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Syntheses and spectroscopic characterization of some phosphoramidates as reversible inhibitors of human acetylcholinesterase and determination of their potency

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Abstract

The ability of phosphoramidates $Me_2NP(O)(Cl)(p\text{-}NHC_6H_4NO_2)$ **1**, $Me_2NP(O)(p\text{-}NHC_6H_4NO_2)_2$ **2**, $(CH_3C_6H_4O\text{-}p)P(O)(p\text{-}NHC_6H_4NO_2)_2$ **3** and $(CH_3C_6H_4O\text{-}p)_2P(O)(p\text{-}NHC_6H_4NO_2)$ **4** to inhibit human acetylcholinesterase (hAChE) has been evaluated by a modified Ellman's method and spectrophotometric measurements. Results showed that compounds **1** and **2** do not have any inhibitory potency, whereas compounds **3** and **4** were reversible mixed inhibitors. The IC_{50} values for inhibitors **3** and **4** were 0.143 and 0.581 mM, respectively. The previously unknown compounds **3** and **4** were synthesized and characterized by ^1H, ^{13}C, ^{31}P NMR and IR spectroscopy and elemental analysis.

Keywords: *Ellman's method, human acetylcholinesterase, IC_{50} values, phosphoramidates, reversible mixed inhibitors, inhibition*

Introduction

Many phosphoramidate compounds inhibit human acetylcholinesterase (hAChE, EC 3.1.1.7) by phosphorylation of a serine hydroxyl group in the active site of this enzyme leading to its inactivation. This inhibition increases acetylcholine's levels in cholinergic synapses of both peripheral and central nervous systems [1–8]. During the inhibition, phosphorylated enzyme undergoes a postinhibitory process and its spontaneous reactivation is very slow [5,8]. The mechanism of inhibition by these compounds has been widely studied. Many papers have reported the irreversible inhibition of human acetylcholinesterase by phosphoramidates [2–4,7,9–19] and some authors have reported reversible inhibitors [1,5,6,8,20–26]. Drug design is a matter of great interest based on reactivation of hAChE with site-directed nucleophiles such as 2-pralidoxime (2-PAM) and its analogs. The nucleophile binds to the active site and reacts with the phosphorylated hydroxyl group to release free and active enzyme which in turn becomes phosphorylated [1,5,8,21,22].

Bollinger et al. studied the inhibitory effect of $(Me_2N)_2P(O)(p\text{-}NHC_6H_4NO_2)$, **5**, and $Me_2P(O)(p\text{-}NHC_6H_4NO_2)$, **6**, on acetylcholinesterase [27]. They found that only molecule **5** with a $(O)PN_3$ moiety has inhibitory potency and acts as reversible inhibitor. To extend investigation in this area, we designed and synthesized compounds **1–4**, which have a $XY(O)P(p\text{-}NHC_6H_4NO_2)$ skeleton and examined their inhibitory potency and inhibition mechanism with hAChE using the spectrophotometric method based on Ellman's procedure [28].

Materials and methods

All reactions for synthesis of the phosphoramidates were carried out under an argon atmosphere. Melting points were determined on a Gallenkamp apparatus. ^1H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker (Avance DRS) 500 spectrometer and chemical

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shifts were determined relative to TMS and 85% H_3PO_4, respectively, as external standards. IR spectra (KBr pellets) were obtained with a Shimadzu, IR-60 model spectrometer. Elemental analysis was performed using a Heraeus CHN-O-RAPID instrument. UV measurements were performed on a Shimadzu UV-2100 spectrophotometer. All chemicals and solvents for syntheses were from Merck. Phosphoramidodichloridicacid-4-methylphenylester was prepared by the literature method [29].

Synthesis

N,N-(dimethyl)-N'-(4-nitrophenyl)phosphoramidochloride, 1 and N,N-(dimethyl)-N',N''-bis(4-nitrophenyl)phosphoramide, 2. were synthesized and characterized by ^1H, ^{13}C, ^{31}P NMR and IR spectroscopy and elemental analysis [30].

