Bisphosphonate Prodrugs: Synthesis and in Vitro Evaluation of Novel Acyloxyalkyl Esters of Clodronic Acid

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Novel tetra-, tri-, and *P*,*P*′-dipivaloyloxymethyl esters of clodronic acid were synthesized, and their properties as possible prodrugs of clodronate were evaluated in vitro. All pivaloyloxymethyl esters were significantly more lipophilic (log P_{app} ranged from −2.1 to 7.4) than clodronate (log $P_{app} \leq -5.4$), which suggests that it may be possible to change the intestinal absorption mechanism of clodronate from a paracellular to a transcellular pathway by a prodrug approach. Pivaloyloxymethyl esters degraded rapidly in 10% rabbit liver homogenate, and half-lives of tri- and *P*,*P*′-diesters were 1.1 and 14 min, respectively. The intermediate degradation products were further degraded, and clodronic acid was released in quantitative amounts. In human serum, the stability of pivaloyloxymethyl esters was comparable to their stability in phosphate buffer (pH 7.4), which suggests that their degradation in human serum is mostly due to the chemical hydrolysis. Benzoyloxypropyl esters of clodronic acid were also synthesized, but they did not release clodronic acid due to the enzymatic and chemical stability of the formed 3-hydroxypropyl phosphonate esters and are, therefore, not prodrugs.

Introduction

Clodronate (disodium (dichloromethylene)bisphosphonate) belongs to the group of bisphosphonates (BPs), which are synthetic analogues of endogenous pyrophosphate. Although a detailed mechanism has not been elucidated, it is clear that bisphosphonates inhibit bone resorption, bone turnover, and therefore, bone loss. Clodronate is also used in the treatment of various other bone diseases and as a regulator in calcium metabolism.[1−3] The therapeutic use of clodronate is limited due to its poor oral bioavailability (1−2%),[4,5] which also shows a great inter- and intraspecies variation.[1] The poor oral bioavailability of clodronate is mostly attributable to its very low lipophilicity due to high ionization at physiological pH values. Due to its low lipophilicity, it is assumed that the main route for the intestinal absorption of clodronate is by a paracellular pathway.[2] Absorption of clodronate is also hindered due to its strong complexation with Ca^{2+} or other divalent cations in the intestinal lumen.[2]

An oral formulation of clodronate with improved absorption properties would be of significant benefit, and a prodrug approach is considered herein to reach this goal. Masking one or more ionizable groups of clodronate by using the prodrug approach would increase the lipophilicity of the molecule and decrease the complexation with cations. A prodrug should release the active drug in the body after absorption by enzymatic and/or chemical hydrolysis.[6,7]

Various alkyl/aryl esters of clodronic acid have been studied in order to modify the physicochemical properties of clodronate.[8,9] However, simple alkyl/aryl esters of clodronic acid are not prodrugs because they are hydrolytically stable.[10] Amides of clodronic acid release clodronic acid, but this takes place via chemical hydrolysis, which limits use of that approach.[11] We have recently reported that clodronic acid dianhydrides are sufficiently stable toward chemical hydrolysis in aqueous solutions (pH 7.4 and 2.0) and rapidly undergo complete enzymatic hydrolysis to clodronic acid in human serum.[12] In that approach, only two of the four ionizable groups of clodronic acid can be derivatized, and therefore, the lipophilicity may not be enhanced enough to change the intestinal absorption mechanism of clodronate from a paracellular to a transcellular pathway.

Acyloxymethyl esters of phosphonates have been reported to be hydrolytically more labile than simple esters,[13] and acyloxymethyl esters as bioreversible prodrugs of phosphonates have been prepared from PMEA,[14] 9-[2-(phosphonomethoxy)ethoxy]adenine,[15] PMPA,[16] and phosphonoformate.[17] However, reports concerning the design of acyloxyalkyl esters of bisphosphonates have very rarely been published. Atack et al. have synthesized tetra-, tri-, di-, and monopivaloyloxymethyl esters of the inositol monophosphatase inhibitor L-690,330 to enhance the entry of the parent drug into cells. Pivaloyloxymethyl esters were more potent than the parent drug when tested in vitro, but unfortunately no data of chemical or enzymatic stability was provided to give better insight into the hydrolytic properties of those pivaloyloxymethyl esters.[18]

The aim of the present study was to synthesize and evaluate in vitro acyloxyalkyl esters of clodronic acid as possible bioreversible prodrugs of clodronate. We synthesized tetra-, tri-, and *P*,*P*′-dipivaloyloxymethyl esters of clodronic acid and evaluated their lipophilicities and hydrolytic properties in phosphate buffer (pH 5.0 and 7.4), in serum, in liver homogenate, and in

