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Long-Chain Functional Bisphosphonates: Synthesis, Anticalcification, and Antiresorption Activity

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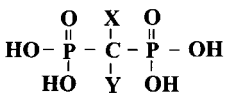
ABSTRACT: *Bisacylphosphonates have previously been shown to possess in vitro activity with regard to hydroxyapatite (HAP) formation and dissolution, and in vivo on tissue calcification and bone resorption. This work was aimed at elucidating the role of the keto groups in the biological activity of these compounds. For this purpose, we have synthesized a series of long-chain bisphosphonates possessing different functional groups adjacent to the phosphonic functions and examined them in some in vitro and in vivo models. The functional groups introduced into the newly synthesized bisphosphonates (hydroxyl, amide, and difluoromethylene) were chosen as to represent certain isolated aspects of the keto groups in the original compounds. None of the functional groups introduced into the long chain bisphosphonates bestowed higher activity on the molecules than the carbonyl groups. The unique effect of the α-keto groups in rendering long-chain bisphosphonates active is presumably attained through a combination of chelating ability with sufficiently low pKa values to enable the bisacylphosphonate molecules to be fully ionized at physiological pH.* © 2000 John Wiley & Sons, Inc. Heteroatom Chem 11:470–479, 2000

INTRODUCTION

Geminal bisphosphonates (BPs), such as etidronate, clodronate, pamidronate, and alendronate (see Figure 1), characterized by a P–C–P structure, were found to be effective anticalcification [1,2] and an-



Name	X	Y
Etidronate	OH	CH_3
Clodronate	Cl	Cl
Pamidronate	OH	$CH_2CH_2NH_2$
Alendronate	OH	$CH_2CH_2CH_2NH_2$

FIGURE 1 Structures of geminal bisphosphonates in clinical use.

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tiresorption [3] agents in vitro and in vivo. Considerable progress has been made recently in elucidating the biochemical mechanisms by which BPs inhibit the activity of bone-resorbing osteoclasts. While certain BPs such as clodronate and etidronate are metabolized inside the osteoclasts to nonhydrolyzable ATP analogs, which poison the cells [4], nitrogen-containing BPs such as pamidronate and alendronate were shown to inhibit cholesterol biosynthesis essential for osteoclast action [5]. A prerequisite to these activities is the affinity of BPs to the bone's hydroxyapatite (HAP) [6,7], resulting in high concentration in the bone, and subsequently, in osteoclasts. A hydroxyl group on the geminal carbon atom of BPs increases not only their affinity to HAP, but also directly affects their activity [8].

Until recently it has been known that nongeminal BPs P–$(CH_2)_n$–P with $n > 2$ were biologically inactive [6]. However, we have demonstrated that, by introduction of keto groups into the α-positions relative to the phosphonic groups (see Figure 2, left-hand side), the bisacylphosphonates obtained are active as anticalcification [9,10] and antiresorption [10,11,12] agents. Subsequently, bisacylphosphonates have been shown to inhibit membrane-mediated calcification, albeit with lower potency than by geminal BPs [13,14].

The present work was undertaken in an attempt to elucidate the roles of (1) the chain linking the two termini of the molecules, and (2) the keto groups, in the biological activity of bisacylphosphonates. In previous articles, we have reported that lengthening the central carbon chain of bisacylphosphonates was accompanied by a decrease in biological activity and an increase of toxicity [9]. One possible way to rationalize this was by assuming that the increase in toxicity was caused by the increased hydrophobicity of the long carbon chain. To shed light on this question, we have decided to synthesize thiobispropionylphosphonate (**3**) and compare its properties with those of its oxidized, and less hydrophobic, sulfone derivative, while retaining the same chain length.

The approach chosen to clarify the roles of the keto groups was to substitute them by different atoms or groups placed on the chain next to the phosphonic functions, each of which would represent certain selected characteristics associated with keto groups (e.g., unshared electrons, polarity, and electron-withdrawing ability). The role of the keto groups in the biological activity of bisacylphosphonates could presumably be related to improved Ca^{2+} binding by chelation and/or to increased acidity of the phosphonic groups due to the electron-withdrawing effect of the α-keto groups.

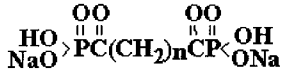
In order to clarify the possible relationship between acidity of the phosphonate groups and the biological activity, we synthesized and evaluated an $\alpha,\alpha,\alpha',\ \alpha'$-tetrafluorobisphosphonate (**6,** see Figure 4), which is a long-chain BP of comparable acidity to that of bisacylphosphonate, but with no chelating capabilities.

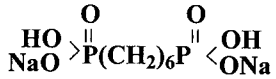
In order to elucidate the chelating effect of the oxygen atoms in the α-position of the phosphonic group, we synthesized and examined (1) long-chain bis(carbamoylphosphonates) (**9** and **11**) in which the carbonyl oxygen atoms should be more strongly nucleophilic than those of the keto groups, owing to the resonance contribution by the nitrogen lone pair in the former; and (2) α,α'-dihydroxypolymethylene-α,ω-bisphosphonic acids (**12, 13, 14**) corresponding in chain lengths to some of the bisacylphosphonates.

