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1-NAPHTHYLMETHYLPHOSPHONIC ACID DERIVATIVES AS OSTEOCLASTIC ACID PHOSPHATASE INHIBITORS

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Abstract. Inhibition of the enzyme, osteoclastic acid phosphatase (OAP) may be a viable approach to the treatment of osteoporosis. A series of arylmethylphosphonic acids were synthesized and shown to be inhibitors of OAP. The most potent inhibitor, bis-benzoyl-1-naphthylmethylphosphonic acid (**7a**) had an IC_{50} = 1.4 μM.

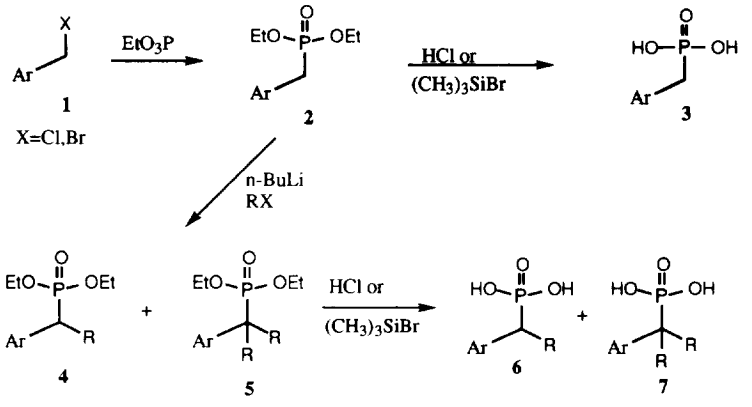
Osteoporosis is a bone wasting disease in which there is an imbalance between the rate of bone formation and resorption resulting in a decrease in total bone mass. Current therapies for osteoporosis include hormone replacement, increased dietary calcium, 1,25-dihydroxyvitamin-D_3, and calcitonin. Bisphosphonates are a promising new class of potent anti-resorptive agents but their mechanism of action remains unclear.[2,3]

The cells responsible for bone resorption, osteoclasts, are known to secrete type 5 tartrate-resistant acid phosphatase (EC 3.1.3.2, TR-ACP) which belongs to a family of iron-containing proteins with acid phosphatase activity.[4] This type 5 TR-ACP activity has long been associated with bone resorption.[5] Inhibition of this enzyme by either antibody or molybdate has been shown to abolish bone resorption.[6] Thus, osteoclastic acid phosphatase (OAP) appears to be a viable target in the treatment of osteoporosis. This paper reports the synthesis and preliminary structure-activity relationship of a novel class of potential bone resorption inhibitors, arylmethylphosphonic acids, and their ability to in vitro inhibit the activity of the type 5 TR-ACP purified from human hairy cell leukemic spleen, a member of the multigene OAP family.[5]

Chemistry

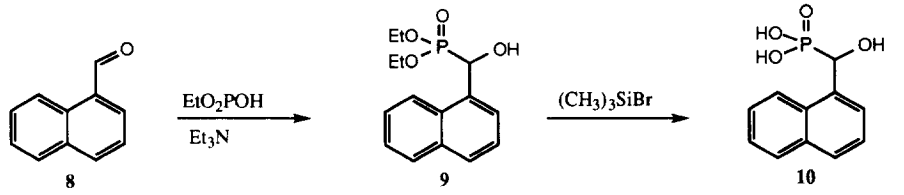
Scheme 1 shows the synthesis of target compounds **3**, **6**, and **7**. The key intermediate esters, **2**, were prepared by Arbuzov reaction of the commercially available arylmethyl halides with triethylphosphite. The target phosphonic acids, **3**, were made directly from **2** either by hydrolysis in refluxing concentrated hydrochloric acid or by treatment with bromotrimethylsilane in methylene chloride. Monobenzyl substituted esters, **4**, were synthesized by reacting the phosphonate ester, **2**, with one molar equivalent of n-butyllithium in tetrahydrofuran at -78 °C followed by addition of one molar equivalent of the appropriate benzyl chloride. While addition of one molar equivalent of 2-naphthoyl chloride to this reaction gave the mono 2-naphthoyl product, addition of one equivalent of benzoyl chloride gave only the bis-substituted ester as product. Synthesis of the bis 4-chlorobenzyl ester involved reacting the mono substituted ester, **4**, with n-butyllithium and one equivalent of 4-chlorobenzyl chloride. Reacting the 1-naphthyl phosphonate, **2**, directly with two equivalents of butyllithium and benzyl chloride gave predominantly the mono substituted ester, **4**,with only trace amounts of **5**. Hydrolysis of the ethyl esters was accomplished in the fashion described in the synthesis of **3** to give target compounds, **6** and **7**.

