References and Notes

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(3) For reviews see (a) B. V. Rama Sastry in "Medicinal Chemistry", A. Burger, Ed., 3rd ed, Wiley-Interscience, New York, N.Y., 1970, p 1544; (b) R. W. Brimblecombe, "Drug Actions on Cholinergic Systems", University Park Press, Baltimore, Md., 1974.
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Potent Reversible Anticholinesterase Agents. Bis- and Mono-N-substituted Benzoquinolinium Halides

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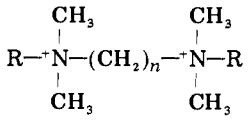
A number of bis- and mono-N-substituted benzoquinolinium salts and their analogues were prepared and evaluated as inhibitors of acetylcholinesterase (AcChE) and butyrylcholinesterase (BuChE). These compounds were also used to help identify some of the morphologic characteristics of the surface at or near the active sites of the cholinesterases. The shape, size, configuration, and conformation of the onium moieties of the quaternary ammonium compounds were found to be the important factors in their anticholinesterase activity. A high concentration of the positive charge of the quaternary ammonium compound is not a critical factor for the cholinesterase inhibitory activity. The order of decreasing potency of cholinesterase inhibition of the benzoquinolinium compounds was found to be acridinium > phenanthridinium > 5,6-benzoquinolinium > 7,8-benzoquinolinium. The inhibitory activity of the monobenzoquinolinium halides against cholinesterases is influenced by the N-substituent. A bis-quaternary ammonium compound with a flexible bridge that links the two nitrogen atoms was found to be more potent in inhibiting AcChE and less potent in inhibiting BuChE than a bis-quaternary ammonium compound with a rigid bridge. The acridinium and phenanthridinium derivatives of the benzoquinolinium compounds are very potent reversible inhibitors against both AcChE and BuChE.

A variety of mono- and bis-quaternary ammonium compounds has been described as reversible inhibitors of the esterolytic activity of both acetylcholinesterase (AcChE) and butyrylcholinesterase (BuChE).[1] It is assumed that these compounds interact by coulombic attraction with one or more of the anionic sites that exist on the enzymes. The more potent inhibitors are believed[1-4] to interact with the anionic site associated with the esteratic site while other inhibitors such as decamethonium, *d*-tubocurarine, and gallamine have been indicated to act on other sites on AcChE designated as β- or γ-anionic sites.[5-7] The mono-quaternary compounds are generally weaker inhibitors than their bis-quaternary counterparts. This lends support to the view that more than one anionic site may exist on the cholinesterases. In general, polymethylene bis-quaternary ammonium salts, **1**, show an increase in anticholinesterase activity as the length of the methylene chain separating the nitrogen atoms increas-

es.[8-12] Although the increase in chain length enhances enzyme inhibitory activity, there is often a marked increase in anticholinesterase activity associated with an increase in lipophilic character of the terminal substituents on the onium nitrogen atoms of the bis-quaternary salts. For



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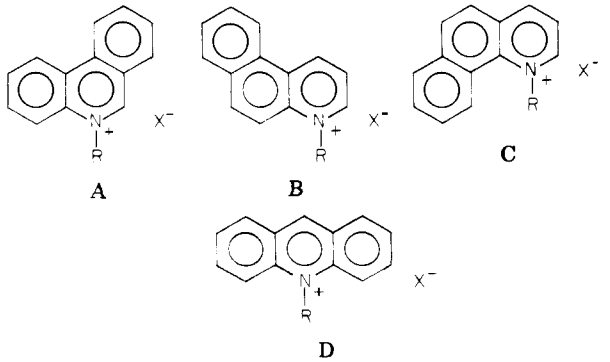
example, when R in 1 is increased from methyl to benzyl to fluorenyl for n = 3, 6, or 10, the anticholinesterase activity increases by a factor of 10 for each change.[8,13] Cavallito and Sandy[8] have suggested that the increase in total lipophilic bulk alone is not the only factor which contributes to the high anticholinesterase activity of hexafluorenium (1, R = fluorenyl). They reported that the trimethylenebis(phenanthridinium) quaternary [I_{50} (AcChE) = 3.8×10^{-6} M] was far more potent than the corresponding bis(4-benzylpyridinium) [I_{50} (AcChE) = 4.9×10^{-4} M] or bis(4-styrylpyridinium) [I_{50} (AcChE) = 4.0×10^{-4} M) or bis(4-diphenylmethylpyridinium) [I_{50} (AcChE) = 6.6×10^{-4} M] salts. These latter three bis quaternaries have approximately the same mass as the phenanthridinium bis quaternary but differ in the configurational aspects of the lipophilic mass.

