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Prostaglandin E_2-Bisphosphonate Conjugates: Potential Agents for Treatment of Osteoporosis

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Dedicated to the Memory of Professor Sir Derek H. R. Barton

Abstract—Conjugates of bisphosphonates (potential bone resorption inhibitors) and prostaglandin E_2 (a bone formation enhancer) were prepared and evaluated for their ability to bind to bone and to liberate, enzymatically, free PGE_2. The conjugate **3**, an amide at C-1 of PGE_2 proved to be too stable in vivo while conjugate **6**, a thioester, was too labile. Several PGE_2, C-15 ester-linked conjugates (**18**, **23**, **24** and **31**) were prepared and conjugate **23** was found to bind effectively to bone in vitro and in vivo and to liberate PGE_2 at an acceptable rate. A 4-week study in a rat model of osteoporosis showed that **23** was better tolerated and more effective as a bone growth stimulant than daily maximum tolerated doses of free PGE_2.

Introduction

Osteoporosis is the most common metabolic bone disease which affects 40–50% of the elderly female and 10–15% of the elderly male population.[1] The disease involves the gradual loss of bone mass as a result of an imbalance between the bone resorption activity of osteoclasts and the bone formation activity of osteoblasts.[2] A number of pharmaceutical agents have been developed to treat this disease. These compounds can be divided into two groups: bone resorption inhibitors such as bisphosphonates[3] (e.g. Fosamax®) and bone formation stimulants.[4–8] Bisphosphonates are analogues of pyrophosphates which are absorbed tightly onto hydroxyapitite surfaces and, due to this process, bisphosphonates are targetted to bone.[9] While bisphosphonates represent an important class of drugs for the treatment of osteoporosis, their value is generally manifested by prevention of bone loss and thus, for treatment of more advanced disease, there has been great interest in the discovery of safe and effective bone formation stimulants. Examples of bone-activating agents may include parathyroid hormone,[4] growth hormone,[5] fluoride,[6] possibly certain vitamin D metabolites[7] and prostaglandin E_2 (PGE_2).[8] Indeed, a number of studies have demonstrated that bone formation can be stimulated in vivo by systemic injection of PGE_2.[10] Furthermore, substantial new bone formation has been observed on the controlled release of PGE_2 from implanted PGE_2-containing polymers[11] indicating that PGE_2 acts locally in bone. Unfortunately, such implants are impractical in a normal therapeutic setting and the pharmaceutical utility of systemic PGE_2 is greatly reduced due to side effects and metabolic instability.

Conjugates of PGE_2 and bisphosphonates described in this report represent a new class of compounds which could circumvent the problems associated with PGE_2. PGE_2 (or an analogue) chemically coupled to a bisphosphonate could be effectively delivered to bone due to the property of the bisphosphonate to bind to bone. Gradual hydrolysis of the conjugate could then liberate a bone resorption inhibitor (the bisphosphonate moiety) and a bone formation enhancer (the PGE_2 moiety). To test this hypothesis, it was necessary to devise methods to couple bisphosphonates and PGE_2 in a way compatible with the chemical and biochemical instability of PGE_2 through a linkage which was suitably stable for the conjugate to survive intact during the time necessary

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for uptake into bone in vivo, and suitably labile to be subsequently released by metabolic hydrolysis. Ideally, a hydrolytic half-life of 1 to 4 days was sought. In order to monitor effectively both the uptake and release of the prostaglandin moiety in the bone, it was decided to utilize radioactive-labelled PGE_2. In early experiments, we also utilized radioactive-labelled bisphosphonate and thus double label analysis of plasma and bone samples over time could be used to follow the uptake and cleavage of the conjugate. Finally, when conjugates with appropriate properties were discovered, more complex in vivo experiments to measure their effect on bone formation were undertaken.

Results and Discussion

Synthesis and studies of conjugates linked via the C-1 carboxyl group of PGE_2[12]

Synthesis of PGE_2-alendronate conjugate 3. With the commercial availability of tritium-labelled PGE_2 (**1**) and the in-house availability of ^{14}C-labelled alendronate (**2**), we first explored the direct coupling of alendrolate with PGE_2 to provide the corresponding amide (**3**). The *N*-hydroxysuccinimide ester of PGE_2 was readily prepared using DCC as coupling agent and the active ester reacted efficiently with alendronate in dioxane–water with careful control of the pH at 8–9 in order to ensure the integrity of the PGE_2 (higher pH led to competing elimination of the 11-hydroxyl group to the corresponding enone) (Scheme 1). Purification of the conjugate was difficult but could be achieved first by evaporation to dryness and then dissolution of the mixture in water and absorption onto a C18 cartridge followed by elution first with water and then with acetonitrile–water to provide fractions that were essentially pure conjugate **3**. In this manner, quantities both of unlabelled and double-labelled conjugate **3** could be prepared.

In vitro and in vivo evaluation of conjugate 3. The conjugate **3** was studied first in vitro to determine its binding to bone powder. These experiments showed that irreversible in vitro binding of **3** in fetal bovine serum (FBS) to bone powder occurred to the extent of 77% of the ^{14}C moiety (alendronate) and 53% of the ^{3}H moiety (PGE_2) within 1 h. Thereafter, disassociation of the PGE_2 moiety could be followed during incubation with FBS by measuring the residual $^{14}C/^{3}H$ ratio. These experiments indicated that disassociation of the ^{3}H moiety occurred at the rate of approximately 5% per day at 37 °C. Conjugate **3** was then studied for its ability to be taken up in the bone in vivo and the subsequent liberation of PGE_2 as measured by ^{3}H loss from bone over time. An initial indication of the effect on bone degradation/formation was also obtained by measure of the release of lysylpyridinolenes (LP) over time. A high level of urinary LP is normally associated with the accelerated breakdown of bone collagen.[13] For the in vivo experiments, rats were dosed i.v. with a single dose of dual-labelled compound **3** and then animals were sacrificed at 24 h, 14 and 28 days after administration. The level of $^{14}C/^{3}H$ was measured after an incineration of the long bones. These studies indicated about 15% of ^{14}C and 12% of ^{3}H associated with conjugate **3** were taken up at 24 h compared with about 33% for ^{3}H-alendronate itself. At 14 and 28 days, there was no significant change in the ratio of ^{14}C to ^{3}H, indicating the stability of the conjugate in situ (Fig. 1). A statistically significant reduction of LP was observed on day 12 for animals treated with the conjugate, although not as great as was observed for the alendronate itself dosed at 1 mg/kg (see Fig. 2). The results of these experiments suggested that hydrolysis of the amide was inefficient in vivo and thus an alternative, more labile coupling methodology was sought.

Synthesis of PGE_2-thioester-alendronate conjugate 6. It was felt that a thioester would be significantly more labile and perhaps more suitable. The synthesis of such a conjugate was complicated by the instability of PGE_2 and the highly polar nature of the alendronate moiety. It was necessary to have a method to bring the two

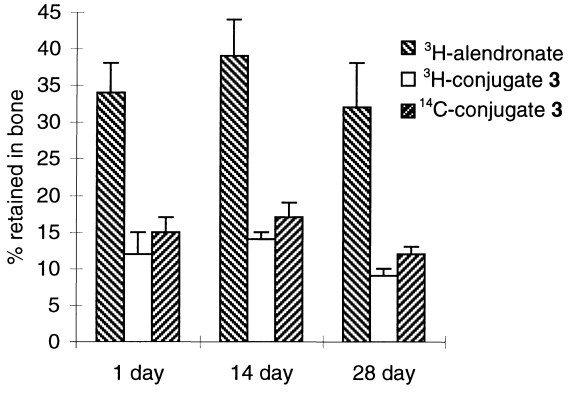
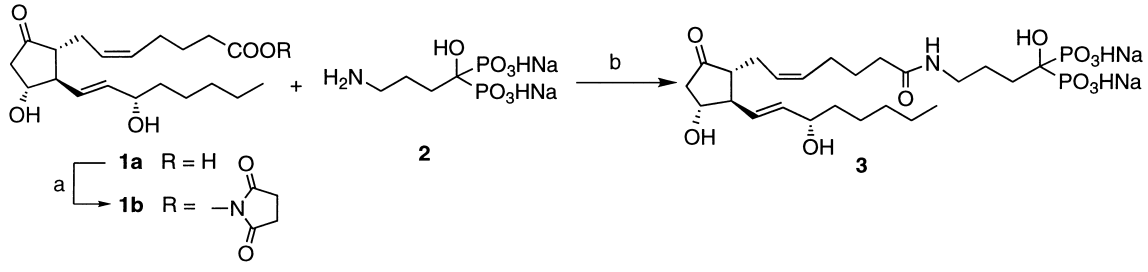


Figure 1.



Scheme 1. Reagents: (a) *N*-hydroxysuccinimide, DCC; (b) dioxane, H_2O, pH 8–9.

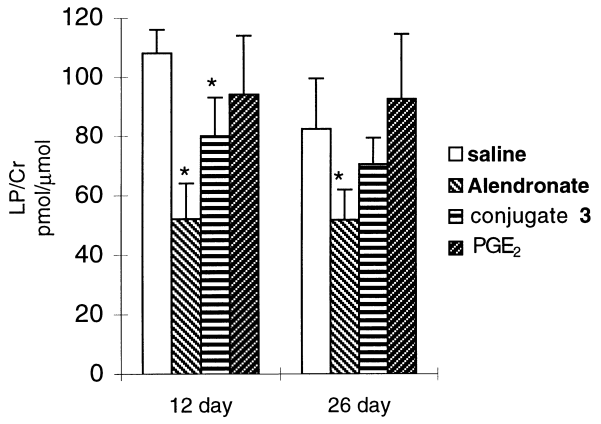


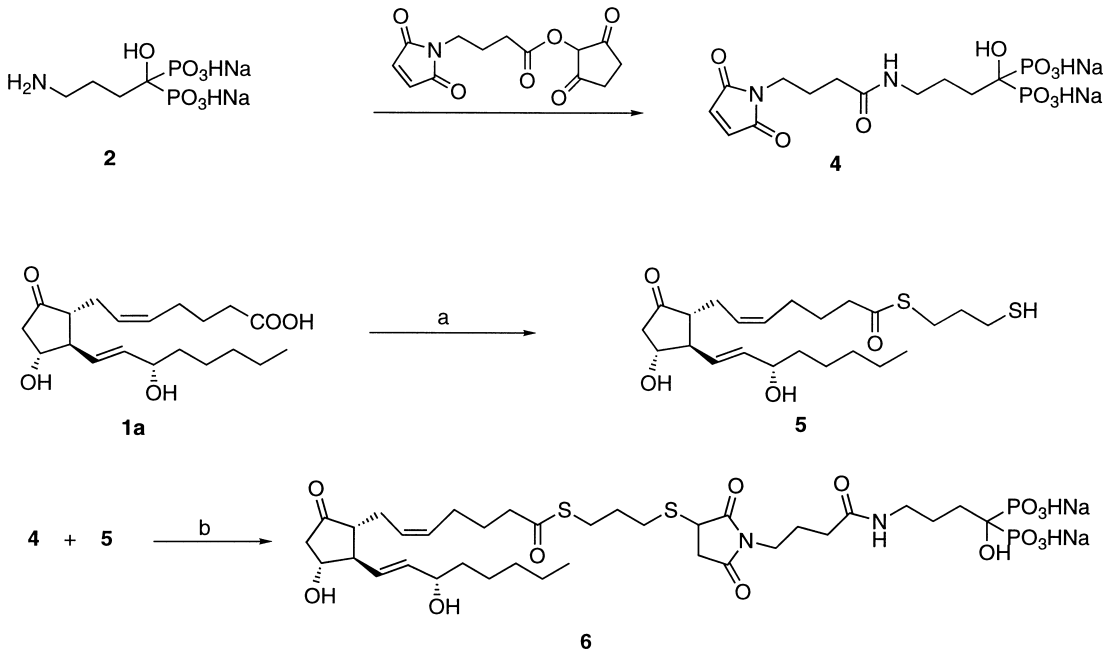
Figure 2.

components together in an aqueous environment whereby coupling would be instantaneous and essentially quantitative to facilitate purification. It was decided to use a bifunctional reactive linker, δ-maleimidobutyric acid *N*-hydroxysuccinimide ester, an agent that has been used for the conjugation of proteins.[14] This agent reacted effectively with alendronate bis-sodium salt to provide the amide **4** containing the maleimido group. PGE_2 was then transformed to its 3-mercaptopropyl-thioester **5** by reaction of PGE_2, 1,3-propanedithiol and DCC. The thioester, in methanol, reacted with **4** in methanol–water to rapidly form the conjugate **6** which could be purified using C18 cartridges (Scheme 2). Again, the corresponding dual-labelled compound incorporating ^{3}H-labelled PGE_2 and ^{14}C-labelled alendronate was prepared.

In vitro studies with conjugate 6. The dual-labelled conjugate **6** bound to bone powder effectively with about 80% of ^{3}H and ^{14}C bound within 5 min and tritium label was released at a more rapid rate of 10% after 24 h and 17% after 48 h in FBS. The ^{14}C label remained bound. This was very encouraging, suggesting **6** could be highly suitable. Unfortunately, when **6** was dosed in vivo in rats, only the ^{14}C label was taken up in the bones with 42.5% of ^{14}C label bound while only traces (0.6%) of the tritium label was taken up after 24 h. This indicated that in vivo, the thioester bond was too labile to be useful. It was therefore necessary to prepare conjugates with intermediate stability. Although the results with conjugate **3** were somewhat encouraging, there was concern that conjugates in which the 15-hydroxyl group was free might be substrates for PGE_2 dehydrogenase (an active enzyme in mammalian systems)[15] and therefore the PGE_2 moiety might be modified into an inactive 15-keto form before it reached the bone or was released. It was therefore decided to redirect our efforts to the formation of conjugates where linking of bisphosphonate was effected through the 15-hydroxyl group of PGE_2.

Synthesis and studies of conjugate bisphosphonates linked through the 15-hydroxyl group of PGE_2

Synthesis of 15-hydroxy-linked conjugates. A variety of methodologies were envisaged for achieving a linkage through the 15-hydroxyl group. All of these approaches were complicated by the need to derivatize selectively the 15-hydroxyl group and not the 11-hydroxyl group of PGE_2 and to bring the two moieties together under conditions where PGE_2 is stable and where the bisphosphonate component would have appropriate solubility and reactivity. We also wished to prepare conjugates where the coupling linkage was the only bond vulnerable to hydrolysis so that the liberation of tritium from the bone or in plasma could be unambiguously assigned



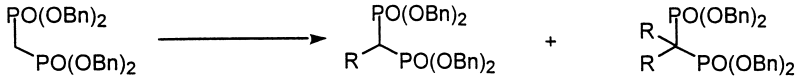
Scheme 2. Reagents: (a) $HS(CH_2)_3SH$, DCC; (b) MeOH, H_2O.

to liberation of PGE_2 itself. The formation of esters appeared to be the best option but initially it was apparent that if PGE_2 were to be directly acylated by a bisphosphonate containing an acylating functionality, then the bisphosphonate moiety would have to be suitably protected and then the conjugate subsequently deprotected to yield the free bisphosphonate. Previous studies on the preparation of steroid and methotrexate bisphosphonate conjugates[16,17] have utilized bisphosphonates protected as linear or branched alkyl (methyl, ethyl, isopropyl) esters. The deprotection of such esters following conjugation was carried out in both cases by treatment with bromotrimethylsilane at room temperature for 2 to 3 days. We confirmed that PGE_2 was unstable under these conditions. We chose instead to prepare benzyl-protected bisphosphonates and considered that the alkylation of tetrabenzyl methylenediphosphonate (**7a**) was an appropriate route for synthesis of the required reagents.

