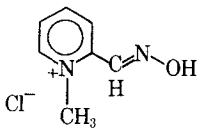
Aryl-*N*-methyl-2-pyridiniumketoxime Acetylcholinesterase Reactivators

C. F. BARFKNECHT, J. P. LONG, and F. W. BENZ

Abstract □ *syn*- and *anti*-Phenyl-*N*-methyl-2-pyridiniumketoximes (II and III) were synthesized as reactivators of organophosphate-inhibited acetylcholinesterase, and the *anti*>*syn* potency relationship was confirmed. 2′-Pyridyl-*N*-methyl-2-pyridiniumketoxime (IV) was synthesized and found to be more potent than II or III and equipotent with *syn*-4-pyridiniumaldoxime methochloride. IV was more potent than II but less potent than III as an inhibitor of acetylcholinesterase. NMR evidence indicates that the phenyl groups of II and III are in different conformations.

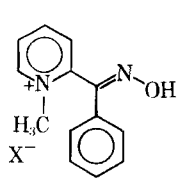
Keyphrases □ Phenyl-*N*-methyl-2-pyridiniumketoximes (*syn* and *anti*)—acetylcholinesterase reactivators, synthesis, potency relationship □ Acetylcholinesterase reactivators—synthesis, potency relationship, *syn*- *anti*-phenyl-*N*-methyl-2-pyridiniumketoximes □ NMR spectroscopy—structure

The standard of comparison for compounds intended as acetylcholinesterase (AChE) reactivators is 2-pyridiniumaldoxime methochloride (2-PAM) (I). Some controversy has existed about which configurational isomer (*syn* or *anti*) is more potent, but there is conjecture that the *syn* is more potent and is the isomer sold as an antidote for AChE inhibition (1–3). It has been proposed that in *syn*-I, the oxime hydroxyl group is positioned to attack the phosphorylated (or carbamylated) esteratic site when the pyridinium nitrogen binds to the anionic site of AChE.



I

In the study of other pyridinium-2-oximes, one might expect the configurational isomers corresponding to *syn*-2-PAM to be more potent than their *anti*-isomers. It must be assumed that these pyridinium-2-oximes can attain the same conformation as *syn*-2-PAM when interacting with AChE. Kitz *et al.* (4) reported the synthesis and *in vitro* biochemical evaluation of *syn*- and *anti*-phenyl-*N*-methyl-2-pyridiniumketoxime iodides (II and III, respectively). Their data showed the *anti*-isomer (III) to be 20 times more potent than the *syn* (II) and approximately equipotent to *syn*-4-PAM (Table I). These results are the opposite to what is expected for 2-PAM-like compounds. Examination of molecular models suggests difficulty for II and III to attain some conformations of I and thus supports the results obtained by Kitz *et al.* (4). Efforts to verify their data and extend the series to the 2-pyridyl analog (IV) are reported in this communication.



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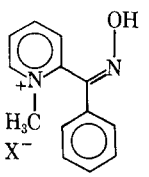

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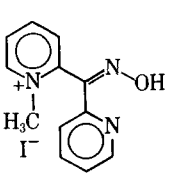

IV

Table I—Biochemical Data of Aryl-*N*-methyl-2-pyridiniumketoximes

Compound	ID_{50} (*M*)	k_2, l./mole/min.	Relative Potency (95% Fiducial Limits)
2-PAM	8.1×10^{-4}	3.3×10^{4}[a]	1.00
syn-4-PAM	16.4×10^{-4}	4.9×10^{2}	1.4×10^{-2} (1.2–1.7)
II Iodide	4.3×10^{-4}	2.9×10^{1}	9.4×10^{-4}[b] (8.8–10.0)
II Bromide	4.4×10^{-4}	3.0×10^{1}	9.9×10^{-4}[b] (9.5–10.3)
III Bromide	1.2×10^{-4}	2.6×10^{2}	8.1×10^{-3} (6.8–9.8)
IV Bromide	2.3×10^{-4}	6.8×10^{2}	2.0×10^{-2} (1.8–2.3)

[a] Mean value of 24 determinations. [b] Relative rate calculated by dividing k_2 test/k_2 standard in *paired* experiments.

