxindole, an intermediate of nitrogenous semi-acetal is favored and readily leads to ring-C formation following the intramolecular elimination of water or amine. In synopsis, oxindoles allow the generation of the ring-C in high yield, whereas 3*H*-benzofuran-2-ones are associated with a low yield.

An exception to the latter occurs when the side chain of the 3 position does not require reduction to allow cyclization, as in the case of $-\text{CH}_2\text{SCOMe}$, $-\text{CH}_2\text{SH}$ ^{11,15} or $-\text{CH}_2\text{NHBoc}$.¹¹ As the yield of intermediate **10** from **9** is 64%, the amide, **7**, may be generated in part by a secondary route involving the rearrangement of the imidic acid (**Scheme 3** - right hand side). This premise is supported by our prior synthesis and characterization of mechanisms underpinning the generation of tetrahydrofurobenzofuran and dihydromethanobenzodioxepine.⁹ In addition, as shown in **Scheme 1**, lactone **12** can be formed from lactone **11**, bearing resemblance to the generation of intermediate (ii).

Table 3. Effect of Heteroatom X on Reduction of the 2-Carbonyl and Unsaturated Substituted Group in the Side Chain of the 3 Position

						
No.	X	Y	X	Z	yield	Ref.
9a	NMe	CN	NMe	NH	98	12,15,16,17
9b	NMe	CO ₂ Me	NMe	O	86	10,14
9c	NMe	CH ₂ SCOMe, CH ₂ SH	NMe	S	72	11,15
9	O	CN	O	NH	no success ^a	-
9d	O	CO ₂ Me	O	O	10	9
9e	O	CH ₂ SCOMe	O	S	no report	-
9f	S	CH ₂ NHBoc	S	NMe	68	11
9g	S	CO ₂ Me	S	O	no report	-
9h	S	CH ₂ SCOMe	S	S	no report	-

R=H, Me, Et, Bn and Tetrahydropyranyl.

^a See **Scheme 2**.

In synopsis, the carbonyl group of a 3H-benzofuran-2-one is more easily reduced, compared to an oxindole derivative. Hence, ring-C formation occurs in far higher yield for the latter and allowed the generation of the novel ring-C open carbamates, **4** and **5**, of the former. However, in the event that no reduction of the side chain of the 3 position is required, ring-C formation can readily proceed for both a 3H-benzofuran-2-one and oxindole.

BIOLOGICAL RESULTS AND DISCUSSION

The novel carbamates **4** and **5** that lack a ring-C have lower anticholinesterase activities than their corresponding tricyclic carbamate counterparts, **1-3** (**Table 1**), but nevertheless possess potency within

the realm of clinically efficacious compounds (**Table 2**). In our recent molecular modeling of this carbamate class with AChE and BChE¹³, a C-H--- π interaction between the ring-C of the inhibitor and the indole system of a critical Trp₈₄ within the enzyme represents a key factor to favor inhibitor-enzyme binding and subsequent inhibition. The ring-C open structure of carbamates **4** and **5**, unlike tricyclic compounds **1-3**, will not support such an interaction. The absence of this carbamate-enzyme interface likely underpins the lower anticholinesterase potency of carbamates of **4** and **5**, versus their tricyclic counterparts. Our recent studies indicate that specific tricyclic carbamates of physostigmine, physovenine and tetrahydrofurobenzofuran have non-cholinergic action to lower production of the AD peptide, β -amyloid (A β), by reducing the rate of synthesis of amyloid precursor protein (APP).¹⁹⁻²¹ Actions of **4** and **5** are currently being assessed on APP and A β in cell culture, as compounds with significant but reduced anticholinesterase potency have been found to be better tolerated in animal models of AD.²²

EXPERIMENTAL

Chemistry Melting points (uncorrected) were measured with a Fisher-Johns apparatus. ¹H NMR and ¹³C NMR spectra were recorded on a Bruker (Bellevica, MA) AC-300 spectrometer. MS spectra (*m/z*) were recorded on a Hewlett-Packard 5973 gas chromatograph-mass spectrometer with chemical ionization [GC-MS (CI)]. High resolution mass spectrum (HRMS) was performed by the UCR Mass Spectrometry Facility, Department of Chemistry. All reactions involving nonaqueous solution were performed under an inert atmosphere.