We wish to report the synthesis and anticholinesterase activity of a number of mono- and bis-quaternary onium compounds for the purpose of studying the steric features of the cationic head needed for maximal cholinesterase inhibition. Using hexafluorenium bromide (1, R = fluorenyl) as a model, a series of hexamethylene bis(onium) compounds was prepared and studied as inhibitors of both AcChE and BuChE. The data are summarized in Table I. In addition, a series of 3,4-benzoquinolinium (phenanthridinium) (A), 5,6-benzoquinolinium (B), 7,8-benzoquinolinium (C), and acridinium (D) mono-quaternary halides was also studied to investigate the effect of the shape of the onium head on inhibitory activity. The data of the mono-quaternary salts are summarized in Table II. All the inhibitors in both Tables I and II showed mixed competitive inhibition kinetics with the exception of the acetyl esters XVI, XXI, and XXVI which were competitive inhibitors. The mono- and bis-quaternary ammonium



compounds were prepared by reacting the readily available tertiary amines with the appropriate alkyl or aralkyl halide.

The active center of the AcChE contains two subsites, esteratic and anionic. The hydrolysis of AcCh by AcChE is regarded to proceed through an acetyl enzyme intermediate. Since deacetylation is assumed to be the slowest step in the hydrolysis process, the inhibitor molecule may form an acetyl enzyme–inhibitor complex. Wilson and Alexander[14] have identified the formation of this complex

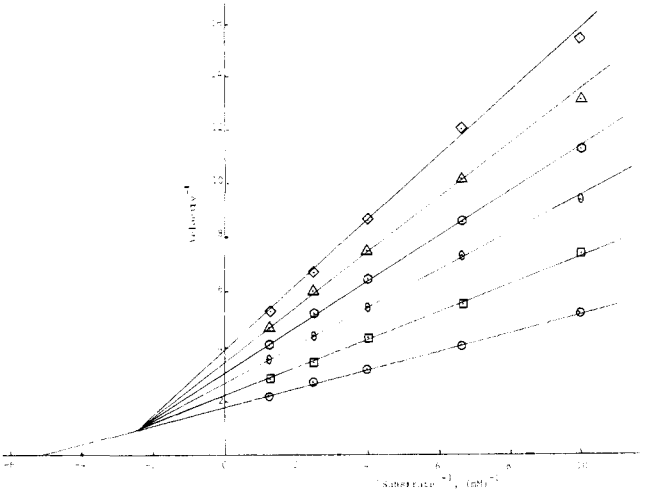


Figure 1. Lineweaver–Burk plot for reversible inhibition of acetylcholinesterase by hexamethylene-1,6-bis(acridinium) diiodide (II). Velocity is the rate of acetylcholine hydrolysis in relative units. Inhibitor (II) concentration (nM): (⊙) 0, (⊡) 1, (⊙) 2, (⊙) 3, (△) 4, (◇) 5.

as providing a noncompetitive component to the observed kinetics of AcCh hydrolysis. AcChE also possesses a large number of negative charges in addition to the one associated with the esteratic site.[15] Many of these charges could serve as sites for reversible binding of inhibitors and substrates and alter the rate of AcCh hydrolysis. AcChE inhibitors such as acridinium methiodide and quaternary ammonium compounds containing certain bulky cationic heads bind to anionic site receptors and to surface areas adjacent to the active sites.[16,17] These compounds have a noncompetitive component as inhibitors of AcChE and have been classed as mixed competitive–noncompetitive inhibitors.[17]

The enzyme kinetic studies with AcChE and BuChE were carried out by automatic titration with a pH-Stat under steady-state conditions and pH 7.5, and the data were plotted according to Lineweaver and Burk.[18] According to Webb[19] if the straight lines of the Lineweaver-Burk plot intersect at neither the x nor on the y axis, the inhibition is of the mixed competitive–noncompetitive type. The coordinates of the point of intersection of a family of lines obtained by using varying concentrations of inhibitors make it possible to estimate the proportion of the two types of inhibition (Figure 1). Competitive inhibition results in lines which intersect on the y axis (Figure 2).

The equations of Wilson and Alexander[14] were used to obtain $K_{I,i}$ and $K_{I,s}$ values, obtained from the intercept and slope. $K_{I,s}$ is the dissociation constant of the (free) en-

$$\frac{1}{V} = \frac{1}{V_{max}}\left[1 + \frac{I}{K_{I,i}}\right] + \frac{K_m}{V_{max}}\left[1 + \frac{I}{K_{I,s}}\right]\frac{1}{S}$$

zyme–inhibitor complex. The displacement of the ordinate intercept of the Lineweaver–Burk plot in the presence of an inhibitor indicates a noncompetitive component in the inhibition and is expressed by $K_{I,i}$.

