Structure–Activity Relationship of the Ficin Hydrolysis of Phenyl Hippurates. Comparison with Papain, Actinidin, and Bromelain[1]

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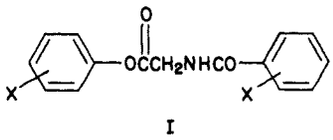
A study of the hydrolysis of 30 substituted-phenyl hippurates by the enzyme ficin has been made. From the results the following quantitative structure–activity relationship (QSAR) has been derived: $\log 1/K_m = 0.79\pi'_3 + 0.58\sigma + 0.28\ MR_{4,5} + 3.70$. In this expression K_m is the Michaelis constant, π'_3 refers to the more hydrophobic of the two meta substituents, and $MR_{4,5}$ is the molar refractivity of substituents in the 4- and 5-positions of the phenyl ring. This QSAR is compared with those from papain, actinidin, bromelain B, and bromelain D.

The continuous advance in enzyme catalysis,[2a,b] kinetics, and mechanism[2c–e] are a source of new ideas for medicinal chemists attempting a more rational and efficient way of designing drugs.[3] The crucial role of many enzymes in various physiological and/or pathological processes and the enzymic nature of most receptors[4] prompt many scientists to study more intensely to obtain more relevant and significant insight into the molecular mechanisms by which enzymes act. Even where very sophisticated analytical and spectroscopic techniques[5] have been successfully applied in mechanistic studies, very often they suffer from limited applicability, especially when fast processes are involved. For these reasons we have begun a systematic study of enzyme–ligand interactions making combined use of QSAR and molecular graphics.[6] To date reactions of the following enzymes have been studied in this manner: various dihydrofolate reductases,[6a] chymotrypsin,[6c] papain,[7] actinidin,[8] trypsin,[9] and carboxypeptidase.[9] The cysteine proteases, structurally similar to the more important serine proteases,[10] were selected for this study because they are readily available and for some of them X-ray crystallographic coordinates are available. From the pharmacological point of view it is important to note that some proteases are widely used in food technology as meat tenderizers[11] and bromelain is currently used as an antiinflammtory agent.[12,13] It is conceivable that from a knowledge of QSAR more effective commercial variations of the serine proteases could be developed via genetic engineering.

The sulfhydryl proteases, papain from papaya, ficin from figs, bromelain from pineapple, and actinidin from kiwi fruit, are closely related to the serine proteases. Of these four very similar enzymes, papain has been the most intensively studied; however, there has long been a strong interest in other members of the group.[14] Recently the mechanism of hydrolysis of both the serine and cysteine proteases has been reviewed by Polgár and Halász.[15]

In this report we compare the structure–activity relationship for the hydrolysis of phenyl esters I by ficin with earlier results obtained with the three other cysteine hydrolases[7,8,16] on congeners I and II.



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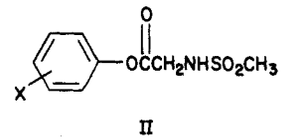

II

Kortt et al. have studied the ficin hydrolysis of several derivatives of I.[17] Recently Brocklehurst and his colleagues have been studying the structure–function relations of ficin.[18–21]

In our earlier studies of the hydrolysis of I and II by papain and actinidin interest was centered on under-

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Table I Parameters Used To Derive Equations 7–11 for the Ficin Hydrolysis of X-$C_6H_4OCOCH_2NHCOC_6H_5$[a] at pH 6, 25 °C