RESULTS

Syntheses

Thiobis(propionylphosphonic) acid (**3**) was synthesized using the Arbuzov reaction of thiobispropionyl dichloride and trimethyl phosphite, followed by demethylation, similarly to the method used for the synthesis of other long-chain bisacylphosphonates (see Figure 3). To approach the corresponding 2,2′-bisphosphonoformyldiethylsulfone, we oxidized thiobispropionic acid to the sulfone by bromine [15] and converted the latter to the dichloride, which was then subjected to the Arbuzov reaction with trimethyl phosphite. However, instead of the desired product, this reaction yielded a complex mixture. Examination by ^{31}P NMR spectroscopy revealed the presence of several signals, some of which could be attributed to trimethyl phosphate ($\delta \sim 0$ ppm) and





n = 4 Na_2 Adipoylbisphosphonate (**AdBP**) Na_2 1,6-Hexanebisphosphonate (**HBP**)
n = 5 Na_2 Pimeloylbisphosphonate (**PiBP**)
n = 4 Na_2 Suberoylbisphosphonate (**SuBP**)

FIGURE 2 Structures of bisacylphosphonates examined in previous studies.

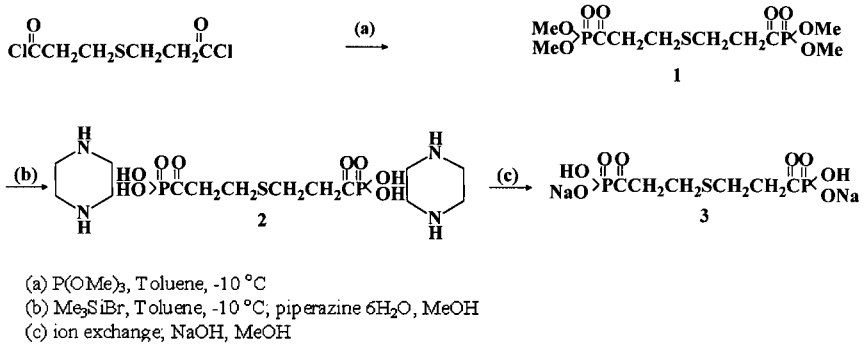


FIGURE 3 The synthesis of thio(bispropionylphosphonic) acid **(3)**.

trimethyl thionophosphate ($\delta \sim 73$ ppm). It is reasonable to assume that trimethyl phosphite has acted as a reducing and a desulfurizing agent in this reaction. The synthesis of the sulfone bisphosphonic acid by oxidation of thio(bispropionylphosphonic) acid (**3**) or its precursor ester, **1**, was not considered because of the existing knowledge on the Baeyer-Villiger oxidation of acylphosphonates [16].

Our first approach to the synthesis of 1,1,6,6-tetrafluorohexane-1,6-bis-phosphonic acid (**6**) was by reacting tetramethyl adipoylbisphosphonate with diethylaminosulfurtrifluoride (DAST), which was reported suitable for the conversion of a benzoylphosphonate to an α,α-difluorobenzylphosphonate [17]. However, in contrast to benzoylphosphonates, the carbonyl groups of this aliphatic acylphosphonate, apparently, are not sufficiently reactive for this reaction to take place. The successful approach to **6** consisted of reaction of diethyl lithium difluoromethylphosphonate with 1,4-butane ditriflate (**4**, see Figure 4). Berkowitz et al. [18] reported that primary alkyl triflates give higher yields in such displacement reactions than the corresponding bromides. After the completion of this work, Blackburn has reported the synthesis of **6** by the reaction of diisopropyl lithium difluoromethylphosphonate with 1,4-dibromobutane [19].

Two bis(carbamoylphosphonates) were synthesized by two different approaches. The reaction of S-ethyl diisopropoxyphosphinylthiolformate (**7**, see Figure 5) with 1,2-ethanediamine [20] gave tetraester **8**, which was dealkylated by bromotrimethylsilane at 50°C for 3 hours [21] to give dihydrogen disodium *N,N′*-bisphosphonoformylethylenediamine (**9**). In contrast to dialkyl acylphosphonates, in which nucleophiles cause fission of the P–C bond [22], in trialkyl phosphonothiolformates, the C–S bond is cleaved preferentially, presumably due to improved leaving group characteristics of RS^- as compared to the dialkyl phosphite anion.

The synthesis of the longer chain tetraethyl *N,N′*-bis(phosphonoformyl)-1,4-butanediamine (**10**) was based on the commercially available 1,4-tetraisocyanatobutane (see Figure 6). It is well known that dialkyl H-phosphonates add rapidly to isocyanates in the presence of base, with the formation of carbamoylphosphonates [23].

α,ω-Dihydroxyalkane-α,ω-bisphosphonic acids (**12, 13, 14**) were synthesized by the direct reduction of bisacylphosphonic acids by sodium borohydride (see Figure 7). We have recently described this reaction, along with the structures of the resulting dihydroxybisphosphonates [24].

