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RESEARCH ARTICLE

Design, synthesis and anticholinesterase activity of some new α-aminobisphosphonates

Khodayar Gholivand[1], Fatemeh Ghaziani[1], Rouhollah Yaghoubi[1], Zahra Hosseini[1], and Zahra Shariatinia[2]

[1]*Department of Chemistry, Faculty of Basic Sciences, Tarbiat Modares University, Tehran, Iran, and* [2]*Department of Chemistry, Amirkabir University of Technology, Tehran, Iran*

Abstract

Some new *α*-aminomethylenephosphonic acids **1**–**11** were synthesised and characterised by ^{1}H, ^{13}C, ^{31}P NMR, IR spectroscopy and elemental analysis. The potencies of these compounds to inhibit human erythrocyte acetylcholinesterase (hAChE, EC 3.1.1.7) were studied by a modified Ellman's method. In addition, the log P values were computed by Hyperchem software. Here, alendronate was used as a reference inhibitor. Results showed that the IC_{50} values ranged from 9.11 to 28.72 mM. The half maximal inhibitory concentration (IC_{50}) value decreased with an increasing number of carbon atoms of the amine group in compounds **1**–**5**. Also, in most cases, increasing the number of carbon atoms led to enhancement of the toxicity as predicted by the log P values. Using Lineweaver-Burk and Dixon analysis, it was indicated that compounds **1**–**10** are mixed inhibitors while compound **11** is a coupling or uncompetitive inhibitor. The results showed that the electronic changes have ignorable effects, steric influence is important in some cases, but the lipophilicity parameter is the most significant factor in hAChE inhibition by bisphosphonates.

Keywords: *Bisphosphonate; human erythrocyte acetylcholinesterase; IC_{50}; Ellman's method; log P*

Introduction

Aminoalkylphosphonic acids, a class of bisphosphonates are structural analogues of amino acids in which the carboxylic group is substituted by a phosphonic or related moiety. Aminobisphosphonic acids and their derivatives have received considerable attention because of their potential biological activity in treating various human diseases such as osteoporosis [1–6], cancer [7–10], Alzheimer's [11,12], and HIV [13]. Moreover, there are several significant applications for these compounds as plant growth regulators [14], antiparasitics [15], herbicides [16–18], pesticides [19], and antiviral agents [20]. The effect of cholesterol-lowering bisphosphonate drugs (e.g. alendronate, Scheme 1) on the activity of cholinesterases (ChE) in rat brain and blood has been reported [21]. Also, it has been shown that cholesterol-modifying drugs modulate AChE activity and it is better to use a blood-brain barrier (BBB) penetrating drug [21]. Additionally, the effect of alendronate on lowering cholesterol levels in the central nervous system of rats has been investigated [22].

We have previously evaluated the inhibition potency of many phosphoramidate compounds on human erythrocyte AChE activity [23–26]. The existence of the phosphoryl group in such molecules is important for biological activity. Acetylcholinesterase inhibitors represent the standard treatment of Alzheimer´s disease and cholesterol plays a key role in the development of this disease. Since the cholesterol synthesis may be inhibited by bisphosphonates, investigations on these types of compounds have been developed [21]. The differences in the inhibition potencies of organophosphorus agents are a manifestation of the differing molecular properties of the inhibitors involved in the interaction with the active site of the enzyme. To study these parameters, we have prepared and characterised a series of new α-aminomethylenebisphosphonic acids **1**–**11** in which the electronic properties of the phosphorus atom and the hydrophobicity of the surrounding substituents have been changed. It should be noted that molecules **1**–**5** had been reported earlier, but they were only characterised by melting point and CHN elemental

Address for Correspondence: K. Gholivand, Department of Chemistry, Faculty of Basic Sciences, Tarbiat Modares University, Tehran, Iran. Tel: (+98)-21-82884422. Fax: (+98)-21-82883455 E-mail: gholi_kh@modares.ac.ir

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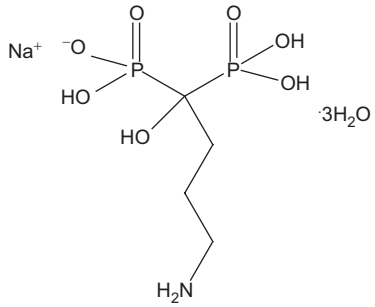


Scheme 1. Alendronate structure.

analysis [27]. Here, full characterisations were made by ^{1}H, ^{13}C, ^{31}P NMR, IR spectroscopy and elemental analysis. Also, the inhibitory potencies of these compounds were determined using a modified Ellman's method [28]. Furthermore, the inhibition mechanisms of these compounds were evaluated by obtaining the Lineweaver-Burk and Dixon plots.

Experimental

Materials and methods

All of the compounds were commercial products (for synthesis) from Merck (Tehran, Iran); purified human plasma erythrocyte acetylcholinesterase (hAChE, EC 3.1.1.7) (50 units / 785µl) from Sigma (Tehran, Iran), acetylthiocholine (ATCh) and 5,5′-dithio bis(2-nitrobenzoic acid) (DTNB) from Fluka (Tehran, Iran). ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker (Avance DRS, Germany) 500 MHz spectrometer (Tehran, Iran). ^{1}H, ^{13}C and ^{31}P chemical shifts were obtained in D_2O relative to TMS and 85% H_3PO_4 (as external standard), respectively. IR spectra were obtained using KBr pellets in the range 400-4000 cm^{-1} on a Shimadzu (Japan) IR-60 model spectrometer (Tehran, Iran). Elemental analyses were performed using a Heraeus CHNO RAPID instrument (Tehran, Iran) and UV measurements were obtained with a Shimadzu UV-2100 (Japan) spectrophotometer (Tehran, Iran).

Synthesis

Compounds **1**–**11** were prepared by a Mannich type reaction according to the procedure previously described [26]. The corresponding amine (1 mmol) was mixed with 37% hydrochloric acid (5 mL), deionised water (5 mL) and phosphorous acid (3 mmol). The mixture was allowed to reflux at 100–120°C for 1.5 h, then paraformaldehyde (4 mmol) was added in small portions over a period of 1 h, and the mixture was refluxed for an additional hour. Removal of the solvents afforded a white powder with a product yield of 89.2%. Its purity was confirmed by NMR measurements and elemental analysis.

