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Design and synthesis of novel bone-targeting dual-action pro-drugs for the treatment and reversal of osteoporosis

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ABSTRACT

There is an important medical need for effective therapies to redress the general bone loss associated with advanced osteoporosis. Prostaglandin E_2 and related EP4 receptor agonists have been shown to stimulate bone regrowth but their use has been limited by systemic side effects. Herein is described the design and synthesis of novel dual-action bone-targeting conjugate pro-drugs where two classes of active agents, a bone growth stimulating prostaglandin E_2 EP4 receptor subtype agonist (**5** or **6**) and a bone resorption inhibitor bisphosphonate, alendronic acid (**1**), are coupled using metabolically labile carbamate or 4-hydroxyphenylacetic acid based linkers. Radiolabelled conjugates **9**, **11a**/**b** and **25** were synthesized and evaluated in vivo in rats for uptake of the conjugate into bone and subsequent release of the EP4 agonists over time. While conjugate **11a**/**b** was taken up (9.0% of initial dose) but not released over two weeks, conjugates **9** and **25** were absorbed at 9.4% and 5.9% uptake of the initial dose and slowly released with half-lives of approximately 2 weeks and 5 days respectively. These conjugates were well tolerated and offer potential for sustained release and dual synergistic activity through their selective bone targeting and local release of the complimentary active components.

1. Introduction

Osteoporosis is a degenerative disease of the bone resulting from an imbalance between the bone-resorptive action of osteoclasts and the bone-generating action of osteoblasts. As a disease of the elderly, this imbalance leads to a slow loss of bone density and a greatly increased risk of bone fracture in this population.

The treatment of osteoporosis can be accomplished in two ways: by inhibiting the rate of bone resorption or enhancing the rate of growth of new bone. The standard antiresorptive treatments for this disease are bisphosphonate drugs such as alendronic acid **1**, RANK ligand inhibitors such as denosumab and selective estrogen receptor modulators (SERMs). A new class of antiresorptive agent, cathepsin-K inhibitors such as odanacatib **2**[1] (Fig. 1), are in late stage development and may offer improved antiresorptive therapy. The bisphosphonate functionality in alendronate mimics the structure of pyrophosphate, both of which chelate strongly with calcium. As bone is the largest reservoir of calcium in the human body bisphosphonates are site-selective for accumulation there, and bisphosphonate antiresorptives tend to have very long half-lives because of this.

While these therapies represent vital tools in treating osteoporosis by halting further progression of the disease, it is normally only treated pharmacologically after considerable bone loss has already taken place. Thus, it is also of interest to stimulate the growth of new bone in place of that which has been lost. To this end, parathyroid hormone can be used to stimulate bone growth but its use has been limited as treatment requires a daily subcutaneous injection and it has been associated with osteosarcoma.[2] It has also been shown that prostaglandin E_2 (PGE_2, **3**) can induce bone growth in animals and humans when given via systemic injection[3] or site-specific delivery to bone[4] and studies have indicated that this activity is mediated by activation of the PGE_2 EP4

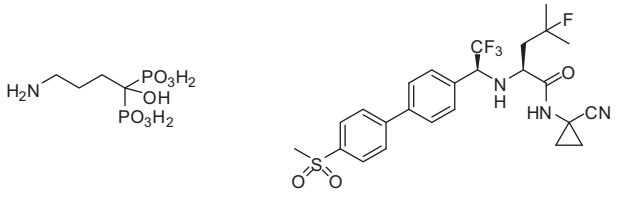

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Figure 1. Inhibitors of bone resorption, alendronic acid (**1**) and odanicatib (**2**).

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receptor subtype.[5] However, the use of PGE_2 as a therapeutic agent is limited by metabolic instability, an extremely short half-life in vivo and intolerable side effects (at least in part due to its action on the other EP receptors (EP1, EP2 and EP3)).

