J. Enzyme Inhibition, 1990, Vol. 3, pp. 211-217
Reprints available directly from the publisher
Photocopying permitted by license only

ACETYLCHOLINESTERASE INHIBITION BY TWO PHOSPHORIC 4-NITROANILIDES

JEAN-CLAUDE BOLLINGER* and JOSIANE LEVY-SERPIER

Laboratoire de Chimie générale et analytique, Faculté des Sciences, 123 avenue Albert Thomas, 87060 Limoges Cedex, France

JEAN DEBORD

Laboratoire d'Histologie, Faculté de Médecine, 2 rue du Docteur Marcland, 87025 Limoges Cedex, France

BERNARD PENICAUT

Laboratoire de Chimie analytique, Faculté de Pharmacie, 2 rue du Docteur Marcland, 87025 Limoges Cedex, France

(Received 22 May, 1989)

Two phosphoric 4-nitroanilides $Z_2P(O)NH\text{-}\Phi\text{-}NO_2$ (**A**, Z = Me; **B**, Z = NMe_2) have been prepared and purified by chromatographic techniques. Their spectral data (uv, ir and ^{1}H-nmr) have been determined, and compared with those of other similar compounds. Their ability to inhibit acetylcholinesterase has been measured by a modification of Ellman's method. The data, as computed according to the Michaelis scheme, indicate that **A** is not an inhibitor, whereas **B** is a reversible mixed one. These differences are discussed in terms of hydrophobic interactions.

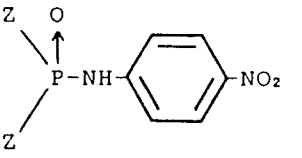
KEY WORDS: Acetylcholinesterase, inhibition, phosphoric anilides.

INTRODUCTION

Acetylcholinesterase (AChE, EC 3.1.1.7) is a well-studied enzyme, hydrolysing ester substrates such as acetylcholine (ACh) or acetylthiocholine (ASCh).[1] Many organophosphorus compounds inhibit AChE by reversible formation of an enzyme-inhibitor complex followed by irreversible phosphorylation of the enzyme.[2] The phosphoryl-enzyme cannot destroy ACh whose accumulation in the organism is fatal to the nervous system. This is the basis for the pesticide activity of organophosphorus compounds[2-4] and their use as nerve incapacitating weapons.

The potency and mechanism of this enzymatic inhibition can be studied by various techniques including microcalorimetry[5] and spectrophotometry.[6] In this paper we describe the AChE-inhibitory power of two phosphoric 4-nitroanilides **A** and **B** (with ASCh as substrate) using a spectrophotometric method based on Ellman's procedure.[7]

*Correspondence.



(A : Z = Me ; B : Z = NMe_2)

MATERIAL AND METHODS

Analytical Methods

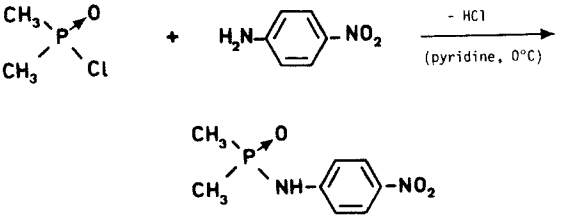
Melting points (m.p.) were determined on a Köfler apparatus. IR spectra (KBr pellets) were obtained with a Perkin-Elmer 297 spectrometer, ^{1}H-NMR spectra with a Varian EM 360 (60 MHz) spectrometer, using tetramethylsilane as internal standard. The solvents were of Merck "Uvasol" grade.

Preparative TLC of compound **B** was performed on Merck pre-coated plates with silica gel G60-F254 (migration solvent = acetone; detection: fluorescent lamp). HPLC was performed with a system comprising a Bischoff pump (mobile phase: methanol/water 50/50 v/v; pressure = 50 mbar; flow = 1.7 mL/min), a μ-Bondapack column (grafted C_{18}, 10 μm; length = 25 cm) and a DuPont Instruments detector operating at 366 nm. The solvents were of HPLC grade.