We were unable to find reports on the alkylation of tetrabenzylmethylene bisphosphonate and thus we undertook a study on the alkylation of **7a** with electrophiles. A variety of bases, solvents and electrophiles were studied and yields were poor to moderate, often leading to a mixture of mono- and dialkylated products (**8** and **9**) (Table 1). However, reactive electrophiles such as benzylbromide and 4-carbomethoxy- or 4-*t*-butyloxycarbonylphenylmethylbromide proceeded relatively efficiently at room temperature in about 30 min. Similarly, bromomethylacetate and alkyliodides such as methyliodide could be reacted efficiently using the anion prepared from **7a** and sodium hydride in DMF.

With the reagents in hand, we turned our attention to the selective acylation of the C-15 hydroxy group of PGE_2 (as its *t*-butyldiphenylsilyl (TBDPS) ester (**12**)). Model studies utilizing acid chlorides such as **11** in pyridine indicated that it was possible to acylate selectively the C-15 hydroxy group of **12** (see Scheme 3). However, as had been predicted from our previous stability studies (*vide supra*), all attempts to deprotect the resulting conjugate **13** lead to decomposition. Attempts to hydrolyse the *t*-butyldiphenylsilyl ester in

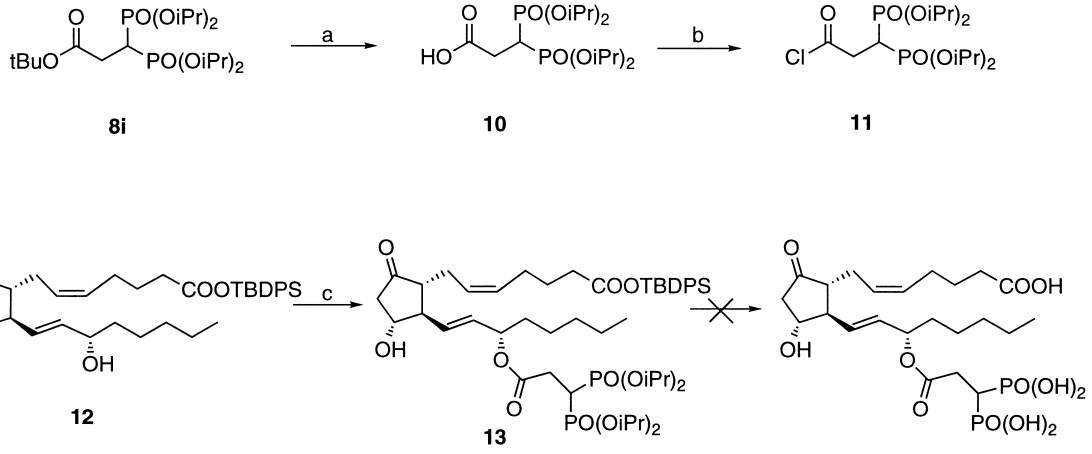
Table 1. Alkylation of tetrabenzyl methylenediphosphonate (**7a**)



7a **8** **9**

RX	Base	Solvent	Time (T°)	Products	Yield **8** (%)	Yield **9** (%)
$Br(CH_2)_4CO_2Et$ 4 eq	NaH	DMF	4 h (35 °C)	R = $(CH_2)_4CO_2Et$ (**8a**)/(**9a**)	5–10	0
$Br(CH_2)_4CO_2Et$ 5 eq	TiOEt	DMF	45 min (35 °C)	R = $(CH_2)_4CO_2Et$ (**8a**)/(**9a**)	0	0
$Br(CH_2)_4CO_2Et$ 4 eq	Cs_2CO_3	DMF	8 h (35 °C)	R = $(CH_2)_4CO_2Et$ (**8a**)/(**9a**)	0	0
$THPO(CH_2)_2I$ 3 eq	NaH	DMF	30 min (80 °C)	R = CH_2CH_2OTHP (**8b**)	5	0
$BrCH_2Ph$ 1.5 eq	NaH	DMF	30 min (23 °C)	R = CH_2Ph (**8c**)/(**9c**)	56	12
p-$CH_2(C_6H_4)CO_2Me$ 1.5 eq	NaH	DMF	30 min (23 °C)	R = $CH_2(C_6H_4)CO_2Me$ (**8d**)/(**9d**)	50	14
p-$CH_2(C_6H_4)CH_2CO_2Me$ 1.5 eq	NaH	DMF	30 min (23 °C)	R = $CH_2(C_6H_4)CH_2CO_2Me$ (**8e**)	10	0
$BrCH_2CO_2Me$ 1.1 eq	NaH	THF	30 min (23 °C)	R = CH_2CO_2Me (**8f**)	53	0
$BrCH_2CO_2tBu$ 1.1 eq	NaH	THF	30 min (23 °C)	R = CH_2CO_2tBu (**8g**)	77	0
CH_3I 5 eq	NaH	DMF	45 min (23 °C)	R = Me (**8h**)/(**9h**)	52	9
$BrCH_2CO_2tBu$[a] 1.1 eq	NaH	THF	45 min (23 °C)	R = CH_2CO_2tBu	62	0

[a]Tetraisopropyl methylenediphosphonate (**7b**) was used in this reaction.

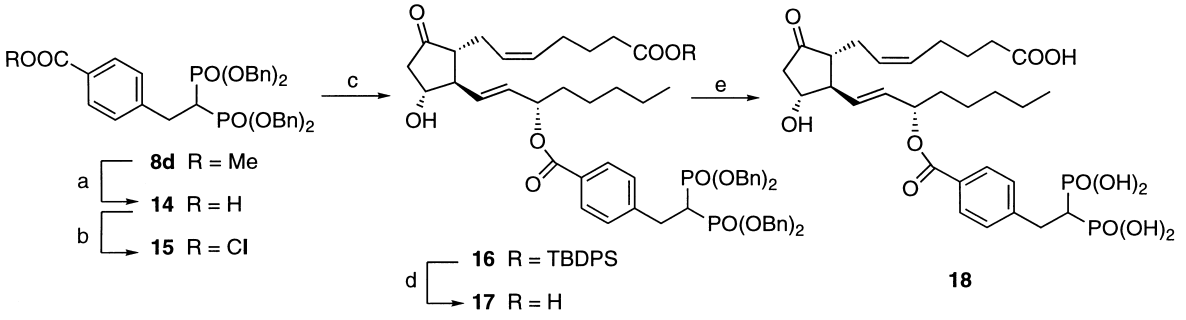


Scheme 3. Reagents: (a) TFA; (b) $SOCl_2$; (c) **11**, pyr.

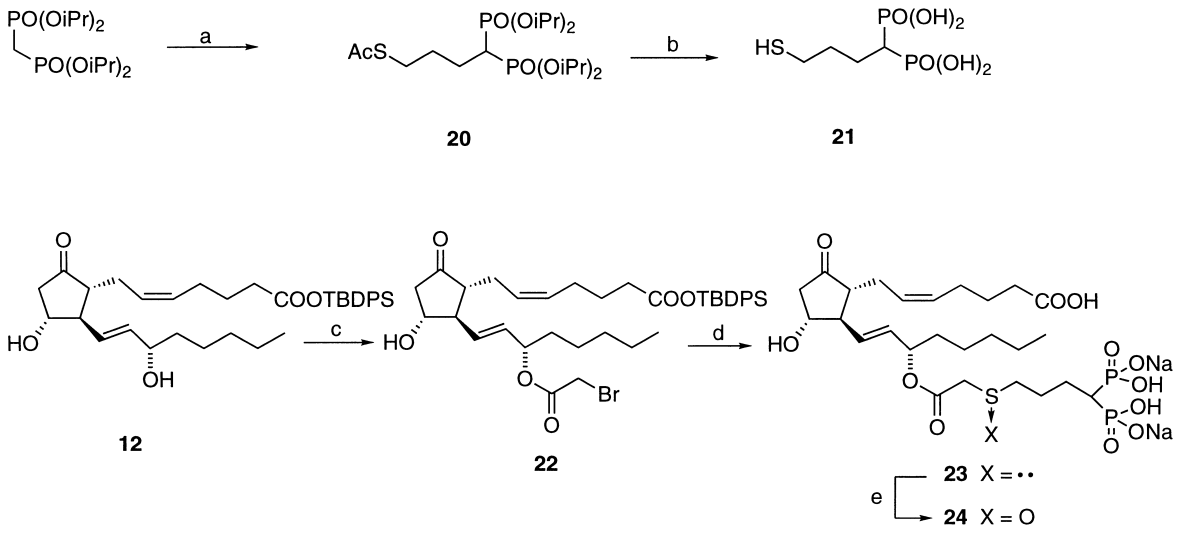
the corresponding tetrabenzyl bisphosphonate (**8g**) by treatment with trifluoracetic acid (TFA) lead to partial debenzylation, suggesting that this reagent was too unstable to be useful.

We next directed our efforts to the study of functionalization of bisphosphonate **8d**. The desired acid chloride was prepared in good yield by hydrolysis of **8d** with lithium hydroxide to give the corresponding acid (**14**) (70% yield) and formation of the acid chloride (**15**) utilizing freshly distilled oxalyl chloride in DMF. The crude acid chloride **15** was used directly for acylation of PGE_2-TBDPS in pyridine at −20 °C to 0 °C to provide the desired C-15 acylated adduct **16** in 42% yield accompanied with 31% recovered PGE_2-TBDPS. Hydrolysis of the silyl ester proceeded smoothly to **17** (94% yield) but the final debenzylation proved to be very difficult and capricious. At first, model reactions were used to evaluate conditions using an equimolar mixture of tetrabenzyl methylene diphosphonate (**7a**) and PGE_2. Acidic conditions such as HBr (48%), DMF, water or nucleophilic debenzylation with lithium iodide in DMF resulted in decomposition of PGE_2 before complete debenzylation of the phosphonate ester. More mild conditions such as HBr (4 equiv) in DMF–water were extremely slow and unusable. A variety of hydrogenations were evaluated including Lindlar conditions; Pd/$BaSO_4$, $Pd(OH)_2$ in EtOAc or ethanol. All gave partial or total reduction of PGE_2 in parallel with the debenzylation. Conditions described by Noyori[18] for the reduction of acetylenes in the synthesis of PGE_2 led to no debenzylation. Gratifyingly however, transfer-hydrogenation with 1,4-cyclohexadiene and Pd^0 was remarkably selective.[19] For example, treatment of a 1/1 mixture of PGE_2 and bisphosphonate **8d** with Pd^0 (10% on charcoal, 1 equivalent w/w of **8d**) and 1,4-cyclohexadiene (10 equivalents) in ethanol at room temperature for 12 h resulted in the recovery of 91% of PGE_2 and 95% of the diphosphonic acid. *This remarkable selectivity finds no precedent and may be specific to bisphosphonate benzyl esters*. Application of these conditions to conjugate **16** apparently gave the desired product (**18**) in low to moderate yield (20–44%). Unfortunately, careful analysis of this product indicated that considerable isomerization of the double bonds in the PGE_2 moiety had taken place, presumably under the mediation of the paladium catalyst. Careful optimization of the conditions, however, utilizing 10% paladium on charcoal (15 mol%) in ethanol/ethyl acetate at 20 °C followed by the addition of 1,4-cyclohexadiene (40 equiv) gave material which appeared to be about 90% pure by NMR and could be purified using either C18 cartridge or C18 HPLC. With the synthesis of this conjugate in hand, a radioactive sample derived from ^3H-PGE_2 was also prepared (Scheme 4).

While this synthesis was successful, subsequent efforts to scale up the synthesis of **18** were still capricious and this led us to evaluate an alternative conjugation method wherein PGE_2 could be coupled with a bisphosphonate in its unprotected (free acid) form as the final step and thus avoiding deprotection. We considered that a thiol-containing bisphosphonate should react efficiently and selectively with a PGE_2 derivative containing the highly reactive 15-bromoacetyl group. The thiol-containing bisphosphonate (**21**) was prepared by alkylation of the anion derived from tetraisopropyl methylenediphosphonate (NaH, DMF) with 3-acetylthiopropyliodide (**19**) to provide the thioacetate (**20**) in 90% yield. Hydrolysis of the thioester and the isopropyl phosphonate esters proceeded concomitantly by refluxing in HCl followed by concentration to dryness under high vacuum. The resulting thiol **21** was used directly without further purification. Selective esterification of PGE_2-TBDPS (**12**) with bromoacetylbromide in THF/pyridine at −25 °C provided the mono-acylated bromoacetate (**22**) in 49% yield. The reaction of the bromide with a slight excess of the thiol **21** and triethylamine in dioxane/water led to the efficient coupling and concomitant desilylation of the PGE_2 ester. Purification using C18 reverse-phase silica gel followed by cation exchange (Dowex 50 Na^+ form) gave the product conjugate (**23**) in 66% yield. The conjugate could be further oxidized using 32% peroxyacetic acid followed by dimethyl sulfide workup to provide the corresponding sulfoxide (**24**) in quantitative yield (Scheme 5). Subsequent studies on conjugate **23** indicated it had properties appropriate for optimal in vivo activity (*vide infra*). It was necessary to prepare an authentic sample of the bisphosphonate carboxylic acid (**26**) that would be liberated on hydrolysis of the conjugate for determination of its intrinsic in vivo activity. This was achieved by alkylation of **21** with the hexyl ester of bromoacetic acid to provide the corresponding ester **25** which could be separated from an unreacted bisphosphophosphonate thiol and other bisphosphonate derived by-products by C18 chromatography. The product, obtained in 62% yield, was then hydrolyzed by



Scheme 4. Reagents: (a) LiOH; (b) $(COCl)_2$, DMF; (c) **12**, pyr, −20°C to 0°C; (d)HCl, THF; (e) Pd°/C, cyclohexadiene, 20°C.



Scheme 5. Reagents: (a) NaH, DMF, $AcS(CH_2)_3I$ (**19**); (b) HCl, reflux; (c) $BrCH_2COBr$, THF, pyr, −25°C; (d) 1) **21**, Et_3N, dioxane, water. 2) DOWEX (Na^+); (e) CH_3COOOH.

HCl/reflux to provide the thioacetic acid **26** in quantitative yield (Scheme 6). Using ^3H-labelled PGE_2, ^3H-labelled analogues of **18**, **23**, **24** and **31** were prepared.