no.	X	log $1/K_m$ obsd	log $1/K_m$ calcd[b]	$\lvert\Delta \log 1/K_m\rvert$	σ	MR_4	π'_3	$MR_{4,5}$
1	H	3.90	3.68	0.22	0.00	0.10	0.00	0.20
2	3-CH_3	4.17	4.11	0.06	−0.07	0.10	0.56	0.20
3	3-F	3.81	3.99	0.18	0.34	0.10	0.14	0.20
4	3-Cl	4.43	4.49	0.06	0.37	0.10	0.71	0.20
5	3-Br	4.75	4.63	0.12	0.39	0.10	0.86	0.20
6	3-I	4.64	4.82	0.19	0.35	0.10	1.12	0.20
7	3-CF_3	4.85	4.67	0.18	0.43	0.10	0.88	0.20
8	3-OCH_3	4.09	4.03	0.06	0.12	0.10	0.00	0.89
9	3-$COCH_3$	4.11	4.32	0.21	0.38	0.10	0.00	1.22
10	3-NO_2	4.36	4.35	0.01	0.71	0.10	0.00	0.84
11	3-CN	4.15	4.22	0.07	0.56	0.10	0.00	0.73
12	3-NH_2	3.72	3.77	0.05	−0.16	0.10	0.00	0.64
13	3-$NHCOCH_3$	3.74[c]	4.37	0.63	0.21	0.10	0.00	1.59
14	3-$CONH_2$	3.99	4.20	0.21	0.28	0.10	0.00	1.08
15	3-$C(CH_3)_3$	5.14	5.30	0.16	−0.10	0.10	1.98	0.20
16	4-CH_3	3.82	3.77	0.05	−0.17	0.56	0.00	0.66
17	4-$CH(CH_3)_2$	4.33	4.18	0.15	−0.13	1.50	0.00	1.60
18	4-F	3.53	3.71	0.18	0.06	0.09	0.00	0.19
19	4-Cl	3.91	4.02	0.11	0.23	0.60	0.00	0.70
20	4-Br	4.13	4.14	0.01	0.23	0.89	0.00	0.99
21	4-OCH_3	3.82	3.81	0.01	−0.27	0.79	0.00	0.89
22	4-$COCH_3$	4.30	4.39	0.09	0.50	1.12	0.00	1.22
23	4-NO_2	4.50	4.39	0.11	0.78	0.74	0.00	0.84
24	4-CN	4.27	4.28	0.01	0.66	0.63	0.00	0.73
25	4-$CONH_2$	4.17	4.25	0.08	0.36	0.98	0.00	1.08
26	3,5-$(CH_3)_2$	4.54	4.26	0.28	−0.14	0.10	0.56	0.66
27	3-CH_3, 5-C_2H_5	4.79	4.65	0.14	−0.14	0.10	1.02	0.66
28	3,5-$(Cl)_2$	4.98	4.91	0.07	0.74	0.10	0.71	0.70
29	3,5-$(NO_2)_2$	4.69	4.52	0.17	1.42	0.10	−0.28	0.84
30	3,5-$(OCH_3)_2$	4.52[c]	4.09	0.43	0.24	0.10	−0.02	0.89

[a] For four new hippurates (**8**, **9**, **17**, **20**) carbon, hydrogen, and nitrogen analyses were within 0.4% of theoretical values. [b] Calculated using eq 11. [c] These points not used in the derivation of eq 11.

standing how the properties of X affect the enzymatic reactions. These two enzymes were selected because X-ray crystallographic structures have been established for both papain[22,23] and actinidin.[24]

For papain quantitative structure–activity relationships (QSAR) were formulated for ester sets I and II.

Papain Hydrolysis of Congeners I[16]

$$\log 1/K_m = 1.03\pi'_3 + 0.57\sigma + 0.61MR_4 + 3.80 \quad (1)$$

$$n = 25,\ r = 0.907,\ s = 0.208$$

Papain Hydrolysis of Congeners II[7]

$$\log 1/K_m = 0.61\pi'_3 + 0.55\sigma + 0.46MR_4 + 2.00 \quad (2)$$

$$n = 32,\ r = 0.945,\ s = 0.178$$

A corresponding structure–activity relationship for actinidin is shown in eq 3.