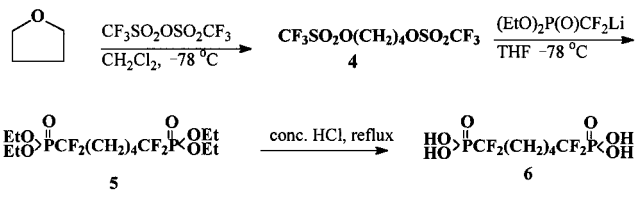


FIGURE 4 The synthesis of 1,1,6,6-tetrafluorohexane-1,6-bisphosphonic acid **(6)**.

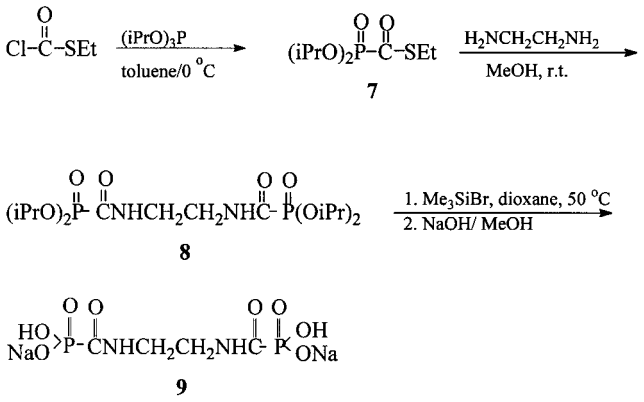


FIGURE 5 The synthesis of 1,2-(bisphosphonoformyl)diaminoethane **(9)**.

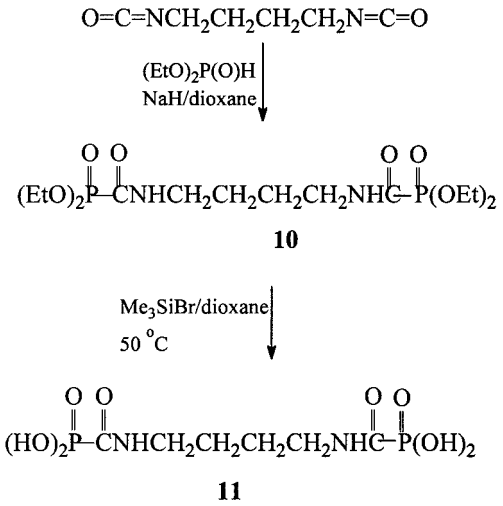
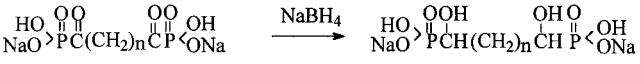


FIGURE 6 The synthesis of 1,4-(bisphosphonoformyl)diaminobutane **(11)**.



n = 4, Disodium 1,6-dihydroxyhexane-1,6-bisphosphonate (**12**)

n = 5, Disodium 1,7-dihydroxyheptane-1,7-bisphosphonate (**13**)

n = 6, Disodium 1,8-dihydroxyoctane-1,8-bisphosphonate- (**14**)

FIGURE 7 Synthesis of dihydroxybisphosphonates.

Evaluation of the Activities of Bisphosphonates

In Vitro Studies. The effect of the various BPs on the inhibition of HAP formation was determined by a method described previously [25]. The results are presented in Figure 8. From this figure it can be seen that at a low drug concentration (0.1 mM) AdBP, **3, 9, 11** and Etidronate significantly inhibited HAP formation. Compounds **12, 13,** and **14** showed slight activity. Compounds **6** and HBP were found to be completely inactive.

In Vivo Studies. The results from study of inhibition of bioprosthetic heart valve tissue calcification [12,26] by the various compounds are depicted in Figure 9. From the tissue calcium content values plotted in this figure, it is seen that tissue calcification was completely inhibited by AdBP, 3,3′-thiobis(propionylphosphonic) acid (**3**) and etidronate. The calcium concentration values in tissues coimplanted with the mini-osmotic pumps containing compounds **6** and HBP were not significantly different from the control group mean value.

DISCUSSION

In order to screen novel BPs for anticalcification activity, it is very useful to examine the inhibition of HAP formation in calcium phosphate solutions. It was shown [10] that there is a good correlation between the antimineralization effect in vitro and the anticalcification effect in vivo. The biological effects of the BPs stem from their incorporation in bone, enabling direct interaction with osteoclasts and/or osteoblasts through a variety of biochemical pathways. [27] After oral or parenteral administration of BP, osteoclasts take up the drug adsorbed on bone mineral. Therefore, the extent of a given BP to adsorb on HAP is one of the factors determining activity in vivo. It was also found [10] that compounds that were not active in the antimineralization experiments in vitro (inhibition of HAP formation in metastable calcium phosphate solutions) were also not active as antiresorptive agents in vivo. Therefore, the structure-activity relationship of long chain BPs could be examined in experimental models: (1) the inhibition of HAP formation in vitro and (2) the inhibition of dystrophic calcification of bioprosthetic tissue.

It is a generally accepted view [6] that geminal BPs are bound to calcium/HAP by both phosphoryl groups with the formation of bidentate or tridentate chelates. This is probably one of the reasons why monophosphonates are not active in calcium-related disorders. It is also known [6] that bisphosphonates of P–$(C)_n$–P type, in which $n > 2$ (e.g., HBP, $n = 6$), are inactive. It is probable, that due to the distance between the two phosphorus atoms, only separate binding of each phosphoryl group to calcium/HAP is obtained.