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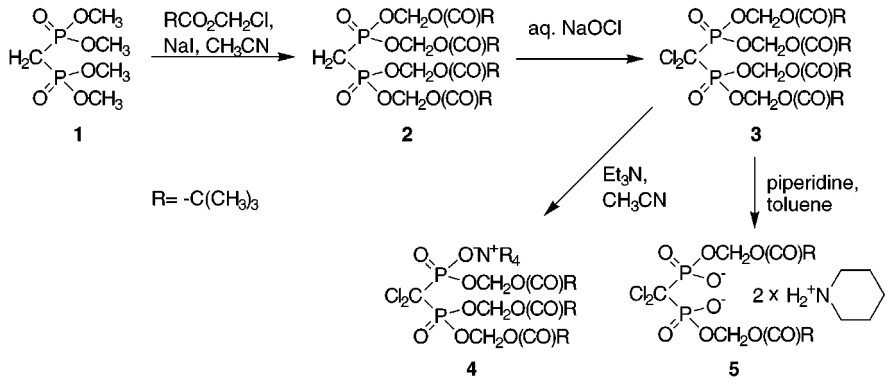
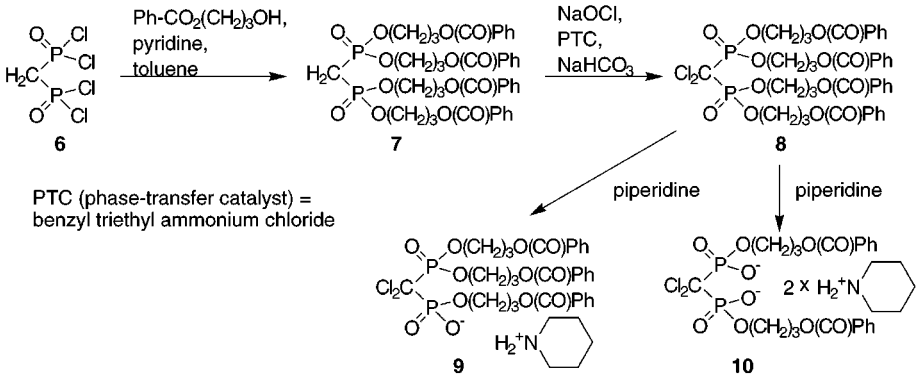
Scheme 1



Scheme 2



specific enzyme solutions. Acyloxymethyl esters of phosphonates should, after cleavage of the carboxylate ester moiety by esterases, release the chemically unstable hydroxymethyl phosphonate ester, which should spontaneously hydrolyze to the corresponding phosphonic acid and formaldehyde. Phosphodiesterases have also been suggested to hydrolyze acyloxymethyl esters of phosphates and phosphonates.[19,20] The action of phosphodiesterases should not yield hydroxymethylated compounds as intermediates. Instead, parent phosphonates should be directly released by the catabolism of the phosphonate ester. We also synthesized and evaluated tetra-, tri-, and *P*,*P*′-dibenzoyloxypropyl esters of clodronic acid. Theoretically, 3-hydroxypropyl phosphonate esters, formed after enzymatic cleavage of the carboxylate moiety, should be chemically stable and would need another enzymatic step to release the parent phosphonate. However, if benzoyloxypropyl esters or 3-hydroxypropyl esters of clodronic acid serve as substrates for the phosphodiesterases, they should release the parent clodronic acid.

Chemistry

Pivaloyloxymethyl Esters. The synthesis of compounds **3**, **4**, and **5** was accomplished by the method outlined in Scheme 1. Tetra(pivaloyloxymethyl) methylenebisphosphonate (**2**) was prepared by known procedure from commercially available tetramethyl methylenebisphosphonate (**1**) and pivaloyloxymethyl chloride (POM-Cl).[21] The halogenation of the methylene carbon of **2** to obtain compound **3** was carried out by using aqueous NaOCl as a chlorinating agent.[21] Corresponding tri- and diester derivatives **4** and **5** were prepared as described earlier using tertiary and secondary amines as dealkylating reagents.[22]