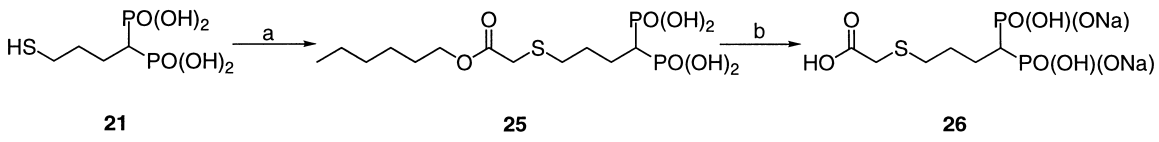
Synthesis of conjugates incorporating the two moles of PGE_2 per mole of bisphosphonate. All PGE_2-bisphosphonate conjugates described above contain an equivalent molar amount of PGE_2 per mole of bisphosphonate. It was considered potentially advantageous if multiple molar quantities of PGE_2 could be delivered per mole of bisphosphonate. We therefore investigated the possibility of incorporating more than one equivalent of PGE_2 utilizing a polysubstituted bisphosphonate. This was achieved utilizing 1,3,5-tris(bromomethyl)-benzene (**27**) as a common coupling unit. Reduction of trimethyl 1,3,5-benzenetricarboxylate with borane/dimethyl sulfate complex provided the corresponding 1,3,5-tris(hydroxymethyl)benzene in quantitative yield which was brominated with phosphorus tribromide in ether to provide the tribromide **27**. The anion derived from tetraisopropyl methenediphosphonate was alkylated with the tribromide to provide the corresponding monoalkylated bisphosphonate **28** in 63% yield. Reaction of **28** with potassium thioacetate gave the bisthioacetate **29** in 70% yield. Hydrolysis of **29** with 6 N HCl proceeded to **30** in essentially quantitative yield (98%). The bromoacetate **22** (2 equiv in dioxane) was added to **30** in water/triethylamine to provide the bisalkylated bisphosphonate **31** which was purified by C18 chromatography and lyophylization (52% yield) (Scheme 7). With these four conjugates now in hand, we undertook a series of in vitro and in vivo biological experiments to evaluate their potential for stimulation of bone growth.

Enzymatic hydrolysis of conjugates 18, 23, 24, and 31. The rate of release of PGE_2 from the conjugates **18**, **23**, **24**, **31** when incubated with rat plasma was measured in two sets of experiments. In the first, [^3H]-**18** was incubated with fresh rat plasma (50%) at 37 °C and liberated tritium was measured by separation of unreacted **18** and liberated 'PGE_2' using silica gel chromatography.

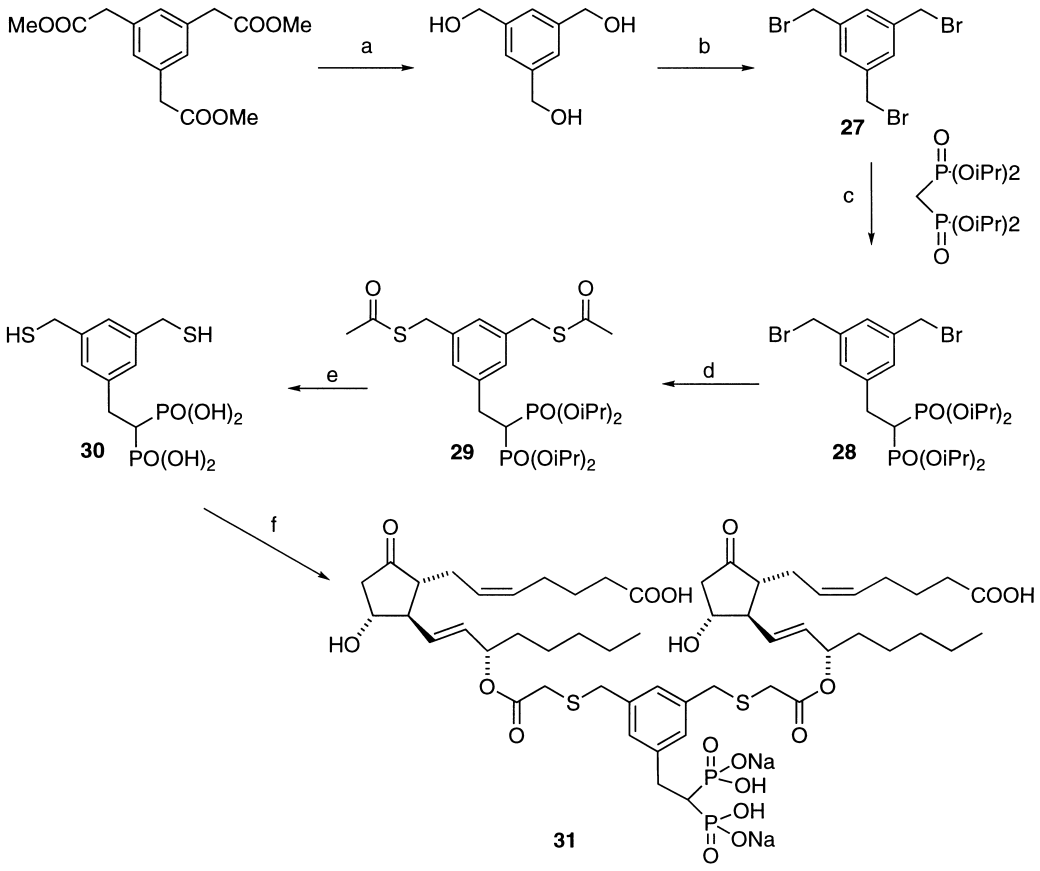
As can be seen in Figure 3, a significant amount of label (20%) was liberated in 2 h relative to incubations with boiled plasma controls or with pH 7.2 phosphate buffered saline (PBS) alone.

In a second set of experiments, conjugates **23** and **24** (0.1 μCi each) were compared with conjugate **31** (0.2 μCi or molar equivalent bisphosphonate) in 100% rat plasma at 37 °C for up to 24 h. As can be seen in Figure 4, both conjugates **23** and **24** released tritium at a reasonable rate with conjugate **31** releasing 51% after 24 h compared to 17.5% in a PBS control incubator and 25.4% in a boiled plasma control (Fig. 5).

It was concluded that esterase activity in rat plasma can hydrolyze each of these conjugates at an acceptable rate. There remained some question as to the nature of the liberated tritium in these experiments. It was possible that label could be liberated by elimination of the 15-ester rather than hydrolysis. In a preliminary incubation of conjugate **18** with rat plasma, direct radioimmunoassay analysis of the released radioactive contents revealed that only ~10% of radioactivity could be accounted for as PGE_2 (data not shown). We thus



Scheme 6. Reagents: (a) $BrCH_2COOC_6H_{13}$, $EtN(iPr)_2$, dioxane; (b) 1) HCl, reflux. 2) NaOH.



Scheme 7. Reagents: (a) BH_3-SMe_2; (b) PBr_3; (c) NaH, DMF; (d) HSAc, DMF; (e) HCl, reflux; (f) 1) **22**, Et_3N, dioxane, water. 2) DOWEX(Na^+).

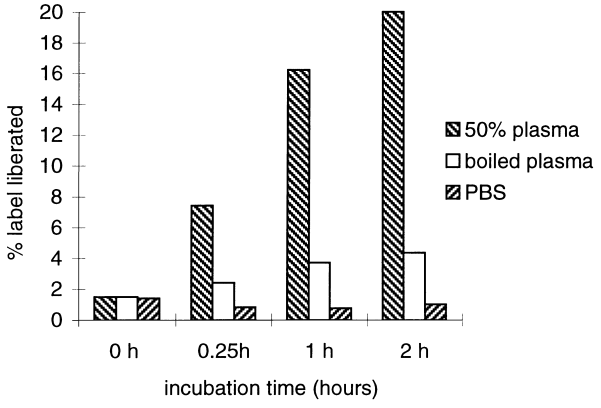


Figure 3.

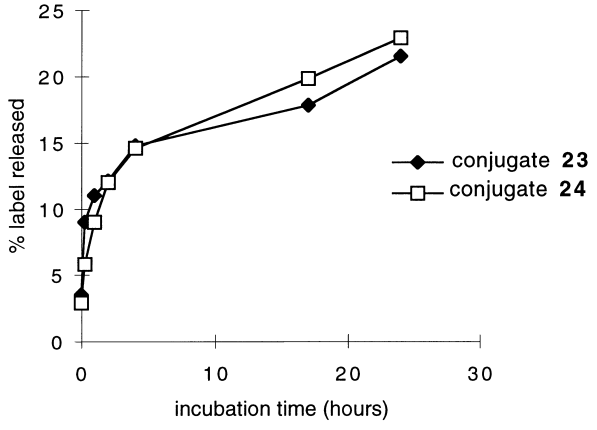


Figure 4.

sought to characterize the nature of the liberated label by HPLC. [^{3}H]-PGE_2 or [^{3}H]-conjugate **31** was incubated with PBS, boiled plasma or fresh rat plasma for 4 or 24 h and HPLC analysis showed that the products in each case were similar. Thus PGE_2 in PBS or boiled plasma was converted to PGA_2 while in fresh plasma PGB_2 and unidentified label at the solvent front (presumed to be tritiated water due to its volatility) were observed together with unreacted PGE_2. Conjugate **31** liberated the same products but little or no PGE_2 was observed. These results were in keeping with the liberation of PGE_2 from **31** and subsequent conversion by albumin to PGB_2 with concomitant liberation of tritium with water by exchange (Fig. 6). Fitzpatrick and Wynalda[20] have shown that albumin sequesters PGE_2 and accelerates dehydration and isomeration into PGB_2 (due to an alkaline microenvironment associated with the binding site). The lack of significant PGE_2 observed to be liberated from **31** can be considered reasonable in that it is being slowly released in minute quantities and concomitantly decomposing to PGA_2 or PGB_2. Clearly no elimination of the 15-ester group was taking place.

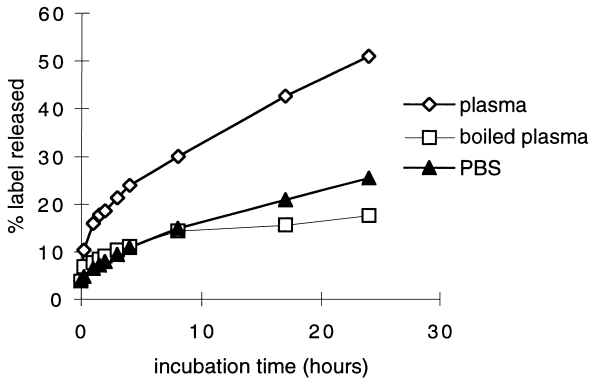


Figure 5.

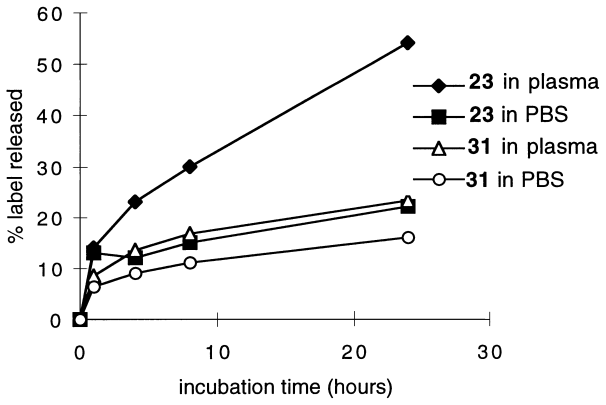


Figure 7.

Binding of conjugates [^{3}H]-23 and [^{3}H]-31 to bone powder and release of label. Vortexing [^{3}H]-**23** or [^{3}H]-**31** with human bone powder demonstrated rapid fixation to bone with 80–90% of label being removed from a PBS solution within 5–15 min. When conjugate **31** was first placed in rat plasma and then vortexed with bone powder, only 20–35% of label was extracted by the bone indicating either competition with plasma elements for binding sites on bone or that plasma protein binding of the conjugates **31** inhibited binding to bone. Bone powder bound [^{3}H]-**23** and [^{3}H]-**31** were incubated at 37 °C with rat plasma or PBS and liberated label was monitored over time in the supernatant (Fig. 7). The release of label from both bound conjugates was significant and elevated in plasma relative to PBS. Release rates, if reproduced in vivo, could liberate potentially therapeutic amounts of PGE_2 to bone.

In vivo uptake and release of [^{3}H]-23 and [^{3}H]-31. The conjugates [^{3}H]-**23** and [^{3}H]-**31** were dosed intravenously in rats at 1 mg/kg and sets of rats ($n = 5$ or 6) were sacrificed at 6 h, 48 h and 7 days post dose and tritium levels in long bones were determined. As can be seen in Figure 8, uptake was moderate (3–3.5%) at 6 h but release was smooth and consistent in each case with about 30–50% release over the 7 day period. Based on these results, it was decided to take conjugate **23** into a long-term (4 week) trial in ovariectomized rats to measure effects of the conjugate relative to PGE_2.

In vivo effects of conjugate 23 in a rat model of osteoporosis. The conjugate **23** was evaluated during a 4-week study in ovariectomized (ovx) osteopenic rats for effects on bone formation at doses of 10 or 100 mg/kg iv once weekly. Controls included (i) intravenous saline vehicle, (ii) the mixture of the core bisphosphonate **26** plus PGE_2 each dosed once weekly at 5 mg/kg iv, (iii) a positive control of rats dosed daily at 6 mg/kg PGE_2 five times per week subcutaneously (s.c.) and (iv) a negative control of sham-ovariectomized rats dosed s.c. with saline vehicle. The results are presented in Tables 2 and 3. The anti-resorptive effect of **26**, the core bisphosphonate, was also evaluated alone in the growing rat model (Schenk assay[21]) in rats dosed s.c. at 0, 3 and 30 mg/kg per day for 10 days.

Eight weeks post-ovariectomy animals developed the expected osteopenia due to estrogen deficiency. Compared to the sham-ovx group femoral bone mineral content (BMC) decreased by 14%. Tibial structural indices of bone volume (BV/TV) and trabecular number (TbN) decreased by 58% while trabecular separation increased threefold. There was also a non-significant decrease in trabecular thickness. Mineralizing surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR/BS) were significantly increased due to ovariectomy (Table 2).

The group treated with **23** (10 mg) and its two relevant controls (groups: OVX-VEH, **26** + PGE_2) were further analyzed. Both the **26** plus PGE_2 and **23** groups showed small but significant increases in femoral BMC compared to the vehicle treated group (Table 3). There were no significant differences in tibial bone volume (BV/TV) or mineral apposition rate (MAR) in either of the three groups. However, the **23** treated group did have significantly increased mineralizing surface (MS/BS) of 25–50% and bone formation rate (BFR/BS) of 33–54% compared to the other two treated groups. Treatment with **23** did produce significant increases in mineralizing surface, mineral appositional rate, and bone formation rate compared to the combined **26** + PGE_2 treated group alone; however, the structural indices of cancellous

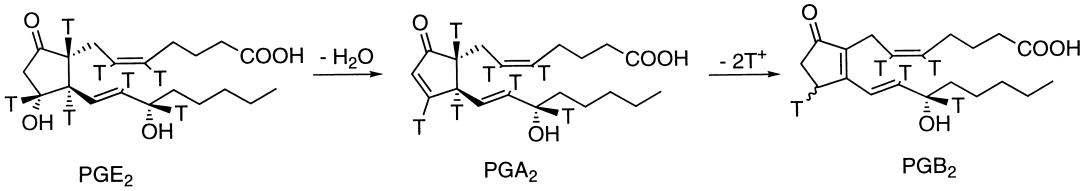


Figure 6.