We have reported previously that bisacylphosphonates, in contrast to other long-chain bisphosphonates (e.g., HBP), are active anticalcification [9,10] and antiresorption [10,11] agents. Bisacylphosphonates were developed for calcium-related disorders based on the hypothesis that the introduction of additional keto groups in positions adjacent to the phosphonic function would compensate for the long distance between the two phosphonic groups [9]. The chelating ability of bisacylphosphonates has been demonstrated recently by X-ray crystallography [28]. On the other hand, the electron-withdrawing keto groups increase the acidity of the adjacent phosphonic groups. Indeed we found that the pKa_3 and pKa_4 values of bisacylphosphonates are in the ranges of 5.17–5.31, and 6.06–6.16, respectively, indicating that these compounds are practically fully ionized at pH = 7.4. In contrast, the four p*K*a values of 1,6-hexanebisphosphonic acid were

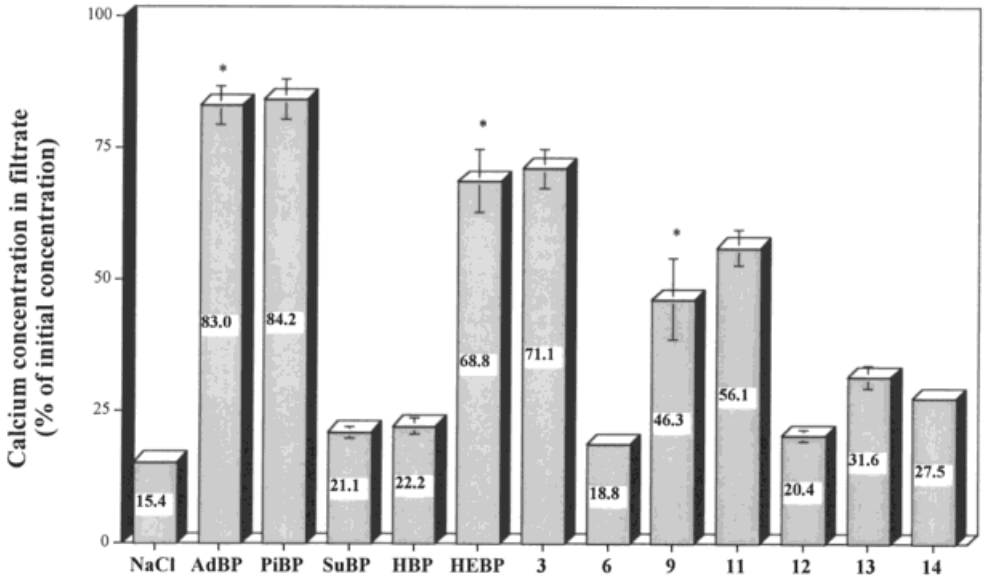


FIGURE 8 The inhibition of HAP formation in a 9mM^2 calcium phosphate metastable solution by bisphosphonates (0.1 mM). New bisphosphonates are compared to a clinically used geminal bisphosphonate (etidronate), to a dideoxobisphosphonate (HBP) and to a bisacylphosphonate (AdBP). (*) Differences were termed statistically significant by the paired *t*-test ($n = 10$, $P < 0.05$).

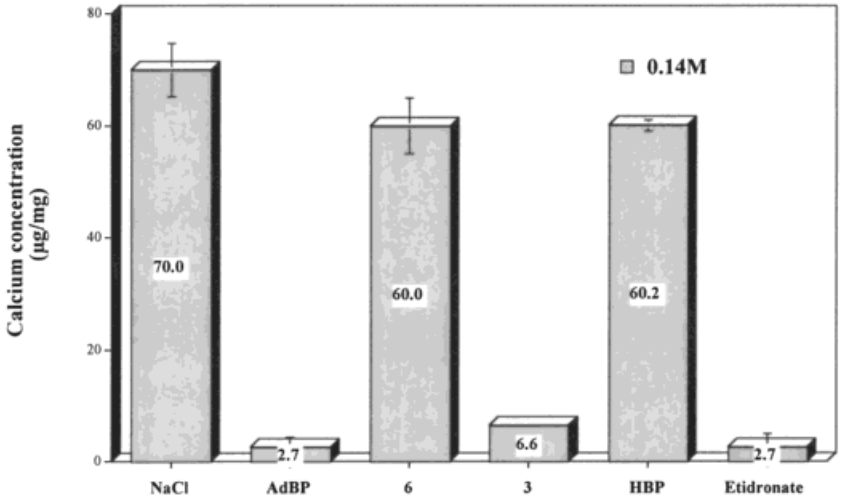


FIGURE 9 The effect of bisphosphonates delivered by mini-osmotic pumps on bioprosthetic heart-valve tissue calcification implanted subcutaneously in rats, for 14 days. (*) Differences were termed statistically significant by the paired *t*-test ($n = 6$, $P < 0.05$).