N,N'-bis(4-nitrophenyl)phosphoramidicacid-4-methylphenylester, 3. To a solution of phosphoramidodichloridicacid-4-methylphenylester (2.25 g, 10 mmol) in dry benzene (20 ml), the sodium salt of 4-nitroaniline (2.76 g, 20 mmol) was added under an argon atmosphere. After 4 h stirring, the precipitate was removed and washed with dry chloroform (20 ml). The purity of the product was up to 98%. (yield 65%), m. p. = 174–176°C. Anal. Calc. for $C_{19}H_{17}N_4O_6P$: C, 53.27; H, 3.97; N, 13.08. Found: C, 53.20; H, 3.93; N, 12.99%. ^1H NMR (DMSO-d_6), δ (ppm): 2.24 (s, 3H, p-CH_3), 6.63 (d, $^3\mathcal{J}_{H-H} = 8.28$ Hz, $4H_{ortho}$),7.0 (d, $^3\mathcal{J}_{H-H} = 7.97$ Hz, $2H_{meta}$), 7.1 (d, $^3\mathcal{J}_{H-H} = 7.93$ Hz, $2H_{ortho}$), 7.86 (s, 2 N–H), 7.92 (d, $^3\mathcal{J}_{H-H} = 8.28$ Hz, $4H_{meta}$); ^{13}C NMR (DMSO-d_6), δ (ppm): 20.28 (s, p-CH_3), 113.02 (s), 119.94 (s), 126.32 (s), 129.86 (s), 133.57 (s), 136.07 (s), 148.76 (s), 155.03 (s); ^{31}P NMR (DMSO-d_6), δ (ppm): −19.09 (s); IR (KBr), ν (cm^{-1}): 2855 (w, N-H), 1962 (w), 1589 (s), 1514 (vs, NO_2), 1342 (s, NO_2), 1309 (m), 1198 (m, P=O), 1121 (m), 990 (m, P-N), 919 (m), 855 (m), 824 (m), 735 (m), 672 (m), 499 (m), 493 (s).

N-(4-nitrophenyl)phosphoramidicacid-bis(4-methylphenyl)ester, 4. The sodium salt of 4-nitroaniline (1.6 g, 10 mmol) was added to a solution of phosphoramidodichloridicacid-4-methylphenylester (2.25 g, 10 mmol) in dry benzene (20 ml) under an argon atmosphere and the mixture was stirred at room temperature for 5 h. The yellow precipitate obtained was washed with dry chloroform (20 ml). The purity of the product was up to 98%. (yield 58%), m.p. = 165–168°C. Anal. Calc. for $C_{20}H_{19}N_2O_5P$: C, 60.3; H, 4.77; N, 7.03. Found: C, 60.1; H, 4.72; N, 6.988%. ^1H NMR (DMSO-d_6), δ (ppm): 2.24 (s, 6H, p-CH_3), 6.61 (d, $^3\mathcal{J}_{H-H} = 7.58$ Hz, $2H_{meta}$), 6.75 (s, NH), 7.01 (d, $^3\mathcal{J}_{H-H} = 7.3$ Hz, $4H_{meta}$), 7.07 (d, $^3\mathcal{J}_{H-H} = 7.36$ Hz, $4H_{ortho}$), 7.91 (d, $^3\mathcal{J}_{H-H} = 7.47$ Hz, $2H_{ortho}$); ^{13}C NMR (DMSO-d_6), δ (ppm): 20.74 (s, p-CH_3), 112.93 (s), 120.44 (s), 126.82 (s), 130.12 (s), 133.37 (s), 136.06 (s), 149.82 (s), 156.25 (s); ^{31}P NMR (DMSO-d_6), δ (ppm): −17.64 (s); IR (KBr), ν (cm^{-1}): 3400 (w, N–H), 2920 (m), 1598 (w), 1499 (s, NO_2), 1337 (m, NO_2), 1255 (s), 1203 (vs, P=O), 1163 (m), 1080 (m), 1031 (s), 990 (m), 949 (s, P–N), 920 (s), 820 (m), 705 (w), 548 (w), 499 (m), 475 (m).