Benzoyloxypropyl Esters. The synthesis of compounds **8**, **9**, and **10** was accomplished by the method outlined in Scheme 2. The required alcohol, 3-hydroxypropyl benzoate, was synthesized following a literature procedure[23] with some modifications. Benzaldehyde was allowed to react with propylene glycol to give the cyclic acetal, 2-phenyl-1,4-dioxolane.[24] In the subsequent step, the cyclic acetal was opened in the presence of *tert*-butylhydroperoxide and vanadyl acetate to afford 3-hydroxypropyl benzoate.[23] The prepared alcohol was then introduced to **6**, which was prepared by treating tetraisopropyl methylenebisphosphonate with sublimed phosphorus pentachloride in an anhydrous atmosphere,[25] yielding **7** in moderate yield (60% after silica column chromatography). The methylene carbon of the tetra(benzoyloxyalkyl) ester **7** was then chlorinated in a slightly basic solution with sodium hypochlorite in the presence of benzyltriethylammonium chloride as a phase-transfer catalyst to yield **8** in quantitative amounts. Tri- and *P*,*P*′-dibenzoyloxypropyl esters, **9** and **10**, were prepared from the tetraester **8** using piperidine as a selective dealkylating agent.[22] Depending on the temperature and reaction time, piperidine selectively hydrolyzes, stepwise, one ester group from each phosphorus atom. By optimizing the reaction conditions and reaction time, it is possible to stop this hydrolysis quite selectively at the trisubstituted state, as the piperidinium salt, with a purity of 85%.

Results and Discussion

Pivaloyloxymethyl Esters of Clodronic Acid. Lipophilicity. The apparent partition coefficients (log P_{app}) for compounds **3**, **4**, and **5** are shown in Table 1. The log P_{app} value for **3** was determined by using the

Table 1. Apparent Partition Coefficients (log P_{app}) and Hydrolysis Half-Lives (in Human Serum, Rabbit Liver Homogenate, and 50 mM Phosphate Buffer) for Pivaloyloxymethyl Esters of Clodronic Acid

		rate data (phosphate buffer)				rate data (enzymatic hydrolysis)			
	log P_{app} (mean ± SD, $N = 3$)	pH 5.0		pH 7.4		80% serum		10% liver homogenate	
compd	pH 7.4	$t_{1/2}$	k_{obs} (min^{-1})	$t_{1/2}$	k_{obs} (min^{-1})	$t_{1/2}$	k_{obs} (min^{-1})	$t_{1/2}$ (min)	k_{obs} (min^{-1})
3 (tetra)	7.4[a]	nd[b]		nd		nd		nd	
4 (tri)	1.56 ± 0.02	99 min	7.0×10^{-3}	102 min	6.8×10^{-3}	78 min	8.9×10^{-3}	1.1 min	6.5×10^{-1}
5 (di)	−2.13 ± 0.01	stable[c]		47 d	1.0×10^{-5}	stable[d]		14 min	5.1×10^{-2}

[a] Determined from HPLC capacity factors. [b] nd = not determined. [c] No degradation observed in 2 weeks. [d] No degradation observed in 24 h.

capacity factors of RP-HPLC, because the traditional shake-flask method was not useful due to the high lipophilicity and low aqueous solubility of **3**. The log P_{app} value of clodronate can be estimated to be less than −5.4, which has been reported for clodronic acid monoethyl ester at pH 7.4.[8] The results show that the lipophilicity of clodronate can be increased several orders of magnitude by substituting the phosphonic OH groups and suggest that at least three phosphonic OH groups must be masked to increase the lipophilicity enough to significantly enhance transcellular absorption of clodronate. Trisubstituted clodronic acid may be an even more promising candidate than tetrasubstituted for the improvement of oral bioavailability, due to its higher water solubility in the gastrointestinal tract. Moreover, the trisubstituted derivative has a log P_{app} value of about 2, which is considered optimal for gastrointestinal absorption.[26]

Chemical Hydrolysis. Compound **5** showed high chemical stability in phosphate buffer solutions, pH 5.0 and pH 7.4, at 37 °C (Table 1). However, the addition of a single additional substituent reduced chemical stability significantly. The half-lives of **4** in phosphate buffers pH 5.0 and pH 7.4 were 99 and 102 min, respectively. The chemical stability of **3** was not determined due to its very low aqueous solubility (i.e., below the detection limit of the HPLC method), but it probably is not better than the stability of **4**.

Enzymatic Hydrolysis. 1. Serum. The susceptibility of pivaloyloxymethyl esters of clodronic acid to enzymatic hydrolysis was first studied in 80% human serum. The shorter half-life of **4** in serum (pH 7.4) than in phosphate buffer pH 7.4 suggests that **4** may be susceptible to serum esterases (Table 1). However, its hydrolysis in serum appears to be mostly due to chemical hydrolysis, judging from the small difference between the half-lives (78 min vs 102 min). Compound **4** was degraded to **5**, which was determined to be stable in serum. Although **3** has a very low aqueous solubility, its degradation was also studied by suspending it in 80% serum. The main degradation products after 4 h at 37 °C were **4** and **5**, and no clodronic acid was detected.