found to be: 1.38, 2.74, 7.53, and 8.32. These values clearly indicate that the third acidic group in this compound can be only partially ionized, while the fourth one is almost completely unionized at pH = 7.4. Ebetino and coworkers [29] and we [10] have shown independently in separate systems, that for activity in calcium-related disorders, a bisphosphonate should be at least triionized. There are conflicting results regarding the role of increased acidity on activity. A correlation between increasing acidity and antiresorptive activity of BPs has been reported [30]. Yet a strongly acidic BP such as 1,1,2,2-tetrafluoroethane-1,2-bisphosphonate ($pKa_1 = 1.07$; $pKa_2 = 1.97$, $pKa_3 = 4.49$, $pKa_4 = 6.30$) [31], was not found active as an inhibitor of bone resorption [32]. We synthesized bisphosphonate **6,** with a chain length similar to that of AdBP, with difluoro groups replacing the keto groups in positions next to the phos-

phonic groups. The pKa_3 and pKa_4 values of **6** were both reported to be 4.95 [19], which is similar to the pKa_2 of difluoromethylphosphonic acid [33]. Although bisphosphonate **6** is completely ionized at physiological pH, it was found not different from HBP both in vitro and in vivo tests. Clearly, a high dissociation constant per se is not sufficient to render a BP active in inhibiting HAP formation.

The electron density on the carbonyl oxygens in amides is higher than in ketones, as a result of electron donation by the amide nitrogen, resulting in polarization of the C=O group. The increased activity of amides as compared to esters in phospholipase A_2 inhibitors was attributed previously to stronger binding of calcium by the amides [34]. In order to examine the role of electron density of the carbonyl oxygens on activity, we have synthesized two biscarbamoylphosphonates **9** and **11,** to compare them with AdBP and SuBP, which correspond to them in chain lengths, respectively. Our results show that, although both biscarbamoylphosphonates are more active than HBP in vitro, **9** is significantly less active than AbBP, while compound **11** is more active than SuBP. It is possible that, in addition to charge density on the carbonyl oxygens, conformational effects also play a role in determining activity. It seems reasonable to assume that hindrance to free rotation around the nitrogen-carbonyl bonds may prevent the shorter molecule **9** from attaining a conformation necessary for activity, which is reached easily in the longer, more flexible **11.**

The hydroxy group on a geminal carbon in BPs (e.g., etidronate, pamidronate, or alendronate, Figure 1) is assumed to play a major role in determining the binding of BPs to mineralized matrix, probably because of tridentate binding to active binding sites [8,35]. Therefore, we synthesized a series of α,ω-dihydroxypolymethylene-α,ω-bisphosphonic acids (**12, 13, 14**) [24]. It was found that compounds **12** and **13,** corresponding in lengths to AdBP and PiBP, respectively, are inactive at low concentrations (0.1 mM) in vitro. In contrast, compound **14** has a low in vitro activity at (0.1 mM), similar to SuBP, the keto derivative of comparable length. However, at high concentrations (1 mM), **12, 13,** and **14** are active in inhibiting HAP formation in vitro (data not shown) similarly to EDTA [7], while HBP remains inactive even at these concentrations [9]. We can conclude that hydroxy groups, although they do contribute to activity, are significantly inferior to keto groups in nongeminal BPs.

Concurrently with this work, the absorption of certain BPs on HAP was studied by solid-state NMR spectroscopy [36]. Due to the broadness of the signals obtained in the solid state, this method is limited to compounds having ^{31}P chemical shifts sufficiently different from that of HAP. Therefore, it was only possible to study by this method α,ω-dihydroxypolymethylene-α,ω-bisphosphonic acids of the compounds reported in this article, but not bisacylphosphonates. The molar ratio of phosphonates adsorbed on hydroxyapatite determined by ^{31}P spectra without cross-polarization (CP) was found to be approximately twice as high for geminal BPs than for 1,1-dihydroxypolymethylene-1,1-bisphosphonates. Interestingly, disodium 1,1-dihydroxypolymethylene-1,1-bisphosphonates in the solid state showed characteristic ^{13}C chemical shifts, which were indicative of either odd or even numbers of CH_2 groups [36].

Additional mechanisms by which a keto group may contribute to interaction with calcium/HAP are through hydration or enolization. ^{31}P NMR spectroscopy is a sensitive tool for detecting changes in hybridization of carbon atoms adjacent to phosphorus. It was previously reported from our laboratory [37] that the formation of tetrahedral addition products, as transient reaction intermediates, could be monitored in several cases. Such tetrahedral addition products show signals in the neighborhood of 18 ppm in the ^{31}P NMR spectrum. In comparison, acylphosphonates resonate at about 0 ppm. Enolphosphonates can also be recognized through their ^{31}P NMR signals (δ = 11–13 ppm) [38]. However, we were not able to detect by ^{31}P NMR the presence of enols or hydrates in aqueous solutions of bisacylphosphonates in the presence or in the absence of calcium.

CONCLUSION

From this study it appears that the α-keto groups are unique in rendering long-chain BPs active. Presumably, this is attained through a combination of chelating ability with sufficiently low p*K*a values that enable the bisacylphosphonate molecules to be fully ionized at physiological pH.