Kinetic experiments

All reagents for enzymatic experiments were from Fluka. Human acetylcholinesterase (hAChE) from SIGMA (50 units/785 µl) was diluted 25-fold in phosphate buffer (Na_2HPO_4/NaH_2PO_4, 70 mM, pH = 7.8).

The activity of the enzyme was measured at 25°C by a modified Ellman's method [28]. The reaction mixture for determination of IC_{50} values consisted of: DTNB solution, 50 µl; Inhibitor, x µl ($5 < x < 400$); acetylthiocholine (ASCh) solution, 15 µl; phosphate buffer, (835-x) µl; AChE solution, 100 µl. The concentrations of substrate (s_0), DTNB and inhibitors **1**–**4** were 1.35×10^{-4}, 10^{-4} and 0.019, 0.014, 0.012 and 0.013 M, respectively, and the enzyme concentration under the assay conditions was 33.4×10^{-9} M. K_m and V_{max} were obtained in the absence and presence of inhibitor from double-reciprocal Linweaver-Burk plots [31]. A control solution containing all of above materials except inhibitor was used to determine the activity of the enzyme.

Results

Synthesis and spectral data

The interesting point in compounds **1** and **2** is their $^3\mathcal{J}_{PNCH} = 0$. Usually, in compounds with the general formula $Me_2NP(O)XY$ (X = Y = halide or amine or X = halide, Y = amine) a doublet with $^3\mathcal{J}_{PNCH}$ of about 10–14 Hz is observed [27,32–34].

The ^1H NMR spectra of compounds **3** and **4** showed that $^7\mathcal{J}_{P-H}$ is zero. This phenomena was has also been observed in some phosphoramidates [32,33]. The reason for the vanishing $^7\mathcal{J}_{P-H}$ coupling was described as the formation of partial multiple bonds between phosphorus and nitrogen in phosphoramidates [33].

It has been demonstrated that the crystalline state of compound $(MeO)_2P(O)(p\text{-}NHC_6H_4NO_2)$ exists as a network of linear hydrogen bonds [35]. In compounds **3** and **4**, we expected the formation of intermolecular hydrogen bonding. The existence of 4-nitroaniline as an electron withdrawing group in these molecules increased the acidity of the amine hydrogen.

The highly acidity of these protons caused the exchange with the moisture of DMSO-d_6 and appeared in the ^{1}H NMR spectra as a singlet downfield peak. The ^{13}C NMR spectra of molecules **3** and **4** did not show any coupling of carbons with phosphorus ($^n\mathcal{J}_{P-C} = 0$). ^{31}P NMR indicated that the phosphorus atom in compound **4** was more deshielded than in compound **3**. This may be attributed to greater electronegativity of oxygen compared to nitrogen. The IR spectra showed a stronger $\nu_{P=O}$ and a weaker ν_{PN} in compound **4** than in compound **3**.

Human acetylcholinesterase inhibition

Compounds **1** and **2**: In experiments with these compounds, the activity of the enzyme showed negligible changes (Figure 1) and their inhibitory potency was negligible.

Compounds **3** and **4**: By plotting the V_I/V_0 (V_I and V_0 are the activity of the enzyme in the presence and absence of inhibitors, respectively) against log [I], where [I] is the inhibitor concentration, the IC_{50} values of compounds **3** and **4** were obtained as 0.143 and 0.581 mM, respectively (Figure 2). Usually, a plot of remaining activity of the enzyme versus time for irreversible inhibiting phosphoramidates shows a linearly decrease from which the rate constant for inhibition of the enzyme may be obtained [16,36]. These plots for the inhibitors **3** and **4** indicated that the activity of the enzyme remains constant with time and demonstrating that the inhibition process is reversible.