Results and Discussion

Hexafluorenium bromide (I) and hexamethylene-1,6-bis(4-phenylbenzyldimethyl)ammonium dibromide (III) are essentially identical insofar as their mass, concentration of positive charge on quaternary nitrogen, and overall lipophilic–hydrophilic balance. Yet, a comparison of $K_{I,s}$ values of these compounds (see Table I) shows that I is

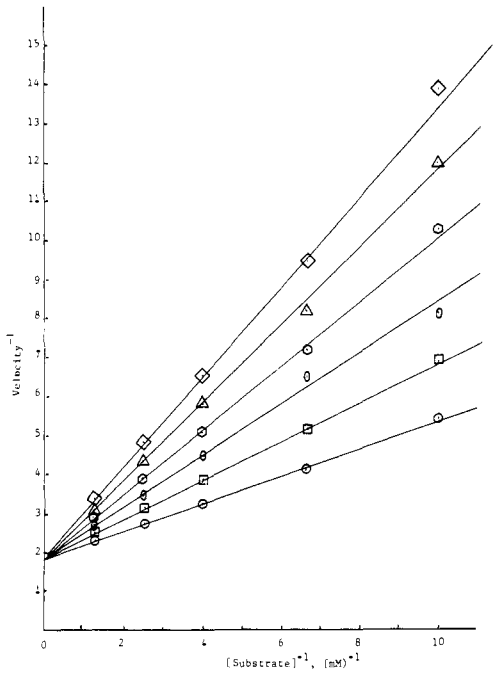


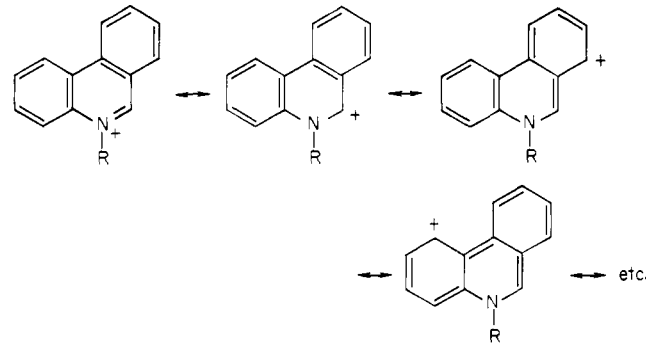
Figure 2. Lineweaver-Burk plot for reversible inhibition of acetylcholinesterse by *N*-acetoxyethylacridinium bromide (XXVI). Velocity is rate of acetylcholine hydrolysis in relative units. Inhibitor (XXVII) concentration (nM): (⊙) 0, (⊡) 0, (θ) 20, (⊙) 30, (△) 40, (◈) 50.

a better inhibitor than III of both AcChE and BuChE. The planar configuration of the fluorenyl moiety in I enhances short-range binding forces on the enzymes and contributes to the inhibitory activity. The biphenyl groups of III are not coplanar in the solution state and their ability to interact with the enzyme through short-range binding forces is decreased, resulting in lower inhibition. The aromatic rings of biphenyl have been reported to be 20° out of plane in solution and 40° in the gaseous state.[20,21] The lower potency of the biphenyl analogue, III, is consistent with the assumption that structural planarity results in a more effective summation of the short-range van der Waals forces to a lipoidal surface as well as providing less steric hindrance in the approach to the binding site on the enzyme.[13]

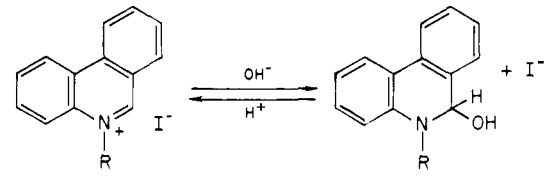
A high concentration of positive charge of bis-quaternary compounds possessing large flat lipophilic moieties is not a critical factor for the anticholinesterase activity. This is demonstrated by comparing the bis(4-phenylbenzyldimethyl)ammonium quaternary, III, with its bis(acridinium) analogue, II, or with the other bis(benzoquinolinium) compounds, IV and V. Both of these two types of compounds contain large hydrophobic end groups. However, acridinium, phenanthridinium, and 5,6-benzoquinolinium derivatives in which the concentration of the positive charge decreases to some extent by either internal resonance stabilization (delocalization) or pseudobase formation in the solution retain high cholinesterase inhibitory activity. Schemes I and Scheme II show the delocalization of the unit positive charge and pseudobase formation properties of the phenanthridinium cation.

Hexamethylene-1,6-bis(pyridinium) (VI) is essentially inactive compared to the bis(acridinium) analogue, II. The much lower potency (1/1 000 000 to 1/100 000) of the bis(pyridinium) compound may be related to a very small contribution of hydrophobic and van der Waals interaction around the anionic site of the enzyme. The shape of the benzoquinolinium moieties influences inhibitory potency. This is evident from a comparison of the K_I values (Table

Scheme I. Delocalization of the Positive Charge



Scheme II. Pseudobase Formation



I) of 1,6-hexamethylenebis(acridinium) (II), bis(phenanthridinium) (IV), and bis(5,6-benzoquinolinium) (V) salts on AcChE and BuChE. The *N*-methyl derivatives (VIII, XVII, XXII, XXIV) and *N*-hydroxyethyl derivatives (XIV, XX, XXIII, XXV) of monobenzoquinolinium salts (Table II) also vary in inhibitor potency on AcChE and BuChE. Both bis- and monobenzoquinolinium compounds show that the order of decreasing potency of their cholinesterase inhibition is acridinium > phenanthridinium > 5,6-benzoquinolinium > 7,8-benzoquinolinium.