Actinidin Hyrolysis of Congeners I[8]

$$\log 1/K_m = 0.50\pi'_3 + 0.74\sigma + 0.24MR_4 + 2.90 \quad (3)$$

$$n = 27,\ r = 0.927,\ s = 0.158$$

For the above hydrolytic reactions of papain and actinidin, k_{cat} is essentially constant regardless of the nature of X.

In these expressions K_m is the Michaelis constant, π'_3 is the hydrophobic substituent constant,[25] σ is the Hammett constant, and MR stands for molar refractivity.[25] The subscript and prime with π means that only the more hydrophobic of the two meta substituents is considered to bind to the enzyme. Therefore the π constant for the more hydrophilic substituent is set equal to zero. It was assumed that, since this yielded a good mathematical model, the more hydrophilic substituent did not contact the enzyme. The construction of a molecular graphics model of papain plus ligand showed this to be a correct assumption.[16]

The subscript with MR_4 means that this term applies only to 4-substituents. Since MR is primarily a measure of bulk,[25] this term indicates that in a rough way the larger X is the better the binding (i.e., the larger log $1/K_m$ is). It was also assumed that, since the substitution of π_4 for MR_4 gave a much poorer correlation, X_4 was not contacting hydrophobic space on the enzyme. This portion of the enzyme was assumed to be polar in nature. The molecular graphics model showed that indeed X_4 contacted polar space by interacting with the amide moiety of Gln-142.

For papain hydrolysis of congeners I and II the coefficients with σ are essentially identical and the difference in the MR terms may or may not be significant, but in any case is not important. The different coefficients with π'_3 in eq 1 and 2 are important. From the study of graphics models[7,8] it has been proposed that the coefficient of 1.03 in eq 1 indicates complete desolvation of X in the formation of the enzyme substrate complex (ES). The smaller value of 0.61 in eq 2 would seem to mean that less effective desolvation is occurring in the ES complex with congeners

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Table II. Physical Properties of Previously Unreported Substituted-Phenyl Hippurates[a] and Spectral Data for Their Ficin-Catalyzed Hydrolysis[b]

X	formula	mp,[c] °C	% bound to preribosomal RNP particles[c]	% bound to preribosomal RNP particles by protein[d]
4-Br	$C_{15}H_{12}BrNO_3$	145–46	280	1094
4-CH-$(CH_3)_2$	$C_{18}H_{19}NO_3$	133–34	275	1295
3-OCH_3	$C_{16}H_{15}NO_4$	120–21	280.5	886
3-$COCH_3$	$C_{17}H_{15}NO_4$	93–94	310	1862

[a] IR (KBr pellets) and ^1H NMR spectra ($CDCl_3$, 1% Me_4Si, 90 MHz) were fully consistent with the proposed chemical structures. [b] At pH 6.0 in 0.30 M NaCl–0.01 M EDTA at 25.0 °C. [c] All products were crystallized from chloroform–hexane. [d] Optimum wavelength for spectrophotometric study of ester hydrolysis. [e] Change in molar absorptivity on complete hydrolysis at optimum wavelength (average of two runs at least).

II. From the graphics analysis[7] it has been suggested that X of II binds on the surface of the enzyme rather than in a hydrophobic pocket so that X is only about 50% desolvated.

Equation 3 for the hydrolysis of I by actinidin has a coefficient with σ significantly higher than those for eq 1 or 2, but it is still a rather small coefficient. Since the coefficient with π'_3 is close to that of eq 2, it is presumed that X of I binds on the surface of actinidin. A molecular graphics model has been constructured rationalizing this hypothesis.[8]

Hawkins and Williams have studied the hydrolysis of congeners I and II using two types of bromelain.[26] From their study QSAR were formulated that contained terms in σ and MR only. At that time,[27] the lipophilic effect of 3-substituents was not appreciated, and since only two such substituents were included in the study, they had little effect on the QSAR. These QSAR have now been redone with use of our previously published parameters but omitting the two meta compounds so that eq 4 and 5 can be compared with the other QSAR.