2. Liver Homogenate. In 10% rabbit liver homogenate, **5** degraded with a half-life of 14 min and released clodronic acid quantitatively. Compound **5** released clodronic acid via an intermediate degradation product, which was identified to be the monopivaloyloxymethyl ester of clodronic acid (based on retention time equivalence with a genuine standard). The degradation of **4** was faster, with a half-life of 1.1 min, and released **5**, which was hydrolyzed to the monopivaloyloxymethyl ester and then further quantitatively to clodronic acid (Figure 1). Clodronic acid was also eventually released when **3** was suspended in 10% rabbit liver homogenate.

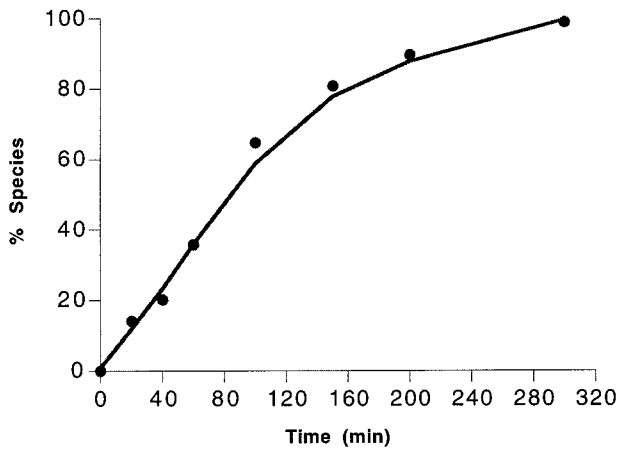


Figure 1. Plot showing the formation of clodronic acid (●) upon hydrolysis of compound **4** in 10% rabbit liver homogenate (pH 7.4) at 37 °C.

3. Enzyme Solutions. The hydrolysis of **5** and **4** was also studied in specific enzyme solutions to gain additional insight into which enzymes were responsible for the degradation. Carboxylate esterase (EC 3.1.1.1) and phosphodiesterase I (EC 3.1.4.1) were chosen as the most probable candidates for the enzymatic degradation of pivaloyloxymethyl esters of clodronic acid, based on previously published reports concerning pivaloyloxymethyl esters of FdUMP and PMEA.[19,20] Compounds **4** and **5** were not hydrolyzed by the action of carboxyl esterase, which is consistent with earlier reports that negatively charged compounds tend to be poor substrates for carboxylate esterase.[27,28] Phosphodiesterase I was also ineffective in hydrolyzing **4** and **5**.

The Mechanism of Bioconversion. Bioconversion of **3** to clodronic acid is suggested in Figure 2. The enzyme(s) responsible for this action could not be identified by carrying out hydrolysis assays in specific enzyme solutions. Therefore, it still remains unclear whether this hydrolysis proceeds via the hydroxymethylated states or not. In the case of FdUMP, Farquhar et al.[19] proposed that phosphodiesterase is responsible for the degradation of the monopivaloyloxymethyl ester of FdUMP. However, that proposition was not confirmed by experimentation. In the present study, pivaloyloxymethyl esters of clodronic acid were found out to be stable in a phosphodiesterase solution, which suggests that phosphodiesterase may not be responsible for their enzymatic hydrolysis. If phosphodiesterase would be responsible for the degradation of pivaloyloxymethyl esters of clodronic acid in rabbit liver homogenate, it should also attack the phosphonate esters of benzoyloxypropyl esters of clodronic acid (unless phosphodiesterase is specific to acyloxymethyl esters). Because that was not observed (see below), the