To further characterize the reversible process and to obtain the K_m and V_{max} values in the absence and in the presence of inhibitor, $1/V$ was plotted against $1/[S]$, where the $V$and $[S]$ are the enzyme activity and substrate concentration, respectively. These Line-weaver-Burk plots [31] indicated that compounds **3** and **4** were mixed inhibitors (Scheme 1) and their K_m^{app}

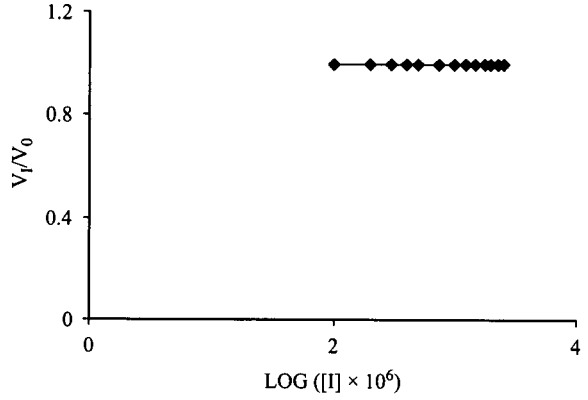


Figure 1. The plot of V_I/V_0 against log ([I] × 10^6) for inhibitors $(Me_2N)P(O)(Cl)(p\text{-}NHC_6H_4NO_2)$, **1**, and $(Me_2N)P(O)(p\text{-}NHC_6H_4NO_2)_2$, **2**. V_I and V_0 are the enzyme activity (OD min^{-1}) and [I] is the inhibitor concentration (μM).

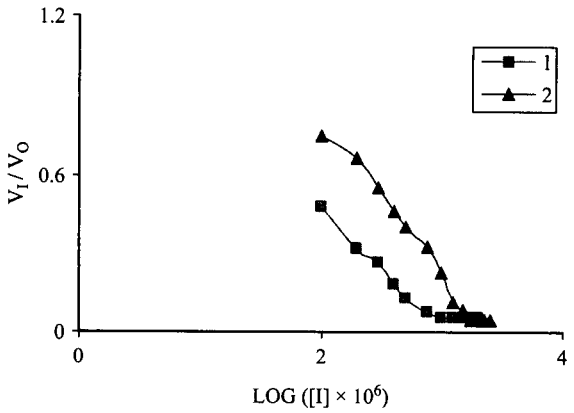


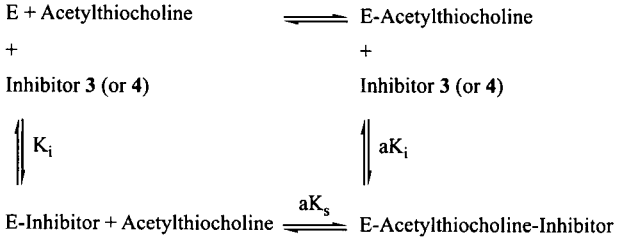
Figure 2. The plot of V_I/V_0 against log ([I] × 10^6) for inhibitors: 1-$(p\text{-}CH_3C_6H_4O)P(O)(p\text{-}NHC_6H_4NO_2)_2$, **3**, 2-$(p\text{-}CH_3C_6H_4O)_2P(O)(p\text{-}NHC_6H_4NO_2)$, **4**. V_I and V_0 are the enzyme activity (OD min^{-1}) and [I] is the inhibitor concentration (μM).

values were 23.19×10^{-5} and 10.79×10^{-5} mol L^{-1} and V_{max}^{app} values were 9.16×10^{-6} and 9.97×10^{-6} mol L^{-1} min^{-1}, respectively. Also, the K_m and V_{max} values for enzyme were 108.20×10^{-5} mol L^{-1} and 10.03×10^{-6} mol L^{-1}min^{-1}, respectively.

Discussion

Usually, the inhibition of acetylcholinesterase by phosphoramidates has an irreversible mechanism [2–4,7,9–19] and only a few show a reversible inhibitory effect such as $(Me_2N)_2P(O)(p\text{-}NHC_6H_4\text{-}NO_2)$, **5** [27]. Compounds **1–4** were designed and synthesized to further investigate the influence of these phosphoramidates on human acetylcholinesterase.