3-Cyanomethylene-5-hydroxy-3-methyl-3H-benzofuran-2-one (18): Under a nitrogen atmosphere, sodium hydride (0.365g, 15.2mmol) was added to a solution of compound **17** (2.50g, 15.2mmol) and chloroacetonitrile (1.26g, 16.7mmol) in 9mL of dry DMF at 0 °C in portions over 1 h. The mixture was stirred for another 1 h at 0 °C and then reacted for 67 h at rt. The reaction mixture then was poured onto 38g of ice and extracted with Et₂O (3x40mL). The extract was washed with brine (2x40mL) and dried over magnesium sulfate. After filtering and removing solvents, 3.7g of crude product was afforded. It was recrystallized with EtOAc to give yellow crystals **18** (1.93g, 62.5%): mp 150.0-152.5 °C; ¹H NMR (DMSO-*d*₆) δ 9.57 (s, 1H, OH), 7.11-6.75 (m, 3H, ArH), 3.27, 3.23 (AB, *J*_{gem}=16.2Hz, 2H, CH₂CN) and 1.50 (s, 3H, CH₃) ppm; ¹³C NMR (CDCl₃) δ 176.5, 152.1, 144.9, 129.0, 14.7, 14.5, 11.2, 109.8, 43.4, 25.8 and 22.1 ppm; CI-MS (CH₄), *m/z* 204 (MH⁺), 176 and 164; HRMS *m/z* calcd for C₁₁H₉NO₃, 203.0582; found 203.0574.

5-Benzoyloxy-3-cyanomethylene-3-methyl-3H-benzofuran-2-one (9): A mixture of compound **18** (384.0mg, 1.890mmol), potassium carbonate (262.2mg, 1.897mmol), benzyl bromide (323.3mg, 1.890mmol) and 5mL of MeCN was stirred for 66.5 h at rt. After filtration, drying

and removal of solvent, the residue was chromatographed on silica gel (AcOEt/CH₂Cl₂=1/7) to give product **9** as pale-yellow needle crystals (397.5mg, 71.7%): mp 103.0-105.5 °C; ¹H NMR (CDCl₃) δ 7.40-6.89 (m, 8H, ArH), 5.01 (s, 2H, CH₂Ph), 2.81, 2.68 (AB, *J*_{gem}=16.2Hz, 2H, CH₂CN) and 1.58 (s, 3H, CH₃) ppm; ¹³C NMR (CDCl₃) δ 178.3, 157.1, 146.9, 137.1, 130.7, 129.4, 129.0, 128.4, 117.0, 16.2, 112.7, 111.3, 71.7, 45.2, 27.6 and 23.9 ppm; CI-MS (CH₄), *m/z* 294 (MH⁺), 266, 216, 181, 131 and 91; HRMS *m/z* calcd for C₁₈H₁₅NO₃, 293.1052; found 293.1048.