N-Methyliminobis(methylenephosphonic acid) 1

Yield 89.2%; mp 223°C; Anal. Calc. for. $C_3H_{11}NO_6P_2$: C, 16.45; H, 5.06; N, 6.39; found: C, 16.44; H, 5.07; N, 6.39%; ^{31}PNMR (202.46 MHz, D_2O), δ (ppm) = 7.81 (t, $^{2}J(P,H)$ = 12.6 Hz); ^{1}H NMR (500.13 MHz, D_2O), δ (ppm) = 3.08 (s, 3 H, CH_3), 3.47 (d, 4H, $^{2}J(P,H)$ = 12.6 Hz, CH_2-N); ^{13}C NMR (125.77 MHz, D_2O), δ (ppm) = 44.08 (s), 53.82 (dd, $^{3}J(P,C)$ = 5.082 Hz, $^{1}J(P,C)$ = 137.5 Hz); IR (KBr), ν (cm^{-1}): 3425 (w, NH^+), 3065 (m, CH), 2850 (m, CH), 2735(m, P-OH), 2335 (m, C-N), 1084-1235 (s, $\nu_{as}PO_3$), 956-1045 (s, ν_sPO_3), 749 (s, P-C), 540 (s, δP-O), 465 (m), 434 (m).

N-Ethyliminobis(methylenephosphonic acid) 2

Yield 87.5%; mp 205°C; Anal. Calc. for $C_4H_{13}NO_6P_2$: C, 20.61; H, 5.62; N, 6.01; found: C, 20.60; H, 5.61; N, 6.00%; ^{31}PNMR (202.46 MHz, D_2O), δ (ppm) = 8.15 (t, $^{2}J(P,H)$ = 12.1 Hz); ^{1}H NMR (500.13 MHz, D_2O), δ (ppm) = 1.22 (m, 3H, CH_3), 3.42 (d, 6H, $^{2}J(P,H)$ = 12.1 Hz, -CH_2-N); ^{13}C NMR (125.77 MHz, D_2O), δ (ppm) = 5.67 (s), 47.81 (d, $^{1}J(P,C)$ = 131.5 Hz, CH_2), 49.84 (s); IR (KBr), ν (cm^{-1}): 3450 (m, NH^+), 3040 (m, CH), 2755 (m, P-OH), 2585 (m), 1278 (m, C-N), 1107-1221 (s, $\nu_{as}PO_3$), 972-1038 (s, ν_sPO_3), 709 (m, P-C), 587 (m, δP-O), 527 (w), 416 (m).

N-Propyliminobis(methylenephosphonic acid) 3

Yield 86.3%; mp 183°C; Anal. Calc. for $C_5H_{15}NO_6P_2$: C, 24.30; H, 6.12; N, 5.67; found: C, 24.29; H, 6.11; N, 5.66%; ^{31}P NMR (202.46 MHz, D_2O), δ = 8.14 (t, $^{2}J(P,H)$ = 12.5 Hz); ^{1}H NMR (500.13 MHz, D_2O), δ (ppm) = 0.78 (t, 3H, CH_3), 1.61 (m, 2H, CH_2), 3.28 (m,2H, CH_2), 3.40 (d, 4H, $^{2}J(P,H)$ = 12.5 Hz, CH_2); ^{13}C NMR (125.77 MHz, D_2O), δ (ppm) = 7.09 (s), 14.14 (s), 48.5 (d, $^{1}J(P,C)$ = 138.00 Hz,CH_2), 55.84 (s); IR (KBr), ν (cm^{-1}): 3415 (w, NH^+), 3005 (m, CH), 2885 (m, CH), 2765 (m, P-OH), 2640 (m), 1283 (m, C-N), 1145-1230 (s, $\nu_{as}PO_3$), 945-1014 (s, ν_sPO_3), 771 (w, P-C), 583(s, δP-O), 506(m), 433(m).

N-Pentyliminobis(methylenephosphonic acid) 4

Yield 88.1%; mp 205°C; Anal. Calc. for $C_7H_{19}NO_6P_2$: C, 30.55; H, 6.96; N, 5.09; found: C, 30.54; H, 6.95; N, 5.09%; ^{31}P NMR (202.46MHz, D_2O), δ (ppm) = 8.13 (t, $^{2}J(P,H)$ = 12.7 Hz) ; ^{1}H NMR (500.13 MHz, D_2O), δ (ppm) = 0.706 (t, 3H, CH_3), 1.17 (m, 4H, CH_2), 1.60 (m, 2H, CH_2), 3.31 (t, 2H, CH_2), 3.41 (d, 4H, $^{2}J(P,H)$ = 12.7 Hz, CH_2); ^{13}C NMR (125.77 MHz, D_2O), δ (ppm) = 10.26 (s), 18.65 (s), 20.02 (s), 24.86(s), 48.30 (dd, $^{3}J(P,C)$ = 4.00 Hz, $^{1}J(P,C)$ = 138.00 Hz,CH^2), 54.40 (s); IR (KBr), ν (cm^{-1}): 3420 (w, NH^+), 2950 (m, CH), 2750 (m, P-OH), 2640 (m), 2500 (m), 1271 (m, C-N), 1150-1212 (s, $\nu_{as}PO_3$), 940-1014 (s, ν_sPO_3), 775 (w, P-C), 583 (s, δP-O), 517 (m), 484 (m).

N-Isopropyliminobis(methylenephosphonic acid) 5

Yield 89.8%; mp 292°C (decomposed); Anal. Calc. for $C_5H_{15}NO_6P_2$: C, 24.30; H, 6.12; N, 5.67; found: C, 24.30; H, 6.12; N, 5.68%; ^{31}P NMR (202.46 MHz, D_2O), δ (ppm) = 8.55 (t, $^{2}J(P,H)$ = 13.0 Hz); ^{1}H NMR (500.13 MHz, D_2O), δ (ppm) = 1.21 (d, 6 H, $^{3}J(P,H)$ = 6.1Hz, CH_3), 3.98 (m, -CH), 3.34 (d, 4H, $^{2}J(P,H)$ = 13.0 Hz, -CH_2-N); ^{13}C NMR (125.77 MHz, D2O), δ (ppm) =13.05 (s), 45.62 (dd, $^{3}J(P,C)$ = 4.1 Hz, $^{1}J(P,C)$ = 136.8 Hz, CH_2), 56.63 (s); IR (KBr), ν (cm^{-1}): 3420 (w,NH^+), 3010 (m,CH), 2730 (m, P-OH), 2605 (m), 1258 (m, C-N), 1120-1220 (s, $\nu_{as}PO_3$), 948-1004 (s, $\nu_s PO_3$), 781 (w, P-C), 555 (m, δP-O), 504 (m), 453 (w).