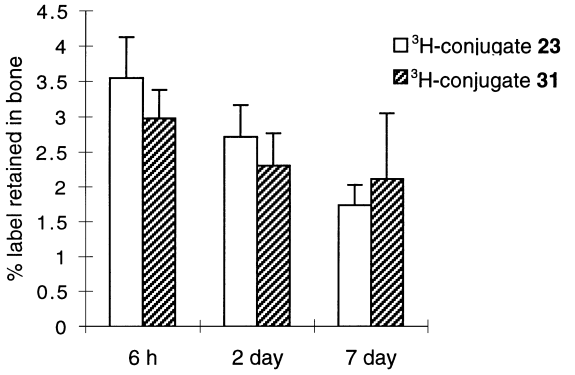


Figure 8.

bone, trabecular thickness, trabecular number, and trabecular separation were not different (data not shown).

An apparent dose response **23** is shown in Table 3. Both the 10 mg/kg and 100 mg/kg doses showed significant increases in femoral BMC of 8% and 14%, respectively, while the 6 mg/kg/d PGE_2 treated group increased by 22% compared to vehicle. A twofold increase in mineralizing surfact (MS/BS) was seen in all treatment groups compared to vehicle. The 100 mg/kg dose group had a significantly higher mineral apposition rate (MAR) and the bone formation rate (BFR/BS) was also significantly increased two- to threefold compared to the vehicle treated group. Significant increases BFR/BS in the 10 mg/kg and PGE_2 treated groups albeit not as great were also seen. However, there were no significant differences in tibial bone volume (BV/TV) between groups. It appears that in these animals, the major effect of PGE_2 was on cortical bone, a major contributor to total BMC. In the Schenk assay, treatment with **26**, the bisphosphonate core of **23**, at doses up to 30 mg/kg for 10 days did not produce significant increases in femoral ash weight indicating that it is not an effective inhibitor of bone resorption (data not shown).

Conclusions

Studies on the synthesis of a variety of conjugates of the bone growth stimulating PGE_2 with bone targeting bisphosphonates have lead to the preparation of conjugates with covalent coupling via the C-1 carbonyl group and the C-15 hydroxyl group of PGE_2. Amides at C-1 proved to be too stable and, while taken up in bone efficiently, were not sufficiently labile to be hydrolyzed in vivo. A thio-ester conjugate proved to be too labile and was hydrolyzed rapidly in the bloodstream before it could be taken up in bone. Methods were developed to conjugate PGE_2 to bisphosphonate via the C-15 hydroxyl group. The conjugate **23** was taken up moderately well and was subsequently released at a slow but steady rate (~50% over 1 week) suggesting it could deliver PGE_2 in situ at an acceptable rate. The conjugate was very well tolerated at high doses (up to 100 mg/kg, equivalent to ~50 mg/kg PGE_2) with ~5% deposited in bone and PGE_2 liberated at a rate of about 6–7% per day or the equivalent of ~200 μg PGE_2/kg/day at the highest dose. The bone formation stimulation of this dose was comparable or superior to PGE_2 dosed at its maximum tolerated dose of 6 mg/kg/day (5 days per week) administered subcutaneously, with respect to increase in bone mineral content, mineralizing surface, mineral apposition rate and bone formation rate. However, neither treatment overcame the cancellous bone osteopenia produced by 2 months of estrogen

Table 2. Effect of ovariectomy on bone mineral content (BMC) and turnover in ovariectomized rats[a]

	Sham	Ovx
Whole femoral BMC (g)	0.4288 (±0.0260)	0.3691 •(±0.0243)[b]
Tibial bone volume (BV/TV, %)	32.56 (±9.27)	13.77 •(±4.10)
Trabecular thickness (μm)	44.92 (±11.93)	44.31 (±7.67)
Trabecular number (#/mm)	7.56 (±1.99)	3.15 •(±0.98)
Trabecular separation (μm)	101.29 (±57.47)	302.67 •(±110.23)
Mineralizing surface (MS/BS, %)	1.39 (±0.82)	4.66 •(±3.42
Mineral apposition rate (MAR, μm/day)	1.49 (±0.15)	1.82 •(±0.37)
Bone formation rate (BFR/BS, μm3/μm2/year)	7.73 (±4.75)	31.65 •(±22.90)

[a]Data represents mean ± SD (n = 6–8 per group).
[b]•Significantly different from Sham group (Student's T test, $P < 0.05$).

Table 3. Effect of treatment with **23** on bone mineral content (BMC) and turnover in ovariectomized rats[a]

	Ovx vehicle	Conjugate **23** 10 mg/kg/wk i.v.	Conjugate **23** 100 mg/kg/wk i.v.	PGE_2 6 mg/kg/d s.c.	**26** + PGE_2 i.v. 5 mg/kg/wk
Whole femoral BMC (g)	0.3691 (±0.0243)	0.3969 Δ (±0.0265)[b]	0.4201 •(±0.0274)[c]	0.4519 •(±0.0391)	0.3967 •(±0.0165)
Tibial bone volume (BV/TV, %)	13.77 (±4.10)	14.16 (±3.21)	12.10 (±4.44)	15.93 (±5.34)	15.24 (±6.67)
Mineralizing surface (MS/BS, %)	4.66 (±3.42)	10.13 •(±3.07)	10.54 •(±2.17)	10.09 •(±3.44)	7.65 (±3.71)
Mineral apposition rate (MAR, μm/day)	1.82 (±0.37)	1.89 (±0.25)	2.88 •(±0.28)	1.91 (±0.15)	1.60 (±0.15)
Bone formation rate (BFR/BS, μm 3/year)	31.6 (±22.9)	67.9 •(±17.2)	111 •(±24.0)	69.9 •(±21.6)	45.5 (±24.7)

[a]Data represent mean ± SD (n = 7–8 per group).
[b]Δ Significantly different from vehicle treated group (Fisher PLSD, $P < 0.08$).
[c]• Significantly different from vehicle treated group (Fisher PLSD, $P < 0.05$).

deficiency. A longer treatment period or higher doses of PGE_2 possibly deliverable via the bis-conjugate **31** may translate these changes in bone formation rates into bone volume increases. Alternatively, conjugation to an active bisphosphonate could combine increased bone formation with inhibition of bone resorption. Femoral bone mineral content (BMC) increased dose dependently compared to vehicle and this effect was not due to an anti-resorptive effect of the bisphosphonate core (**26**). Thus as proof of concept experiments, these results support bone formation by local release of prostaglandin E_2 from a conjugate compound.

Experimental

General methods

All reagents and dry solvents were obtained from commercial sources and used without further purification. ([5,6,8,11,12,14,15-^{3}H(N)]-PGE_2 was purchased from New England Nuclear and 3-[^{14}C]-3-amino-1-hydroxypropane-1,1-diphosphonate (^{14}C-alendronate) (^{14}C-ABP) was synthesized by Merck Research Laboratories, Rahway, NJ). All reactions were carried out under a positive pressure of nitrogen. Flash chromatography was performed on silica-gel (Merck, 230–400 mesh). Bond Elute C18 pack cartridges were obtained from Varian Inc. and washed with CH_3CN, methanol and water before use. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARX-400 or AMX-300 instrument. Infrared spectra were recorded on a Perkin–Elmer 681 spectrometer. Melting points were taken on a Mettler FP61 apparatus and are uncorrected. Low resolution mass spectra and elemental analyses were obtained from Oneida Research Services. High resolution mass spectra were obtained at the Biomedical Mass Spectrometry Unit, McGill University using a ZAB 2F HS instrument.

PGE_2-alendronate conjugate (3). 1,3-Dicyclohexylcarbodiimide (3.6 mg) was added to a stirred solution of PGE_2 (**1a**) (3.1 mg) and *N*-hydroxysuccinimide (3.0 mg) in dry acetonitrile (200 μL) and stirred at room temperature (25 °C) until thin-layer chromatography indicated that the reaction was complete. The solvent is removed under an inert atmosphere (nitrogen) and the residue was dissolved in CH_2Cl_2 and applied to a small column of silica gel in a pasteur pipette. The pipette was then eluted with EtOAc to afford the hydroxysuccinimide ester (**1b**) and a small quantity of dicyclohexylurea. ^{1}H NMR ($CDCl_3$): δ 5.45–5.7 (2H, m, H-13,14), 5.37 (2H, m, s, H-5,6), 3.95–4.15 (2H, m, H-11,15), 2.85 (4H). A solution of this ester in 1,4-dioxane was added to a stirred solution of alendronate disodium salt (**2**) (ABP) (2.4 mg) in water (150 μL) and 1.0 NaOH (10 μL). After 10 min the pH of the reaction mixture was adjusted to approximately 9 with 1.0 M aqueous NaOH, and then 1 h later the pH was adjusted to 7 with 0.1 M HCl. The solution is filtered and the filtrate was concentrated to dryness. The residue was then dissolved in water and applied to a Varian Bond Elute C18 pack which was eluted with water. When the product began to elute, the solvent system on the C18 column was changed to acetonitrile/water (50/50). Evaporation of fractions containing the product afforded the target amide (**3**) (3.7 mg). ^{1}H NMR (D_2O): δ (2H, m, H-5,6), 5.1–5.4 (2H, m , H-13,14), 3.9–4.1 (2H, m, H-11,15), 3.0 (2H, m, $HN-CH_2$).

^{3}H-^{14}C-conjugate 3. The identical procedure was followed with tritiated PGE_2 and ^{14}C-labelled alendronate monosodium salt to produce the dual-labelled **3** (specific activity 7 mCi/mmol each in ^{3}H and ^{14}C).

Synthesis of PGE_2-thioester-alendronate conjugate (6). Dicyclohexylcarbodiimide (0.2 g) was added to a stirred solution of *N*-(4-carboxybutyl)maleimide (0.12 g) (mp 87–89 °C prepared in the same way as the procedure in Coleman et al.[22]) in dichloromethane (10 mL) containing *N*-hydroxysuccinimide (0.38 g). After 2 h, the reaction mixture was poured onto a silica gel column which was eluted with EtOAc affording bifunctional reagent *N*-(4-maleimidobutyric acid) *N*-hydroxysuccinimide ester (0.086 g). ^{1}H NMR ($(CD_3)_2CO$) δ 6.85 (2H, s), 3.50 (2H, 5), 2.87 (4H, s), 2.72 (2H, t), 1.98 2H, dt).

A solution of the active ester (12 mg) in 1,4-dioxane (200 μL) was added to a stirred solution of alendronate (ABP) (7 mg) in water (400 μL) and 1 N sodium hydroxide (25 μL). After 15 min the solution was adjusted to pH 7 with 0.1 N HCl and then lyophilized. The resulting powder was dissolved in water and eluted through two Varian 6 mL C18 'bond elute' cartridges with water, collecting the first 4 mL from each cartridge. This solution was lyophilized and the resulting colorless powder contained the maleimide derivative (**4**) as well as *N*-hydroxysuccinimide and, perhaps, some unreacted ABP. ^{1}H NMR (D_2O) δ 6.72 (2H, s), 3.40 (2H, t), 3.01 (2H, t), 2.13 (2H, t), 1.9–1.6 (6H, m).

A solution of PGE_2 (**1a**) (5 mg) in CH_2Cl_2 (500 μL) was stirred under nitrogen and treated with 1,3-propanedithiol (14 μL) and dicyclohexylcarbodiimide (8 mg). The reaction was followed by thin-layer chromatography (TLC) and when complete (~4 h) the reaction mixture was poured onto a small silica gel column in a pasteur pipette. Elution with deoxygenated EtOAc afforded the thiolester (**5**). This was immediately dissolved in methanol (500 μL) and added to a solution of **4** in aqueous methanol (1 mL, 1/1 v/v). The solution was allowed to stand for 15 min, then most of the methanol was evaporated and the residual aqueous solution was lyophilized. The crude product was dissolved in water and absorbed onto a Varian 6 mL C18 bond elute cartridge. This was eluted with water (9 mL), 30% MeOH/H_2O (6 mL), then 60% MeON/H_2O (6 mL). The first 3 mL of the 60% MeOH fraction contained all the product (**6**) obtained as a white powder (4.6 mg) after lyophilization. mp >260 °C (dec). ^{13}C NMR (D_2O) δ (ppm) 215.7 (C=O), 198.2 (C−S), 176.0, 176.1, 172.2 (C−N), 133.8, 129.6, 127.7, 124.5 (HC=), 71.1 (t, Jclp = 134 Hz, C-p), 70.2, 68.4 (CH−O), 51.7, 50.6, 37.0 (CH), 43.2, 40.6, 37.4, 35.9, 34.0, 33.4, 30.3, 28.9, 28.4, 27.4, 26.1, 24.8, 23.6, 22.5, 20.8, 20.6, 19.9 (CH_2), 11.3 (CH_3).

[^{3}H]-[^{14}C]-conjugate 6. The identical procedure as described above was followed with tritiated PGE_2 and ^{14}C labelled ABP monosodium salt to produce the dual labelled conjugate **6** (specific activity 4.48 mCi/mmol tritium and 5.76 mCi/nM ^{14}C).

Tribenzyl orthoformate. Benzyl alcohol (390 mL, 3.6 mol) was added to a solution of triethyl orthoformate (150 mL, 0.9 mol) in benzene (350 mL) at room temperature. Trifluoroacetic acid (6.8 mL, 0.09 mol) was then added at room temperature and the mixture was slowly distilled under reduced pressure (35 °C, 20 mm Hg) until the volatiles (EtOH, C_6H_6, TFA) had distilled. Excess benzyl alcohol was distilled (75 °C, 0.1 mm Hg) and the residue consisted mainly of tribenzyl orthoformate which could be distilled (170–185 °C, 0.1 mm Hg) although it could be used crude in the next step. ^{1}H NMR ($CDCl_3$): δ 7.40 (15H, s), 5.50 (1H, s), 4.74 (6H, s); ^{13}C NMR ($CDCl_3$): δ 137.8, 128.9, 128.1, 111.8, 66.5.

Tetrabenzyl methylenediphosphonate (7a). A mixture of methylenediphosphonic acid (14.8 g, 0.08 mol) and tribenzyl orthoformate (226 g, 0.68 mol) was heated to 150 °C for 2 h, cooled down, diluted with ethyl acetate (125 mL) and poured onto a silica gel column (4.5 L) column. Elution with ethyl acetate gave 34.6 g (77%) of tetrabenzyl methylenediphosphonate **7a** as an oil. IR (neat) 3100–2900 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.29 (20H, m), 4.98 (8H, m), 2.50 (2H, t, J = 24.0 Hz); ^{13}C NMR ($CDCl_3$): δ 136.1, 128.9, 128.8, 128.2, 128.1, 68.4, 26.4 (t, J = 138.4 Hz). MS (FAB, NaI) m/z (relative intensity): 537 (MH^+, 96), 447 (7), 181 (100). HRMS (FAB, NaI): calcd for $C_{29}H_{31}P_2O_6$ (MH^+) 537.1596; found 537.1594. Anal. calcd for $C_{29}H_{31}P_2O_6$: C, 64.91; H, 5.64; P, 11.55; found: C, 64.69; H, 5.85; P, 11.26.