Scheme 1



Reaction of 1-naphthaldehyde with diethylphosphite in the presence of triethylamine gave 9 or O,O-diethyl-1-naphthylhydroxymethylphosphonate which gave **10** after treatment with bromotrimethylsilane. (Scheme 2)

Scheme 2

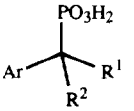


RESULTS AND DISCUSSION

Initially, identification of a protoype inhibitor was approached through screening a number of phosphonates as substrate mimetics. The activity of the present series of arymethylphosphonic acids are presented in Table 1. Significant inhibition was observed with 1-naphthylmethylphosphonic acid, **3b**. Both 2-naphthyl and phenyl analogs exhibited little or no inhibition of OAP. Modest increases in potency were made by mono substitution at the α-carbon by large lipophilic substituents such as benzyl, substituted benzyl or cyclohexylmethyl. On the other hand, α-hydroxy group substitution abolished activity. Interestingly, the highly bulky 2-Naphthoyl, **6k** and the bis-benzoyl substituted **7a**, were the most potent inhibitors of this series suggesting an extremely large hydrophobic or sterically tolerant region near the phosphate binding site of the enzyme as well as a possible preference for the acyl (versus the benzyl) derivatives. Additional studies concerning the nature and size of α-substitution and aryl substituent effects should further elucidate the inhibitor requirements of OAP.

Table 2 shows that **6j**, Clodronate and vanadate all inhibited bone resorption at concentrations that did not adversely affect the viability of the cells as measured by thymidine incorporation. Concerns related to the phosphatase-specific inhibition of the present simple phosphonate analogues are also being explored.

Table 1 INHIBITION OF OSTEOCLASTIC ACID PHOSPHATASE



Compd	Ar	R1,R2	OAP %inh @ 100μM[a]	IC_{50}(μM)
3a	Phenyl	H,H	0	
3b	1-Naphthyl	H,H	64	30
3c	2-Naphthyl	H,H	4	
3d	2-Methyl-1-naphthyl	H,H	20	
6a	Phenyl	Benzyl,H	15	
6b	1-Naphthyl	Benzyl,H	92	21
6c	2-Naphthyl	Benzyl,H	19	
6d	1-Naphthyl	Cyclohexylmethyl,H	73	11.4
6e	2-Methyl-1-naphthyl	Cyclohexylmethyl,H	90	11
6f	1-Naphthyl	4-Methylbenzyl,H	75	
6g	1-Naphthyl	4-Methoxybenzyl,H	73	
6h	1-Naphthyl	4-Nitrobenzyl,H	80	
6i	1-Naphthyl	4-Phenylbenzyl,H	100	6.3
6j	1-Naphthyl	4-Chlorobenzyl,H	94	21
6k	1-Naphthyl	2-Naphthoyl,H	95	6
7a	1-Naphthyl	bis-Benzoyl	96	1.4
7b	1-Naphthyl	bis-4-Chlorobenzyl	87	9.3
9	1-Naphthyl	OH,H	10	
Orthovanadate				34

[a] Compounds were initally screened in duplicate at 100 μM according to the protocol outlined in the experimental section. Compounds which demonstrated greater than 50% inhibition of the control enzyme activity were retested with a dose response determination to generate IC_{50} values. These measurements were also made in duplicate.

Table 2: INHIBITION OF BONE RESORPTION[a]

Compd	% Inh. of PTH stimulated bone resorption	Thymidine inc. % of control
Clodronate (10 μM)	95.6±2.3*	104±10
6j (100μM)	31.9±12.3	ND
6j (500 μM)	101.8±3.4*	76±7
Orthovanadate (1 μM)	31.3±7.8*	127±10
Orthovanadate (10 μM)	72.7±3.3*	94±14
Orthovanadate (100μM)	130.3+1.4*	0.4+0.01*

[a] Neonatal mouse calvaria were placed in culture and bone resorption measured in the presence of the compounds indicated as described in the experimental section. Thymidine incorporation was measured as described in the experimental section. The data represent an average of 8 determinations ± S.E.M. ND=not determined.
* $p \leq 0.01$.

EXPERIMENTAL

Melting points were determined on a Meltemp melting point apparatus and are uncorrected. Proton nmr spectra were recorded on a Bruker WP100SY spectrometer. Mass spectra were recorded on a Finnigan 8230 spectrometer in the DCI mode using isobutane as the ionizing gas. The NMR, and MS of the compounds in Tables 3 and 4 were consistent with the chemical structures. Elemental analysis, determined using a Perkin Elmer 2400 CHN elemental analyzer, were within 0.4% of theory for all of the target compounds **3**, **6**, **7** and **10**.