RESULTS

The phenyl-2-pyridylketoximes (*syn* and *anti*) were synthesized and separated by the method of Huntress and Walker (5). Completeness of separation was demonstrated by TLC. The correctness of structural assignments, made by classical methods, was confirmed by the NMR spectra. While Kitz *et al.* (4) had succeeded in quaternizing both isomers utilizing methyl iodide, in the present study only II could be prepared. Both II and III were synthesized by reaction with methyl bromide in a sealed tube, demonstrating that configurational isomerization does not occur under these conditions. The NMR data (Table II) are consistent with the configurational assignments (6).

2,2′-Dipyridylketoxime was readily prepared by standard methods and easily quaternized with methyl iodide to form IV. All attempts to form the diquaternary compound (*N*-methyl-*N*′-methyl) with methyl iodide failed. A sealed-tube reaction with methyl bromide also yielded the monoquaternary compound. The configurational assignment of IV (*syn*-2′-pyridyl) is based upon the inability to diquaternize with methyl iodide, the NMR data, and examination of molecular models which indicated that an interaction between the oxime hydroxyl and the 2′-pyridyl nitrogen is likely.

The pH stat method was used for the biochemical evaluation of the compounds. Diethylphosphoryl thiocholine-inhibited acetylcholinesterase from the electric eel was used for the reactivation experiments. Detailed methodology appears in the *Experimental* section. Results of the reactivation and inhibition experiments are summarized in Table I.

DISCUSSION

The reactivation data demonstrate that in aryl-*N*-methyl-2-pyridiniumketoximes, the *anti*-phenyl isomer is more potent than the *syn*-phenyl isomer and thus *anti*-2-PAM may be more active than *syn*-2-PAM. This is the conclusion reached by Kitz *et al.* (4), and the present authors are now in full agreement with it. The inhibition data reinforce the notion that the phenyl group of II and III is interacting with AChE in a detrimental fashion. That the

phenyls of II and III are not in the same conformation is demonstrated by the NMR spectra. The protons of the phenyl group of II are observed as a multiplet, while the protons of the III phenyl group are observed as a singlet. To be observed as a singlet, all protons must be influenced equally by the remainder of the molecule. This could be accomplished by rotating the phenyl ring of III out-of-plane. The phenyl protons of both *syn*- and *anti*-phenyl-2-pyridylketoxime are observed as a singlet. From examination of space-filling models of these compounds, it is clear that free rotation of the phenyl groups is possible. It is not clear why, when quaternized, the conformation of the phenyl groups of II and III should differ.

That IV interacts differently than II and III is established by the reactivation and inhibition data. Compound IV exhibits higher reactivation than II and III and higher inhibition than II but lower inhibition than III. Compound IV's increased potency as a reactivator is undoubtedly linked to its favorable pKa. Whether the conformations of I and IV are identical cannot be determined from the available information. The 2′-pyridyl-2-pyridiniumketoxime group (present in IV) warrants further investigation with diquaternary systems that should enhance its reactivator potency.

EXPERIMENTAL

Phenyl-2-pyridylketoximes—The method of Huntress and Walker (5) was used for the synthesis and separation of the isomers. *syn*-Phenyl-2-pyridylketoxime (m.p. 151–152°) and *anti*-phenyl-2-pyridylketoxime (m.p. 166–167°) were used.

***syn*-Phenyl-*N*-methyl-2-pyridiniumketoxime Iodide and Bromide (II)**—The method of Kitz *et al.* (4) was followed for the preparation of the II iodide, m.p. 206–208° (lit. m.p. 212–213°).

The II bromide was synthesized by reacting the phenyl-2-pyridylketoxime, dissolved in the minimum isopropyl alcohol, with a slight excess of methyl bromide in a sealed tube on a steam bath for 2 days. The product was obtained in near quantitative yield and recrystallized from absolute ethanol–ethyl acetate, m.p. 212–214°.

Anal.—Calcd. for $C_{13}H_{13}BrN_2O$: C, 53.44; H, 4.11; N, 9.59. Found: C, 53.60; H, 4.01; N, 9.67.

***anti*-Phenyl-*N*-methyl-2-pyridiniumketoxime Bromide (III)**—The procedure employed for the synthesis of the II bromide was used. A nearly quantitative yield of product, m.p. 190–193°, was obtained (absolute ethanol–ethyl acetate).

Anal.—Calcd. for $C_{13}H_{13}BrN_2O$: C, 53.44; H, 4.11; N, 9.59. Found: C, 53.32; H, 3.99; N, 9.72.