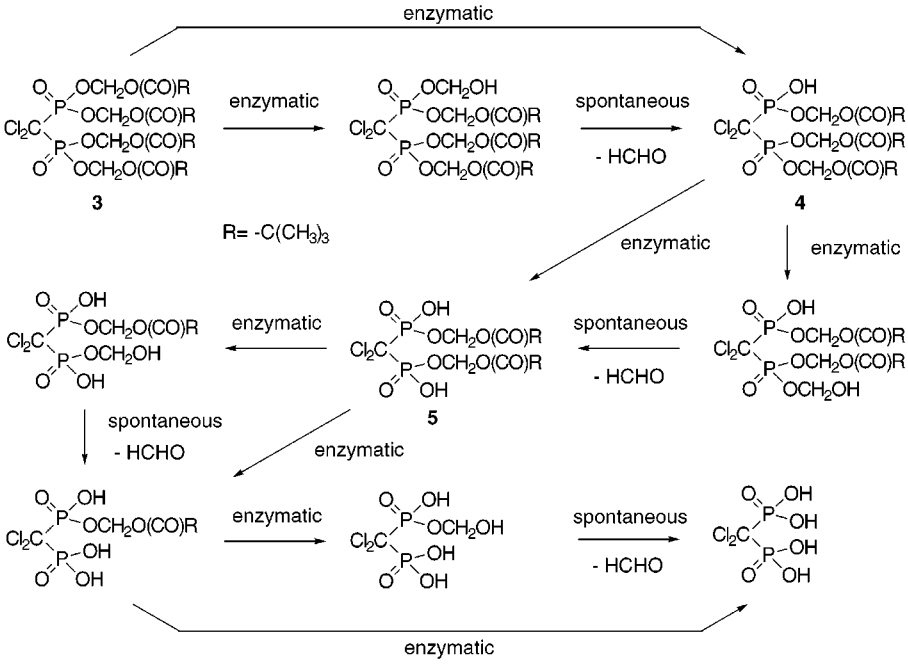


Figure 2. Suggested degradation pathway of tetrapivaloyloxymethyl ester of clodronic acid to clodronic acid. The two possible routes in each degradation step are shown.

pivaloyloxymethyl esters of clodronic acid appear to be hydrolyzed to clodronic acid via hydroxymethylated intermediates by esterases abundant in the rabbit liver homogenate.

Benzoyloxypropyl Esters of Clodronic Acid. The apparent partition coefficients (log P_{app}) of compounds **8**, **9**, and **10** were determined to be 6.9, 2.1, and −0.9, respectively. These values are in accordance to the lipophilicities determined for pivaloyloxymethyl esters **3**, **4**, and **5**. Compounds **9** and **10** were stable against enzymatic hydrolysis in serum. In rabbit liver homogenate, half-lives of triester **9** and diester **10** were 12 min and 17 h, respectively, but no clodronate was detected. It was confirmed by HPLC-MS that one carboxylate ester of **10** was degraded, revealing the hydroxypropyl group, but the degradation did not go any further during the hydrolysis assay (24 h). The benzoyloxypropyl esters synthesized and evaluated in the present study are not bioreversible prodrugs because the hydroxypropyl phosphonate esters formed in the hydrolysis process are too stable to undergo further enzymatic or chemical conversion to clodronic acid.

Conclusions

The lipophilicity of clodronate can be significantly increased by masking the phosphonic OH groups by acyloxyalkyl groups. Pivaloyloxymethyl esters of clodronic acid hydrolyzed rapidly to clodronic acid in quantitative amounts in rabbit liver homogenate. Benzoyloxypropyl esters of clodronic acid did not release clodronic acid due to the enzymatic and chemical stability of the formed 3-hydroxypropyl phosphonate esters and are therefore not likely to be prodrugs of clodronic acid.

Tripivaloyloxymethyl ester of clodronic acid has the potential to improve the oral bioavailability of clodronate because it is more lipophilic than clodronic acid, has adequate water solubility, and it releases clodronic acid via enzymatic hydrolysis.

Experimental Section

Chemistry. All solvents and reagents were high-purity reagent-grade materials and used without further purification. ^{1}H, ^{31}P, and ^{13}C NMR spectra were recorded on a Bruker AM 400 WB spectrometer operating at 400 MHz, 162 MHz, and 100.6 MHz, respectively, using TMS or TSP (for D_2O solutions) as a reference for ^{1}H and ^{13}C, and 85% H_3PO_4 as an external standard for ^{31}P. TLC analyses were run on silica gel 60 F_{254} plates (Merck), and column chromatography was performed with silica gel 60 (Merck) (0.063−0.200 mm).

Tetra(pivaloyloxymethyl) Methylenebisphosphonate (2). Compound **2** was prepared by a known method from tetramethyl methylenebisphosphonate and pivaloyloxymethyl chloride (POM-Cl):[21] ^{1}H NMR ($CDCl_3$) 5.73 (8H, m), 2.72 t (2H, $^2J_{HP}$ = 21.7 Hz), 1.24 (36H, bs); ^{31}P NMR 18.74.

Tetra(pivaloyloxymethyl) (Dichloromethylene)bisphosphonate (3). Aqueous NaOCl (10%, 110 mL) in saturated $NaHCO_3$ solution (40 mL) was added at 20 °C with efficient stirring to a mixture of **2** (5.7 g, 9.0 mmol) and chloroform (200 mL). Stirring was continued for 30 min, and 250 mL of ice-cold water was added. Organic phase was separated, washed with water (50 mL), dried ($MgSO_4$), and evaporated to dryness. The residue was suspended to hexane (20 mL) at 40 °C for two times, and solids were collected and dried to a constant weight to give **3** (3.85 g, 61%) as yellow crystals: ^{1}H NMR ($CDCl_3$) 5.82 (8H, m), 1.25 (36H, s); ^{31}P NMR 6.75. Anal. ($C_{25}H_{44}Cl_2O_{14}P_2$) C, H, N.