Orientation of the two lipophilic groups of bis(onium) salts with respect to one another seems to affect relative activity between AcChE and BuChE. The flexible hexamethylene chain in IV is more favorable for the inhibitory activity of AcChE ($K_{I,s}$ = 6.6 × 10^{-9} M) than BuChE ($K_{I,s}$ = 2.7 × 10^{-8} M). However, replacement of the hexamethylene with the more rigid *p*-xylylenic moiety as in VII decreases AcChE inhibitory activity ($K_{I,s}$ = 1.8 × 10^{-7} M) much more than anti-BuChE activity ($K_{I,s}$ = 5.7 × 10^{-8} M).

The inhibitory activity of the monobenzoquinolinium halides against cholinesterases is influenced by their N-substituents. N-Substituted *n*-hexyl (e.g., XI), benzyl (e.g., XII and XVIII), or phenethyl (e.g., XIII and XIX) groups of the benzoquinolinium derivatives enhance the inhibitory activity, while the N-substituted β-hydroxyethyl (e.g., XIV, XX, XXIII, and XXV) or carboxymethyl (XV) group reduce the inhibitory activity against the enzyme. The *n*-hexyl, benzyl, and phenethyl groups are believed to enhance the hydrophobic binding of the benzoquinolinium moiety onto the hydrophobic area around the anionic site of the enzyme. The hydroxyl group, in contrast, interferes with binding and therefore the inhibitory activity of the hydroxyl derivatives is decreased. Attachment of a carboxyl group to the quaternary nitrogen of the benzoquinolinium moiety such as compound XV decreases the inhibitory activity to some extent and suggests that the electrostatic forces orienting quaternary ammonium ions to the anionic site of the enzyme have been decreased due to the repulsion between the carboxyl group of the inhibitor and the functional groups at the anionic site of the enzyme. This indicates that the total binding forces include both hydrophobic and anionic components. The N-substituted propyl and isopropyl groups do not show a significant effect on the binding of the benzoquinolinium moiety onto the enzyme surface. If any, the slightly lower potency of the isopropyl derivative

on anti-AcChE activity may be the result of steric hindrance of the ionic binding of the benzoquinolinium moiety to the anionic site of the enzyme.

The ratio of $K_{I,i}$ to $K_{I,s}$ for each mono- and bis-onium compound showing mixed noncompetitive–competitive inhibition kinetics is greater than unity but with only one exception less than tenfold. The proportionality between the two types of inhibition by these onium compounds is essentially the same for both enzymes. The mean values are 4.4 ± 0.3 and 4.8 ± 0.4 for AcChE and BuChE, respectively. These values are surprisingly close despite the diversity of compounds studied and the variation of inhibitor potencies observed.

The *N*-acetoxyethyl derivatives of phenanthridinium (XVI), 5,6-benzoquinolinium (XXI), and acridinium (XXVII) are not substrates of either AcChE or BuChE but instead are inhibitors of these enzymes. Since the corresponding choline derivatives, XV, XXII, and XXV, are weaker inhibitors by a magnitude of 10^2, it can be assumed that the acetyl moiety contributes to the binding of the molecule to AcChE and BuChE. The inhibition of AcChE by the acetoxyethyl derivatives, XVI, XX, and XXIII, is competitive (Figure 2) and differs from the mixed inhibition of the corresponding hydroxyethyl analogues, XIV, XX, and XXIII. The poor inhibitory activity of *N*-acetoxyethylpyridinium bromide [$K_{I,s}$ (AcChE) = 3.2 ± 0.2 × 10^{-4} M, $K_{I,s}$ (BuChE) = 1.2 ± 0.1 × 10^{-4} M] may result from the much smaller onium moiety which does not allow the formation of sufficient short-range bonding forces at the hydrophobic region adjacent to the anionic site of the enzyme.

Experimental Section

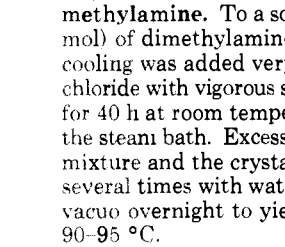
Preparation of the Quaternary Ammonium Compounds. As a general procedure, the tertiary amine was dissolved or suspended in acetonitrile and the alkylating agent such as alkyl halide, aralkyl halide, or its analogues then added. The mixture was refluxed until a precipitate formed after an aliquot of the reaction mixture was added into ether or when crystals formed in the boiling solution. The product was recrystallized and finally dried in vacuo at 80 °C over anhydrous $CaSO_4$. The melting points were taken with a Fisher-Johns melting point apparatus and are uncorrected. Ultraviolet spectra were measured on a Cary 15 spectrophotometer. Infrared spectra were obtained on a Perkin-Elmer Model 257 spectrophotometer. Elemental analyses were done by M-H-W Laboratories, Garden City, Mich., or Atlantic Microlab, Inc., Atlanta, Ga.