Bromelain B Hydrolysis of Congeners I

$$\log k_o/K_m = 0.70\sigma + 0.50MR_4 + 2.62 \quad (4)$$

$$n = 8,\ r = 0.962,\ s = 0.137$$

Bromelain D Hydrolysis of Congeners I

$$\log k_o/K_m = 0.63\sigma + 0.46MR_4 + 2.21 \quad (5)$$

$$n = 8,\ r = 0.996,\ s = 0.041$$

Bromelain B Hydrolysis of Congeners II

$$\log k_o/K_m = 0.68\sigma + 0.60MR_4 + 1.16 \quad (6)$$

$$n = 8,\ r = 0.978,\ s = 0.107$$

Since only k_o/K_m values were reported for the bromelain studies, we cannot produce QSAR *strictly* equivalent to eq 1–3. However, for papain and actindin it is known that k_o (k_{cat}) is essentially constant. Assuming this to be so, eq 4–6 can be compared with eq 1–3 except for the intercepts. The results are very similar, showing that QSAR provides an excellent means for studying the effect of different enzymes on the same kind of substrates.

The intellectually satisfying mathematical and graphics structure–activity models obtained for papain, actinidin, and bromelain encouraged us to extend our study to the reaction of congeners I with ficin. We assume that the enzyme ficin (E) reacts with phenyl hippurate to first form an ES complex, which then acylates the enzyme as governed by k_2. The acylenzyme then reacts with water to regenerate enzyme plus product as follows:

$$E + ROCOR' \underset{}{\overset{k_1}{\rightleftarrows}} EROCOR' \xrightarrow{k_2} ECOR' + HOH \xrightarrow{k_3} \underset{ROH}{+} \quad E + R'COOH$$

Results and Discussion

From the parameters for hydrolysis of variations of compounds I in Table I by ficin, we have derived the following equations:

$$\log 1/K_m = 0.57(\pm0.23)\pi'_3 + 4.12(\pm0.13) \quad (7)$$

$$n = 30,\ r = 0.695,\ s = 0.299,\ F_{1,28} = 26.1$$

$$\log 1/K_m = 0.66(\pm0.17)\pi'_3 + 0.56(\pm0.23)\sigma + 3.94(\pm0.12) \quad (8)$$

$$n = 30,\ r = 0.854,\ s = 0.221,\ F_{1,27} = 24.5$$

$$\log 1/K_m = 0.72(\pm0.18)\pi'_3 + 0.60(\pm0.23)\sigma + 0.21(\pm0.22)MR_4 + 3.84(\pm0.15) \quad (9)$$

$$n = 30,\ r = 0.875,\ s = 0.209,\ F_{1,26} = 3.97$$

$$\log 1/K_m = 0.79(\pm0.19)\pi'_3 + 0.58(\pm0.21)\sigma + 0.28(\pm0.22)MR_{4,5} + 3.70(\pm0.22) \quad (10)$$

$$n = 30,\ r = 0.885,\ s = 0.200\ F_{1,26} = 6.9$$

Equation 9 is similar to eq 1 and 2 in terms of the essential parameters; however, the quality of fit is not as good.

A slight improvement in correlation is obtained if MR_4 is replaced by $MR_{4,5}$ as seen in eq 10. Although the F test shows the MR_4 term to be significant at the 0.90 level ($F_{1,30\alpha=10} = 2.88$) the $MR_{4,5}$ term is significant at 0.975 level of significance ($F_{1,30\alpha=0.25} = 5.57$). The 5 in $MR_{4,5}$ refers to the more hydrophilic of the two meta substituents (hydrogen is considered as a substituent), which is assumed not to be contacting hydrophobic space. With papain and actinidin the 5-substituents did not appear to make contact with the enzyme in any fashion. However, with ficin this does not seem to be true; the single coefficient with MR_4 and MR_5 suggests that contact of the enzyme by groups in these positions is very much the same, and since MR cannot be replaced by π, polar space must be involved. If two poorly fit points [3-$NHCOCH_3$ and 3,5-$(OCH_3)_2$] are dropped, eq 11 is obtained. The 3,5-$(OCH_3)_2$ congener