N-2-Ethyl hexyliminobis(methylenephosphonic acid) 6
Yield 88.2%; mp 216°C; Anal. Calc. for $C_{10}H_{25}NO_6P_2$: C, 37.86; H, 7.94; N, 4.42; found: C, 37.85; H, 7.94; N, 4.41%; ^{31}PNMR (202.46 MHz, D_2O), δ (ppm) = 7.59 (t, ^{2}J (P,H) = 12.4 Hz); ^{1}H NMR (500.13 MHz, D_2O), δ (ppm) = 0.88 (t, 6H, J(P,H) = 12.6Hz, CH_3), 1.29 (m, 4H, CH_2),1.40 (m, 4H, CH_2), 1.87 (m, 1H, CH), 3.46 (m, 2H, CH_2), 3.54 (d, 4H, ^{2}J(P,H)= 12.4Hz, CH_2); ^{13}C NMR (125.77 MHz, D_2O), δ = 8.73 (s), 12.81 (s), 21.75 (s), 22.31(s), 26.87(s), 28.80 (s), 33.81 (s), 52.01 (d, ^{1}J(P,C) = 135.2 Hz, CH_2), 61.02 (s); IR (KBr), ν (cm^{-1}): 3405 (w, NH^+), 2930 (s, CH), 2735 (m, P-OH), 2585 (m), 2295 (m), 1254 (m,C-N), 1134-1225 (s, $\nu_{as}PO_3$), 933-1009 (s, ν_sPO_3), 763 (w, P-C), 570 (s, δP-O), 508 (m), 444 (m).

N-Benzyliminobis(methylenephosphonic acid) 7
Yield 88.8%; mp 247°C (decomposed); Anal. Calc. for $C_9H_{15}NO_6P_2$: C, 36.62; H, 5.12; N, 4.74; found: C, 36.61; H, 5.12; N, 4.73%; ^{31}PMR (202.46MHz, D_2O), δ (ppm) = 6.20 (t, ^{2}J(P,H) = 12.1 Hz); ^{1}H NMR (500.13 MHz, D_2O), δ (ppm) = 3.06 (d, 4H, ^{2}J(P,H) = 12.1 Hz, CH_2), 4.20 (s, 2H, CH_2), 7.31-7.48 (m, 5H, C_6H_5); ^{13}C NMR (125.77MHz,D_2O), δ (ppm) = 50.61 (dd, ^{3}J(P,C) = 5.1 Hz, ^{1}J(P,C) = 143.6 Hz ,CH_2), 59.99 (s), 127.93 (s),128.25 (s),130.06 (s), 135.18 (s); IR (KBr), ν (cm^{-1}): 3405 (w, NH^+), 2965 (s, CH), 2845 (s, CH),2720 (s, P-OH), 2545 (s), 1284 (m, C-N), 1166-1229 (s, $\nu_{as}PO_3$), 935-1008 (s, ν_sPO_3), 746 (s, P-C), 573(s, δP-O), 485 (m), 421 (m).

N-2-Phenyl ethyliminobis(methylenephosphonic acid) 8
Yield 89.6%; mp 255°C (decomposed); Anal. Calc. for $C_{10}H_{17}NO_6P_2$: C, 38.85; H, 5.54; N, 4.53; found: C, 38.84; H, 5.54; N, 4.52%; ^{31}P NMR (202.46 MHz, D_2O), δ (ppm) = 7.27 (t, ^{2}J(P,H) = 12.5 Hz); ^{1}H NMR (500.13 MHz, D_2O), δ (ppm) = 3.07 (t, 2H, CH_2), 3.52 (d, 4H, ^{2}J(P,H) = 12.5 Hz, CH_2), 3.68 (d, 2H, ^{2}J(P,H) = 13.0 Hz, CH_2), 7.29-7.33(m, 5H, C_6H_5); ^{13}C NMR(125.77 MHz, D_2O), δ (ppm) = 26.7 (s), 48.7 (d, ^{1}J (P,C) = 136.00 Hz,CH_2), 55.46 (s), 124.62 (s), 126.25 (s), 126.33 (s), 133.15 (s); IR (KBr), ν (cm^{-1}): 3415 (w, NH^+), 3040 (m, CH), 2870 (m, CH), 2775 (m, P-OH), 2590 (m), 1450 (w), 1237 (m,C-N), 1161-1200 (s, $\nu_{as}PO_3$), 940-1004 (s, ν_sPO_3), 744 (w, P-C), 575 (m, δP-O), 493 (m), 405 (m).

N-Cyclopentyliminobis(methylenephosphonic acid) 9
Yield 88.7%; Anal. calcd for $C_7H_{17}NO_6P_2$: C, 30.78; H, 6.27; N, 5.13; found: C, 30.77; H, 6.27; N, 5.12%; ^{31}PNMR (202.46MHz, D_2O): δ (ppm) = 8.60 (t, ^{2}J(P,H) = 13 Hz); ^{1}H NMR (500.13MHz, D_2O), δ (ppm) = 1.54 (m, 2H, CH_2), 1.66 (m, 4H, CH_2), 2.07 (m, 2H, CH_2), 3.51 (d, 4H, ^{2}J(P,H) = 13 Hz, CH_2), 4.1 (m, 1H, CH); ^{13}CNMR (125.77MHz, D_2O), δ (ppm) = 21.09 (s), 25.12 (s), 46.72 (d, ^{1}J(P,C) = 137.4 Hz,CH_2), 65.97 (s); IR (KBr), ν (cm^{-1}): 3435 (w, NH^+), 2980 (m, CH), 2755 (m, P-OH), 2295 (m), 1274 (m, C-N), 1078–1192 (s, $\nu_{as}PO_3$), 913-1015 (s, ν_sPO_3), 759 (w, P-C), 521 (s, δP-O), 461 (m), 411 (m).