We originally postulated that conjugation of PGE_2 through metabolically hydrolysable linkers to bisphosphonate antiresorptive compounds might mitigate the issues seen with PGE_2 dosing by taking advantage of the bone-directing effects of the bisphosphonate, allowing for the slow release of PGE_2 at its site of action and thus limiting systemic exposure. The use of bisphosphonates to target drugs to bone has been widely explored[6] and in particular to deliver antitumor agents to bone.[7] In our previous studies some success was achieved in preparing conjugates (for example, compound **4**, Fig. 2) using novel but biologically ineffective bisphosphonate carriers. Compound **4** was shown to be well tolerated in rats (up to 100 mg/kg), to be preferentially taken up into bone, to slowly release the PGE_2 component and to provide enhanced efficacy in promoting bone growth relative to an equivalent dose of PGE_2 itself.[4] However, owing to the instability of PGE_2 we were unable to prepare conjugates with the preferred, bioactive bisphosphonates such as alendronic acid with the intention of generating a compound with dual bone resorption inhibitor/bone growth stimulating properties. It was thought that preparation of these dual action conjugates would require a new synthetic approach to generating the conjugates and/or more stable analogs of PGE_2.

Subsequently a series of more stable and selective EP4 receptor agonists (exemplified by compounds **5**[8] and **6**[9]) (Fig. 2) were identified that are at least as potent and efficacious as PGE_2 at the EP4 receptor. Unfortunately, while EP4-selective agonists were found to be effective in stimulating bone growth in rats[10], they still retained some undesirable gastrointestinal side effects making them likely unsuitable for development as chronic therapies for osteoporosis. Compounds such as **5** and **6** are much more chemically stable and accessible than PGE_2 and they have considerably longer systemic half-lives in vivo and thus they could meet the above-noted conditions necessary to prepare the next generation of bone-targeting conjugate.

The goal of this work was to prepare conjugates of both the EP4 agonist acid **5** and tetrazole **6** with the bioactive bisphosphonate alendronic acid **1** and to evaluate their in vivo uptake and release from bone. Such conjugates would need to be relatively stable in the blood steam, survive intact in the systemic circulation long enough to bind efficiently with bone and then to release the two active components slowly over time. Our preferred half-life for release was on the order of 4–7 days, similar to that previously found for the efficacious conjugate **4** and so that once weekly dosing could be supported. The conjugates themselves would need to be well tolerated and compatible with intravenous (IV) dosing.

2. Results and discussion

The obvious points of connection to attach **5** or **6** to **1,** through a slowly hydrolysable linker that will liberate the EP4 agonists intact, are through the C-15 hydroxyl of either compound, the acid moiety of **5** or the tetrazole moiety of **6**. It is known (and demonstrated in the previous pro-drug studies) that the acidic functionality and free 15-(*R*)-hydroxyl group are *both* required for biological activity at the EP receptors[11] and thus the conjugates themselves were not anticipated to manifest any EP activity in vivo prior to cleavage. As our previous study demonstrated[4] that a simple amide coupling between the PGE_2 and bisphosphonate **1** yielded a conjugate that was too robust to allow for liberation of PGE_2 once bound to bone, we began a program to prepare carbamate based linkers that should be chemically stable but would hopefully undergo faster cleavage in vivo.

A conjugate linked through the C-15 hydroxyl of **5** was prepared from ester **7**, first by generating a 15-*p*-nitrophenylcarbonate and then reacting it with the tetra-*n*-butylammonium salt of alendronic acid to give **9** (Scheme 1). Notably, while alendronate itself has little or no solubility in any solvent other than water, the mono-tetra-*n*-butylammonium salt is freely soluble in dry DMF which allows one to work in non-aqueous environments. The ethyl ester of **7** (or a conjugate derived from **7**) was expected to hydrolyze rapidly to liberate the free carboxylic acid upon exposure to rat plasma esterases to liberate the free acid in vitro and in vivo and this was confirmed by model incubation of **7** in rat plasma. The novel tetrazole conjugate **11** was synthesized by exposing the mercury salt of **6** to iodomethyl-4-nitrophenylcarbonate[12] to form the methylene carbonate as a mixture of separable regioisomers (**10a** and **10b**), favouring the 2,5-disubstituted tetrazole. Reaction with tetra-*n*-butylammonium alendronate gave conjugates **11a** and **11b** (Scheme 2). Purification of these conjugates was tedious, requiring careful extraction, ion-exchange chromatography to remove the tetra-*n*-butylammonium ion and final isolation by reverse-phase chromatography. While the compounds were initially prepared and characterized separately, the conjugates **11a** and **11b** were subsequently prepared and evaluated as the mixture.