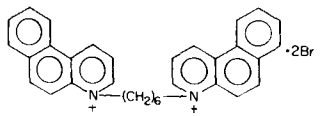
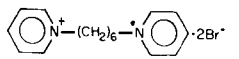
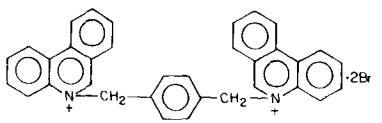
Method A. Hexamethylene-1,6-bis(4-phenylbenzyldimethylammonium) Dibromide (III). (a) Reaction of 4-Phenylbenzoic Acid with Thionyl Chloride. To 50 g (0.25 mol) of 4-phenylbenzoic acid was added slowly 13.3 g (1.04 mol) of thionyl chloride with stirring. The mixture was refluxed on a steam bath for 1.5 h with occasional stirring. The unreacted thionyl chloride was removed by use of a rotary flash evaporator. After cooling the reaction mixture, 48 g (87%) containing the crude acid chloride was collected and the product recrystallized from petroleum ether, mp 100–103 °C.

(b) Reaction of 4-Phenylbenzoyl Chloride with Dimethylamine. To a solution prepared by bubbling in 13.5 g (0.3 mol) of dimethylamine into 75 mL of anhydrous pyridine with cooling was added very slowly 40 g (0.22 mol) of the crude acid chloride with vigorous stirring. The mixture was allowed to stand for 40 h at room temperature and then heated gently for 1 h on the steam bath. Excess (900 mL) water was added to the reaction mixture and the crystals which separated were filtered, washed several times with water and 5% Na_2CO_3, and dried at 50 °C in vacuo overnight to yield 37.3 g (50%) of the crude amide, mp 90–95 °C.

(c) Reaction of Biphenyl-*N*,*N*-dimethylcarboxamide with Lithium Aluminum Hydride. A mixture of 6.46 g (0.17 mol) of lithium aluminum hydride suspended in 250 mL of anhydrous ether was refluxed overnight in a flask fitted with Soxhlet extractor in which 33.4 g (0.15 mol) of biphenyl-*N*,*N*-dimethylcarboxamide

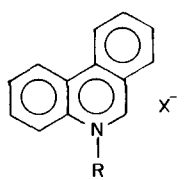
Table I. Physical Constants and Cholinesterase Inhibitory Activities of Bis-Quaternary Ammonium Salts

No.	Structure	Mp, °C	Method	Yield, %	Anticholinesterase activities: AcChE $K_{I,s}$, μM	AcChE $K_{I,i}$, μM	BuChE $K_{I,s}$, μM	BuChE $K_{I,i}$, μM
I		188–189[a]			1.3 ± 0.04	6.8 ± 0.2	0.34 ± 0.01	0.89 ± 0.04
II		271–274	B	46	0.0021 ± 0.00007	0.0092 ± 0.00001	0.0073 ± 0.0002	0.024 ± 0.005
III		260–270	A	16	39.0 ± 3.0	120.0 ± 5.0	1.5 ± 0.2	7.9 ± 0.6
IV		250–255	B	22	0.0066 ± 0.0005	0.018 ± 0.003	0.027 ± 0.004	0.091 ± 0.002

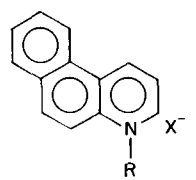
V		258-260	B	34	0.010 ± 0.0008	0.072 ± 0.0008	0.36 ± 0.03	1.5 ± 0.2
VI		238-241	B	75	16 000[b] ± 9 000		120 ± 10	620 ± 30
VII		259-260	B	48	0.18 ± 0.006	0.71 ± 0.03	0.057 ± 0.003	0.23 ± 0.01

[a] Cavallito et al., *J. Am. Chem. Soc.*, 76, 1862 (1954). [b] The concentration of the inhibitor required for 25% inhibition of AcChE.

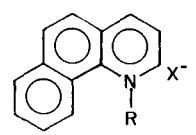
Table II. Physical Constants and Cholinesterase Inhibitory Activities of Mono-N-substituted Benzoquinolinium Halides