Alkylation of tetrabenzyl methylenediphosphonate (7a)

Typical procedure. Sodium hydride (60%) (291 mg, 7.3 mmol) was added portionwise to a solution of tetrabenzyl methylenediphosphonate (**7a**) (3.0 g, 5.5 mmol) in DMF (10.0 mL) at room temperature. The mixture was stirred at room temperature for 60 min and a solution of methyl *p*-bromomethylbenzoate (1.9 g, 8.4 mmol) in THF (2.0 mL) was added. The mixture was stirred at room temperature for 30 min and a solution of saturated ammonium chloride (15 mL), water (100 mL), and a (1/1) mixture of ether/hexanes (100 mL) were added. The separated aqueous layer was extracted with a (1/1) mixture of ether/hexanes (3×100 mL) and the combined organic layers were washed (brine), dried ($MgSO_4$ anh.), filtered and evaporated. Flash-chromatography (EtOAc/hexanes, 1/1) of the residue gave monoalkylated product **8d** (1.8 g, 47%) along with dialkylated product **9d** (670 mg, 14%). Spectral data for compounds **8** and **9** are given below.

Tetrabenzyl 6-carboethoxyhexane-1,1-diphosphonate (8a). IR (neat) 3100–2880, 1730 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.29 (20H, s), 5.00 (8H, m), 4.07 (2H, q, J = 7.0 Hz), 2.34 (1H, tt, J = 24.0, 6.1 Hz), 2.12 (2H, m), 1.90 (2H, m), 1.46 (4H, m), 1.20 (3H, t, J = 7.0 Hz); ^{13}C NMR ($CDCl_3$): δ 173.3, 136.1 (d, J = 4.6 Hz) 128.6, 128.5, 128.4, 128.1, 128.0, 68.6, 68.2, 60.1, 37.4 (t, J = 134.3 Hz), 34.0, 28.5 (t, J = 6.8 Hz), 25.4 (t, J = 4.8 Hz), 24.6, 14.2. MS (FAB, NaI) m/z (relative intensity): 665 (71), 575 (7), 181 (100). HRMS (FAB, NaI): calcd for $C_{36}H_{43}P_2O_8$ (MH^+) 665.2433; found 665.2434.

Tetrabenzyl 3-(tetrahydropyran-2yl)oxy-propane-1,1-diphosphonate (8b). IR (neat) 3100–2880, 1730 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.29 (20H, s), 5.01 (8H, m), 4.40 (1H, t, J = 3.3 Hz), 3.83, 3.70, 3.53, 3.35 (4H, 4 m), 2.79 (1H, tt, J = 24.0, 6.1 Hz), 2.26 (2H, m), 1.90 (2H, m), 1.40 (6H, m); ^{13}C NMR ($CDCl_3$): δ 136.3, 128.5, 128.3, 128.1, 128.0, 98.6, 68.2, 68.1, 68.0, 64.9 (t, J = 4.8 Hz), 62.2, 33.5 (t, J = 133.7 Hz), 30.6, 25.9 (t, J = 7.0 Hz), 25.4, 19.5.

Tetrabenzyl 2-phenylethane-1,1-diphosphonate (8c). IR (neat) 3100–2900, 1960, 1880, 1820 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.19 (25H, m), 4.92 (8H, m), 3.28 (2H, td, J = 16.4, 6.1 Hz), 2.77 (1H, tt, J = 24.0, 6.1 Hz). MS (FAB, NaI) m/z (relative intensity): 627 (50), 181 (100). HRMS (FAB, NaI): calcd for $C_{36}H_{37}P_2O_6$ (MH^+) 627.2065; found 627.2067.

Tetrabenzyl 1-phenylmethyl-2-phenylethane-1,1-diphosphonate (9c). IR (neat) 3100–2900 cm^{-1}. ^{1}H NMR ($CDCl_3$): 7.20 (30H, m), 4.80 (8H, m), 3.41 (4H, t, J = 16.0 Hz). MS (FAB, NaI) m/z (relative intensity): 717 (65), 519 (20), 181 (100). HRMS (FAB, NaI): calcd for $C_{43}H_{43}P_2O_6$ (MH^+) 717.2532; found 717.2535.

Tetrabenzyl 2-(4-carbomethoxyphenyl)ethane-1,1-diphosphonate (8d). IR (neat) 3100–2890, 1720 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.80 (2H, d, J = 7.0 Hz), 7.33 (20H, m), 7.10 (2H, d, J = 7.0 Hz), 4.92 (8H, m), 3.88 (3H, s), 3.26 (2H, td, J = 16.7, 6.4 Hz), 2.71 (1H, tt, J = 24.0, 6.4 Hz); ^{13}C NMR ($CDCl_3$): δ 166.8, 144.4, 136.0, 129.5, 128.9, 128.5, 128.4, 128.2, 128.1, 68.1 (dd, J = 24.1, 6.6 Hz), 51.9, 40.8, 39.6 (t, J = 132.5 Hz), 31.3 (t, J = 6.2 Hz). MS (FAB, NaI) m/z (relative intensity): 685 (42), 301 (10), 181 (100). HRMS (FAB, NaI): calcd for $C_{38}H_{39}P_2O_8$ (MH^+) 685.2120; found 685.2122.

Tetrabenzyl 1-(4-carbomethoxyphenylmethyl)-2-(4-carbomethoxy)phenyl)ethane-1,1-diphosphonate (9d). IR (neat) 3100–2890, 1725 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.79 (4H, d, J = 7.0 Hz), 7.40 (2H, d, J = 7.0 Hz), 7.33 (20H, m), 4.85 (8H, m), 3.86 (6H, s), 3.40 (4H, t, J = 16.0 Hz); ^{13}C NMR ($CDCl_3$): δ 168.9, 141.5, 135.9, 131.7, 128.8, 128.7, 128.5, 128.4, 128.2, 68.2 (t, J = 2.9 Hz), 51.9, 49.3 (t, J = 130.9 Hz), 38.3 (t, J = 6.2 Hz). MS (FAB, NaI) m/z (relative intensity): 833 (23), 603 (16), 449 (11), 181 (100). HRMS (FAB, NaI): calcd for $C_{47}H_{47}P_2O_{10}$ (MH^+) 833.2645; found 833.2642.

Tetrabenzyl 2-(4-carbomethoxymethylphenyl)ethane-1,1-diphosphonate (8e). IR (neat) 3100–2890, 1735 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.30–7.08 (24H, m), 4.95 (8H, m), 3.61 (3H, s), 3.53 (2H, s), 3.27 (2H, td, J = 16.7, 6.4 Hz), 2.75 (1H, tt, J = 24.0, 6.4 Hz); ^{13}C NMR ($CDCl_3$): δ 171.9, 138.1–126.1 (m), 8.1 (dd, J = 21.0, 6.6 Hz), 51.9, 40.8, 39.9 (t, J = 132.1 Hz), 30.9 (t, J = 6.2 Hz). MS

(FAB, NaI) *m/z* (relative intensity): 699 (21), 537 (15), 271 (15), 205 (16), 197 (16), 193 (24), 181 (100). HRMS (FAB, NaI): calcd for $C_{39}H_{41}P_2O_8$ (MH^+) 699.2277; found 699.2276.

Tetrabenzyl 2-carbomethoxyethane-1,1-diphosphonate (8f). IR (neat) 3100–2860, 1740 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.27 (20H, m), 5.02 (8H, m), 3.43 (3H, s), 3.25 (1H, tt, $J=24.0$, 6.1 Hz), 2.83 (1H, td, $J=16.4$, 6.1 Hz. MS (FAB, NaI) *m/z* (relative intensity): 609 (42), 181 (100). HRMS (FAB, NaI): calcd for $C_{32}H_{35}P_2O_8$ (MH^+) 609.1807; found 609.1807.

Tetrabenzyl 2-*t*-butoxycarbonylethane-1,1-diphosphonate (8g). IR (neat) 3100–2890, 1740 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.28 (20H, m), 5.02 (8H, m), 3.30 (1H, tt, $J=24.0$, 6.1 Hz), 2.82 (2H, td, $J=16.4$, 6.1 Hz); ^{13}C NMR ($CDCl_3$): δ 169.6 (t, $J=9.1$ Hz), 136.1 (d, $J=4.6$ Hz), 128.5, 128.4, 128.1, 128.0, 81.5, 68.2 (dd, $J=14.8$, 6.4 Hz), 33.2 (t, $J=135.7$ Hz), 31.4, 27.9.

Tetrabenzyl ethane-1,1-diphosphonate (8h). IR (neat) 3080–2900 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.26 (20H, s), 5.03 (8H, m), 2.52 (1H, tq, $J=24.0$, 6.8 Hz), 1.48 (3H, td, $J=16.4$, 6.8 Hz); ^{13}C NMR ($CDCl_3$): δ 136.9, 128.5, 128.3, 128.1, 98.6, 68.1, 32.0 (t, $J=134.4$ Hz), 20.0 (t, $J=6.8$ Hz).

Tetraisopropyl 2-*t*-butoxycarboxylethane-1,1-diphosphonate (8i). Compound **8i** was obtained in 62% yield following the typical procedure described above with tetraisopropyl methylenediphosphonate **7b** as starting material. IR (neat) 3060–2880, 1730 cm^{-1}. ^1H NMR ($CDCl_3$): δ 4.75 (4H, m), 2.94 (1H, tt, $J=24.0$, 6.1 Hz), 2.69 (2H, td, $J=16.4$, 6.1 Hz), 1.44 (9H, s), 1.33 (24H, m); ^{13}C NMR ($CDCl_3$): δ 169.9, 80.9, 71.1 (dd, $J=24.7$, 6.7 Hz), 33.9 (t, $J=137.7$ Hz), 31.7 (t, $J=4.2$ Hz), 27.9, 23.9. MS (FAB, NaI) *m/z* (relative intensity): 459 (29), 403 (41), 361 (25), 319 (27), 277 (40), 235 (100), 217 (97). HRMS (FAB, NaI): calcd for $C_{19}H_{41}P_2O_8$ (MH^+) 459.2276; found 459.2276.

Tetraisopropyl 2-carboxyethane-1,1-diphosphonate (10). Trifluoroacetic acid (5.5 mL) was added to *t*-butyl ester **8i** (1.1 g, 2.4 mmol) at room temperature and the mixture was stirred for 60 s at room temperature and evaporated under reduced pressure. Flash-chromatography (EtOH/EtOAc, 1/9) of the residue gave carboxylic acid **10** (802 mg, 83%). IR (neat) 3700–2300, 1735 cm^{-1}. ^1H NMR ($CDCl_3$): δ 4.69 (4H, m), 2.94 (1H, tt, $J=24.3$, 6.1 Hz), 2.71 (2H, td, $J=16.4$, 6.1 Hz), 1.26 (24H, m); ^{13}C NMR ($CDCl_3$): δ 169.9 (t, $J=8.6$ Hz), 71.9 (dd, $J=21.6$, 7.0 Hz), 34.0 (t, $J=138.2$ Hz), 30.6, 23.9 (m). MS (FAB, NaI) *m/z* (relative intensity): 827 ($2M+Na^+$, 8), 425 ($M+Na^+$, 44), 403 (56), 361 (15), 319 (22), 277 (36), 235 (100), 217 (73). HRMS (FAB, NaI): calcd for $C_{15}H_{33}P_2O_8$ (MH^+) 403.1650; found 403.1652.

Acid chloride (11). Thionyl chloride (182 μL, 2.5 mmol) was added to a solution of carboxylic acid **10** (201 mg, 0.5 mmol) in dichloromethane (2.5 mL) at room temperature. The mixture was heated to reflux for 3 h, cooled and evaporated under reduced pressure to give 200 mg (95%) of acid chloride **11** used directly in the next step. IR (neat) 1800 cm^{-1}.

PGE_2-*t*-butyl-diphenylsilyl ester (PGE_2TBDPS) (12). To a solution of PGE_2 (352.5 mg, 1 mmol) in CH_2Cl_2 (5 mL) at 0 °C was added *t*-butyldiphenylsilylchloride (275 μL, 1.1 mmol) and triethylamine (278 μL, 2.0 mmol) consecutively via microsyringe. The mixture was stirred at 0 °C for 2 h, then the solvent was evaporated and the residue purified by flash chromatography on silica gel eluting with ethyl acetate to provide PGE_2-TBDPS ester (**12**) (592 mg, 100%). ^1H NMR ($CDCl_3$): δ 7.65, 7.37 (10H, m), 5.59 (1H, dd, $J=15$, 7 Hz), 5.48 (1H, dd, $J=15$, 8 Hz), 5.38 (1H, m), 5.29 (1H, m), 4.05–3.97 (2H, m), 2.68 (1H, dd, $J=15$, 7 Hz), 2.44 (2H, dd, $J=7$, 7 Hz), 2.38–2.28 (6H, m), 2.14 (1H, dd, $J=17$, 9 Hz), 2.05 (3H, m), 1.70 (2H, m), 1.56–1.40 (2H, m), 1.35–1.24 (5H, m), 1.08 (9H, s), 0.86 (3H, t, $J=7$ Hz).

A sample of [5,6,8,11,12,14,15-^3H(N)]-PGE_2-TBDPS ester was prepared by diluting [5,6,8,11,12,14,15-^3H(N)]-PGE_2 (1 mCi, 100–200 Ci/mmol) into 100 mg PGE_2 to provide a final specific activity of 3.53 mCi/mmol. The PGE_2 was converted to [^3H]-PGE_2-TBDPS ester (**12**) as above in 89% yield.

PGE_2-TBDPS-bisphosphonate ester conjugate (13). A solution of PGE_2-TBDPS ester (61 mg, 0.1 mmol) in pyridine (150 μL) was added to a mixture of acid chloride **9** (110 mg, 0.25 mmol) and pyridine (150 μL) at room temperature. The mixture was stirred at room temperature for 3 h and a solution of saturated ammonium chloride (2 mL), water (10 mL), and ethyl acetate (20 mL) were added. The separated aqueous layer was extracted with ethyl acetate (3×20 mL) and the combined organic layers were washed (brine), dried ($MgSO_4$ anh.), filtered and evaporated. Flash-chromatography (EtOAc (100%) then EtOH/EtOAc, 1/9) of the residue gave PGE_2-TBDPS bisphosphonate ester conjugate **13** (22 mg, 22%). IR (neat) 3380, 3080–2860, 1750–1720 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.63, 7.31 (10H, 2m), 5.78–5.16 (5H, m), 4.70 (4H, m), 3.94 (1H, m), 2.89 (1H, tt, $J=24.0$, 6.1 Hz), 2.85–1.45 (23H, m), 1.31 (24H, m), 1.09 (9H, s), 0.85 (3H, m). MS (FAB, NaI) *m/z* (relative intensity): 997 ($M+Na^+$, 8), 559 (7), 515 (15), 499 (38), 497 (36), 477 (60), 459 (40), 423 (46), 404 (100). HRMS (FAB, NaI): calcd for $C_{51}H_{80}P_2SiNaO_{12}$ ($M+Na^+$) 997.4792; found 997.4788.