$$\log 1/K_m = 0.84(\pm0.14)\pi'_3 + 0.57(\pm0.16)\sigma + 0.41(\pm0.18)MR_{4,5} + 3.60(\pm0.17) \quad (11)$$

$$n = 28,\ r = 0.941,\ s = 0.147$$

is poorly fit in QSAR for both papain and actinidin. If the two poorest points of eq 9 are dropped [3-I and 3,5-$(OCH_3)_2$], the improvement in correlation is not as great as seen in eq 11 ($r = 0.914$ and $s = 0.178$). The intercepts of eq 11 and 1 are almost the same, showing that the intrinsic activity of papain and ficin are very close and about 10 times greater than actinidin.

An interesting aspect of eq 11 is that the coefficient with π'_3 approaches that of eq 1, indicating that desolvation of X is more or less complete as in the case of papain. This suggests that X binds in a ficin hydrophobic pocket much like that in papain.[16]

The $MR_{4,5}$ term uncovers a difference between ficin and the two other cysteine proteases papain and actinidin. The more hydrophilic meta substituent appears to be engaged in a buttressing effect by a surface amino acid residue that is the same for both meta and para substituents. Unfortunately we shall have to await the outcome of X-ray

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crystallographic studies before we know which residue is involved.

An impressively constant feature of eq 1–11 is the coefficient with the σ term, whose small size shows that electronic effects are not very important in setting the value of K_m. In fact, it is the hydrophobic effect that is the most important factor in the formation of the ES complex for papain, actinidin, and ficin. The compounds of Table I bring this out clearly since only 11 have meta substituents with π values significantly different from zero while all show variation in σ, yet despite the large variation in σ, π'_3 accounts for most of the variance in log $1/K_m$.

Note that compounds **29** and **30** have been assigned negative π values. Although these values are so small that the results have little affect on whether or not they are given the value of zero, we have assumed that when two meta substituents are present one will be forced into hydrophobic space.

Since k_{cat} (V_{max}) shows little variation [(largest V_{max} = 13.1 (3-$COCH_3$) and the smallest = 4.01 (4-CN) 10^{-5} M^{-1} min^{-1}] and since all substituent effects have little effect on k_{cat} in the hydrolysis of I and II by papain, actinidin, and ficin, it would seem that when the ester is in the ES complex its structure must rather closely resemble that of the transition state in the acylation step. That is, once in the ES complex the ester has more or less acylated the enzyme. At present, nothing can be said about deacylation since all of congeners I have the same acyl group.

Hollaway et al.[28] showed that in the ficin hydrolysis of I, X = 4-NO_2 at pH 3.91 $k_3 > k_2$, but at pH 5.6 $k_2 > k_3$. They present evidence that the formation of ES is a pseudo-equilibrium process. The value of k_3 does not vary significantly between pH 3.9 and 6.6, but k_2 does. This change in rate-limiting step with pH illustrates the danger in the assumption that k_{cat} vs. pH profiles for thiol protease action on substrates with good leaving groups are equivalent to k_3 vs. pH profiles.

In the case of papain hydrolysis of benzoylarginine ethyl ester it has been shown that $k_2 > k_3$ while in the hydrolysis of benzoylarginine amide $k_3 > k_2$.[29] It thus seems difficult to make generalizations about the relative importance of k_2 and k_3 in the thiol proteases; however, one must be careful about drawing analogies between ficin and papain. Brocklehurst and Malthouse[20] have shown that ficin and papain show greatly different pH profiles in their reaction with 2,2'-dipyridil disulfide. Kortt et al. made the first reasonably comprehensive study of the specificity of ficin toward synthetic substrates.[17] They noted the importance for hydrophobic bonding by the acyl group to ficin. They observed that both ficin and papain require an acyl group for enzymatic hydrolysis since $H_2NCH_2COOC_6H_5$-4'-NO_2 is not hydrolyzed by these enzymes. Also $C_6H_5CON(CH_3)CH_2COOC_6H_4$-4'-$NO_2$ is not hydrolyzed, which shows the importance of an NH unit. The NH group of such amide esters has been postulated to bind to the carbonyl group of Gly-66 in papain.