These conjugates were first examined for their stability in rat plasma. If they were not sufficiently robust to survive delivery to bone after IV injection they clearly would not be suitable candidates for further development. 100 μg/mL samples of conjugate were incubated in fresh rat plasma at 37 °C. Aliquots were removed after various time periods and were diluted with equal volumes of acetonitrile and centrifuged to remove precipitated proteins and the concentrations of liberated EP4 agonists **5** and **6** was monitored using LC/MS. It was found that the C-15 hydroxyl conjugate **9** was stable over 96 h under these conditions while tetrazole conjugate **11** was liberated slowly (ca. 5% after 96 h). Both conjugates hydrolyzed completely to liberate **5** or **6** respectively when treated at pH 10 for a few minutes. As it was expected that uptake into bone would be relatively facile should they survive in the circulatory system, both conjugates were taken forward for in vivo evaluation.

In order to measure the in vivo uptake of the conjugates and the release of the EP4 agonists from bone it was necessary to radiolabel the conjugates. By dosing a conjugate of known specific activity labelled on the EP4 agonist portion, the initial uptake into bone and

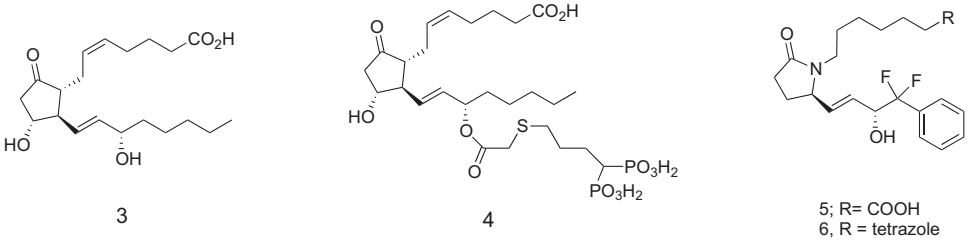
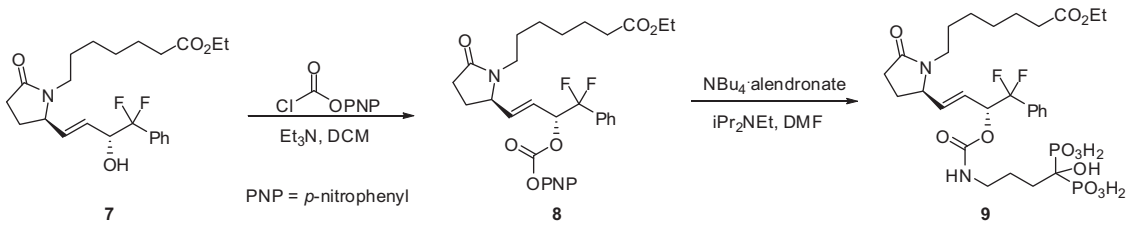
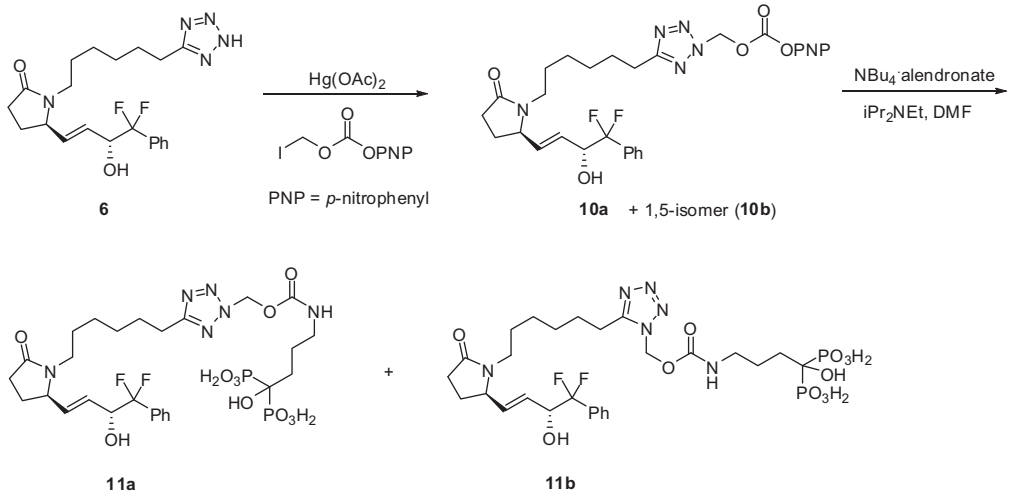