Tetrabenzyl 2-(4-carboxyphenyl)ethane-1,1-diphosphonate (14). A solution of lithium hydroxide (84 mg, 2.0 mmol) in water (1.0 mL) was added to a solution of methyl ester **8d** (455 mg, 0.6 mmol) in 1,4-dioxane (1.0 mL) at room temperature. The mixture was stirred at room temperature for 5 h and a 1 N solution of HCl (10 mL) was added. Dioxane was evaporated under reduced pressure and the mixture was diluted with EtOAc (20 mL). The separated aqueous layer was extracted with EtOAc (3×50 mL) and the combined organic layers were washed (brine), dried ($MgSO_4$ anh.), filtered and evaporated. Flash-chromatography (HOAc/EtOH/EtOAc, 0.1/1/9) of the residue gave carboxylic

acid **14** (210 mg, 48%). ^{1}H NMR ($CDCl_3$): δ 9.65 (1H, br. s), 7.98 (2H, d, J = 8.1 Hz), 7.26 (20H, s), 7.13 (2H, d, J = 8.1 Hz), 4.95 (8H, m), 3.31 (2H, td, J = 16.7, 6.4 Hz), 2.82 (1H, tt, J = 24.0, 6.4 Hz). ^{13}C NMR ($CDCl_3$): δ 170.0, 144.6 (t, J = 7.6 Hz), 135.9, 135.8, 130.1, 129.0, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 68.3 (dd, J = 19.6, 6.5 Hz), 39.4 (t, J = 133.2 Hz), 31.2 (br. s).

PGE_2-TBDPS ester bisphosphonate conjugate 17. Freshly distilled oxalyl chloride (1.5 equiv) was added to a solution of the acid **14** (177 ng, 0.264 mmol) and DMF (10 μL, 0.132 mmol) in dichloromethane (1 mL) at 0 °C. After stirring 10 min the volatiles were evaporated under high vacuum and the residue acid chloride **15** used directly. IR (neat): 1770, 1740 cm^{-1}. Compound **15** was dissolved in dichloromethane (100 μL), cooled to −20 °C and pyridine 50 μL was added followed by PGE_2-TBDPS in pyridine (350 μL) and dichloromethane (100 μL). After stirring 10 min at −10 °C and 0.5 h at 0 °C. A solution of saturated ammonium chloride was added and the mixture was extracted with EtOAc acetate (3×5 mL). The organic extracts were washed with brine, dried over magnesium sulfate and evaporated to dryness. The residue was purified by HPLC (ZORBAX, 21–5×25 cm, 20 mL/min EtOAc: hexane, (80/20) as eluant). The first fraction corresponded to the C-11 regioisomer (**16b**) (66.4 mg; 18%). The second fraction was the desired C-15 regioisomer **16a** (156.4 mg, 42%). Ethyl acetate elution provided the bis-acylated product (20 mg) and recovered PGE_2-TBDPS (55.3 mg, 31.4%). C-15 isomer **16b**: IR (neat) 3400, 3060–2860, 1740–1720 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.78 (2H, d, J = 8.2 Hz), 7.66, 7.64 (4H, 2d, J = 7.9 Hz), 7.43 7.18 (26H, m), 7.11 (2H, d, J = 8.2 Hz), 5.67, 5.39 (4H, 2m), 5.28 (1H, m), 4.93 (8H, m), 4.05 (1H, m), 3.28 (2H, td, J = 16.6, 6.4 Hz), 2.72 (1H, tt, J = 24.0, 6.4 Hz), 2.71 (1H, m), 2.44 (2H, t, J = 7.5 Hz), 2.41–1.22 (19H, m), 1.09 (9H, s), 0.86 (3H, m). ^{1}H NMR (CD_3COCD_3): δ 7.85 (2H, d, J = 8.3 Hz), 7.73, 7.72 (4H, 2d, J = 7.7 Hz), 7.48–7.26 (28H, m), 5.87 and 5.78 (2H, 2dd, J = 15.5, 7.8 Hz and J = 15.5, 6.5 Hz, respectively), 5.52, 5.40 (3H, m), 5.03 (8H, m), 4.31 (1H, d, J = 5.1 Hz), 4.16 (1H, m), 3.33 (2H, td, J = 16.4, 6.6 Hz), 3.05 (1H, tt, J = 23.7, 6.6 Hz), 2.67 1.20 (19H, m), 1.10 (9H, s), 0.87 (3H, m); ^{13}C NMR (CD_3COD_3): δ 214.3, 173.0, 165.9, 145.7 (t, J = 7.8 Hz), 137.5 (dd, J = 9.0, 6.9 Hz), 136.0, 130.1, 129.2, 75.5, 72.4, 68.5 (m), 54.8, 53.9, 39.8 (t, J = 131.1 Hz), 47.5, 35.9, 35.4, 33.9, 32.3, 27.3, 25.8, 25.7, 23.2, 19.6, 14.3. MS (FAB, NaI) m/z (relative intensity): 1265 ($M + Na^+$, 24), 1243 (13), 1192 (17), 819 (13), 761 (16), 671 (100), 581 (62). HRMS (FAB, NaI): calcd for $C_{73}H_{85}P_2SiO_{12}$ (MH^+) 1243.5286; found 1243.5287. C-11 isomer **16b**: IR (neat) 3400, 3060–2860, 1740–1720 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.75 (2H, d, J = 8.3 Hz), 7.64 (4H, m), 7.46–7.18 (26H, m), 7.11 (2H, d, J = 8.3 Hz), 5.62–5.21 (5H, 4m), 5.28 (1H, m), 4.93 (8H, m), 4.06 (1H, d, J = 6.6 Hz), 4.00 (1H, m), 3.27 (2H, td, J = 16.7, 6.5 Hz), 3.00 (1H, dd, J = 18.3, 6.8 Hz), 2.79–1.11 (19H, m), 1.09 (9H, s), 0.81 (3H, br. t, J = 6.7 Hz).

PGE_2 bisphosphonate ester conjugate 17. A solution of PGE_2-TBDPS ester conjugate **16** (145 mg, 0.117 mmol) in THF (4 mL) and 0.2 N HCl (1 mL) was stirred at room temperature for 4 h and diluted with brine, extracted with EtOAc (4×10 mL). The extracts were combined, concentrated in vacuo and the residue was purified by circular chromatography (EtOAc/hexane = 80/20) to furnish the corresponding acid (110 mg, 94%). **17**: IR (neat) 3680–3200, 3000–2840, 1740 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.79 (2H, d, J = 8.0 Hz), 7.25 (20H, m), 7.13 (2H, d, J = 8.0 Hz), 5.68 (2H, 2m), 5.37 (3H, m), 4.92 (8H, m), 4.07 (1H, m), 3.26 (2H, td, J = 16.6, 6.2 Hz), 2.84 (1H, tt, J = 24.0, 6.2 Hz), 2.68 (1H, br. dd, J = 18.4, 7.4 Hz), 2.41–1.22 (19H, m), 1.09 (9H, s), 0.86 (3H, m); ^{13}C NMR ($CDCl_3$): δ 214.8, 176.7, 165.2, 144.4 (t, J = 7.6 Hz), 135.8 (dd, J = 9.0, 6.9 Hz), 131.5, 129.3, 128.6, 128.5, 128.2, 74.9, 72.0, 68.3, 54.5, 53.2, 39.2 (t, J = 132.9 Hz), 46.2, 34.5, 33.4, 31.6, 31.1, 26.6, 25.1, 24.9, 24.6, 22.5, 14.0. MS (FAB, NaI) m/z (relative intensity): 1027 ($M + Na^+$, 31), 671 (100). HRMS (FAB, NaI): calcd for $C_{57}H_{67}P_2O_{12}$ (MH^+): 1005.4108; found: 1005.4106.

PGE_2 bisphosphonate conjugate 18. In a 3 mL borosilicate test tube a solution of the acid **17** (36 mg, 0.036 mmol) in EtOH (420 mL) and EtOAc (80 mL) under nitrogen was immersed in a 20 °C water bath. To the solution was added Pd/C (5% Pd content, 5.7 mg, 0.036 mmol) followed by 1,4-cyclohexadiene (136 mL, 1.44 mmoL) and the resultant mixture was stirred at room temperature for 4.5 h and transferred to a 1.5 mL plastic Eppendorf vial and centrifuged. The supernatant was separated and the residue rinsed twice with ethanol (1 mL). The supernatants were combined, neutralized with 0.5 N ammonium acetate (144 mL, 0.072 mmol) and concentrated. The crude product (~90% pure by ^{1}H NMR) could be purified in two ways: (1) by C18 mini-columns (6 mL Varian Bond Elute) using water (5 mL), 30% MeOH/water (5 mL), 60% MeOH/water (5 mL) and MeOH (5 mL). The desired product eluted with the 30% MeOH/water fraction. The fraction was lyophilized to afford the compound **18** (21 mg, 76%); as a light-yellow fluffy powder. (2) by HPLC using Waters PrepPak μbondapak® C18 column (25×100 mm, 10 mL/min, gradient composition: 0.5 N NH_4OAc/CH_3CN = 90/10 to 70/30 in 10 min and 70/30 for 10 min, UV detection: 254 nM). The fractions thus obtained were lyophilized to give the desired product, **18**. ^{1}H NMR (D_2O): δ 7.82 (2H, d, J = 7.9 Hz), 7.37 (2H, d, J = 7.9 Hz), 5.65 (2H, m), 5.37 (2H, m), 5.19 (1H, m), 4.05 (1H, m), 3.03 (2H, m), 2.65 (1H, dd, J = 18.8, 7.5 Hz), 2.42–1.16 (21H, m), 0.71 (3H, m). ^{13}C NMR (D_2O): δ 222.1, 180.0, 169.5, 133.0, 130.4, 130.3, 77.2, 72.1, 53.2, 42.1, 40.4, 35.1, 34.6, 34.5, 32.4, 32.1, 31.8, 25.2, 25.1, 22.9, 14.3. MS (FAB, NaI) m/z (relative intensity): 667 ($M + Na^+$, 4), 645 (2), 399 (4), 311 (11), 293 (14), 177 (60), 136 (100). HRMS (FAB, NaI): calcd for $C_{29}H_{43}P_2O_{12}$ (MH^+): 645.2231; found: 645.2230.

Preparation of 4-mercaptobutane-1,1-diphosphonic acid (21)

3-acetylthiopropyliodide (19). To a solution of 1,3-diiodopropane (10 g, 33.8 mmol) in 10 mL of anhydrous DMF at 0 °C under nitrogen was added, via a cannula over 15 min, a solution of potassium thioacetate (1.3 g,

11.3 mmol) in 5 mL of DMF and the mixture was stirred at 0 °C for 0.5 h, quenched with water (20 mL) and extracted with ether (3×20 mL). The extracts were combined, washed with brine and dried over $MgSO_4$, filtered and concentrated. The residue was purified by flash chromatography (silica gel, EtOAc/hexane, 5/95–10/90) to yield iodide **19** (2.5 g, 90%) as a light-yellow oil. IR (neat) 2960, 2920, 1689, 1418, 1350, 1210, 1130 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.00 (2H, m), 2.26 (3H, s), 2.87 (2H, t, $J=7$ Hz), 3.13 (2H, t, $J=6.9$ Hz); ^{13}C NMR ($CDCl_3$) δ 4.35, 29.70, 30.63, 32.97, 195.09.

Tetraisopropyl 4-acetylthiobutane-1,1-diphosphonate (20). To a solution of tetraisopropyl methylenediphosphonate (9.35 g, 27 mmol) in anhydrous DMF (30 mL) was added NaH (0.96 g, 32 mmol) portionwise and the resulting suspension was stirred at room temperature for 1 h. To the above solution was then introduced dropwise a solution of iodide **4** in DMF (7 mL) and the mixture was stirred at room temperature for 2 h, quenched with saturated aqueous ammonium chloride and extracted with EtOAc (3×60 mL). The extracts were combined, washed with brine and dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was subjected to Kughrör distillation to remove the unreacted starting material. The residue of the distillation was purified by flash chromatography (silica gel, EtOH/CH_2Cl_2, 0/100–3/97) to furnish bisphosphonate **20** (4.5 g, 36%) as a colorless oil. IR (neat) 2980, 2930, 2875, 1692, 1381, 1370, 1248 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.27 (24H, m), 1.73–1.92 (4H, m), 2.06 (1H, tt, $J=24.1$, 5.8 Hz), 2.23 (3H, s), 2.80 (2H, t, $J=7$ Hz), 4.70 (4H, m); ^{13}C NMR ($CDCl_3$): δ 23.06, 23.11, 23.16, 23.42, 24.30 (t, $J=5$ Hz), 27.85, 28.06 (t, $J=6.6$ Hz), 29.71, 37.18 (t, $J=135$ Hz), 70.10 (d, $J=6.9$ Hz), 70.25 (d, $J=7$ Hz), 194.11; MS (FAB) *m/z* (relative intensity) 461 (MH^+, 46), 251 (100); HRMS calcd for $C_{18}H_{39}O_7P_2S$ (MH^+) 461.1891, found 461.1892.

4-Mercaptobutane-1,1-diphosphonic acid (21). A solution of bisphosphonate **20** (2.07 g, 4.5 mmol) in 40 mL of 6 N HCl was heated to reflux under nitrogen for 6 h and cooled to room temperature. The solution was concentrated under high vacuum to afford **21** (1.1 g, 98%) as a yellowish oil. ^1H NMR (D_2O, 400 MHz) δ 1.54–1.78 (4H, m), 2.08 (1H, tt, $J=23.6$, 5.9 Hz), 2.31 (2H, t, $J=6.7$ Hz); ^{13}C NMR (D_2O, 100 MHz) δ 24.13, 24.63 (t, $J=4.5$ Hz), 33.46 (t, $J=6.6$ Hz), 37.75 (t, $J=128$ Hz); MS (FAB) *m/z* (relative intensity) 251 (MH^+, 47), 217 (39), 136 (100); HRMS calcd for $C_4H_{13}O_6P_2S$ (MH^+) 250.9908, found 250.9908.

15-Bromoacetyl PGE_2-TBDPS ester (22). To a solution of PGE_2-TBDPS (3.3 g, 5.58 mmol) in anhydrous THF (9 mL) at −25 °C was added pyridine (0.54 mL, 6.7 mmol) and bromoacetylbromide (0.54 mL, 6.14 mmol) and the suspension was stirred 10 min at −25 to −20 °C. The mixture was quenched with saturated aqueous ammonium chloride, warmed to room temperature and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The crude product was purified by flash chromatography (silica gel, EtOAc/hexane, 10/90–40/60) to yield the desired compound **22** (1.93 g, 49%) as a colorless oil. IR (neat) 3460, 2950, 2928, 2855, 1725, 1460, 1424, 1270 cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.86 (3H, t, $J=6.7$ Hz), 1.08 (9H, s), 1.22–1.35 (6H, m), 1.52–1.77 (4H, m), 1.88 (1H, b), 2.04–2.12 (3H, m), 2.17 (1H, dd, $J=18.5$, 9.4 Hz), 2.31 (1H, m), 2.35–2.50 (2H, m), 2.44 (2H, dd, $J=7.7$, 7.3 Hz), 2.71 (1H, ddd, $J=18.4$, 7.3, 1 Hz), 3.77 (2H, s), 4.07 (1H, ddd, $J=9.3$, 9.3, 8.5 Hz), 5.21 (1H, d, $J=6.9$ Hz), 5.25–5.44 (2H, m), 5.55 (1H, dd, $J=15.4$, 7.1 Hz), 5.66 (1H, dd, $J=15.4$, 8.3 Hz), 7.34–7.47 (6H, m), 7.65 (4H, m); ^{13}C NMR ($CDCl_3$): δ 13.99, 19.16, 22.50, 24.75, 24.87, 25.18, 26.30, 26.68, 26.96, 31.42, 34.25, 35.50, 46.18, 53.25, 54.35, 71.90, 76.90, 126.45, 127.73, 130.07, 131.08, 131.33, 131.95, 133.87, 135.32, 166.82, 172.76, 213.92; MS (APCI) *m/z* (relative intensity) 730 (^{81}Br) ($[M+NH_4]^+$, 15), 477 (63), 149 (100); MS (FAB) *m/z* (relative intensity) 711 (MH^+, 1), 135 (100); HRMS calcd for $C_{38}H_{52}O_6SiBr$ (MH^+) 711.2716, found 711.2715.