Human Osteoclastic Acid Phosphatase Assay.

Human osteoclastic-like acid phosphatase used in this study was purified from the spleen of a patient with hairy cell leukemia according to the procedure of Stepan *et al.*.[7] The enzyme preparation was purified up to the Superose 12 FPLC column chromatography step and contained a mixture of both TR-ACP 5a and 5b isoenzymes. Previous biochemical characterization showed that both isoforms of the hairy cell leukemia spleen TR-ACP have similar kinetic properties[4] and are very similar to the OAP[4] purified from human osteoclastomas.[8] Therefore, hairy cell leukemic spleen TR-ACPs are members of the OAP multigene family. The enzyme was stored at -80 °C in sodium acetate buffer (50mM, pH 5.0) prior to use.

The assay for OAP activity measures its ability to dephosphorylate 4-methylumbelliferyl phosphate to 4-methylumbelliferone. The assay was performed as follows. Test compounds were dissolved in ethanol, DMSO, or buffer. Each compound (5 µL) was added to 150 µL of enzyme. The reaction was initiated by addition of 50 µL of substrate (4-methylumbelliferyl phosphate) making the final substrate concentration equal to 25 µM. The reaction was performed at ambient temperature for 15 minutes and was terminated by addition of 50 µL of 4N NaOH. 4-Methylumbelliferone was measured by fluorescence using a Dynatech microfluorescence detector. The potency of the test compounds were calculated as the percent inhibition of control enzyme activity. The IC_{50} values were determined in duplicate using several inhibitor concentrations.

Mouse Calvaria Bone Resorption and Thymidine Incorporation Assays

The method used was similar to one reported by Linkhart et al .[9] Newborn mice (up to 5 days old) were injected subcutaneously over the skull with 2 uCi of ^{45} Ca in a 2 µL volume. After 4 days the mice were sacrificed using CO_2 asphyxiation and the calvaria were harvested aseptically. The calvaria were dissected free of sutures and separated into two halves. Half calvaria were placed, one per ml, in Dulbecco's Modified Eagles Medium containing 0.1% Bovine Serum Albumin, 0.01% ascorbic acid, with 100 units/ml penicillin G-sodium and 100 ug/ml streptomycin for 24 hours at 37 °C in a 95% air/5% CO_2 mixture. Medium was then replaced and calvaria were incubated for an additional hour. Calvaria were placed (one half per 2 ml) in the experimental media. To induce bone resorption, some cultures were incubated in the presence of PTH. Media were replaced on days one and three and calvaria were harvested on day six. Calvaria were rinsed with HBSS. Formic acid (0.5 ml of 96% solution) was then added and the calvaria were then incubated on a Fisher orbital shaker, model #361 at 120 rpm overnight at 40 °C to dissolve the bone matrix. Ecolume scintillation cocktail was added to each sample and the radioactivity was determined. Radioactivity in 0.5 ml aliquots of media from days one, three and six were determined by liquid scintillation spectroscopy. The total calvarial ^{45}Ca content at the beginning of the experiment was calculated as follows: the ^{45}Ca content of the dissolved calvaria plus the ^{45}Ca released during the incubation period. The amount of bone resorbed was determined to be the percent of the total ^{45}Ca released during the time period in question (days 3-6). Viability of the cultures were determined by measuring the incorporation of ^{3}H thymidine. Radiolabelled thymidine (1 µCi/ml) was added to each culture following 5 days incubation with compound. Following a 24 hour incubation, media were removed and the cultures were incubated with 2 ml of unlabelled thymidine (0.20 mM) in Hanks balanced salt soultion for 15 minutes. This solution was removed and the DNA was precipitated with 10% TCA. The calvaria were then dissolved in formic acid as above and the radioactivity measured by liquid scintillation spectroscopy.

General Method A. The preparation of arymethylphosphonate esters, **2**.

The commercially available arylmethyl halides were heated to 130 °C in excess triethylphosphite for 3- 20 h (monitored by tlc). The excess phosphite was removed by distillation and the crude products obtained were purified if necessary with silica gel flash chromatography using ethyl acetate-hexane 1:1 as the eluent. Yields and molecular ions of the resulting oils are shown in Table 3.

General Method B for the synthesis of α-substituted esters, **4** and **5.**

The ester **2** or **4j** was dissolved in anhydrous THF and cooled to -78 °C. To this was added n-butyllithium (1 molar equivalent). After stirring for 20 minutes, one molar equivalent of the appropriate acid chloride, cyclohexylmethyl bromide or benzyl chloride was added. The dry ice-acetone bath was removed and the reaction was stirred at room temperature for a period of 2 h after which time the reaction mixture was washed with water. The organic layer was dried over sodium sulfate, evaporated *in vacuo* to an oil and purified, if necessary, on a silica gel column eluted with ethyl acetate-hexane (1:1). Yields and molecular ions are listed in Table 3.