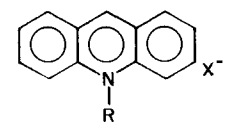
VIII-XVI



XVII-XXI



XXII, XXIII



XXIV-XXVII

						Anticholinesterase activities			
						AcChE[a]		BuChE	
No.	R	X	Mp, °C	Method	Yield, %	$K_{I,s}$, μM	$K_{I,i}$, μM	$K_{I,s}$, μM	$K_{I,i}$, μM
VIII	CH_3	Br	244-246[b]			5.60 ± 0.4	25.5 ± 1.0	1.10 ± 0.02	6.4 ± 0.5
IX	$CH_3CH_2CH_2$	Br	240-242	C	44	3.20 ± 0.2	13.6 ± 0.8	1.30 ± 0.1	7.5 ± 0.6
X	$(CH_3)_2CH$	Br	225	C	23	22.00 ± 3.0	97.0 ± 6.0	1.5 ± 0.2	4.8 ± 0.2
XI	$CH_3(CH_2)_5$	Br	103-104	D	35	0.53 ± 0.02	1.8 ± 0.5	0.12 ± 0.01	0.9 ± 0.05
XII	$C_6H_5CH_2$	Br	248-250	C	61	0.25 ± 0.03	0.95 ± 0.04	0.061 ± 0.002	0.26 ± 0.03
XIII	$C_6H_5CH_2CH_2$	Br	234-236	C	47	0.28 ± 0.05	0.78 ± 0.01	0.054 ± 0.005	0.31 ± 0.02
XIV	$HOCH_2CH_2$	Br	245-246	C	72	31.00 ± 2.00	45.00 ± 4.0	37.00 ± 9.0	98.4 ± 7.0
XV	$HOOCCH_2$[c]	Br	162	E	24	12.00 ± 1.00	72.4 ± 3.0	41.00 ± 2.0	86.0 ± 4.0
XVI	$CH_3COOCH_2CH_2$	Br	178-180	F	71	0.38 ± 0.02		0.12 ± 0.01	
XVII	CH_3	Br	259[b]			8.9 ± 0.5	25.4 ± 0.9	5.4 ± 0.2	34.0 ± 0.7
XVIII	$C_6H_5CH_2$	Br	226-228	C	70	9.3 ± 0.2	37.4 ± 1.0	0.86 ± 0.05	5.6 ± 0.9
XIX	$C_6CH_2CH_2$	Br	225-230	C	51	1.8 ± 0.3	18.5 ± 0.8	0.92 ± 0.01	4.9 ± 0.4
XX	$HOCH_2CH_2$	Br	227-229	C	73	83.0 ± 0.3	160 ± 30	79.00 ± 4.00	210 ± 7.0
XXI	$CH_3COOCH_2CH_2$	Br	180	E	68	0.95 ± 0.03		0.82 ± 0.01	
XXII	CH_3	I	167-171	C	30	18.0 ± 4.0	140 ± 3.0	32.00 ± 1.0	230 ± 20
XXIII	$HOCH_2CH_2$	Br	250	C	22	150 ± 10	360 ± 5.0	110 ± 7.0	410 ± 7.0
XXIV	CH_3	I	227	D	36	0.5 ± 0.02	2.8 ± 0.2	0.24 ± 0.03	1.7 ± 0.5
XXV	$HOCH_2CH_2$	Br	225	C	44	2.9 ± 0.80	9.6 ± 0.7	1.30 ± 0.20	7.2 ± 0.6
XXVI	$CH_3COOCH_2CH_2$	Br	228-230	F	66	0.021 ± 0.005		0.011 ± 0.001	
XXVII	*N*-Acetoxyethylpyridinium	Br	280	G	70	320 ± 20		120 ± 10	

[a] Physostigmine salicylate was used as the reference compound; $K_{I,s}$(AcChE) is 0.21 ± 0.01 μM. [b] Cavallito et al., *J. Med. Chem.*, 12, 134 (1969). [c] K_I values were determined in the dark as compound XV was photosensitive.

was placed in the extractor thimble. After cooling, the reaction mixture was transferred to a beaker and 80 mL of ethyl acetate was added to it over a period of 30 min to decompose the excess $LiAlH_4$. The white solid was filtered off and rinsed several times with anhydrous ether and the washings were combined with the initial filtrate from the reaction. The ether solution was dried over Na_2SO_4 (anhydrous) and the solvent removed on rotary evaporator. The residue was dissolved in 134 mL of anhydrous ether and the HCl salt precipitated by adding a solution of HCl in ether. The HCl salt was collected by filtration and recrystallized from isopropyl alcohol. The free base was obtained by dissolving the HCl salt in a minimum amount of water and adding 0.5% NaOH until no additional material separated. The mixture was extracted several times with anhydrous ether and the ether extracts were combined. The ether was dried over anhydrous Na_2SO_4 and the solvent removed on a rotary evaporator to yield 14 mL of crude amine.

(d) Reaction of 4-Phenylbenzyl-*N*,*N*-dimethylamine with 1,6-Dibromohexamethylene. A mixture of 2.3 mL of 4-phenylbenzyl-*N*,*N*-dimethylamine and 1 mL of (0.0076 mol) of 1,6-dibromohexane in 30 mL of acetonitrile was refluxed for 54 h. The resulting yellow solid was filtered off hot and washed several times with hot acetonitrile. This was recrystallized four times from a mixture of ethanol and water and then dried at 90 °C in vacuo overnight to afford 0.78 g (15.6%), mp 260–270 °C.

Method B. Hexamethylene-1,6-bis(acridinium) Diiodide (II). A mixture of 3.58 g (0.02 mol) of acridine, 3.38 g (0.01 mol) of 1,6-diiodohexane, and 10 mL of acetonitrile was boiled under reflux for 40 h. The crystals which formed in the boiling solution were filtered out while hot, washed three times with hot acetonitrile, recrystallized twice from distilled water, and finally dried over anhydrous $CaSO_4$ in vacuo at 80 °C for 6 h. The yield was 3.2 g, mp 271–274 °C. Anal. ($C_{32}H_{30}N_2I_2$) C, H.