PGE_2 bisphosphonate conjugate 23. To a solution of bromide **22** (4.39 g, 6.16 mmol) in dioxane (50 mL) at room temperature and under nitrogen was added dropwise via a cannula a solution of thiol **21** (2.23 g, 8.92 mmol) and triethylamine (4.95 mL, 35.68 mmol) in water (20 mL) and the clear solution was stirred at room temperature for 2 h and concentrated. The residue was partitioned between EtOAc and water. The aqueous layer was washed twice with EtOAc and concentrated. The residue was purified by flash chromatography (silica gel C-18, MeOH/water, 0/100–60/40). The desired product came out in the 30% MeOH/water fractions which were filtered on a cation exchange (DOWEX 50 Na^+ form, 35 g). The filtrate was lyophilized to give the desired conjugate **23** (2.8 g, 66%) as a white sticky solid. ^1H NMR (D_2O): δ 0.70 (3H, m), 1.16 (6H, m), 1.40–1.60 (4H, m), 1.67–1.80 (5H, m), 1.88 (2H, m), 2.01 (2H, dd, $J=8$, 7.4 Hz), 2.08 (1H, dd, $J=18.7$, 9.6 Hz), 2.22 (3H, m), 2.35 (1H, m), 2.51 (2H, m), 2.66 (1H, dd, $J=18$, 7.3 Hz), 3.26 (2H, s), 4.03 (1H, m), 5.10–5.20 (2H, m), 5.37 (1H, m), 5.52 (1H, dd, $J=15.4$, 7 Hz), 5.61 (1H, dd, $J=15.4$, 8.3 Hz); ^{13}C NMR (D_2O): δ 14.20, 22.81, 24.96, 25.25, 25.63 (t, $J=5$ Hz), 26.59, 27.56, 29.47 (t, $J=7.5$ Hz), 31.55, 32.75, 34.29, 34.35, 37.78, 39.60 (t, $J=116$ Hz), 46.93, 53.33, 55.11, 71.87, 77.92, 126.84, 132.25, 132.92, 134.54, 173.33, 183.81, 221.32; MS (FAB) *m/z* (relative intensity) 709 ($[M+Na]^+$, 1.5), 687 ($[M+H]^+$, 2.7), 665 ($[M+2H-Na]^+$, 1.5), 115 (100); HRMS calcd for $C_{26}H_{42}O_{12}P_2SNa_3$ ($[M+Na]^+$) 709.1565, found 709.1564.

PGE_2 bisphosphonate sulfoxide conjugate 24. To a solution of conjugate **23** (10 mg, 0.0145 mmol) in 1 mL of MeOH was added at room temperature a 32% peracetic acid (3.37 μL, 0.016 mmol) solution and the mixture was stirred for 10 min. Dimethyl sulfide was then added and after 5 min the solvents of the reaction were removed to give sulfoxide **24** (10.2 mg, 100%). ^1H NMR (D_2O): δ 0.69 (3H, m), 1.08–1.23 (6H, m), 1.43–1.65 (4H, m), 1.75–2.00 (7H, m), 2.07 (1H, dd, $J=18.3$, 9.7 Hz), 2.14–2.25 (5H, m), 2.34 (1H, m), 2.65 (1H, dd, $J=19$, 7.4 Hz), 2.89 (2H, m), 3.71 (1H, d, $J=14.6$ Hz), 3.90 (1H, d, $J=14.6$ Hz), 4.04 (1H, m), 5.13–5.27 (2H, m), 5.32 (1H,

m), 5.51 (1H, dd, $J=15.4$, 6.8 Hz), 5.61 (1H, dd, $J=15.4$, 8.3 Hz); ^{13}C NMR (D_2O): δ 14.45, 23.05, 25.18, 25.35, 25.47, 25.67, 27.22, 31.87, 34.49, 34.63, 39.47 (t, $J=117$ Hz), 39.59, 46.73, 51.92, 53.73, 55.12, 55.97, 71.77, 78.68, 127.47, 131.92, 132.00, 135.83, 167.19, 179.20, 179.38; MS (FAB) m/z (relative intensity) 747 ($[M-H+2Na]^+$, 3.5), 725 ($[M+Na]^+$, 4), 703 ($[M+H]^+$, 3), 115 (100); HRMS calcd for $C_{26}H_{43}O_{13}P_2SNa_2$ (MH^+) 703.1695, found 703.1696.

Preparation of 4-carboxymethylthiobutane-1,1-diphosphonic acid (26)

Bromomethylcarbonyloxyhexane. To a solution of *n*-hexyl alcohol (4 mL, 31.8 mmol) in dichloromethane (20 mL) at 0 °C was added pyridine (2.83 mL, 35 mmol) and dropwise bromoacetylbromide (3.05 mL, 35 mL). The mixture was stirred at room temperature for 2 h, quenched with water and extracted with dichloromethane. The organic layer was dried over Na_2SO_4, concentrated to give bromomethyl carbonyloxyhexane (7 g, 99%) as a yellow liquid. IR (neat) 2955, 2925, 2855, 1733, 1280 cm^{-1}; ^1H NMR ($CDCl_3$): δ 0.87 (3H, t, $J=6.9$ Hz), 1.25–1.36 (6H, m), 1.64 (2H, m), 3.80 (2H, s), 4.14 (2H, t, $J=6.8$ Hz); ^{13}C NMR ($CDCl_3$): δ 13.89, 22.44, 25.36, 25.88, 28.33, 31.30, 66.33, 167.23.

4-(Hexyloxycarbonylmethylthio)butane-1,1-diphosphonic acid (25). To a solution of bromomethylcarbonyloxyhexane (0.98 g, 4.4 mmol) in dioxane (16.5 mL) at room temperature and under nitrogen was added via a cannula a solution of thiol **21** (1.1 g, 4.4 mmol) and Hünig's base (3.06 mL, 17.6 mmol) in water (8.5 mL). The mixture was stirred at room temperature for 1.5 h, concentrated, washed three times with EtOAc and concentrated in vacuo. The residue was filtered on a cation exchange resin (DOWEX 50 Na^+ form, 20 g) and the filtrate was evaporated. The crude was purified by flash chromatography (silica gel C-18, methanol/water, 0/100–60/40). The desired product eluted with 20% MeOH/water fractions. Lyophilization of the fractions afforded the bisphosphonate **25** (1.2 g, 62%) as a white sticky solid. ^1H NMR (D_2O): δ 0.70 (3H, m), 1.12–1.25 (6H, m), 1.50 (2H, m), 1.60–1.75 (5H, m), 2.51 (2H, m), 3.26 (2H, s), 4.01 (2H, t, $J=6.6$ Hz)); ^{13}C NMR (D_2O): δ 14.31, 22.86, 25.70, 25.76, 28.69, 29.57 (t, $J=7.3$ Hz), 31.67, 32.80, 34.08, 39.60 (t, $J=117$ Hz), 67.17, 173.91; MS (FAB) m/z (relative intensity) 459 ($[M+Na]^+$, 17), 437 ($[M+H]^+$, 12), 115 (100); HRMS calcd for $C_{12}H_{25}O_8P_2SNa_2$ (MH^+) 437.0540, found 437.0540.

4-Carboxymethylthiobutane-1,1-diphosphonic acid disodium salt (26)

A solution of bisphosphonate **25** (0.98 g, 2.24 mmol) in 30 mL of 6 N HCl was heated to reflux for 2 h and cooled to room temperature. The solvent was evaporated (wash twice with water) and the residue was solubilized in water and neutralized with an aqueous solution of NaOH. Lyophilization of the aqueous solution gave the acid **26** (0.79 g, 100%) as a white sticky solid. ^1H NMR (D_2O): δ 1.68–1.90 (5H, m), 2.47 (2H, t, $J=7$ Hz), 3.09 (2H, s); ^{13}C NMR (D_2O): δ 25.77 (t, $J=4.4$ Hz), 29.71 (t, $J=7.3$ Hz), 32.66, 37.49, 39.53 (t, $J=116.9$ Hz), 179.26.

Preparation of 2-(3,5-bis(mercaptomethyl)phenyl)ethane-1,1-diphosphonic acid (30)

1,3,5-Tris(hydroxymethyl)benzene. To a stirring solution of trimethyl 1,3,5-benzenetricarboxylate (10.45 g, 41.4 mmol) in 70 mL of anhydrous THF was added at room temperature a 10 M solution of borane–methyl sulfide complex (25 mL, 248 mmol) and the solution was heated to reflux for 3 h. The mixture was then added slowly to 50 mL of MeOH and the resulting mixture was heated at 70 °C for 10 min to remove the methyl sulfide. Evaporation of solvent, washing twice with 50 mL of MeOH and evaporation of MeOH gave 1,3,5-tris(hydroxymethyl)benzene (6.96 g, 100%). ^1H NMR (D_2O): δ 4.52 (6H, s), 7.15 (3H, s).

1,3,5-Tris(bromomethyl)benzene (27). To a suspension of 1,3,5-tris(hydroxymethyl)benzene (3.19 g, 18.98 mmol) in 75 mL of anhydrous ether at 0 °C was added dropwise a solution of phosphorus tribromide (7 mL, 74.4 mmol) in 7 mL of ether and the mixture was stirred for 1.5 h at 0 °C and 4 h at room temperature. The mixture was poured onto ice and extracted with ether. The combined ether extracts were dried over Na_2SO_4 and evaporated to give 1,3,5-tris(bromomethyl)benzene **27** (6.35 g, 94%) as a white solid. ^1H NMR ($CDCl_3$): δ 4.42 (6H, s), 7.33 (3H, s).

Tetraisopropyl 2-(3,5-bis(bromomethyl)phenyl)ethane-1,1-diphosphonate (28). NaH (0.216 g, 5.4 mmol) was added at room temperature to a solution of tetraisopropyl methylenediphosphonate (1.77 g, 5.14 mmol) in 7 mL of anhydrous DMF and the suspension was stirred for 30 min under nitrogen. The resulting solution was transferred via a cannula to a solution of 1,3,5-tris(bromomethyl)benzene **27** (3.658, 10.2 mmol) in 8 mL of anhydrous DMF. The mixture was stirred for 1.25 h, quenched with a saturated solution of ammonium chloride and extracted with EtOAc (twice). The extracts were combined, washed with brine and dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by flash chromatography (silica gel, $MeOH/Ch_2Cl_2$, 0/100–2/98) to furnish bisphosphonate **28** (2 g, 63%) as a colorless oil. IR (neat) 2975, 2930, 2870, 1721, 1673, 1602, 1450, 1380, 1370 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.11 (6H, d, $J=6.3$ Hz), 1.14 (6H, d, $J=6.2$ Hz), 1.19 (12H, d, $J=6.2$ Hz), 2.37 (1H, tt, $J=24$, 6.2 Hz), 3.07 (2H, td, $J=16.4$, 6.2 Hz), 4.31 (4H, s), 4.62 (4H, m), 7.12 (3H, s); ^{13}C NMR ($CDCl_3$): δ 23.63, 23.66, 23.69, 23.73, 23.79, 23.93, 24.00, 31.17 (t, $J=4.9$ Hz), 32.66, 40.42 (t, $J=135$ Hz) 70.94 (d, $J=4$ Hz), 70.98 (d, $J=4$ Hz), 71.11 (d, $J=4$ Hz), 71.28 (d, $J=4$ Hz), 127.57, 129.76, 138.00, 141.24 (t, $J=7.5$ Hz); MS (APCI) m/z (relative intensity) 623 ($^{81}Br^{81}Br$), 621 ($^{81}Br^{79}Br$), 619 ($^{79}Br^{79}Br$) (MH^+, 58, 100, 59), 579 (53), 537 (42), 495 (35), 453 (43); MS (FAB) m/z (relative intensity) 623 ($^{81}Br^{81}Br$), 621 ($^{81}Br^{79}Br$), 619 ($^{79}Br^{79}Br$) (MH^+, 36, 71, 36), 453 (82), 371 (100); (MH^+, 46); HRMS calcd for $C_{22}H_{39}O_6P_2Br_2$ (MH^+) 619.0588, found 619.0589.

Tetraisopropyl 2-(3,5-bis(acetylthiomethyl)phenyl)ethane-1,1-diphosphonate (29). To a solution of bisphosphonate **28** (2 g, 3.2 mmol) in 12 mL of anhydrous DMF under nitrogen was added at 0 °C via a cannula a solution of potassium thioacetate (1.1 g, 9.6 mmol) in 15 mL of anhydrous DMF. The mixture was stirred for 1.5 h at 0 °C, quenched with water and extracted with EtOAc. The organic layer was dried over Na_2SO_4, filtered and evaporated. The residue was purified by flash chromatography (silica gel, $MeOH/CH_2Cl_2$, 0/100–2/98) to give bisthioacetate **29** (1.38 g, 70%) as a light-yellow oil. IR (neat) 2980, 2932, 2230, 1692, 1600 cm^{-1}; ^1H NMR ($CDCl_3$): δ 1.16 (6H, d, $J=6.2$ Hz), 1.18 (6H, d, $J=6.2$ Hz), 1.23 (12H, d, $J=6.2$ Hz), 2.26 (6H, s), 2.40 (1H, tt, $J=24$, 6.3 Hz), 3.07 (2H, td, $J=16.5$, 6.3 Hz), 4.00 (4H, s), 4.67 (4H, m), 6.95 (1H, s), 6.99 (2H, s); ^{13}C NMR ($CDCl_3$): δ 23.72, 23.75, 23.78, 23.84, 23.87, 23.90, 24.14, 30.25, 31.35 (t, $J=4.8$ Hz), 33.17, 40.59 (t, $J=134$ Hz), 70.98 (d, $J=3$ Hz), 71.01 (d, $J=3$ Hz), 71.15 (d, $J=3$ Hz), 71.28 (d, $J=3$ Hz), 127.24, 128.55, 137.62, 140.99 (t, $J=7.6$ Hz), 194.77; MS (APCI) *m/z* (relative intensity) 611 (MH^+, 100), 569 (63), 527 (58), 485 (37), 453 (31)); MS (FAB) *m/z* (relative intensity) 611 (MH^+, 100); HRMS calcd for $C_{26}H_{45}O_8P_2S_2$ (MH^+) 611.2031, found 611.2029.