Tri(pivaloyloxymethyl) (Dichloromethylene)bisphosphonate *N,N,N*-Triethyl-*N*-(pivaloyloxymethyl) Ammonium Salt (4). A mixture of **3** (300 mg, 0.43 mmol) and triethylamine (47.5 mg, 0.47 mmol) in acetonitrile (4 mL) was refluxed for 2 h. The residue was evaporated to a constant weight to give **4** as a brown oil with quantitative yield: ^{1}H NMR ($CDCl_3$) 5.72 (4H, m), 5.60 (2H, m), 5.36 (2H, s), 3.38 (6H, q, $^3J_{HH}$ = 7.3 Hz), 1.17 (9H, t), 1.07 (9H, s), 1.05 (27H, bs); ^{31}P NMR 11.34 d ($^2J_{PP}$ = 21.4 Hz), 2.15 d. Anal. ($C_{31}H_{59}Cl_2NO_{14}P_2$) C, H, N.

***P,P′*-Di(pivaloyloxymethyl) (Dichloromethylene)bisphosphonate Dipiperidinium Salt (5).** A mixture of **3** (200 mg, 0.28 mmol) and piperidine (200 mg, 2.3 mmol) in acetonitrile (4 mL) was refluxed for 40 min and evaporated to dryness. The residue was suspended to dry ether (3 mL), and solids were collected and dried to a constant weight to give **5** as white crystals with quantitative yield: ^{1}H NMR ($CDCl_3$)

5.72 (4H, m), 3.14 (8H, m), 1.89 (8H, m), 1.67 (4H, m), 1.22 (18H, s); ^{31}P NMR 5.53 s. Anal. as a potassium salt ($C_{13}H_{22}Cl_2K_2O_{10}P_2 \cdot H_2O$) C, H, N.

2-Phenyl-1,4-dioxolane. The methods have been reported in ref 24: ^{1}H NMR ($CDCl_3$) 1.43−1.55 and 2.24 (2m, 2 H, $OCH_2CH_2CH_2O$), 4.00 and 4.25−4.30 (2m, 4 H, OCH_2), 5.51 (s, 1 H, ArCH), 7.26−7.50 (m, 5 H, aromatic).

3-Hydroxypropyl Benzoate. Prepared by a reported procedure[23] with the following modifications: 5 times more vanadyl acetate was used and the mixture was refluxed.

Tetrakis[3-(phenylmethanoyloxy)propyl] Methylenebisphosphonate (7). Compound **6** (0.83 g, 3.32 mmol) was dissolved in dry toluene (10 mL) and chilled to 0 °C in an ice−water mixture. A mixture of 3-hydroxypropyl benzoate (2.50 g, 13.9 mmol) and dry pyridine (1.05 g, 13.2 mmol) in dry toluene (10 mL) was added dropwise. After the addition was complete, stirring was continued for an additional 45 min at 0 °C and then overnight at room temperature. The reaction mixture was filtered, and the filtercake was washed with toluene. The filtrate was washed with 0.2 N sodium hydroxide (2 × 20 mL), then dried (anhydrous Na_2SO_4) and concentrated in vacuo. The residue was chromatographed with ethyl acetate−methylene chloride (1:1) and ethyl acetate−methanol (9:1). The ethyl acetate−methanol fractions containing **7** were evaporated to dryness to afford 1.63 g of product (60%): ^{1}H NMR ($CDCl_3$) 2.13 (qv, J = 6.2 Hz, 8 H, $CH_2CH_2CH_2$), 2.50 (t, J = 21 Hz, 2 H, PCH_2P), 4.29 (m, 8 H, $POCH_2$), 4.41 (t, J = 6.2 Hz, 8 H, CH_2OCO), 7.41 (m, 8 H, *m*-aromatic), 7.53 (m, 4 H, *p*-aromatic), 7.99−8.02 (m, 8 H, *o*-aromatic); ^{13}C NMR 25.4 t ($^1J_{CP}$ = 140 Hz), 30.1 t ($^3J_{CP}$ = 3.3 Hz), 61.2 s, 63.6 t ($^2J_{CP}$ = 2.8 Hz), 128.6 s, 129.8 s, 130.2 s, 133.3 s, 166.6 s; ^{31}P NMR 20.5.