Method C. *N*-(2-Hydroxyethyl)phenanthridinium Bromide (XIV). A solution of 1.79 g (0.01 mol) of phenanthridine and 2.2 mL (0.03 mol) of 2-bromoethanol in 20 mL of acetonitrile was refluxed for 8 h. The crystals which formed in the boiling solution were filtered out while hot and washed with hot acetonitrile. The product was recrystallized from methanol and dried over anhydrous $CaSO_4$ in vacuo at 80 °C for 6 h. The yield was 2.2 g, mp 245–246 °C. Anal. ($C_{15}H_{14}BrNO$) C, H.

Method D. *N*-(*n*-Hexyl)phenanthridinium Bromide (XI). A mixture of 1.79 g (0.01 mol) of phenanthridine and 8.5 mL (an excess) of 1-bromohexane was heated at 80 °C for 24 h. The product which precipitated in the hot solution was filtered out, recrystallized from isopropyl alcohol and ether, and then dried overnight in vacuo at 43 °C over anhydrous $CaSO_4$. The yield was 1.2 g, mp 103–104 °C. Anal. ($C_{19}H_{22}BrN$) C, H.

Method E. *N*-Carboxymethylphenanthridinium Bromide (XV). A solution of 2.37 g (0.017 mol) of bromoacetic acid and 3.05 g (0.017 mol) of phenanthridine in 25 mL of acetonitrile was refluxed in a dark room (the product was found to be photosensitive) for 2 h. The yellow, solid material which precipitated in the boiling solution and stuck firmly on the flask wall was collected and washed with hot acetonitrile and then with ether. The product was recrystallized twice from methanol and dried over anhydrous $CaSO_4$ in vacuo at 40 °C for 8 h. The yield was 1.3 g, mp 162 °C. Anal. ($C_{15}H_{12}BrNO_2$) C, H.

Method F. *N*-Acetoxyethylphenanthridinium Bromide (XVI). Acetic anhydride (30 mL) was added to a solution of 1 g (0.003 mol) of *N*-(2-hydroxyethyl)phenanthridinium bromide (XV) in 15 mL of dimethylformamide (DMF). The solution was heated at 80 °C with stirring for 44 h. The desired product which precipitated while cooling down to room temperature was filtered out, washed with ether, recrystallized from EtOH–Et_2O, and finally dried over anhydrous $CaSO_4$ in vacuo at 80 °C for 12 h. The yield was 0.82 g, mp 178–180 °C. Anal. ($C_{17}H_{16}BrNO_2$) C, H.

Method G. *N*-Acetoxyethylpyridinium Bromide (XXVII). A mixture of 2.5 g (0.015 mol) of 2-bromoethyl acetate and 15 mL of pyridine (an excess) was heated at 100 °C for 69 h. The flask was cooled and the solution was concentrated in vacuo. The solid residue was washed with dry ether, recrystallized from EtOH–Et_2O, and finally dried over anhydrous $CaSO_4$ in vacuo at 60 °C for 6 h. The yield was 0.71 g, mp 280 °C. Anal. ($C_9H_{12}BrNO_2$) C, H.

Determination of K_I Values of Inhibitors. The initial rates of the enzymatic hydrolysis of acetylcholine bromide in the absence or presence of an inhibitor were measured on a Radiometer pH-Stat at pH 7.5.

In practice, five different concentrations of an inhibitor were prepared. Each of these five inhibitor solutions was then mixed with five different concentrations of substrate (e.g., 0.1, 0.15, 0.25, 0.4, and 0.8 mM acetylcholine bromide in the final inhibitor–substrate mixtures).

To each inhibitor–substrate (10 mL) mixture was added an aliquot (i.e., 20 μL) of the enzyme solution (0.1 unit of acetylcholinesterase or 0.7 unit of butyrylcholinesterase) with stirring at 25 °C, and the initial hydrolysis rates over a 4-min period were then recorded. A K_m was determined by adding the same amount of enzyme as above in the absence of inhibitor. The medium of the reaction solution always contained 0.02 M $MgCl_2$ and 0.1 M NaCl. The reaction vessel was closed off from CO_2 in the surrounding atmosphere.

The experimental initial velocities in the absence or presence of the inhibitor were fitted in the points of a Lineweaver–Burk plot. The inhibition constants (K_I) were obtained from computer fits of the data to the equation for competitive or noncompetitive inhibition. The lines which intersected on the ordinate were fitted (through a computer program) to the equation for competitive inhibition, and those which intersected to the left of the ordinate were fitted to the equation for noncompetitive inhibition. *t* tests were also used to help distinguish between the types of inhibition.

Acetylcholinesterase and butyrylcholinesterase which were prepared from the electric organ of *E. electricus* and from horse serum, respectively. were purchased from Worthington Biochemical Corp. Acetylcholine bromide (Sigma Chemical Co.) was used as a substrate throughout the experiment. The K_m value of acetylcholinesterase for acetylcholine bromide was 0.14 ± 0.06 mM while butyrylcholinesterase for the same substrate was 0.37 ± 0.03 mM.