3-Aminocarbonylmethylene-5-benzyloxy-3-methyl-2,3-dihydrobenzofuran (7): Under a nitrogen atmosphere, a solution of compound **9** (58.7mg, 0.2mmol) in 1 mL of anhydrous THF was dropwise added to a mixture of lithium aluminum hydride (15.2mg, 0.4mmol) and 2 mL of anhydrous THF at 0 °C. The reaction mixture was gradually risen to rt and reacted for another 80 min. A saturated water solution of sodium sulfate was added to this mixture until no bubble occurred. After filtering with celite-salt and extracting with EtOAc, organic layer was evaporated to give 35mg of residue. It was purified by chromatography on silica gel (MeOH/CH₂Cl₂=1/20) to afford product **7** as a white needle crystals (14mg, 24%): mp 126.5-128.5 °C; ¹H NMR (CDCl₃) δ 7.49-6.74 (m, 8H, ArH), 5.21 (s, br, 2H, NH₂), 5.05 (s, 2H, CH₂Ph), 4.64, 4.34 (AB, *J*_{gem}=10.8Hz, 2H, C2-CH₂), 2.56, 2.54 (AB, *J*_{gem}=16.2Hz, 2H, CH₂CONH₂) and 1.50 (s, 3H, CH₃) ppm; ¹³C NMR (CDCl₃) δ 173.0, 154.0, 153.8, 137.6, 135.7, 128.9, 128.3, 128.0, 115.1, 10.9, 10.4, 83.0, 71.5, 46.0, 44.9 and 25.3 ppm; CI-MS (CH₄), *m/z* 298 (MH⁺), 280, 267, 239, 220, 202, 149 and 91; HRMS *m/z* calcd for C₁₈H₁₉NO₃, 297.1365; found 297.1372.

3-Aminocarbonylmethylene-5-hydroxy-3-methyl-2,3-dihydrobenzofuran (8): Under a hydrogen atmosphere, a mixture containing compound **7** (10mg, 0.034mmol) and Pd(OH)₂/C (20%, 5mg) in 0.5mL of MeOH was stirred for 3h at rt. After removal of solution, the residue was taken a chromatography on silica gel (MeOH/CH₂Cl₂=1/16) to give product **8** as a gum (5.8g, 83%): ¹H NMR (CDCl₃) δ 6.62-6.52 (m, 3H, ArH), 5.30 (s, br, 2H, NH₂), 5.12 (s, 1H, OH), 4.53, 4.22 (AB, *J*_{gem}=10.8 Hz, 2H, C2-CH₂), 2.49, 2.41 (AB, *J*_{gem}=16.2Hz, 2H, CH₂CONH₂) and 1.39 (s, 3H, CH₃) ppm; ¹³C NMR (MeOH-d₄) δ 176.4, 154.0, 152.9, 137.4, 116.0, 111.5, 110.9, 84.0, 46.0, 36.7 and 25.6 ppm; CI-MS (CH₄), *m/z* 208(MH⁺), 189, 177 and 149; HRMS *m/z* calcd for C₁₁H₁₃NO₃, 207.0895; found 207.0889.

3-Aminocarbonylmethylene-3-methyl-2,3-dihydrobenzofuran-5-yl N-ethylcarbamate (4): Under a nitrogen atmosphere, two small pieces of sodium (0.5mg per piece) were added to a solution of compound **8** (15mg, 0.072mmol) in 2 mL of anhydrous Et₂O at rt. The mixture was stirred for 1min, and then ethyl isocyanate (5.4mg, 0.076mmol) was added in one portion. The reaction was continued for another 15 min at rt, then 1 mL of water was added, and the ether layer was separated. After the ether solution was dried over sodium sulfate and filtered, the filtrate was

evaporated to provide a residue that was chromatographed on silica gel (MeOH/CH₂Cl₂=1/20) to afford product **4** as a gum (12mg, 60%): ¹H NMR (CDCl₃) δ 6.98-6.77 (m, 3H, ArH), 5.29 (s, br, 2H, NH₂), 4.98 (s, br, 1H, NH), 4.68, 4.34 (AB, *J*_{gem}=12.6Hz, 2H, C2-CH₂), 3.33 (q, 2H, NCH₂CH₃), 2.56, 2.54 (AB, *J*_{gem}=16.2Hz, 2H, CH₂CONH₂), 1.51 (s, 3H, C3-CH₃) and 1.23 (t, 3H, CH₃CH₂N) ppm; ¹³C NMR (CDCl₃) δ 172.6, 157.0, 155.5, 145.1, 135.3, 121.9, 117.1, 110.4, 83.3, 46.3, 44.8, 36.5, 25.3 and 15.5 ppm; CI-MS (CH₄), *m/z* 279 (MH⁺), 262, 220, 207, 177, 149, 133, 100 and 72; HRMS *m/z* calcd for C₁₄H₁₈N₂O₄, 278.1267; found 278.1261.