UV spectra and kinetic results were obtained with a Hewlett-Packard HP 8450 spectrophotometer linked to a HP 9825 calculator and a HP 7225 plotter.

Synthesis

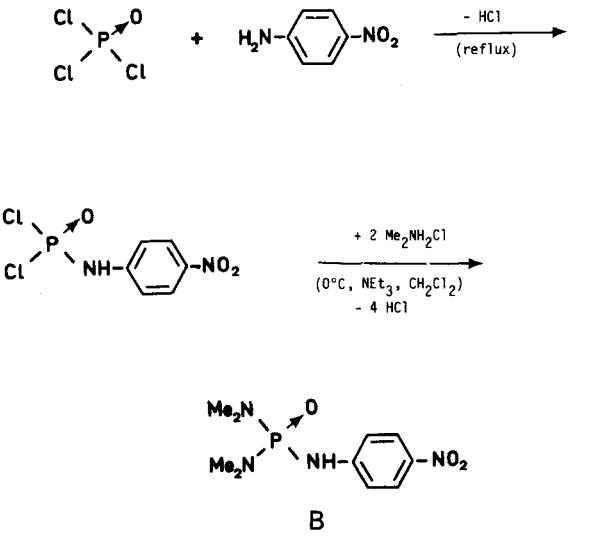
*N-(4-nitrophenyl) dimethylphosphinamide (**A**)* was obtained directly from 4-nitroaniline and dimethylphosphinic chloride in a pyridine/diglyme medium, by the method of Rewcastle[8] (Scheme 1). The yellowish crystals (m.p. = 252°C, yield = 64%, lit.[8] m.p. 235°C, 34%) gave only one peak in HPLC, t_R = 0.3 min. ^{1}H-NMR (DMSO-d_6): δ 1.8 (d, 6H, J = 14 Hz, CH_3), 7.4 (d, 2H, J = 10 Hz, C_{ar} *H*-NHPO), 8.3 (d, 2H, J = 10 Hz, C_{ar} *H*-NO_2).



A

SCHEME 1

*N,N,N',N'-tetramethyl-N''-(4-nitrophenyl) phosphoric triamide (**B**)* was synthesized by a two-stage process as described in Scheme 2.[8] The intermediate 4-nitrophenyl phosphoric dichloride (pink crystalline powder, m.p. = 153°C, yield = 86%; lit.[8] m.p. 152°C, 94%) gave after reaction with dimethylamine hydrochloride a brownish-



SCHEME 2

yellow powder (m.p. not determined, yield = 14%; lit.[8] m.p. 214°C, 34%) from which the phosphoramide **B** was isolated by preparative TLC (solvent = acetone, R_f = 0.5) and checked for purity by HPLC (solvent = methanol/water 50/50 v/v. t_R = 0.45 min). ^{1}H-NMR (CH_2Cl_2): δ 2.7 (d, 12H, J = 10 Hz, NMe_2), 7.2 (d, 2H, J = 14 Hz, C_{ar} *H*-NHPO), 8. (d, 2H, J = 14 Hz, C_{ar} *H*-NO_2).

Kinetic Experiments

All reagents were from Sigma. AChE from *Electrophorus electricus* (200 units/mg protein) was diluted to 0.1 mg/mL in a phosphate buffer (Na_2HPO_4/KH_2PO_4 0.133 M pH 7.4). ASCh (0.069 M or 0.035 M) was prepared in distilled water and the indicator DTNB (0.52 mM) in phosphate buffer.

The enzymatic activity was measured at 37°C by a modification[6] of Ellman's method.[7] The reaction medium was obtained by mixing, in the following order: DTNB solution, 5 mL; inhibitor (**A** or **B**) in methanol, 0.2 mL; ASCh solution, x μL ($0 < x < 100$); phosphate buffer, $(100 - x)$ μL; AChE solution, 25 μL. The absorbance was followed at 412 nm for ca. 5 min. The initial rate was computed from the linear portion of the time-absorbance curve, using the molar absorption coefficient of TNB ($13{,}600\ L \cdot mol^{-1} \cdot cm^{-1}$ at 412 nm[7,9]). Replacing the inhibitor solution by an equivalent volume of methanol and omitting the enzyme gave the rate of spontaneous hydrolysis of the substrate.