2,2′-Dipyridylketoxime—The procedure of Case and McMenamin (7) was employed; yield 83%, m.p. 141–143° [lit. (8) m.p. 141–143°].

2′-Pyridyl-*N*-methyl-2-pyridiniumketoxime Iodide (IV)—A nearly quantitative yield of IV was obtained by stirring the 2,2′-dipyridylketoxime with a slight molar excess of methyl iodide in anhydrous diethyl ether for 2 hr. at room temperature, m.p. 194–196° (ethanol–ethyl acetate).

Anal.—Calcd. for $C_{12}H_{12}IN_3O$: C, 42.40; H, 3.52; N, 12.34. Found: C, 42.16; H, 3.51; N, 12.27.

Enzyme—Acetylcholinesterase [acetylcholine, acetylhydrolase (3·1·1·7)] from the electric eel was used.[1] The enzyme (0.58 mg. = 1000 μM units) was dissolved in 5 ml. of 1% bovine albumin[1] and stored frozen. It had a specific activity of 2.5 mmole acetylcholine hydrolyzed per min./mg.

Inhibition—The enzyme was inhibited by reacting 0.1 ml. (20 μM units) of the enzyme solution with 0.1 ml. (2×10^{-6} M) diethylphosphoryl thiocholine iodide at pH 7.0 and 20°. After 2 hr. the inhibited enzyme was diluted to 4 μM units/ml. with a solution containing 0.03 M NaCl and 0.02 M $MgCl_2$ and was stored at 4°. This stock solution of inhibited enzyme could be used in reactivation experiments for 2–3 days without loss of activity.

Reactivation—The reactivation was carried out at 37° at pH 7.4 in a medium consisting of 20 ml. of 0.03 M NaCl and 0.02 M $MgCl_2$. The final concentrations of enzyme and acetylcholine were 8 μM units/l. and 10^{-3} M, respectively. A Radiometer titrator-type TTT1d with SBR2c recorder and syringe buret SBU1a were used to monitor the reaction rate.

Pseudo-first-order rate constants, k, were calculated from a plot of $\log E_\infty - E_t$ *versus* t, where E_∞ is the total amount of enzyme available and E_t is the total amount of enzyme at any time t. A linear relationship between velocity of hydrolysis (v) and enzyme concentration (E) was verified. The first-order rate constants were proportional to the reactivator concentrations, revealing that the reactivations were bimolecular and that the reactivator concentrations were less than the dissociation constants for the intermediate complexes. Relative potencies were calculated by a 2×2 parallel line bioassay (9). First-order rate constants for two concentrations of the test reactivator were compared with first-order rate constants for two concentrations of the standard reactivator, 2-PAM. All criteria for a valid bioassay were met. In those cases where a 2×2 bioassay was unfeasible, because the reactivator acted as a reversible inhibitor of the enzyme during the reactivation, a relative rate of reactivation as compared to 2-PAM was calculated by the formula, relative rate = k_2 test/k_2 2-PAM, where k_2 is the second-order rate constant obtained by dividing k/[R]. Relative rates are identical to relative potencies if the plots of k *versus* [R] for the test reactivator and the standard reactivator, 2-PAM, are parallel.

ID_{50} Determinations—The ID_{50} was determined from plots of percent inhibition *versus* log inhibitor concentration. At least three concentrations between 30 and 70% inhibition were used.

[1] Obtained from Sigma Chemical Co., St. Louis, Mo.

Table II—NMR and pKa Data on Aryl-*N*-methyl-2-pyridiniumketoximes[a]

Configuration		Aryl Group	Oxime Hydroxyl	*N*-Methyl Group	pKa[b]
II Iodide	*syn*	Phenyl 7.62	13.05	4.23	8.9
II Bromide	*syn*	Phenyl 7.62	13.08	4.22	—
III Bromide	*anti*	Phenyl 7.50[c]	12.83	4.23	8.7
IV Iodide	*syn*	2-Pyridyl-	13.26	4.23	7.9

[a] All samples were run in DMSO-d_6 with tetramethylsilane as the internal standard; chemical shifts are expressed in parts per million. [b] Determined by potentiometric titrations; literature value for 2-PAM (pKa = 8.0) was duplicated. [c] All aryl groups exhibited a complex multiplet except III; both *syn*- and *anti*-phenyl-2-pyridylketoximes showed a singlet for the phenyl group at 7.33 and 7.42 p.p.m., respectively.

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