Tetrakis[3-(phenylmethanoyloxy)propyl] (Dichloromethylene) bisphosphonate (8). Compound **8** was prepared from **7** using a known method[29] with the following modification: benzyltriethylammonium chloride was used as a phase-transfer catalyst, affording **8** (90%): ^{1}H NMR ($CDCl_3$) 2.19 (m, 8 H, $CH_2CH_2CH_2$), 4.42−4.52 (m, 16 H, $CH_2CH_2CH_2$), 7.41 (m, 8 H, *m*-aromatic), 7.54 (m, 4 H, *p*-aromatic), 8.01 (m, 8 H, *o*-aromatic); ^{13}C NMR 29.99 t (δ^3J_{CP} = 6.6 Hz), 60.94 t, 67.10 t (δ^2J_{CP} = 5.6 Hz), 71.61 t ($^1J_{CP}$ = 154.1 Hz), 128.54 d, 129.73 d, 130.11 s, 133.20 d, 166.36 s; ^{31}P NMR 9.0. Anal. ($C_{41}H_{44}Cl_2O_{14}P_2 \cdot H_2O$) C, H, N.

Tris[3-(phenylmethanoyloxy)propyl] (Dichloromethylene)bisphosphonate Piperidinium Salt (9). Compound **8** (100 mg) was suspended to piperidine (800 μL). The mixture was allowed to react 3 h at room temperature. The excess of piperidine was evaporated in vacuo, and the residue was suspended to a small volume of ether (0.5 mL). The product did not crystallize well, and the solvents were evaporated in vacuo to afford a brown oil of 85% purity: ^{1}H NMR ($CDCl_3$) 1.54 (m, 2H, $N[(CH_2)_2]_2CH_2$), 1.73 (m, 4H, NCH_2CH_2), 2.24 (m, 6H, $OCH_2CH_2CH_2O$), 3.00 (m, 4H, NCH_2), 4.20−4.51 (12H, m, $POCH_2CH_2CH_2O$), 7.35−7.45 (m, 6H, *m*-aromatic), 7.48−7.60 (m, 3H, *p*-aromatic), 7.98−8.04 (m, 6H, *o*-aromatic); ^{31}P NMR 4.40 d, 12.42 d ($^2J_{PP}$ = 20.7 Hz). Anal. ($C_{36}H_{45}Cl_2NO_{12}P_2 \cdot$ piperidine) C, H; N: calcd, 3.11; found, 2.52.

***P,P′*-Bis[3-(phenylmethanoyloxy)propyl] (Dichloromethylene)bisphosphonate Dipiperidinium Salt (10).** Compound **8** (600 mg) was suspended to piperidine (4.8 mL). The mixture was placed in an oil bath at 120 °C for 30 min and then evaporated to dryness in vacuo to yield yellowish crystals. The product was recrystallized from ether affording 200 mg of **10** (40%): ^{1}H NMR ($CDCl_3$) 1.53 (m, 4H, $N[(CH_2)_2]_2CH_2$), 1.73 (m, 8H, NCH_2CH_2), 2.22 (m, 4H, $OCH_2CH_2CH_2O$), 3.02 (m, 8H, NCH_2), 4.21 (m, 4H, $POCH_2$), 4.40 (m, 4H, CH_2OCO), 7.41 (m, 4H, *m*-aromatic), 7.52 (m, 2H, *p*-aromatic), 8.00 (m, 4H, *o*-aromatic); ^{13}C NMR 24.38 t, 25.00 t, 32.02 t (δ^3J_{CP} = 6.0 Hz), 47.41 t, 61.03 t, 67.05 t (δ^2J_{CP} = 5.5 Hz), 79.84 t ($^1J_{CP}$ = 136.3 Hz), 128.74 d, 129.66 d, 130.00 s, 133.05 d, 166.50 s; ^{31}P NMR 5.98. Anal. ($C_{31}H_{46}Cl_2N_2O_{10}P_2$) H, N; C: calcd, 50.35; found, 52.03.

HPLC Measurements. HPLC determinations were performed with a Merck LaChrom HPLC system consisting of Model L-7250 programmable autosampler, Model L-7100 HPLC pump, Model D-7000 interface module, Model L-7400 UV-detector, and Model D-7000 HPLC system manager (Hitachi Ltd., Tokyo, Japan) and a Sedex 55 evaporative light scattering detector (Sedere, Vitry-Sur-Seine, France).

The determination of clodronate (limit of detection 37.5 μg/mL) and compounds **4**, **5**, and **10** was carried out by the method described previously.[10] The determination of compounds **8** and **9** was carried out by using a UV detector at wavelength 200 nm. The mobile phase consisted of 20 mM phosphate buffer (pH 5.8) and 80% acetonitrile. During the elution, the organic concentration was increased linearly from 50% to 90% over 6.5 min. The analytical column was a Kromasil 100 RP-C8 (150 × 4.6 i.d., 5 μm) (Higgins Analytical Inc., Mountain View, CA).