RESULTS

Synthesis and Purification of Compounds

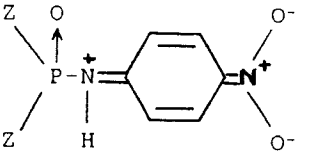
The two phosphoric anilides **A** and **B** were synthesised by the general procedures previously described by Rewcastle *et al.*[8] (see Schemes 1 and 2). Although the monoamide **A** was obtained pure (only one peak in HPLC), the HPLC chromatogram

for the triamide **B** revealed the presence of many impurities. Preparative TLC was used to separate the required compound which then gave a single peak in HPLC and whose structure was ascertained by ^{1}H-NMR spectroscopy.

Spectral Data

UV spectroscopy. Due to p_π–d_π back-donation between the P and N atoms[13] the phosphoryl substituent withdraws electrons from the nitrogen atom so, as previously noted by Cheng and Shaw,[14] the maximum observed in the ultra-violet spectrum moves to shorter wavelength (**B**: λ = 340 nm, **A**: λ = 333 nm, in acetonitrile solution) when compared to the parent 4-nitro-aniline (λ = 365 nm). The electron-attractive power of the phosphorylated substituents is in the order: NH-P(O)NMe_2 < NH-P(O)Me_2 which is in good agreement with the inductive constants of the NH-P(O)Z_2 groups, as calculated by Modro[15] from ^{13}C-NMR data in some Φ-NH-P(O)Z_2 compounds; σ_I = +0.02 for Z = NEt_2 and σ_I = +0.19 for Z = Me.

IR spectroscopy. The spectra for our two solid samples are nearly the same: they present many characteristic bands that can be attributed[16] particularly to ν_{NH} (3175 cm^{-1}), ν_{CN} (1260, 1300, 1340 cm^{-1}), ν_{PN} (915, 980, 1000 cm^{-1}), ν_{PO} (1255 cm^{-1} in compound **A**, 1250 cm^{-1} in compound **B**). The ν_{PO} values can be compared to the range ν_{PO} = 1218 to 1239 cm^{-1} for a series of ring-substituted dimethyl *N*-aryl phosphoramidates X-Φ-NH-P(O) $(OMe)_2$ where hydrogen-bonding effects upon the stretching frequency of the phosphoryl bond were studied,[17] and to the higher values ν_{PO} = 1240 to 1280 cm^{-1} for various *N*-methyl-derivatives X-Φ-N(Me)-P(O) $(OEt)_2$ which are not capable of self-association.[14] Strong hydrogen-bond formation is expected in our phosphoramides because of the electron-withdrawing power of the 4-nitro group that can increase the acidity of the N–H hydrogen atom. Indeed, it is known that NO_2-Φ-NH-P(O) $(OMe)_2$ exists in the crystalline state as a monomer stabilized by a network of linear hydrogen bonds.[18] So, the high value for ν_{PO} is probably due to the contribution of dipolar resonance structures such as,



^{1}H-NMR spectroscopy. The signal corresponding to the NMe_2 group in **B** is only slightly modified (δ = 2.7 ppm, J = 10 Hz) compared to all-aliphatic phosphoramides (δ = 2.6 ppm, J = 10 Hz in various XYP(O)NMe_2 compounds[19]). The influence of the aromatic moiety is more important in the derivative **A** (δ = 1.8 ppm, J = 14 Hz) than in Me_2P(O)NMe_2 (δ = 1.36 ppm, J = 14 Hz) or in MeP(O) $(NR_2)_2$ (δ = 1.28 to 1.33 ppm, J = 14 Hz[19]). As for the protons in the aromatic ring, their behaviour is similar in the two phosphorylated anilides (**A**, dd, δ = 7.4 and 8.3 ppm, J = 10 Hz; **B**, dd, δ = 7.2 and 8.1 ppm, J = 10 Hz), as compared with the parent 4-nitro-aniline (dd, δ = 6.55 and 7.87 ppm, J = 15 Hz). Moreover, it can be observed that the two protons on the phosphoramido side are more deshielded than in the amino compound, while those on the nitro side have essentially the same chemical shift.