Figure 2. Prostaglandin E_2 (PGE_2) (**3**), PGE_2-bisphosphonate conjugate **4** and EP4 agonists **5** and **6.**



Scheme 1. Preparation of conjugate **9**.



Scheme 2. Preparation of conjugates **11a** and **11b**.

its subsequent decrease upon hydrolysis of the EP4 agonist can be assessed by excising the bones from the animals and directly measuring the radioactivity contained within. It has been well established that bisphosphonates bind rapidly and almost irreversibly with bone and bound radioactivity could be taken to indicate bound conjugate. Upon hydrolysis, the label would be liberated, the radioactivity would diminish and the alendronate would remain bound to the bone. While tritium labelled **6** was available as a kind gift from Merck, we prepared [^{3}H]-**7** ourselves according to a previously published procedure.[13]

The radiolabelled EP4 agonists were elaborated to their respective conjugates [^{3}H]-**9** and [^{3}H]-**11** following the routes described for the unlabelled compounds.

Our initial in vitro experiments with [^{3}H]-**9** and [^{3}H]-**11** determined that both conjugates bind quickly and efficiently to bone powder with upwards of 90% binding after 10 min. As expected, free **5**,**6** and **7** showed no significant affinity for bone powder in comparable control experiments. With this information, conjugates [^{3}H]-**9** and [^{3}H]-**11** were then studied for in vivo uptake of the compounds into bone and the subsequent release of the labelled EP4 agonist using rat models. These in vivo experiments were performed by administering the labelled conjugates to female Sprague-Dawley rats via IV injection. After dosing, blood, urine and feces samples were collected and analyzed for radioactivity and at various time intervals thereafter to determine rate and route of elimination of *unbound* conjugate. After extended periods of time (from 6 h up to 4 weeks) the animals were euthanized and the uptake and subsequent release of the conjugates was determined by excising the long bones and measuring radioactivity using a biological oxidiser.

Both conjugates **9** and **11** were well tolerated (dosed at 5 mg/kg and 2.5 mg/kg respectively) and radioactivity was shown to be rapidly cleared from plasma with elimination half-lives of <15 min. As exemplified by **9**, most of the applied doses were excreted in feces during 24 h after dosing with only a small amount found in urine. About 9% of the initial dose of tetrazole conjugate [^{3}H]-**11** was found to be taken up into bone after 24 h. However, subsequently and for up to 14 days, the level of bone labelling remained essentially constant indicating that none of the labelled **6** was liberated from the adsorbed conjugate. The C-15 hydroxyl conjugate [^{3}H]-**9** was similarly taken up, with 24 h bone samples showing a bone labelling equivalent of 9.4% of the initial dose. However, in contrast to [^{3}H]-**11,** the [^{3}H]-**9** conjugate showed a slow release of the label with about 50% lost over 28 days (Fig. 3). These data would predict