O,O-Diethyl-1-naphthylhydroxymethylphosphonate, 9.[10]

Table 3

Compd	Ar	R1,R2	Yield (%)	MH+
2a	1-Naphthyl	H,H	80	279
2b	2-Naphthyl	H,H	73	279
2c	2-Methyl-1-Naphthyl	H,H	96	293
4a	Phenyl	Benzyl,H	75	319
4b	1-Naphthyl	Benzyl,H	80	369
4c	2-Naphthyl	Benzyl,H	53	369
4d	1-Naphthyl	Cyclohexylmethyl,H	59	375
4e	2-Methyl-1-Naphthyl	Cyclohexylmethyl,H	26	389
4f	1-Naphthyl	4-Methylbenzyl,H	27	383
4g	1-Naphthyl	4-Methoxybenzyl,H	15	399
4h	1-Naphthyl	4-Nitrobenzyl,H	3	414
4i	1-Naphthyl	4-Phenylbenzyl,H	35	445
4j	1-Naphthyl	4-Chlorobenzyl,H	91	403
4k	1-Naphthyl	2-Naphthoyl,H	37	433
5a	1-Naphthyl	bis-Benzoyl	50	487
5b	1-Naphthyl	bis-4-Chlorobenzyl	62	527

General Methods of Ester Hydrolysis to Phosphonic Acids.

Method C

The ester was hydrolyzed in refluxing concentrated hydrochloric acid for 17-20 h. Upon cooling, a solid formed which was filtered and washed with water. Recrystallization from ethanol-water afforded analytically pure products.

Method D

The ester was dissolved in methylene chloride. Bromotrimethylsilane (5.0 molar equivalents) was added and the reaction was stirred for 4-24 h. Evaporation *in vacuo* gave an oil which was crystallized from alcohol-water.

Phenylmethylphosphonic acid, 3a.

Compound **3a** was Purchased from Lancaster Synthesis Inc. and used without further purification.

1-Naphthylmethylphosphonic acid, 3b.[11]

2-Naphthylmethylphosphonic acid, 3c.[12]

Table 4

Compd	Method	Yield (%)	mp °C	MS	1H NMR
3 d	D	95	213-217	237 (MH^+)	(DMSO-d_6) δ 3.50 (d, 2H, PCH)
6 a	C	84	164-166	263 (MH^+)	(DMSO-d_6) δ 7.13 (m,10H), 3.21 (m,3H)
6 b	C	62	73-77	313 (MH^+)	(DMSO-d_6) δ 4.25 (m,1H, PCH)
6 c	D	97	190-191	313 (MH^+)	(DMSO-d_6) δ 3.43 (m, 2H), 3.23 (m,1H)
6 d	D	81	154.5-157	319 (MH^+)	(DMSO-d_6) δ 3.86 (m,1H, PCH)
6 e *	D	45	257-259	333 (MH^+)	(acetic acid-d_4) δ 4.11 (d, t, 1H, PCH)
6 f	D	32	198-203	327 (MH^+)	(TFA-d) δ 4.90-4.65 (m, 1H, PCH)
6 g	D	59	170-176	343 (MH^+)	(TFA-d) δ 4.80-4.60 (m, 1H, PCH)
6 h	D	21	197-215 (dec)	358 (MH^+)	(acetic acid-d_4) δ 4.78-4.57 (m, 1H, PCH)
6 i	D	63	196-199	389 (MH^+)	(TFA-d) δ 4.92-4.70 (m, 1H, PCH)
6 j	D	70	204-206	347 (MH^+)	(DMSO-d_6) δ 4.40-4.15 (m, 1H, PCH)
6 k	D	75	185-190	377 (MH^+)	(acetic acid-d_4) δ 6.55 (d, 1H, PCH)
7 a *	D	50	153-155	429 (M-H)	(DMSO-d_6) δ 8.40-7.25 (m, 17H)
7 b	D	83	142-145	471 (MH^+)	(TFA-d) δ 4.08 (d, 4H, CH2)
10	D	45	155-157	239 (MH^+)	(DMSO-d_6) δ 5.52 (d, 1H, PCH)

* Isolated as cyclohexylammonium salt

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1. All future correspondence should be directed to the author at his present address: LeukoSite, Inc., 215 First street, Cambridge, Ma 02142.
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