One of the conclusions that can be drawn from eq 9–11 is that ficin very probably contains a polar residue in the same position of the side chain as Gln-142 in papain and Lys-145 in actinidin. The coefficient of the MR_4 term in the ficin equations is closer in size to that in the actinidin eq 3 than to that in the papin eq 1 and 2. Bromelain, however, has an MR more like that for papain. Whether or not this means that ficin contains a Lys unit and bromelain contains a Gln residue situated to interact with X_4 must await X-ray crystallographic studies.

Equations 1–11 are all very similar and present a self-consistent structure–activity relationship for the cysteine hydrolases. It has long been suspected that these hydrolases have very similar structures and hence similar mechanisms of action. Baker's X-ray diffraction studies on actinidin uncover a structure that is very much like that of papain. Our results show that the hydrolytic mechanisms of ficin and bromelain are also very closely related to papain and actinidin.

It is of course appreciated that MR is a crude measure of the bulk of substituents as well as their polarizability and that the substituents of Table I are by no means all symmetrical. In addition, some have hydrogen bonding capacity and some do not. Also some have rather large dipole moments and some have almost none. Nevertheless, MR does help to rationalize the data and moreover provides a consistent view for the four different enzymes. With the exception of 3-$NHCOCH_3$ and 3,5-$(OCH_3)_2$, no special interactions of significance, unaccounted for by π, σ, and MR, appear to occur with ficin. We believe that these results when taken together with the more convincing data form molecular graphics studies provide solid evidence for both the rationalizing and diagnostic value of MR in QSAR investigations.

The good correlations obtained with these four cysteine proteases, two of whose structures are well established by X-ray crystallography, lends further support for the value of using physicochemical parameters and correlation analysis to map the characteristics of the biological receptors. The next step that needs much more attention, but on which work has started,[30,31] is that of establishing to what degree one can count on QSAR obtained from isolated receptors to hold for receptors in the living cell.

Experimental Section

Materials. Preparations of most of the substituted phenyl hippurates were reported previously.[8,16,32] The new analogues were synthesized in 80–90% yield by the same methods with hippuric acid and the appropriate phenol as the starting material; their analytical and physical properties are collected in Table II. Other materials were of analytical grade, and bidistilled water was used throughout the investigation. Ficin (EC 3.4.22.3) twice crystallized and suspended in 2 M sodium chloride and 0.03 M cysteine solution was purchased from Sigma Chem. Corp. and its catalytic activity in the hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester (obtained from Sigma) was measured according to reported methods.[33]

Kinetics. The procedure followed to obtain kinetic parameters was essentially that of Smith.[16] The optimum wavelength and the change in molar absorptivity on complete hydrolysis were mostly taken from our previous papers;[8,16] for the new substrates they were obtained according to standard method. For the kinetic measurements, the commercial stock suspension of ficin was diluted with the aqueous buffer solution (0.03 M NaCl–0.01 M EDTA, pH 6), activated by saturation with a tiny crystal of 4-thiocresol for about 2 h before use, and then diluted again with the same buffer to give a final ficin solution of suitable concentration. The enzymatic hydrolysis were carried out at 25 °C and pH 6 on a Cary 19 spectrophotometer in a 3.3% acetonitrile–buffer solution. Initial rates were measured for at least eight different concentrations for each substrate. The K_m and V_{max} values were derived by applying the method of least squares to a Lineweav-

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er-Burk plot. Only in three instances were the correlation coefficients less than 0.99; the worst was 0.97.