EXPERIMENTAL

General

The reagents used for syntheses are from Aldrich Chemical Company (Milwaukee, WI) or from Fluka (Buchs, Switzerland). All reagents used in the characterization of bisphosphonates' activity were analytical grade (Sigma, St. Louis, MO). Elemental analyses were performed by the Analytical Laboratories of The Hebrew University of Jerusalem, Givat Ram,

Jerusalem. Infrared spectra were determined on an Analect FTIR spectrometer (Analect Instruments FX-6160, Irvine, CA). Nuclear Magnetic Resonance spectroscopy was performed on a Varian VXR-300S (Palo Alto, CA) or a Bruker-WH-300S (Bruker, Rheinstetten, Germany) instrument; ^{1}H NMR and ^{31}P NMR spectra were recorded in deuteriochloroform or in deuterium oxide solutions. Chemical shifts are reported as parts per million from tetramethylsilane (TMS) as internal standard in ^{1}H NMR and from 10% H_3PO_4 as external standard in ^{31}P NMR; positive chemical shifts are at low field with respect to the standards. Atomic absorption spectroscopy was performed on a Perkin-Elmer 403 instrument (Norwalk, CT).

Syntheses

The syntheses of dihydrogen disodium adipoylbisphosphonate (AdBP), dimethyl dihydrogen bishydroxyiminophosphonate (AdBPDox), and dihydrogen disodium 1.6-hexanebisphosphonate (HBP) were described previously [9].

Tetramethyl 3,3′-Thiobis(*propionylphosphonate*) **(1)**

Trimethyl phosphite (7.7 mL, 0.065 mol) was added dropwise, with stirring, to a solution (6.65 g, 0.039 mol) of 3,3′-thiobis(propionyl chloride) at −10°C under a nitrogen atmosphere. The reaction mixture had been stirred for 1 hour, and the excess trimethyl phosphite was evaporated at reduced pressure to yield an oily product. NMR ^{31}P:δ = −1.13 (sept. J = 11.0 Hz). This compound decomposed upon attempted distillation, but it was sufficiently pure to be characterized and was used for the next step.

Dipiperazine Dihydrogen 3,3′-Thiobis(*propionylphosphonate*) **(2)**

To 0.031 mol of tetramethyl 3,3′-thiobis(propionylphosphonate) bromotrimethylsilane (23.3 mL, 0.180 mol) was added dropwise, and the reaction mixture was stirred at ambient temperature for 1 hour. The excess bromotrimethylsilane was evaporated in vacuo, and the residue was taken up in 90 mL of absolute methanol. A solution of piperazine hexahydrate (13.11 g, 6.75 mmol) in 37.5 mL of absolute methanol and 37.5 mL of water was prepared separately. The piperazine solution was added to the methanol solution, and the resulting mixture was allowed to stand overnight. The crude product was collected by filtration and dried in a desiccator. Yield 10.5 g, 71%. The product was purified by dissolving 2 g of it in water (60 mL), and the solution obtained was decolorized with charcoal, filtered, and concentrated in vacuo to a syrup which was taken up in absolute methanol (20 mL). The resulting suspension was allowed to stand overnight, and the product was filtered off and dried to give a yield of 1.7 g, 85%. NMR (D_2O) ^{1}H: δ = 2.63 (t, 4H, J = 6.3 Hz), 2.98 (t, 4H, J = 6.3 Hz), 3.3 (s, 16H). ^{31}P: δ = −1.1 (s). Anal. Calcd. for $C_{14}H_{32}O_8P_2S_1H_2O$: C, 33.86, H, 6.91, N, 11.29; Found; C, 33.32, H, 6.64, N, 10.92

Disodium dihydrogen 3,3′-Thiobis(*propionylphosphonate*) **(3)**

3,3-Thiobis(propionylphosphonate) dipiperazine salt (0.7 g, 1.46 mmol) was dissolved in distilled water (70 mL) and passed through a Dowex column −50 (H+ − form) which was eluted with water. The eluted solution was neutralized with a solution of sodium hydroxide (0.117 g, 3 mmol) and concentrated in vacuum to a syrup. Absolute methanol (50 mL) was added to the syrup causing the formation of a white precipitate. The disodium salt was filtered off, washed, and dried to give a yield of 0.35 g, 71%. NMR (D_2O) ^{1}H: δ = 2.66 (t, 4H, J = 6.9 Hz), 3.02 (t, 4H, J = 6.9 Hz). ^{31}P: δ = −2.17 (s). FAB Mass: M^+ = 350; $(M\text{-}Na)^+$ = 327.1; $(M+Na)^+$ = 372.

Diethyl Bromodifluoromethylphosphonate [39]

To a magnetically stirred solution of triethyl phosphite (20 g, 0.120 mol) in dry ether (150 mL) at −10°C was added dropwise dibromodifluoromethane (26.8 g, 0.128 mol). The solution was allowed to warm to ambient temperature and then refluxed for 24 hours using an efficient condenser to prevent loss by evaporation of CF_2Br_2 (b.p 23°C). After removal of the solvent, the residue was distilled in vacuo and the product was collected at 99–102°C/16 mm to give a yield of 28.7 g (95%). NMR ^{31}P: δ = 0.05 (t, J = 91.2 Hz).