N-Cyclohexyliminobis(methylenephosphonic acid) 10
Yield 89.0%; mp 240°C (decomposed); Anal. Calc. for $C_8H_{19}NO_6P_2$: C, 33.46; H, 6.67; N, 4.88; found: C, 33.45; H, 6.66; N, 4.88%; ^{31}P NMR (202.46 MHz, D_2O), δ = 8.47 (t, ^{2}J(P,H) = 13.0 Hz); ^{1}H NMR (500.13MHz, D_2O), δ (ppm) = 1.31-1.48 (m, 6H, CH^2), 1.87-2.02 (m, 4H, -CH_2), 3.46(d, ^{2}J(P,H) = 13.0 Hz, CH_2), 3.56 (m, 1H, CH). ^{13}C NMR (125.77MHz, D_2O), δ (ppm) = 21.62 (s), 21.79(s), 23.60 (s), 46.22 (dd, ^{3}J(P,C) = 4.00 Hz, ^{1}J(P,C) = 136.00 Hz, CH_2), 63.86; IR (KBr), ν (cm^{-1}): 3395 (w, NH^+), 3000 (m, CH), 2870 (m, CH), 2735 (m, P-OH), 2570 (m), 1420 (w), 1259 (m, C-N), 1173-1231 (s, $\nu_{as}PO_3$), 928-1000 (s, ν_sPO_3), 784 (w, P-C), 595 (m), 552 (m), 509 (m).

Homopiperazine-1,4-bis(methylenephosphonic acid) 11
Yield 89.2%; mp 236°C (decomposed); Anal. Calc. for $C_7H_{18}N_2O_6P_2$: C, 29.17; H, 6.30; N, 9.72; found: C, 29.16; H, 6.31; N, 9.71%; ^{31}P NMR (202.46 MHz, D_2O), δ (ppm) = 6.80 (t, ^{2}J(P,H) = 12.6 Hz); ^{1}H NMR (500.13 MHz, D_2O), δ = 2.27 (s, 2H, CH_2), 3.38 (d, 4H, ^{2}J(P,H) = 12.6 Hz, CH_2), 3. 63 (s, 4H, CH_2), 3.90 (s, 4H, CH_2); ^{13}C NMR (125.77 MHz, D_2O), δ (ppm) = 19.81 (s), 49.74 (s), 53.21 (d, ^{1}J(P,C) = 136.0 Hz, CH_2), 55.23; IR (KBr), ν (cm^{-1}): 3420 (s, NH^+), 2985 (s, CH), 2875 (s, CH), 2750 (s, P-OH), 2625 (s), 1461 (m), 1235 (m, C-N), 1167 (s, $\nu_{as}PO_3$), 938-1067 (s, ν_sPO_3), 780 (m, P-C), 537 (m), 451 (m).

Measurement of AChE activity
The activity of hAChE was determined by a modified Ellman's method [28], the methodology described by Ellman follows the hydrolysis of acetylthiocholine, by indirect means, that is, the rate of increase of absorbance at 412 nm, after chemical reaction between the residue of thiocholine (resulting from hydrolysis) and the DTNB particle in the solution. This method is based on evaluation of the rate of enzymatic hydrolysis of acetylthiocholine substrate with 5,5′-dithiobis-(2-nitrobenzoic) acid as the thiol group indicator (interaction of this acid with thiocholine results in the formation of 5-mercapto-2-nitrobenzoic acid with maximum absorption at 412 nm). The reaction was carried out at 37°C in 70 mM phosphate buffer (Na_2HPO_4/NaH_2PO_4, pH 7.4) containing the enzyme (20 µl volume, diluted 100 times in phosphate buffer, pH 7.4), DTNB (5,5-dithiobis(2-nitrobenzoic acid)) (10^{-4}M concentration) and ATCh (1.35×10^{-4}M concentration). The absorbance change at 37°C was monitored with the spectrophotometer at 412 nm for 3 min. and three replicates were run in each experiment. In the absence of inhibitor, the absorbance change was directly proportional to the enzyme activity.

Human acetylcholinesterase inhibition experiments
The reaction mixtures for determination of the half maximal inhibitory concentration (IC_{50}) values, the median inhibitory concentration, Consisted of DTNB solution, 50µl; ×µl (5–250); acetylthiocholine (ATCh) solution, 30 µl; phosphate buffer (890-x) µl; hAChE solution, 30 µl. The final concentrations (M) of DTNB, ATCh, and inhibitors **1**–**11** were: 10^{-4}, 1.35×10^{-4}, $(0.91\times10^{-3}\leq x\leq22.8\times10^{-3})$ **1**, $(4.3\times10^{-3}\leq x\leq17.2\times10^{-3})$ **2**, $(4.0\times10^{-3}\leq x\leq20.2\times10^{-3})$ **3**, $(3.6\times10^{-3}\leq x\leq16.7\times10^{-3})$ **4**, $(4.9\times10^{-3}\leq x\leq24.3\times10^{-3})$

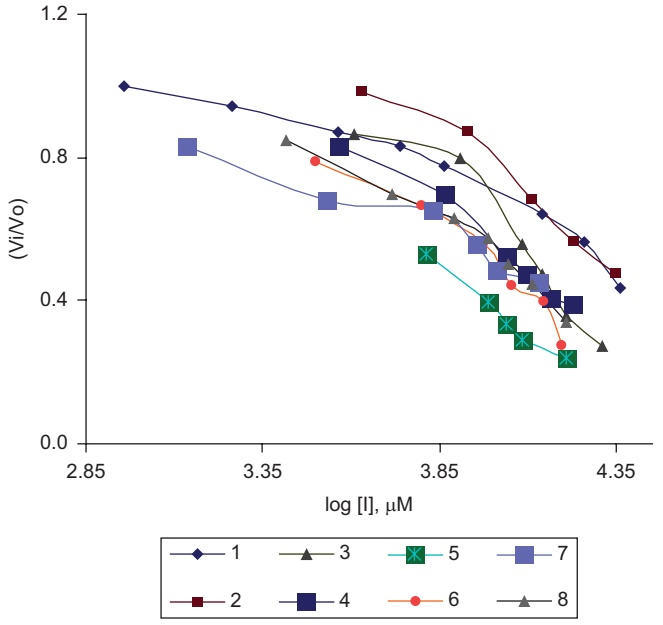


Figure 1. The plot of V_I/V_0 against log ([I], μM) for inhibitors **1**–**8**. V_I and V_0 are the enzyme activity (OD min^{-1}) and [I] is the inhibitor concentration (μM).