3-Aminocarbonylmethylene-3-methyl-2,3-dihydrobenzofuran-5-yl N-phenylcarbamate (5): Under a nitrogen atmosphere, two small pieces of sodium (0.5mg per piece) were added to a solution of compound **8** (19mg, 0.0917mmol) in 2 mL of anhydrous Et₂O and 0.5 mL of EtOAc at rt. The mixture was stirred for 1min, and then phenyl isocyanate (0.011mL, 0.0992mmol) was added in one portion. The reaction was continued for another 16min at rt, then 1.5 mL of water was added, and the organic layer was separated. After the solution was dried over sodium sulfate and filtered, the filtrate was evaporated to provide a residue that was chromatographed on silica gel (MeOH/CH₂Cl₂=1/20) to afford product **5** as a gum (8mg, 27%): ¹H NMR (CDCl₃) δ 7.48-6.80 (m, 8H, ArH), 5.29 (s, br, 2H, NH₂), 4.72, 4.39 (AB, *J*_{gem}=10.8Hz, 2H, C2-CH₂), 2.58, 2.56 (AB, *J*_{gem}=16.2Hz, 2H, CH₂CONH₂) and 1.50 (s, 3H, C3-CH₃) ppm; ¹³C NMR (CDCl₃) δ 172.6, 157.3, 144.7, 137.7, 135.5, 129.6, 124.4, 122.0, 119.0, 117.1, 113.1, 10.5, 83.4, 46.1, 44.8 and 25.3 ppm; CI-MS (CH₄), *m/z* 327 (MH⁺), 207, 149, 100 and 69; HRMS *m/z* calcd for C₁₈H₁₈N₂O₄, 326.1141; found 326.1149.

2-(2-Hydroxymethylene-2-methyl-1-cyanoethyl)-4-benzyloxyphenol (10): Under a nitrogen atmosphere, a solution of compound **9** (500mg, 1.70mmol) in 5 mL of dry THF was dropwise added to a mixture of lithium aluminum hydride (127mg, 3.35mmol) and 20 mL of dry THF at rt. The mixture was reacted for 2 h, and then oxalic acid (760mg, 8.44mmol) was added and continued to react for another 0.5 h. After filtering, the filtrate was evaporated to give a gum crude product, which was taken a chromatography on silica gel (EtOAc/CH₂Cl₂=1/6) or recrystallized with EtOH and water (9/1, v/v) to afford product **10** as white needle crystals (325mg, 64%): mp 133.0-136.0 °C; ¹H NMR (DMSO-*d*₆) δ 7.50-6.70 (m, 8H, ArH), 5.01 (s, 2H, CH₂Ph), 3.79, 3.55 (AB, *J*_{gem}=12.6Hz, 2H, CH₂OH), 3.28, 2.88 (AB, *J*_{gem}=16.2Hz, 2H, CH₂CN) and 1.37 (s, 3H, CH₃) ppm; ¹³C NMR (DMSO-*d*₆) δ 161.3, 151.5, 149.9, 137.8, 129.7, 128.7, 128.1, 119.9, 116.9, 116.3, 113.4, 70.1, 66.2, 42.4, 23.9 and 22.1 ppm; CI-MS (CH₄), *m/z* 281 (MH⁺-17), 273, 261, 247, 233 and 57.