Diethyl Difluoromethylphosphonate [40]

To a mixture of zinc (7.2 g, 0.11 mol) (activated by chlorotrimethylsilane [41]) in THF (20 mL) diethyl bromodifluoromethylphosphonate (28 g, 0.1 mol) in THF (20 mL) was added with stirring at 0°C, and the reaction mixture was refluxed for 24 hours. Water (50 mL) was added, and the reaction mixture was further stirred for a few hours. The solids were filtered off from the reaction mixture, and the solvent was evaporated resulting in a yield of 16.3 g (95%). NMR ^{31}P: δ = 4.83 (t, J = 95.5 Hz).

1,4-Butane Ditriflate **(4)** *[42]*

A solution of 1.08 g (0.015 mol) of THF in CH_2Cl_2 (50 mL) was added dropwise to a solution of 4.95 g (0.015 mol) trifluoromethanesulfonic anhydride in CH_2Cl_2 (50 mL) at −78°C. The reaction mixture was allowed to warm to ambient temperature and then extracted. Evaporation and recrystallization of the residue from CH_2Cl_2 gave a yield of 2.8 g (53%), m.p. 35–37°C. NMR ($CDCl_3$) ^{1}H:δ = 4.63 (4H, m), 2.03 (4H, m). Anal. Calcd. for $C_6F_6H_8O_6S_2$: C, 20.34, H, 2.27; Found: C, 20.02, H, 2.23.

1,1,6,6-Tetrafluorohexane-1,6-bisphosphonic Acid **(6)**

To a solution of diisopropylamine (4.5 mL, 0.032 mol) in dry THF (20 mL) in a three-necked flask equipped with a rubber septum was added BuLi (20 mL, 1.6M in hexane, 0.032 mol) under argon. The resulting solution was allowed to stir for 25 minutes at 0°C and then cooled to −78°C. Diethyl difluoromethylphosphonate (6 g, 0.032 mol) in dry THF (20 mL) was added by a syringe to this solution of LDA at −78°C followed 2 minutes later by a solution of **1** (1.42 g, 0.004 mol) in dry THF (20 mL) added dropwise. After 10 minutes at −78°C, the reaction was quenched by adding aqueous NH_4Cl and ether. The layers were separated and the aqueous layer was further extracted twice with ethyl acetate. The organic layer was dried, filtered and evaporated. To the residue, 10 mL of 6N HCl was added, and the reaction mixture was refluxed for 5 hours. The solvent was evaporated, and the yellowish solid was recrystallized from concentrated HCl. The product was washed with a little acetic acid giving a yield of 0.445 g (35%), m.p. 199–201°C. NMR (D_2O) ^{1}H:δ = 1.78 (4H, m), 1.36 (4H, m); ^{31}P: δ = 5.55 (t, J = 99.8 Hz). Anal. Calcd for $C_6F_4H_{12}O_6P_2.1/2H_2O$: C, 21.88, H, 3.95; Found: C, 21.73, H, 4.05.

Tetraisopropyl N,N′-bisphosphonoformylethylenediamine **(8)**

To a magnetically stirred solution of ethyl chlorothiolformate (0.5 g, 0.004 mol) in dry toluene (3 mL) under nitrogen at 0°C was added dropwise 0.83 g (0.004 mol) of triisopropyl phosphite. After 3 hours, the solvent was evaporated under vacuum, and to the residue dissolved in methanol, a solution of 0.12 g (0.002 mol) of 1,2-ethanediamine in 3 mL methanol was added dropwise, and the reaction mixture was stirred for 72 hours. The solvent was evaporated, and the product was recrystallized from ethyl acetate giving **8** in a yield of 0.75 g (85%), m.p. 83–85°C. NMR (CD_3OD) ^{1}H:δ = 4.70 (2H, m), 3.38 (4H, s), 1.30 (12H, dd); ^{31}P:δ = −2.98 (t, J = 7 Hz). Anal. Calcd. for $C_{16}H_{34}N_2O_{16}P_2$: C, 43.84, H, 7.71; Found: C, 43.67, H, 8.00

Dihydrogen Disodium N,N′-bisphosphonoformyl Ethylenediamine **(9)**

To 0.75 g (0.0017 mol) of **5** in dry dioxane (20 mL) 2.14 g (0.007 mol) of bromotrimethylsilane was added dropwise, and the reaction mixture was stirred at 50°C for 3 hours. The dioxane was evaporated, and the residue was taken up in absolute methanol (20 mL), followed immediately by the addition of a filtered solution of 0.135 g (0.0034 mol) sodium hydroxide in methanol (15 mL). The reaction mixture was stirred for 2 hours, and the solvent was evaporated. The solid was recrystallized from absolute methanol, giving **6** in a yield of 0.49 g (90%). M.p. > 300°C. NMR (D_2O) ^{1}H:δ = 3.27 (4H, s), 3.17 (4H, s); ^{31}P:δ = −3.11 (s).