Cholinesterase Inhibition

Compound A. Graphical treatment of data according to Dixon[10] and Cornish-Bowden[11] shows horizontal parallel lines, indicating negligible inhibition of AChE.

Compound B. Graphical analysis leads to the characterization of phosphotriamide **B** as a mixed reversible inhibitor of AChE (Figure 1). Non-linear least squares analysis[12] gave the kinetic parameters: $V = 97 \cdot 10^{-6}\,mol \cdot L^{-1} \cdot min^{-1}$; $K_m = 5 \cdot 10^{-5}\,mol \cdot L^{-1}$; $\overline{K_i} = 3.4 \cdot 10^{3}\,L \cdot mol^{-1}$; $\overline{K_i'} = 1.0 \cdot 10^{3}\,L \cdot mol^{-1}$. $\overline{K_i}$ and $\overline{K_i'}$ are the association constants for competitive and uncompetitive inhibition, respectively. The Michaelis constant is in good agreement with published values.[21]

DISCUSSION

Although of very similar structures the two organophosphorus compounds described here behave differently towards AChE: phosphoramide **A** is not an inhibitor, whereas

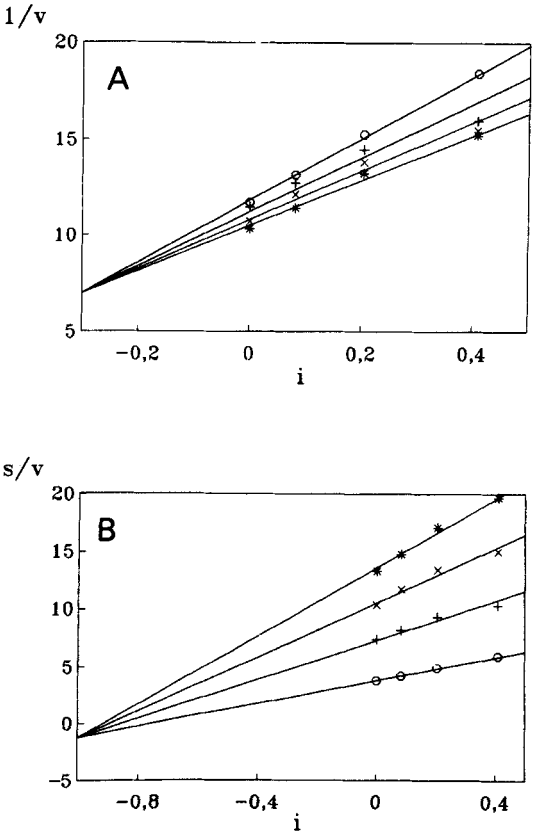


FIGURE 1 Dixon (A) and Cornish-Bowden (B) plots for phosphoramide **B**. i, inhibitor concentration (mM); s, initial substrate concentration (mM), 0.32 (○), 0.65 (+), 0.97 (×), 1.30 (*); v, initial rate of hydrolysis ($mM \cdot min^{-1}$).

phosphoramide **B** is a reversible mixed inhibitor. According to Schrader's rules,[3] an irreversible mechanism is related to the presence of a labile bond in the inhibitor molecule, such as P-OΦ in organophosphate insecticides. Since phosphoramides are stable compounds in basic medium (the P-N bond does not hydrolyse), a reversible mechanism was to be expected.

The competitive inhibition constant $\overline{K_i}$ reflects the affinity of the inhibitor for the free enzyme, while the uncompetitive inhibition constant $\overline{K_i'}$ is thought to reflect the affinity of the inhibitor for the actyl-enzyme (rather than the enzyme-substrate complex).[20] According to our previous analysis of the interaction of aliphatic phosphoramides with butyrylcholinesterase[6,22] the OPN_3 moiety of the phosphoramide interacts with a hydrophilic zone of the catalytic site of the enzyme, probably by dipole/dipole interactions and/or hydrogen bonding of the P → O group to an acidic aminoacyl residue. This zone is surrounded by three hydrophobic regions binding the hydrocarbon substituents, as described by Järv *et al.*[23] for acetylcholinesterase. Some of these hydrophobic regions are free in the acetyl-enzyme and can also bind to the inhibitor, although less tightly than in the enzyme.