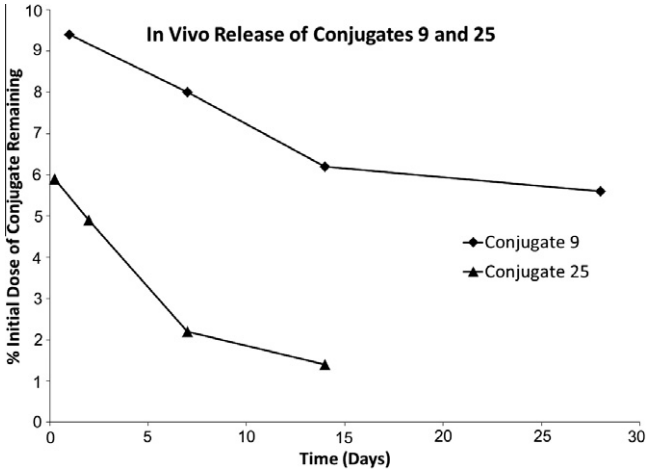


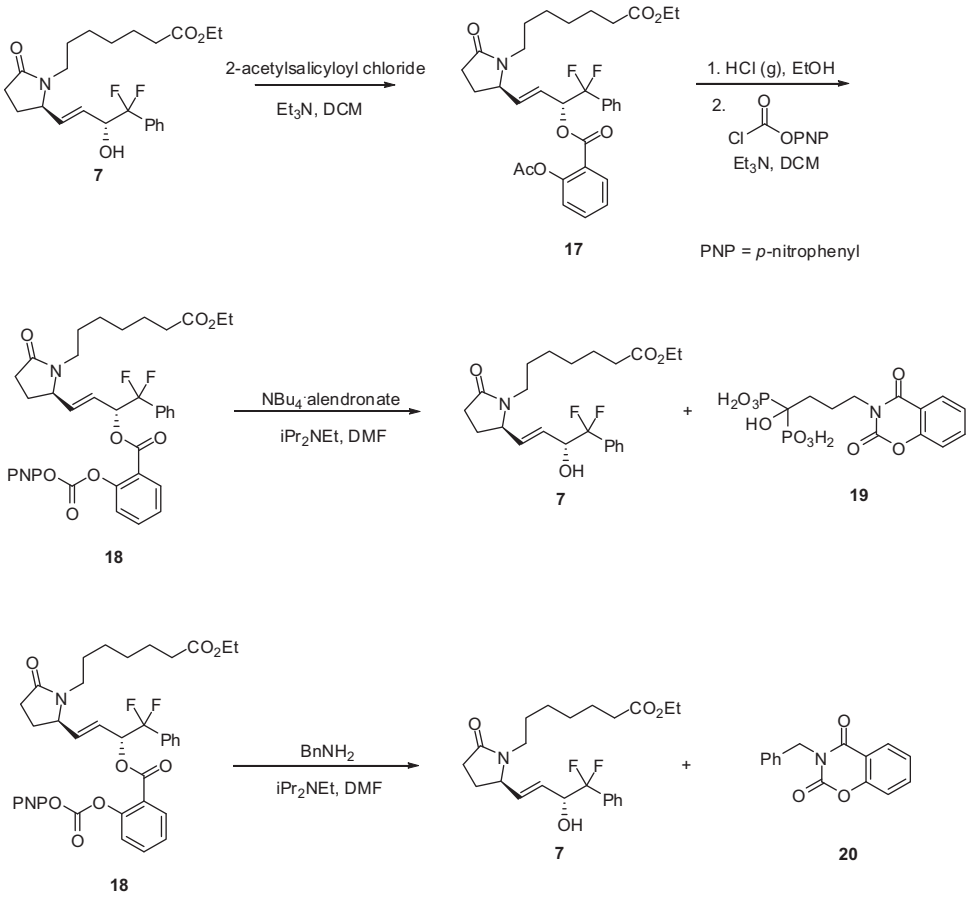
Figure 3. Uptake and release of radiolabelled conjugates **9** and **25** into long bones of SD rats.

sustained release of **5** at a rate of ca. 5 μg/kg/day. While this result was encouraging, this was about two to three times less than the calculated sustained release dose of PGE_2 (calculated to be ca. 12 μg/kg/day) that was shown to be efficacious in earlier experiments.[4] As we were seeking to minimize the dose level needed for efficacy, we felt that the higher rate of release that a half-life of about 7 days would provide would be preferable and so we turned our attention towards developing conjugates that would be designed to be somewhat more labile than **9**.