2-(3,5-Bis(thiomethyl)phenyl)ethane-1,1-diphosphonic acid (30). A solution of bisthioacetate **29** (0.647 g, 1.06 mmol) in 20 mL of 6 N HCl was heated to reflux under nitrogen for 6 h and cooled to room temperature. The solution was directly concentrated under high vaccum to afford the bisthiol diphosphonic acid **30** (0.373 g, 98%) as an amorphous solid. ^1H NMR (D_2O): δ 2.46 (1H, tt, $J=23$, 6.4 Hz), 3.00 (2H, td, $J=16.6$, 6.4 Hz), 3.55 (4H, s), 7.04 (3H, s); ^{13}C NMR (D_2O): δ 28.75, 31.41, 40.47 (t, $J=126$ Hz), 126.73, 127.99, 141.46, 142.74; MS (APCI) *m/z* (relative intensity) 359 (MH^+, 85), 325 (100); HRMS calcd for $C_{10}H_{17}O_6P_2S_2$ (MH^+) 358.9941, found 358.9942.

Bis-(PGE_2)-bisphosphonate conjugate (31). To a solution of bromide **22** (103 mg, 0.144 mmol) in dioxane (1 mL) at room temperature and under nitrogen was added dropwise via a cannula a solution of thiol **30** (25.8 mg, 0.072 mmol) and triethylamine (50 μL, 0.36 mmol) in water (0.5 mL) and the solution was stirred at room temperature for 2 h and concentrated. The residue was partitioned between EtOAc and water. The aqueous layer was washed twice with EtOAc and filtered on a cation exchange (DOWEX 50 Na^+ form). The filtrate was concentrated and the residue was purified by flash chromatography (silica gel C18, MeOH/water, 0/100–60/40). The desired product came eluted in the 30% and 60% methanol/water fractions which after lyophilization gave the desired conjugate **31** (44 mg, 52%) as a light-yellow sticky solid. ^1H NMR (D_2O): δ 0.73 (6H, m), 1.17 (12H, m), 1.43 (4H, m), 1.54 (4H, m), 1.83 (4H, m), 1.98–2.20 (13H, m), 2.36 (2H, m), 2.63 (2H, dd, $J=18.5$, 7.6 Hz), 3.00 (2H, m), 3.05 (4H, s), 3.67 (4H, s), 3.98 (2H, m), 5.07–5.20 (4H, m), 5.32 (2H, m), 5.50 (2H, dd, $J=15.4$, 7.3 Hz), 5.61 (2H, dd, $J=15.4$, 8.4 Hz), 6.87 (1H, s), 7.17 (2H, s); ^{13}C NMR (D_2O): δ 14.57, 23.13, 25.33, 25.60, 26.80, 27.68, 32.03, 32.20, 33.57, 34.80, 36.57, 38.01, 42.15 (t, $J=113$ Hz), 53.41, 53.49, 55.11, 71.82, 77.50, 126.77, 128.04, 129.62, 131.99, 132.86, 134.97, 137.92, 144.66, 172.53, 183.67, 220.66; MS (FAB) *m/z* (relative intensity) 1230 ($[M+2Na]^+$, 0.8), 1208 ($[M+Na]^+$, 0.5), 379 (8), 114 (100).

In vitro binding of conjugate 3 to human bone powder. Dual-labelled conjugate **3** ($[^3H]$-PGE_2/$[^{14}C]$-alendronate) (21.64 μCi of ^{14}C and 19.05 μCi of ^3H) was placed in 1 mL 100% fetal bovine serum to yield a final concentration of 3.5 μM. 200 μM of this solution was incubated with 10 mg bone powder for 1, 2, 3 and 5 min with vigorous shaking. The mixture was centrifuged (20 s), 125 μL aliquot was taken from each sample and counted in 10 mL Atomlight in an LKB liquid scintillation counter, 125 μL of the radioactive sample was also counted at 0 time. The uptake of radioactivity into the bone powder was calculated by subtracting the dpms in the medium counted at the times indicated above from dpms at 0 time and this number was divided by the dpms at 0 time. The data demonstrated that about 76% of the ^{14}C-moiety and 53% of the ^3H-moiety were taken up by bone particles within 1 min. In a separate experiment, 77% ^3H-ABP was taken up by bone in 1 min.

In vitro dissociation of conjugate 3 from human bone powder. Dissociation of $[^3H]$-PGE_2/$[^{14}C]$-alendronate from human bone powder in fetal bovine serum at 37 °C was measured by incubating 10 mg of human bone powder with 1 μL $[^3H]$-PGE_2/$[^{14}C]$-ABP in 1 mL FBS for 5 min. The mixture was centrifuged (20 s), 100 μL aliquot was taken and counted in Atomlight in an LKB liquid scintillation counter. The rest of the 900 μL solution was withdrawn, the bone powder was washed once with 1 mL phosphate buffered saline, 1 mL fresh fetal bovine serum was added and incubated with the bone powder for 15, 24, 39, 48, 59, 79 and 103 h in a shaking bath at 37 °C. 100 μL aliquots were withdrawn at these times and counted in 10 mL Atomlight in an LKB liquid scintillation counter. The release of radioactivity from the human bone powder into the medium was calculated as follows: dpms from 100 μL of the $[^3H]$-PGE_2/$[^{14}C]$-ABP at 5 min were subtracted from dpms at 0 time. The resulting dpms reflect radioactivity taken up by bone powder. The dpms obtained by counting 100 μL aliquots at each time point were then divided by the dpms taken up by bone. 13% of the ^3H-moiety was released into the medium at 15 h and by 103 h 32.9% of the radioactivity was released into the medium. About 5% of the 3H moiety was released per day whereas the dpms of ^{14}C-moiety in the medium were not significantly changed during this time frame.

In vivo uptake and release of dual-labelled conjugate 3 and $[^3H]$-alendronate in rat tibiae and femora

Both compounds were administered i.v. via the tail vein to Sprague–Dawley female rats as a single dose of 28 nmoles of radio-labelled compound, equivalent to 0.2 μCi/animal. $[^3H]$-alendronate which was administered to nine rats corresponds to 0.1 mg/kg and $[^3H]$-PGE_2/$[^{14}C]$-ABP (dual-labelled conjugate **3**), which was administered to seven rats, corresponds to 0.24 mg/kg.

After 1, 14 or 28 days, animals were sacrificed by CO_2 and the tibiae and femora were dissected, weighed and then stored at 20 °C. The amount of radioactivity incorporated into the bone was determined by incineration in a Packard combuster after first air drying the bone for 3 days at ambient temperature. The percent of the compound retained in the skeleton at each time point was calculated on the basis of the radioactivity, converted to nmoles/gm bone on the assumption that the skeleton represents 8% of the body weight. The skeletal retention was expressed as percent administered dose.

Effect of conjugate 3 on bone resorption estimated by urinary excretion of lysylpyridinoline in the rat

Four-week old Sprague–Dawley female rats were injected i.v. via the tail vein with equimolar weekly doses of alendronate (1 mg/kg, $n=5$), conjugate **3** (2.4 mg/kg, $n=5$), PGE_2 (1.4 mg/kg, $n=5$), or saline ($n=4$) each. Filtered urine was collected after 12 and 26 days by housing individual rats in metabolic cages and providing them with food and water ad libitum. The overnight collections of urine were centrifuged at $1000\times g$ for 10 min to remove any particles and the supernatant fluid was stored at −80 °C until analysis. Lysylpyridinoline (LP) was extracted from duplicate 1 mL aliquots by acid hydrolysis and subsequent low pressure CF-1 chromatography according to the method of Beardsworth.[23] LP was further resolved by high pressure liquid chromatography according to the method of Uebelhart[24] and quantitated by comparison with an external standard. Urinary creatinine was measured using the picric acid colorimetric assay (Pharmacia Diagnostics Inc., Fairfield, NJ). Final results were expressed as pmoles LP per μmole creatinine.

In vitro binding and dissociation of conjugate 6 to human bone powder

Incubation of dual-labelled conjugate **6** (specific activity ^{3}H 4.48 mCi/nM) and ^{14}C 5.76 mCi/nM in a manner similar to studies described for conjugate **3** led to 79% uptake of tritium and 81% uptake of ^{14}C label. After uptake, incubation with fetal bovine serum, as previously described for conjugate **3**, led to 10% release of ^{3}H at 24 h and 17% release of ^{3}H at 48 h compared with 2% and 3% of ^{13}C label at the same time points.

In vivo uptake and release of dual-labelled conjugate 6 in rat tibiae and femora

Dual-labelled conjugate **6** (30.8 nmoles/rat; 0.14 μCi ^{3}H, 0.18 μCi ^{14}C) was administered in a manner as described for conjugate **3**. Analysis of tibiae and femora indicated 42.4% uptake of ^{13}C after 1 day and 40.2% after 14 days. Levels of ^{3}H observed were 0.6% after 1 day and 0.3% after 14 days.

Hydrolysis of conjugate 18 in rat plasma

In a typical experiment, a stock solution of conjugate **18** (50 μL, 18 μg, 0.02 μCi) was added to 1 mL solution of rat plasma (diluted to 50% with PBS) at 37 °C and the mixture was vortexed and incubated at 37 °C for 15 min, 1, 2 and 4 h. At each time interval, 200 μL of the incubate was pipetted into a 1 mL Eppendorf vial and diluted with 200 μL acetonitrile. The suspension was centrifuged at 14K rpm for 3 min and 200 mL of the supernatant was pipetted into the silica gel column (preconditioned with either toluene or isopropyl alcohol). The column was then eluted with 2 mL methanol and the collect solution was counted on a Beckmann 2000 β-sintillation counter. The radioactivity obtained divided by the original loading represented the percentage of hydrolysis. The same experiments were carried out using 50% boiled plasma (diluted with PBS) as control and PBS as control.

Hydrolysis of conjugates 23, 24 and 31 in rat plasma

In a set of experiments essentially as described above but utilizing 100% rat plasma ^{3}H-labelled conjugates **23** (36 μg, 0.1 μCi), **24** (38 μg, 0.1 μCi) and **31** (62 μg, 0.2 μCi) were incubated in fresh heperinized rat plasma at 37 °C. Aliquots (100 μL) were worked up as before and the eluted ^{3}H-label counted.

Characterization of ^{3}H liberated on hydrolysis of conjugate 31

A stock solution of 0.4 μCi conjugate **31** or [^{3}H]-PGE_2 (0.4 μCi) was incubated in either fresh rat plasma, boiled plasma or PBS (mL) at 37 °C. After 4 h or 24 h, 100 μL aliquots were removed, diluted with acetonitrile (100 μL), vortexed and centrifuged. 100 μL of supernatant was separated by HPLC (C-18, 0.5% HOAc in water, 66%: acetonitrile 33%, 1 mL/min) with effluent monitored by an on-line scintillation detector and UV detector. No radioactivity was eluted under these conditions when 0.2 μCi, conjugate **31** (62 μg) was applied. (It was necessary to mix 0.4 mg of unlabelled **31** with 0.1 μCi labelled **31** to recover 0.05 μCi from the HPLC.) Radioactive peaks were identified by coelution with authentic [^{3}H]-PGE_2 and cold PGA_2. An authentic sample of PGB_2 was prepared by incubating PGE_2 (2.4 mg) with 1 mL rat plasma at 37 °C for 24 h. The sample, purified by HPLC, had appropriate ^{1}H NMR, UV and MS. To identify radioactive peaks eluting at the solvent front with incubation of conjugate **31** for 24 h in fresh rat plasma, the fraction was collected and distilled. The collected distillate had 60% of the initial counts.

Binding of conjugates [^{3}H]-23 and [^{3}H]-31 to human bone powder and release of label

Studies were carried out essentially as described previously for conjugate **3** using 0.1 μCi conjugate per 10 mg bone powder.

In vivo uptake and release of [^{3}H]-23 and [^{3}H]-31 in rat tibiae and femora

In vivo studies were carried out essentially as previously described for conjugate **3** with Sprague–Dawley rats dosed at 1 mg/kg, ~0.3 μCi/rat. In vivo assay of

conjugate **23** in a rat model of osteoporosis. Briefly, three month old Sprague–Dawley rats were ovariectomized and were kept for 8 weeks prior to the start of treatment to allow the development of osteopenia. Treatment groups received 10 or 100 mg/kg **23**, iv (see Table below). Control groups included: an ovariectomized vehicle treated group, a sham operated non-ovariectomized group, group 4 receiving equimolar doses of non-conjugated bisphosphonate (**26**) plus PGE_2, and group 5 PGE_2 alone. All animals were treated for 4 weeks.

1. Ovx	vehicle tx	saline i.v.	1×per week
2. Ovx	**23**	100 mg/kg i.v.	1×per week
3. Ovx	**23**	10 mg/kg i.v.	1×per week
4. Ovx	**26** + PGE_2	~5 mg/kg each i.v.	1×per week
5. Ovx	PGE_2	6 mg/kg s.c.	5×per week
6. Sham–Ovx	vehicle tx	saline s.c.	5×per week

Animals received the fluorescent bone label calcein (20 mg/kg ip) 14 and 4 days prior to sacrifice. Femora, tibiae and vertebrae were removed and fixed in 70% EtOH. The femoral bone mineral content (BMC) was measured using a HOLOGIC QDR 4500A X-ray densitometer. Femoral length was also measured. Tibiae were processed without decalcification through increasing concentrations of EtOH and embedded in methylmethacrylate using a Hypercenter XP tissue processor. Five micron thick Masson's Trichrome stained sections were used to measure the following static histomorphometric variables of cancellous bone structure. Bone volume/tissue volume (BV/TV, %), trabecular number (Tb.N, #/mm), trabecular thickness (TbTh., μm), trabecular separation (TbSp., μm) were measured or calculated directly from primary measurements of tissue area, trabecular bone area, and trabecular bone perimeter. Ten-micron thick sections were coverslipped unstained for dynamic fluorochrome label measurements. Viewed under epifluorescence the length of calcein labeled bone surfaces and the interlabel distances were measured. The mineralizing surface (MS/BS, %) was calculated as one-half the length of single labeled surface plus the length of the double labeled surface expressed as a percentage of total bone surface. This measures the relative amount of bone surface undergoing formation. The mineral apposition rate (MAR, μm/day) was calculated as the mean of equidistant points between the first and second label divided by the labeling interval (14 days) and estimates the cell based formation rate. Bone formation rate surface referent (BFR, BS, $\mu m^3/\mu m^2$/year) or the estimated 3D volume of bone formed per measured 2-D bone area was calculated as the product of mineral apposition rate (MAR) and the mineralizing surface (MS) expressed per year.

The anti-resorptive effect of **26**, the bisphosphonate core of **23** was also evaluated using the growing rat model (Schenk Assay). Using this model rats were treated sc for ten days at 0, 3, or 30 mg/kg. After necropsy, femora are measured for length and incinerated at 700 °C for 24 h. Inhibition of bone resorption in long bones (femur) of growing rats results in increased bone mineral content measured as femoral ash weight corrected for length (mg/mm).

Statistical analysis was done using the Statview (Macintosh) package. Differences between two groups were tested using Students-*t* test. With three or more groups, differences were tested using one-way analysis of variance (ANOVA). If significance was found, the differences in group means were tested using the Fisher PLSD with a $P < 0.05$ considered significant.

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