This model agrees with our present results. Phosphoramide **B** has a weaker affinity for the acetyl-enzyme than for the enzyme ($\overline{K_i} > \overline{K_i'}$). It has indeed a weak affinity for the enzyme itself, probably due to the presence of the nitro substituent which decreases both the basicity of the P → O group and the lipophilicity of the aromatic chain. Rekker's hydrophobic constant which is a measure of the lipophilicity of a molecular fragment[24] is 1.69 for C_6H_4 and −0.05 for aromatic NO_2.

The affinity is further decreased in phosphoramide **A** by the presence of two methyl groups directly linked to phosphorus. These lipophilic substituents hinder the binding to the hydrophilic zone. Similar results have been obtained with butyrylcholinesterase.[6]

References

1. Quinn, D.M. (1987) *Chem. Rev.*, **87,** 955–979.
2. Main, A.R. (1979) *Pharmacol. Ther.*, **6,** 579–628.
3. Fest, C. and Schmidt, K.J. (1973) *The Chemistry of Organophosphorus Pesticides*, Berlin-Heidelberg, Springer Verlag.
4. Eto, M. (1979) *Organophosphate Pesticides: Organic and Biological Chemistry*, Cleveland, The Chemical Rubber Company.
5. Debord, J., Labadie, M., Bollinger, J.C. and Yvernault, T. (1983) *Thermochim. Acta*, **66,** 323–331.
6. Debord, J., Labadie, M., Bollinger, J.C. and Yvernault, T. (1985) *Phosphorus Sulfur*, **22,** 121–130.
7. Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M. (1961) *Biochem. Pharmacol.*, **7,** 88–95.
8. Rewcastle, G.W., Baguley, B.C. and Cain, B.F. (1982) *J. Med. Chem.*, **25,** 1231–1235.
9. Silverstein, R.M. (1975) *Anal. Biochem.*, **63,** 281–282.
10. Dixon, M. (1953) *Biochem. J.*, **55,** 170–171.
11. Cornish-Bowden, A. (1974) *Biochem. J.*, **137,** 143–144.
12. Marquardt, D.W. (1963) *J. Soc. Indust. Appl. Math.*, **11,** 431–441.
13. Labarre, M.C. and Labarre, J.F. (1971) *J. Chim. Phys.*, **68,** 539–541; Labarre, M.C. and Coustures, Y. (1973) *J. Chim. Phys.*, **80,** 534–543.
14. Cheng, C.Y. and Shaw, R.A. (1987) *J. Cryst. Spectrosc. Res.*, **17,** 331–348.
15. Modro, T.A. (1979) *Phosphorus Sulfur*, **5,** 331–336.
16. Thomas, L.C. (1974) *The Identification of Functional Groups in Organophosphorus Compounds*, London, Academic Press.
17. Foulds, G.A., Modro, T.A. and Rijkmans, B.P. (1981) *S. Afr. J. Chem.*, **34,** 72–75.
18. Du Plessis, M.P., Modro, T.A. and Nassimbeni, L.R. (1982) *Acta Cryst.*, **B38,** 1504–1507.

19. Bollinger, J.C., Yvernault, G. and Yvernault, T. (1985) *Spectrochim. Acta,* **41A,** 399–406.
20. Krupka, R.M. and Laidler, K.J. (1961) *J. Am. Chem. Soc.,* **83,** 1448–1454.
21. Hofer, P. and Fringeli, U.P. (1981) *Biophys. Struct. Mech.,* **8,** 45–59.
22. Debord, J. (1988) *Thèse Univ. Poitiers.*
23. Järv, J., Aaviksaar, A., Godovikov, N. and Lobanov, D. (1977) *Biochem. J.,* **167,** 823–825.
24. Rekker, R.F. and De Kort, H.M. (1979) *Eur. J. Med. Chem.,* **14,** 479–488.