Table I summarizes the spectral and enzymatic data for compounds **1–6**. Compound **1** with a chlorine atom as a suitable leaving group was expected to be an irreversible inhibitor, but to our surprise this compound gave no significant change in the activity of the enzyme hAChE. The ^{31}P NMR spectra of this compound in D_2O indicated two peak at −10.59 and 0.29 ppm with relative ratio 1:1. The P–Cl bond in molecule **1** is labile and hydrolysis in phosphate buffer perhaps produces a P–OH bond. This may be the reason for the negligible inhibitory potency of molecule **1**.



Scheme 1. The mechanism of human acetylcholinesterase inhibition by compounds **3** and **4**.

Table I. The spectral and enzymatic data for compounds **1–6**.

Compound	δ_{31P} (ppm)	$^{3}J_{PNCH}$ (Hz)	$^{7}J_{P-H}$ (Hz)	$^{n}J_{P-C}$ (Hz), (n=2–5)	$\nu_{P=O}$ (cm^{-1})	ν_{PN} (cm^{-1})	IC_{50} (mM)	$K_m \times 10^5$ ($mol\,L^{-1}$)	$V_{max} \times 10^6$ ($mol\,L^{-1}min^{-1}$)	Ref.
1	−0.9	s, 0	–	s, 0	1221	907 (ar) 724 (al)	[a]NI	101.12	9.98	[b]
2	2.17	s, 0	–	s, 0	1300	902 (ar) 746 (al)	[a]NI	103.08	10.01	[b]
3	−19.09	–	s, 0	s, 0	1198	990	0.143	23.19	9.16	[b]
4	−17.64	–	s, 0	s, 0	1203	949	0.581	10.79	9.97	[b]
5	–	d., 10	–	–	1250	980	–	–	–	27
6	–	–	–	–	1255	980	–	–	–	27

[a] Non-Inhibitor.
[b] This work.

The behavior of compound **2** with hAChE was similar to that of compound **1**. Although the structure of compound **2** is similar to that of molecule **5** of Bollinger et al. [27] and contains a $(O)PN_3$ moeity, their inhibition powers are different against hAChE.

Debord et al. [37] showed that the $(O)PN_3$ moeity in aliphatic phosphoramides interacts with the hydrophilic zone of the catalytic site of butyrylcholinesterase. Jarv et al. indicated [38] that in acetylcholinesterase three hydrophobic regions bind to hydrocarbon substituents and surround this zone. In phosphoramide **2**, the existence of two nitroaniline groups decrease the lipophilicity of the aromatic ring. The hydrophobic constants obtained by Rekker et al. [39] are the measure of lipophilicity of a molecular fragment which is 1.69 for C_6H_4 and −0.059 for an aromatic nitro group. Therefore, it is likely that two nitroaniline groups will decrease the lipophilicity of the molecule to a great extent, and this probably leads to the negligible inhibitory effect of this compound.

Surprisingly, compounds **3** and **4** revealed different results. Although the only structural difference between molecules **2** and **3** is the replacement of a Me_2N group by *p*-cresol, the interaction of molecule **3** with hAChE indicated that it was a reversible mixed inhibitor.

To further investigate the relationship between the inhibitory effects of this molecule with a *p*-cresol substituent, we used molecule **4** which has two *p*-cresol groups. The inhibitory potency of this molecule is drastically decreased in comparison to molecule **3**. The IC_{50} values for compounds **3** and **4** are 0.143 and 0.581 mM, respectively. These values show that the inhibitory potency of compound **3** is greater than that of compound **4**.

The noticeable point in molecules **3** and **4** with phenolic substituents is that the P–O–ph groups are stable against hydrolysis and show only a singlet peak in ^{31}P NMR spectra in D_2O.

Finally, it is concluded that changing the substituents X and Y in phosphoramidates (X)(Y)P(O) (p-$NHC_6H_4NO_2$) leads to compounds with different inhibitory potency. The effect of 4-nitroaniline within this research area is interesting. Compounds **1** and **2** showed no inhibitory effect while compounds **3** and **4** possesing $(O)PN_2O$ and $(O)PNO_2$ moieties, respectively, are reversible mixed inhibitors. Spectral data showed that in compounds with a (O)P(p-$NHC_6H_4NO_2$) moiety all phosphorus-hydrogen and phosphorus-carbon couplings disappeared.

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