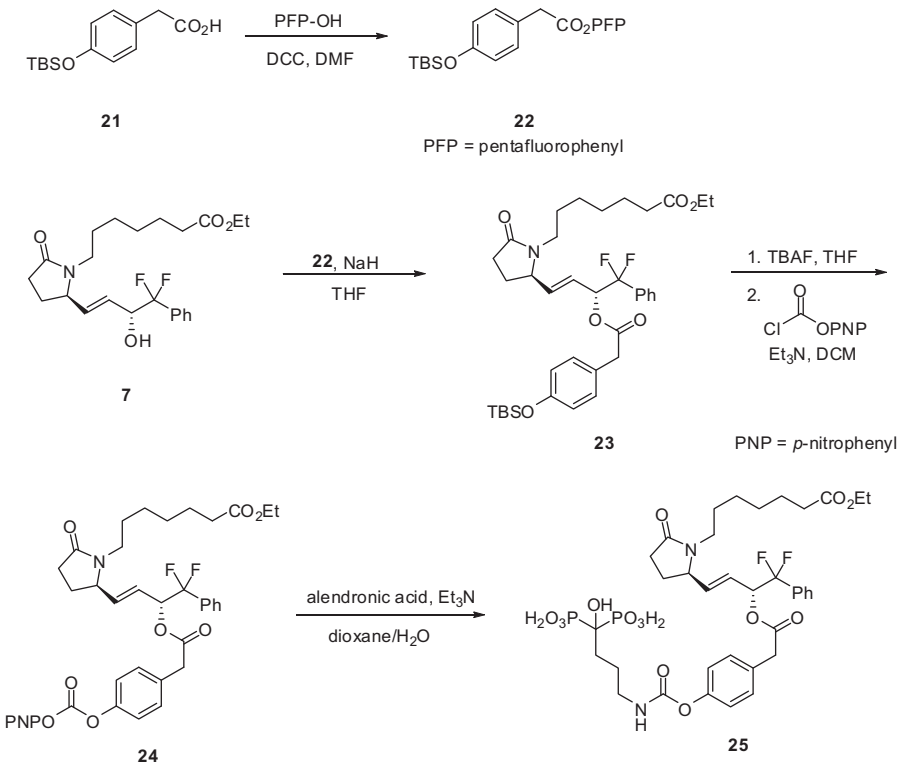
While one can envision many linkers that might fill this role, the C-15 hydroxyl linked esters had been shown to be hydrolyzed in vivo at an acceptable rate in the earlier studied series of PGE_2/inactive bisphosphonate conjugates (such as in conjugate **4**).[4] Though acylation of the C-15 hydroxyl group should be readily achievable, most reaction conditions would not be compatible with free bisphosphonates which are notoriously difficult to manipulate and purify due to high polarity and poor solubility. The use of protected bisphosphonates was not preferred due to the relatively harsh conditions necessary to de-protect such compounds. This implied that the coupling with the bisphosphonate would ideally be designed to use free alendronate at a late or preferably, the last step in the preparation of these new conjugates after an ester-based linking fragment was first attached to the C-15 hydroxyl of the EP4 agonist. A bifunctional linker unit was thus required. This linkage unit must be hydrolysable to liberate both the free EP4 agonist and the free primary amine of alendronic acid. It is known that phenolic carbamates are much more labile in vivo than are alkyl carbamates and indeed there are a number of carbamate prodrugs of phenols (e.g. bambuterol) that are successfully marketed drugs. Also, any linker unit should liberate components that would be expected (or are known) to be non-toxic. Thus a phenolic acid such as salicylic acid seemed ideal where the EP4 agonist could be coupled to the carboxyl function and the alendronate to the phenolic functionality.