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Structure-Activity Relationships for Reactivators of Organophosphorus-Inhibited Acetylcholinesterase: Quaternary Salts of 2-[(Hydroxyimino)methyl]imidazole

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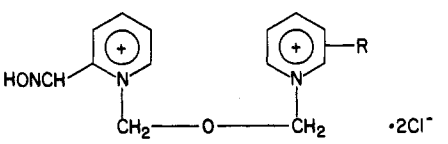
A series of 1,3-disubstituted-2-[(hydroxyimino)methyl]imidazolium halides were prepared and evaluated in vitro with respect to their ability to reactivate acetylcholinesterase inhibited by ethyl *p*-nitrophenyl methylphosphonate (EPMP) and 3,3-dimethyl-2-butyl methylphosphonofluoridate (GD). The compounds conform to the general formula $\overline{N(CH_3)C(CHNOH)N(CH_2OR)CHCH}^{+}\cdot Cl^-$, where R = CH_3, $(CH_2)_3CH_3$, $(CH_2)_7CH_3$, $CH_2C_6H_5$, $CH_2C_{10}H_7$, $(CH_2)_3C_6H_5$, $CH(CH_3)_2$, $CH_2C(CH_3)_3$, and $CH(CH_3)C(CH_3)_3$. For comparison we also evaluated three known pyridinium reactivators, 2-PAM, HI-6, and toxogonin. The imidazolium aldoximes exhibit oxime acid dissociation constants (pK_a) in the range 7.9–8.1, bracketing the value of 8.0, believed to be optimal for acetylcholinesterase reactivation. With imidazolium compound in excess over inhibited enzyme, the kinetics of reactivation are well behaved for EPMP-inhibited AChE and depend on the nature of the alkyl ether group R. For GD-inhibited AChE, maximal reactivation was used to compare compounds because rapid phosphonyl enzyme dealkylation and enzyme reinhibition complicate interpretation of kinetic constants.

Various organophosphorus (OP) compounds are powerful inhibitors of synaptic acetylcholinesterase (AChE).[1] Standard therapy for intoxication by anti-AChE agents consists of coadministration of atropine and an AChE "reactivator".[2-4] Reactivators function as nucleophiles to displace OP moieties from inhibited AChE and thereby restore activity to the enzyme. Although research efforts over the past 25 years have produced dozens of experimental AChE reactivators, only three compounds have found extensive clinical application in managing anti-AChE agent poisoning. These three compounds, 2-PAM [2-[(hydroxyimino)methyl]-1-methylpyridinium halide], TMB4 [1,3-bis[4-[(hydroxyimino)methyl]-1-pyridinio]-propane dichloride], and toxogonin [1,3-bis[4-[(hydroxyimino)methyl]-1-pyridinio]-2-oxapropane dichloride], effectively reverse intoxication symptoms in cases of accidental pesticide or nerve agent poisoning by many OP agents. However, in animals these three pyridinium oximes are ineffective in preventing or treating intoxication by 3,3-dimethyl-2-butyl methylphosphonofluoridate (GD) when GD is administered in quantities exceeding approximately 1.2 times the LD_{50}.[5-7]

In 1970, Oldiges and Schoene[5] reported that certain unsymmetrically bis(substituted pyridinium) dimethyl ether derivatives constitute effective therapy for GD poisoning in mice under conditions where 2-PAM, toxogonin, and TMB4 have insignificant therapeutic efficacy. The findings of Oldiges and Schoene evoked considerable interest[5-19] in the synthesis and evaluation of bis(pyridinium) dimethyl ether derivatives. Several structurally related AChE reactivators have been discovered that are useful in treating GD-intoxicated animals. The reactivators that are effective against GD conform to the general structure 1, where R = $C(O)NH_2$, $C(O)C_6H_5$, or $C(O)C_6H_{11}$



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in the 3- or 4-position of the indicated pyridinium ring.[20]

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