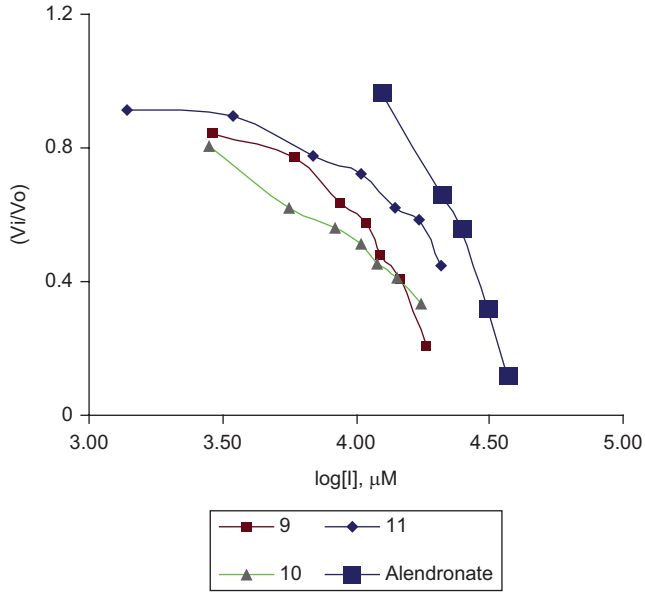


Figure 2. The plot of V_I/V_0 against log ([I], μM) for inhibitors **9**–**11** and alendronate. V_I and V_0 are the enzyme activity (OD min^{-1}) and [I] is the inhibitor concentration (μM).

Table 1. Selected spectroscopic data for compounds **1**–**11**.

Compound	δ(^{31}P) (ppm)	^{2}J(P,H) (Hz)	^{1}J(P,C) (Hz)	^{3}J(P,C) (Hz)	ν(NH$^+$) (cm^{-1})	$\nu_s(PO_3)$ (cm^{-1})	$\nu_{as}(PO_3)$ (cm^{-1})	ν(P-C) (cm^{-1})
1	7.81	12.6	137.5	5.1	3425	956–1045	1084–1235	749
2	8.15	12.1	131.5	-	3450	972–1038	1107–1221	709
3	8.14	12.5	138	-	3415	945–1014	1145–1230	771
4	8.13	12.7	138	4	3420	940–1014	1150–1212	775
5	8.55	13	136.8	4.1	3420	948–1004	1120–1220	781
6	7.59	12.6	135.2	-	3405	933–1009	1134–1225	763
7	6.2	12.1	143.6	5.1	3405	935–1008	1166–1229	746
8	7.27	12.5	136	-	3415	940–1004	1161–1200	747
9	8.6	13	137.4	-	3435	919–1015	1078–1192	759
10	8.47	13	136	4	3395	928–1000	1173–1231	784
11	6.8	12.6	136	-	3420	936–1067	1167	780

Table 2. The enzymatic data for hAChE enzyme, alendranate and compounds **1**–**11**.

Compound	IC_{50}(mM)	log P	K_m(mol L^{-1})	V_m(mol L^{-1} min^{-1})	Inhibition mechanism
Enzyme	-	-	0.098	15.77	-
Alendronate	27.45	0.1	0.062	10.89	Mixed
1	28.09	0.36	0.053	9.52	Mixed
2	22.09	0.7	0.04	9.51	Mixed
3	12.67	1.17	0.035	11.53	Mixed
4	11.66	1.97	0.036	9.98	Mixed
5	9.11	1.12	0.046	9.65	Mixed
6	9.48	3.16	0.042	8.47	Mixed
7	11.93	1.38	0.04	14.19	Mixed
8	10.99	1.63	0.05	14.39	Mixed
9	11.07	1.55	0.043	7.38	Mixed
10	9.96	1.95	0.057	11.63	Mixed
11	28.72	0.22	0.05	6.48	Uncompetitive

5, $(3.2\times10^{-3}\leq x\leq15.8\times10^{-3})$ **6**, $(1.4\times10^{-3}\leq x\leq13.6\times10^{-3})$ **7**, $(2.6\times10^{-3}\leq x\leq16.2\times10^{-3})$ **8**, $(2.9\times10^{-3}\leq x\leq18.3\times10^{-3})$ **9**, $(1.6\times10^{-3}\leq x\leq13.1\times10^{-3})$ **10**, $(1.4\times10^{-3}\leq x\leq20.8\times10^{-3})$ **11**, respectively. The reaction mixtures for determination of the inhibition mechanism were: DTNB (the same as above) and ATCh substrate (10≤x≤40 μl); a solution of enzyme plus inhibitors (inhibitors concentration were adjusted to give about 50% of hAChE inhibition) 150 μl; phosphate buffer, (820-x) μl.

A control solution containing all of above materials except inhibitor was used to determine the activity of the enzyme.

Enzymatic measurements

The plot of 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, gave the IC_{50} values of compounds **1–11** as shown in Figures 1 and 2. K_m and V_m were obtained in the absence and presence of inhibitor from double reciprocal Lineweaver-Burk plots [29]. The IC_{50} (mM), K_m (M) and V_{max} (M min^{-1}) values are given in Table 2.