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Potential Inhibitors of Collagen Biosynthesis. 5,5-Difluoro-DL-lysine and 5,5-Dimethyl-DL-lysine and Their Activation by Lysyl-tRNA Ligase

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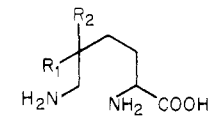
The synthesis of lysine analogues wherein blocking groups are substituted at position 5, the site of hydroxylation by peptidyl lysine hydroxylase, is described. Thus, 5,5-difluorolysine (1) and 5,5-dimethyllysine (2) were synthesized via a four- and six-step sequence, respectively, starting from ketone precursors. The propensity for these lysine analogues to be incorporated into procollagen protein in vivo was assessed by their ability to stimulate the lysine-dependent ATP-PP_i exchange reaction in the presence of lysyl-tRNA ligase in vitro. The difluoro analogue 1 stimulated exchange, but at a K_m (1.3×10^{-3} M) 1000 times greater than that for lysine itself. The dimethyl analogue 2 did not stimulate exchange, but at high concentrations was a competitive inhibitor of lysine, with an apparent K_i of 1.6×10^{-2} M. Thus, electronegative and/or bulky substituents at the 5 position of lysine cannot be tolerated by lysyl-tRNA ligase, and this position must be kept free in lysine analogues specifically designed to block collagen biosynthesis.

A number of reports linking aberrant collagen production with pathologic defects found in a variety of disease states[1] and in tumor growth[2] suggest that agents that can selectively control collagen biosynthesis might be of therapeutic utility. Recent advances in our knowledge of collagen biosynthesis[3] indicate the importance of lysyl and hydroxylysyl residues in collagen for the formation of intra- and intermolecular cross-links, such cross-links being vital to the structural integrity and metabolic stability of collagen. Cross-link formation involves the oxidative deamination of the ϵ-amino groups of lysyl or hydroxylysyl residues in collagen to reactive aldehydes by the enzyme, lysyl oxidase. These aldehydes serve as focal centers for cross-linking via aldol condensation or aldimine formation.

The biosynthesis of hydroxylysine,[4] specific for collagen and one other protein, viz., the Clq component of complement,[5] involves the introduction of a hydroxyl group at the 5 position of lysyl residues in procollagen, a collagen precursor, by an α-ketoglutarate-dependent mixed function oxidase, viz., peptidyl lysine hydroxylase (PLH, E.C. 1.14.11.4; lysine, 2-oxoglutarate dioxygenase[6]). This 5-hydroxyl group on lysyl residues is also a position for polysaccharide attachment. The uniqueness of this hydroxylation step to collagen and the essential role of hydroxylysine in cross-link formation suggest that lysine analogues, wherein hydroxylation and/or cross-link formation can be blocked, might selectively inhibit collagen biosynthesis.

Certain synthetic lysine analogues have been reported to substitute for L-lysine in proteins, viz., 4-thialysine,[7] 4-azalysine,[8] 4-selenolysine,[9] and *trans*-4,5-dehydrolysine.[10] Incorporation of the latter in a collagen synthesizing system has been reported to generate abnormal procollagens which were poorly secreted and thus were subject to ready degradation.[11] Therapeutic attempts to limit collagen production in patients with progressive systemic scleroderma by administration of agents that inhibit cross-linking have met with only limited success due to adverse side reactions. Such agents, e.g., β-aminoproprionitrile[12] or penicillamine,[13] act by either preventing aldehyde formation or by entrapping the reactive aldehydes necessary for cross-link formation.

5,5-Difluoro-DL-lysine (1) and 5,5-dimethyl-DL-lysine (2),



1, $R_1 = R_2 = F$
2, $R_1 = R_2 = CH_3$

the title compounds projected for synthesis, represent lysine analogues bearing geminal blocking groups at the 5 position. This geminal substitution was designed to serve two purposes, viz., (1) to block hydroxylation at this site by PLH and (2) to prevent the aldol condensation of the aldehyde generated by oxidative deamination of the ϵ-amino group by virtue of α,α-disubstitution on the newly generated aldehyde group. Inhibition of collagen biosynthesis by 1 and 2 can therefore be envisioned to take place by formation of abnormal procollagen which would have decreased propensity for extracellular secretion and/or cross-link formation.

Chemistry. The synthesis of 1 (Scheme I) started with the substituted aminomalonic ester derivative 3 described by Zyl et al.[14] Protection of the ω-amino group of 3 was achieved by phthaloylation with *N*-carbethoxyphthalimide[15] to give 4. Oxidation of the unprotected secondary alcohol function of 4 with Me_2SO–DCC in the presence of pyridinium trifluoroacetate[16] gave a product whose elemental analyses and infrared and NMR spectra were consistent with the expected structure 5. Fluorination of 5 with SF_4–HF[17] gave a mixture of products which was resolved into three components by preparative TLC. They were, in the order of their increasing R_f values, the unreacted ketone 5 and two other compounds whose elemental analyses pointed to the desired difluorinated product 6 and a tetrafluorinated product 7. Comparison of the IR spectra of 6 and 7 with 5 suggested that only the ketone function in 6 was fluorinated, whereas in 7 both the