X-Ray Crystallography A clear colorless crystal of **7** with normal dimensions was mounted on glass fiber using a small amount of Epoxy. A colorless crystal of **10** was mounted on an identical manner. Data for compounds **7** and **10** were collected on a Bruker three-circle platform diffractometer equipped

with a SMART 6000 CCD detector. The crystals were irradiated by use of a rotating anode Cu K α source ($\lambda=1.541\ 78$) with incident beam Göbel mirrors. Data collection was performed until cell was initially refined with SMART (version 5.625).²³ Data reduction was performed with SAINT (version 6.36A)²⁴ and XPREP (version 6.12).²⁵ Corrections were applied for Lorentz, polarization and absorption effects by use of SADABS (version 2.03).²⁶ Each structure was solved and refined with the aid of the programs in the SHELXTL-plus (version 6.10) system of programs.²⁷ The full-matrix least-squares refinement on F^2 included atomic coordinates and anisotropic thermal parameters for all non-H atoms. The H atoms were included by use of a riding model.

Quantitation of Anticholinesterase Activity The action of compounds **4**, **5**, **7** and **8** to inhibit the ability of freshly prepared human AChE and BChE to enzymatically degrade their respective specific substrates, acetyl(β -methyl)thiocholine and s-butyrylthiocholine (0.5 mmol/L) (Sigma Chemical Co., St. Louis, MO), was quantified.^{3,5,8,28} Samples of AChE and BChE were derived from freshly collected human whole red blood cells and plasma, respectively. Compounds were dissolved in Tween 80/EtOH 3:1 (v/v; <150 μ L total volume) and were diluted in 0.1 M Na₃PO₄ buffer (pH 8.0) in half-log from 0.3 nM to 30 μ M. Tween 80/EtOH was diluted to in excess of 1:5000. No inhibitory action on either AChE or BChE was detected in separate experiments where the ChEI activity of the known physostigmine was quantified in excess and without Tween 80/EtOH. For the preparation of BChE, freshly collected blood was centrifuged (10000g, 10 min, 4°C) and plasma was removed and diluted 1:125 with 0.1 M Na₃PO₄ buffer (pH 7.4). Plasma was carefully checked to ensure an absence of hemolysis. For AChE preparation, erythrocytes were washed five times in isotonic saline, lysed in 9 volumes of 0.1 M Na₃PO₄ buffer (pH 7.4) containing 0.5% Triton-X (Sigma), and then were diluted with an additional 19 volumes of buffer to a final dilution of 1:200. Analysis of anticholinesterase activity was undertaken by utilizing a 25 μ L sample of each enzyme preparation, at their optimal working pH (8.0), in 0.1 M Na₃PO₄ buffer (0.75 mL total volume). Compounds were preincubated with enzymes (30 min at rt) and then were incubated with their respective substrates and with 5,5'-dithiobis(2-nitrobenzoic acid) (25 min, 37 °C). The substrate/enzyme interaction was immediately halted by the addition of excess enzyme inhibitor (physostigmine, 1×10^{-5} M) and production of a yellow thionitrobenzoate anion was then measured by spectrophotometry at λ 412 nm. To correct for nonspecific substrate hydrolysis, aliquots were coincubated under conditions of absolute enzyme inhibition [by the addition of 1×10^{-5} M physostigmine], and the associated alteration in absorbance was subtracted from that observed through the concentration range of each test compound. Each agent was analyzed on four separate occasions and assayed alongside physostigmine, as a control and external standard whose activity we have previously reported.^{3,5,8,28} The mean enzyme activity at each concentration of test compound was then expressed as a percent of the activity in the absence of compound. This was transformed into a logit format [where logit = $\ln$ (%)

activity/100minus % activity)] and then was plotted as a function of its log concentration. Inhibitory activity was calculated as an IC₅₀, defined as the concentration of compound (nanomolar) required to inhibit 50% of enzymatic activity, which was determined from a correlation between log concentration and logit activity. Only results obtained from correlation coefficients of $r^2 \geq -0.98$ were considered acceptable. Studies that did not obtain this threshold were repeated.

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