Hydrolysis in Human Serum. An appropriate amount of compound was dissolved in one volume (e.g., 1 mL) of phosphate buffer (50 mM, μ = 0.15, pH 7.4) at 37 °C. Four volumes (e.g., 4 mL) of preheated human serum were added, and the solutions were kept in a water bath at 37 °C (initial concentrations were 1.4−1.7 mM). At suitable intervals, 300 μL samples were withdrawn and "deproteinized" with 300 μL of methanol. After mixing and centrifugation, 400 μL of the supernatant was evaporated to dryness under a stream of air. The residue was redissolved in 400 μL of the mobile phase buffer and analyzed by HPLC. If the analyte was relatively labile, the supernatant was injected into the HPLC as such.

Hydrolysis in 10% Rabbit Liver Homogenate. The rabbit liver was homogenized with approximately four equivalent volumes of isotonic phosphate buffer (pH 7.4) using a X-1020 homogenizer (Ystral, Germany). The homogenate was centrifuged for 90 min at 9000*g* at 4 °C with a Biofuge 28 RS-centrifuge (Heraeus Instruments, Germany). The supernatant was stored at −80 °C until used. An appropriate amount of compound was dissolved in one volume of isotonic phosphate buffer (pH 7.4), followed by addition of one volume of preheated 20% liver homogenate (initial concentrations were 1.6−1.8 mM). The solution was then incubated at 37 °C. At appropriate intervals, samples were withdrawn and analyzed by HPLC. Sample pretreatment was done by the method described in the serum hydrolysis studies.

Hydrolysis Studies in the Presence of Specific Enzymes. Porcine liver carboxyl esterase (EC 3.1.1.1) and bovine intestinal mucosa phosphodiesterase I (EC 3.1.4.1) were obtained from Sigma Chemical Co. (St. Louis, MO) and used as received. The specific activity of the carboxyl esterase solution was 2.9 IU/μL, and the phosphodiesterase I solution was 0.014 IU/μL. Substrate (2.6 μmol) was added to the following enzyme solutions maintained at 37 °C in a shaking water bath: (a) carboxyl esterase (26 IU) in 50 mM phosphate buffer, pH 7.4 (1.5 mL), and (b) phosphodiesterase I (0.13 IU) in 0.1 M Tris-HCl buffer−0.01 mM $MgCl_2$, pH 8.0 (1.5 mL). At intervals of 0.08, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, and 24 h, samples were withdrawn and one volume of cold methanol was added. The clear supernatant was analyzed by HPLC.

Chemical Hydrolysis in Phosphate Buffer. An appropriate amount of the compound (initial concentrations were 0.9−1.8 mM) was dissolved in preheated phosphate buffer (50 mM, μ = 0.15, pH 7.4 or pH 5.0). The solution was placed in a thermostated water bath at 37 °C, and at suitable intervals, samples were withdrawn. The samples were then analyzed by HPLC.

Partition Coefficient. The apparent partition coefficients (log P_{app}) for compounds **4**, **5**, **9**, and **10** were evaluated for their distribution between 1-octanol and phosphate buffer (0.16 M, pH 7.4, μ = 0.5) using the so-called shake-flask technique.[30] Determinations were carried out in triplicate for each compound. Log P_{app} values of the lipophilic derivatives of clodronate (tetraesters) were estimated from the capacity factors (k') of reversed-phase liquid chromatography.[31] A Kromasil 100 RP-C8 column (see HPLC Measurements) was used as the stationary phase, and the mobile phase consisted of a mixture of 20 mM phosphate buffer (pH 6.0) and methanol (30:70, v/v). The flow rate was 1.0 mL/min. The column effluent was

monitored at 230 nm (k' value of **3** was determined by using the evaporative light-scattering detector). The calibration curve was performed with nine standard components, which were nonionizable and had log P_{app} values ranging from 1.53 to 6.25. The standard compounds and their log P_{app} values were as follows: anisole 2.11,[32] anthracene 4.54,[33] budesonide 3.55,[34] hydrocortisone 1.53,[35] levonorgestrel 3.70,[36] naphthalene 3.30,[32] nitrobenzene 1.85,[32] perylene 6.25,[33] and progesterone 3.87.[35] The log P_{app} values of the standard compounds were plotted against the measured log k' values, and a linear relationship ($r^2 = 0.988$) was found between these two parameters. The log P_{app} values of the lipophilic clodronate derivatives were determined from this linear relationship.

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