Tetraethyl N,N′-bis(phosphonoformyl)-1,4-butanediamine **(10)**

To a solution of diethyl phosphite (0.75 mL, 5.8 mmol) in dry dioxane (3 mL) in a three-necked flask equipped with a rubber septum was added NaH (catalytic amount) under nitrogen at room temp. After 5 minutes, 1,4-diisocyanatobutane (0.5 mL, 3.95 mmol) was added by a syringe. After the reaction mixture had been stirred overnight at room temperature, the solution was concentrated to half volume, and the product was crystallized from the reaction mixture giving (**10**) in a yield 0.623 g (38%), m.p. 120–122°C. NMR ($CDCl_3$) ^{1}H:δ = 7.54 (2H, m), 4.19 (8H, q), 3.29 (4H, m) 1.55, (4H, m), 1.32 (12H, t). ^{31}P:δ = −0.43 (q, J = 7.8 Hz). Anal. Calcd. for $C_{14}H_{30}N_2P_2O_8$: C, 40.37, H, 7.27, N, 6.73. Found: C, 40.64, H, 7.16, N, 6.71.

N,N′-Bis(phosphonoformyl)-1,4-Butanediamine **(11)**

To **10** (0.0235 g, 0.56 mmol), dissolved in dry dioxane (9 mL) bromotrimethylsilane (0.5 mL, 3.9 mmol) was added dropwise, and the reaction mixture was stirred at 60°C for 4.5 hours. The solvent was evaporated, and the residue was taken up in absolute methanol (10 mL) to yield a white solid product, 0.1 g (60%). The product was recrystallized from 10% HCl solution to give 0.06 g **11,** m. p. 195–196°C, NMR (D_2O) ^{1}H:δ = 3.22 (4H, m), 1.51 (4H, m), ^{31}P:δ = −2.72 (s). Anal. Calcd. for $C_6H_{14}O_8N_2P_2$: C, 23.68, H, 4.64, N, 9.21. Found: C, 23.59, H, 4.70, N, 9.29.

Determination of Dissociation Constants

The dissociation constants (p*K*a) were determined by high precision, potentiometrically controlled titration using MINI T and subsequent iteration of data with the program ITERAX 2.01. The pH was monitored using a glass electrode calibrated by the method of blank titration as described in Ref. [43]. For typical p*K*a determinations, 50 mL of a solution consisting of 0.25 mmol BP, 1.5 mmol NaOH, and 3.5 mmol NaCl were titrated vs. 0.1 M HCl in equidistant steps of 0.1 mL at 25 ± 0.1°C.

In Vitro and In Vivo Characterization of the Activity of the Bisphosphonates

Inhibition of HAP Formation Based on a slightly modified method of Francis et al. [44], the extent of HAP formation in the presence of inhibitors was studied in supersaturated calcium phosphate solutions as described previously [9]. The product of calcium ($CaCl_2.2H_2O$) and phosphate (K_2HPO_4) concentrations in the incubated solutions was 9 mM^2, (calcium 3.87 mM and phosphate 2.32 mM), in a Ca/PO_4 ratio of 1.67, as in hydroxyapatite (HAP). Each salt solution was prepared in 0.05 M Tris buffer, pH 7.4, and the drug being examined was dissolved in the phosphate solution. Equal volumes of doubled concentrations of 3.87 mM calcium and 2.32 mM phosphate were mixed in borosilicate glass tubes (acid- and acetone-washed). The vials were placed in a shaker (100 rpm) at 37°C. Calcium concentrations in the filtrate were determined by atomic absorption spectroscopy after incubation for 24 hours [45].

Inhibition of Ectopic Calcification The anticalcification effect of the bisacylphosphonates in vivo was studied by examining the inhibition of bioprosthetic tissue calcification implanted subdermally in rats [2,46,47]. Bioprosthetic heart valve tissue cusps were prepared from bovine pericardium treated with glutaraldehyde as described previously [48]. Fresh parietal pericardium was obtained at slaughter from 1 to 3-month-old calves and immediately placed in iced sterile saline. After dissection of superficial fat from the external surfaces, 1 × 1 cm pieces were cut and incubated in 0.2% glutaraldehyde (25% for electron microscopy, under nitrogen, Merck, Germany) of 0.05 M HEPES and 0.1 M NaCl, pH 7.4, for crosslinking (for at least a two-week period) and storage at 4°C. Mini osmotic pumps (ALZET, 2001, Alza, Stanford, CA) containing 0.14 M drug solution, delivering 16.8 mMol/kg/day, for 14 days, of tested compound, were placed next to subcutaneous bioprosthetic tissue cusps (1 × 1 cm) implanted in the dorsal part of each ether-anesthetized rat. Two tissue cusps, implanted subcutaneously in the abdominal wall of each animal (separated by 1–2 cm), served as a paired control. An additional group of rats, implanted with bioprosthetic tissue without treatment served as an unpaired control. Euthanasia was carried out by ether 14 days after implantation. The retrieved tissue was rinsed with copious amounts of double distilled water, dried to constant weight, and the amount of calcium was determined by atomic absorption spectroscopy on aliquots of HCl hydrolyzates diluted with a lanthanum solution. The amount of calcium was expressed as microgram of calcium per milligram of dry tissue weight. The control values represent the average of both control groups' data, since there was no difference between these groups.

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