Enzyme inhibition mode

The double reciprocal, or Lineweaver-Burk, plot is the most straightforward method of diagnosing inhibitor modality

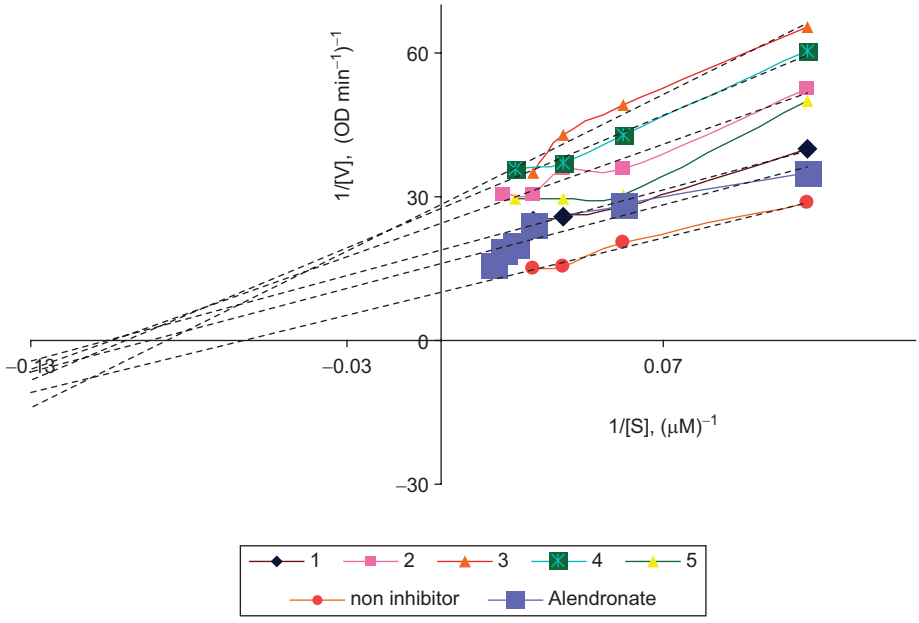


Figure 3. The plot of 1/[V]against 1/[S] for inhibitors **1–5**, alendronate and Enzyme activation without inhibitor. [V] is the enzyme activity (OD min^{-1}) and [S] is the substrate concentration (μM).

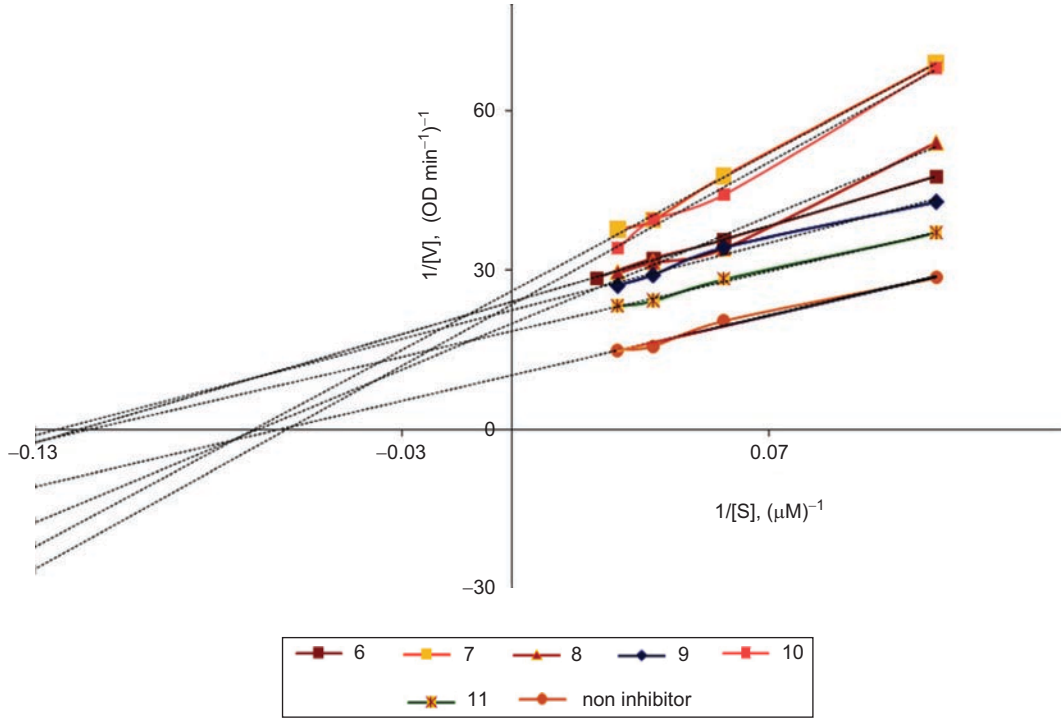


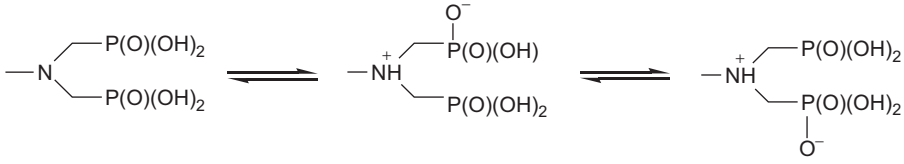
Figure 4. The plot of 1/[V]against 1/[S] for inhibitors **6-11** and Enzyme activation without inhibitor. [V] is the enzyme activity (OD min^{-1}) and [S] is the substrate concentration (μM).

[30]. The kinetic constants K_m and V_{max} can be determined in the absence and presence of inhibitor from the slope and intercept values of the linear fit of the data in a Lineweaver-Burk and Dixon plot (Figures 3 and 4).

Results and discussion

Spectroscopic study

In this work, we have prepared and characterised some new α-aminomethylphosphonic acids **1**–**11** (Scheme 2) to study their inhibition potencies against hAChE enzyme. The selected spectroscopic data of the compounds are listed in Table 1. The ^{1}H NMR spectra of compounds **1**-**11** showed a doublet peak for methylene moiety with a coupling constant $^2J(PCH) = 12.1–13$ Hz, which results from the coupling of methylene protons with the phosphorus atom. The $^{31}P\{^1H\}$ NMR spectra indicated a singlet in the range of 6.20–8.6 ppm for the phosphorus atom, which splits into a triplet in ^{31}P NMR spectra due to coupling of the phosphorus atoms with neighboring CH_2 protons. The formation of aminomethylphosphonic acid moieties was signaled by the appearance of the triplet [26]. The ^{31}P NMR spectra exhibited that $\delta(^{31}P)$ shifts to down fields from compound **1** to compounds **2**–**4**. Interestingly, in compounds **3** and **5** which have same carbon atoms of alkyl groups, the phosphorus atom in **3** is more negative than in **5**, due to the steric effects. Also, the $\delta(^{31}P)$ in **7** is at up field relative to that of **8**. This trend can also be observed for compounds **9** and **10.** These results revealed that in similar compounds, increasing the number of CH_2 moieties produced a more positive phosphorus atom. The ^{13}C NMR spectra displayed a doublet peak for the methylene carbon atom with a $^1J(P,C)$ of 131.5–143.6 Hz. Furthermore, a doublet peak appeared for the CH_2 carbon atom with a $^3J(P,C)$ of 4–5.1 Hz. IR spectra of the bisphosphonates **1**–**11** indicated the $\nu(NH^+)$ in the range of 3395–3450 cm^{-1} due to the resonance interaction between nitrogen and oxygen atom of the P(O) group as follows:



The $\nu_s(PO_3)$ values are observed at lower frequencies than $\nu_{as}(PO_3)$ amounts. In compounds **3** and **5** with the same number of carbon atoms in the alkyl chain, ν(P-C) is stronger in compound **5** that has an isopropyl group. A comparison of the analogous compounds **7**, **8** and **9**, **10** showed that addition of a CH_2 moiety enhances the ν(P-C).

Enzymatic study

Lipophilicity effect

One of the most exciting fields of modern enzymology is the application of enzyme inhibitors as drugs in human and veterinary medicine [31]. In the search for potent and selective inhibitors of the enzyme human acetylcholinesterase (hAChE, EC 3.1.1.7), compounds **1**–**11** were prepared and their ability to inhibit hAChE were examined by a modified Ellman's method [28]. Selected enzymatic data of hAChE enzyme, alendronate and compounds **1**–**11** are presented in Table 2. Alendronate was used as a reference inhibitor. It has been argued that the inhibitory process of organophosphorus inhibitors is dependent upon the charge on the phosphorous atom, stereochemistry, reactivity, substituents on the phosphorous atom and the leaving group [32]. Thus, we examined the effects of ring size, side chain and number of carbon atoms on enzyme activity.

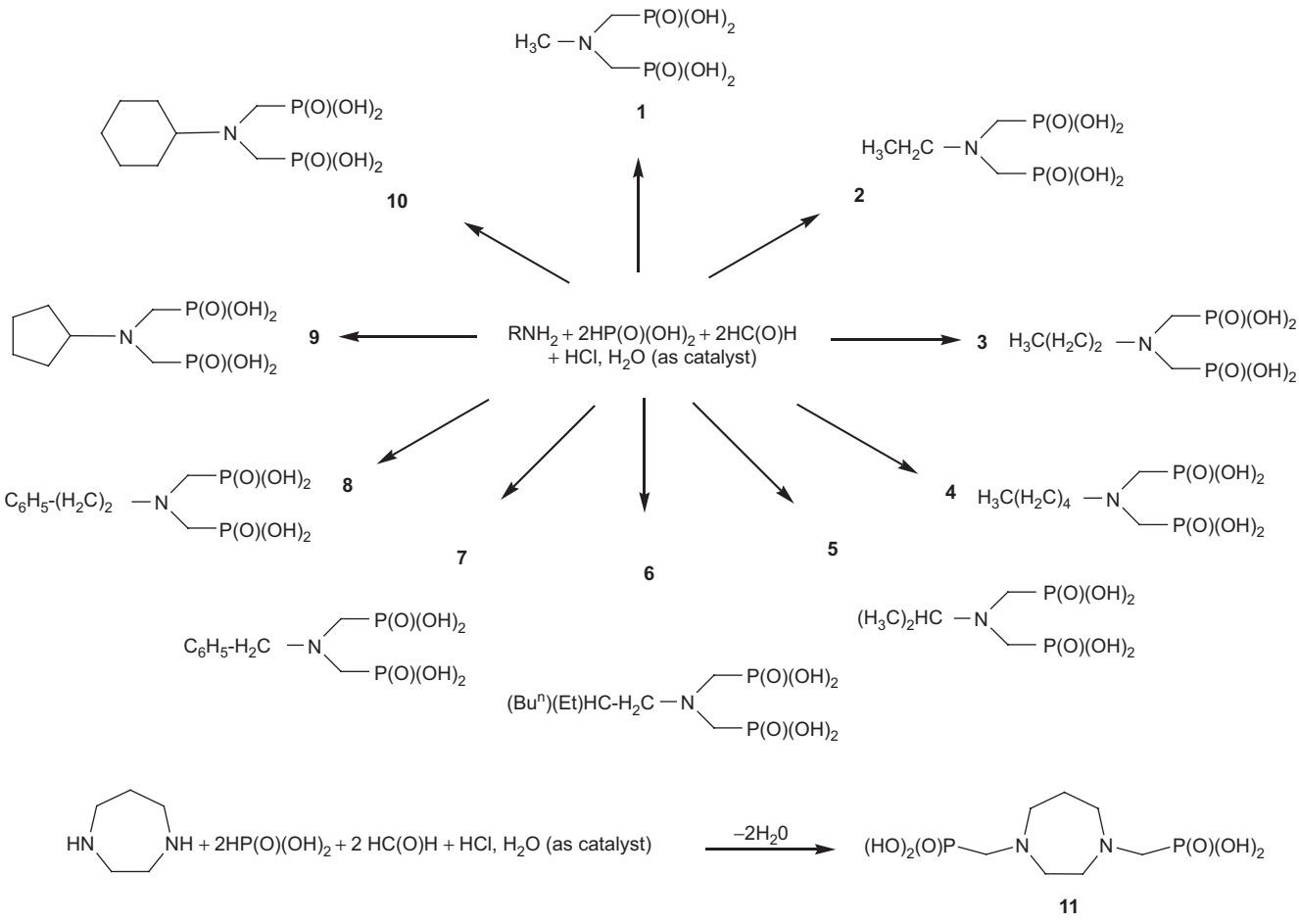
In addition, the log P(o/w) values, the octanol–water partition coefficient, representing the lipophilicity of a molecule, were computed by Hyperchem 7.0 software (Tehran, Iran). Waterhouse determined the lipophilicity and its use as a predictor of blood–brain barrier (BBB) penetration [33]. He noted that there is often a parabolic relationship between measured lipophilicity and *in vivo* brain penetration of drugs, where those that are moderate in lipophilicity exhibit the highest uptake.

Ghadimi et al. considered three electronic, steric and lipophilicity parameters as significant possible factors affecting the inhibition potencies of phosphoramide compounds, and found that liphophilicity has the main influence on IC_{50} (in agreement with the QSAR equations) [34]. Here, the greatest IC_{50} values were observed for compounds **1** and **11** (~28 mM), while the lowest values were for **5**, **6** (~9 mM). The reference inhibitor (alendronate) has a relatively high IC_{50} (27.45 mM) with a small logP value (0.1) and it can be seen that its potency is close to those of compounds **1** and **11**. That is, the least toxic compounds (that are similar to our reference drug) were **1**and **11** while the other inhibitors are much more toxic. Thus, compounds **1** and **11** may be good alternatives for the alendronate drug.

It is interesting that increasing the number of carbon atoms in a linear alkyl chain in the compounds **1**–**4** leads to a decrease in the IC_{50} values (and increasing the log P amounts). The IC_{50} value of compound **7** is greater than that of compound **8** even though they are similar compounds. Therefore, it may be said that addition of one CH_2 group to the alkyl chain increases the toxicity. A comparison of compounds **9** and **10** revealed that the IC_{50} values were dependent on ring size, i.e. by increasing the ring size, the inhibition power is enhanced.

Steric effect

Compounds 3 and 5 with the same number of carbon atoms have nearly the same calculated log P values, but they have different effects on hAChE enzyme (the IC_{50} value



Scheme 2. The synthesis pathways of compounds **1**–**11**.

of **5** (3.56 mM) is less than that of **3**). This effect is perhaps due to the effect of the alkyl side chain against the linear chain (steric effect). Ashani et al. indicated that inhibition potency is influenced by a combined contribution of the electropositivity of the phosphorous atom, size and charge of the O-alkyl or S-alkyl substituents [35]. Thus, the greatest activity is attributed in part to the hydrophobic moieties that can offer favourable interactions with the enzyme which enables a better alignment of the electropositive phosphonyl centre toward nucleophilic attack by the active site serine [35].

The structure of compound **11** is different from those of **1**–**10**. This molecule has two phosphorus atoms (similar to other inhibitors) but three carbon atoms in an aliphatic ring connected to the CH_2 groups. The IC_{50} value for **11** is the greatest one with the least log P=0.22 and $\delta(^{31}P)$=6.8 ppm. If it is assumed that the interaction of hAChE enzyme active site occurs through the P-O bonds, the electronic effects as well as steric ones must be considered simultaneously. Since $\delta(^{31}P)$ of compounds **1**–**11** do not display significant differences, this effect can be attributed to the steric factors. Thus, the type of substituents, their bulkiness and the dihedral angles are important factors for toxicity. That is, the geometry of molecule **11** is not very suitable for the nucleophilic attack of the serine active site.

Enzyme inhibition mode

The relationship between inhibitory potency and substrate concentration was studied initially in a classical way in order to determine the mode of inhibition [36]. Double reciprocal plots for compounds **1**–**10** yielded increasing slopes and intercepts, indicating the inhibitors are mixed ones. Mixed inhibition refers to an inhibitor which displays fixed but uneven binding affinity for both the free enzyme and the enzyme-substrate binary complex. The reciprocal form of the velocity equation for evaluating noncompetitive or mixed inhibitors is: $1/v=K_m/V_{max}(1+ [I]/K_i)1/[S] + 1/V_{max}(1+[I]/\alpha K_i)$, (here α is finite but not equal to 1). As indicated by the above equation, both the slope and the y-intercept of the double-reciprocal plot will be affected by the presence of a noncompetitive inhibitor. The pattern of lines revealed that the plots for varying inhibitor concentrations depend on the value of α. In compounds **7**–**10**, the lines intersect at a value of 1/[S] less than zero and a value of 1/ν of greater than zero (Figure 4) and α exceeds 1 (α>1). In alendronate and compounds **1**–**6**, the lines intersect below the x and y axes at negative values of 1/[S] and 1/ν, α<1 (Figures 3 and 4). Bukowska and Hutnik revealed mixed inhibition mechanism for the inhibition of erythrocyte acetylcholinesterase by 2,4-dichlorophenoxyacetic acid [37].

In compound **11,** the slope of the double-reciprocal plot is independent of inhibitor concentration and the y-intercept increases steadily with an increasing inhibitor concentration (Figure 4). The straight line pattern is the characteristic signature of a coupling or uncompetitive inhibitor. Uncompetitive inhibitors bind exclusively to the ES complex, rather than to the free enzyme. The apparent effect of an uncompetitive inhibitor is to decrease V_{max} and to actually decrease K_m. The reciprocal form of the velocity equation for an uncompetitive inhibitor is given by $1/v=(K_m/V_{max}\times 1/[S]) + 1/V_{max}(1+[I]/\alpha K_i)$.

In Figures 3 and 4, the y-intercept = $1/V_m$, the x-intercept = $-1/K_m$ and the regression coefficients were between 0.96 and 0.99. The K_m and V_{max} values of the bisphosphonates and hAChE were obtained under experimental conditions (Table 2). The K_m^{app} values were between 0.035 and 0.057 mol L^{-1} and the V_{max}^{app} values ranged from 7.38 to 14.39 mol L^{-1} min^{-1}. Also, the K_m and V_{max} values for enzyme were 0.098 mol L^{-1} and 15.77 mol $L^{-1}min^{-1}$, respectively.

Conclusion

In summary, a series of new α-aminomethylene-bisphosphonates **1–11** were synthesised and characterised to study their inhibitory potencies against the hAChE enzyme. Our observations confirm the importance of lipophilicity, steric and electronic factors as significant determinants of the inhibitory potency. These factors can be experimentally estimated from the log P, bulk of molecule and $\delta(^{31}P)$ values. It is deduced from the results that the electronic changes have ignorable effects, steric influence is important in some cases, but the lipophilicity parameter is the most significant factor in hAChE inhibition by bisphosphonates. That is, in most of the cases, increasing the number of carbon atoms leads to increasing the toxicity of compounds, as predicted by the log P values.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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