To this end we attempted to prepare a linkage based on salicylic acid as it has a well-known safety profile. Acylation of **7** proceeded smoothly to give **17**. De-acetylation of **17** and subsequent generation of carbonate **18** also proceeded as expected. Unfortunately when we attempted to prepare the desired acetylsalicylic acid-based conjugate using the conditions developed for the first generation of conjugates, analysis of the crude reaction mixture (^1H NMR, MS) suggested we generated compound **7** and bisphosphonate **19** and not the desired product (Scheme 3). This unproductive pathway is presumably a result of initially forming the desired conjugate followed by reaction of the carbamate nitrogen with the C-15 ester, which upon collapse of the tetrahedral intermediate expulses **7** and cyclized bisphosphonate **19**. This reaction manifold was corroborated by the observation that replacing alendronic acid with benzylamine afforded an easily identified, analogous cyclization product **20**.

While this was a disappointing observation, we were able to avoid this problem by instead using a linkage unit that could not undergo such a cyclization. We chose 4-hydroxyphenylacetic acid, which is a common natural compound found in foods such as olive oil. The15-substituted ester derived from **5** or **6** and 4-hydroxyphenylacetic acid would be expected to be hydrolyzed at a rate similar to that observed for the earlier conjugate **4**. Hydrolysis of the alendronate from the 4-phenolcarbamate of the putative conjugate



Scheme 3. Attempted synthesis of a salicylic acid linked conjugate.



Scheme 4. Preparation of conjugate **25**.

was expected tobe a relatively facile process. The synthesis of this linker began by preparing the pentafluorophenyl-activated ester **22** from known compound **21**.[14] Acylation of the C-15 hydroxyl of **7** again proceeded smoothly to afford **23**, which was converted to carbonate **24** in 2 steps. Using an alternative set of coupling conditions that greatly facilitate purification of the final bisphosphonate products, we were pleased to see that the desired conjugate was isolated in high yield as triethylamine salt **25** (Scheme 4).

With a second generation conjugate in hand the stage was set for its' in vitro and in vivo evaluation for uptake and release from bone. After initially determining that conjugate **25** was sufficiently stable for IV injection (6% hydrolysis after 24 h in rat plasma at 37 °C) it was dosed into rats following the protocol used for our first round of in vivo testing. Although the uptake of **25** into bone was not quite as efficient as seen with conjugates **9** and **11** (5.9% uptake 6 h after dosing), our hypothesis that the 4-hydroxyphenyl-acetic acid based linkage would provide a more labile system was confirmed when we found the bound radiolabelled EP4 agonist was released with a half-life of approximately 5 days (see Fig. 3). This allows calculation that a 5 mg/kg dose of **25** should provide sustained release of **5** at a rate of ca. 15 μg/kg/d or about three times more than achieved with conjugate **9** for the same dose and comparable to the rate of release of PGE_2 previously shown to be efficacious in an OVX rat model of reversal of bone loss.[4] While this experiment does not directly demonstrate the rate at which the 4-hydroxyphenyl-acetic acid moiety is hydrolyzed from the bisphosphonate moiety, we are confident that considering the known lability of phenolic carbamates and the extremely long half-life of alendronic acid when bound to bone, the anti-resorptive effects of the bisphosphonate should also be observed via slow release.

In conclusion, we have developed a versatile method for the preparation of dual action bone targeting pro-drug conjugates by coupling the primary amino group of anti-resorptive bisphosphonates such as alendronic acid **1** and hydroxyl containing EP4 receptor selective agonists such as **5** or **6**. While the first iteration of carbamate-based conjugates proved too stable in vivo the interposition of a dual-functional coupling group, 4-hydroxyphenylacetic acid proved successful. Scale-up of the synthesis of the optimized conjugate **25** is underway and results of longer term in vivo bone growth experiments to determine ultimate biological efficacy of this compound compared to the separate components in established models on bone growth stimulation will be reported in the near future.

3. Experimental section

3.1. General methods

^{1}H and ^{13}C NMR spectra were recorded with a Bruker Avance II 600 MHz spectrometer using a TCI cryoprobe, an Avance III 500 MHz spectrometer using a TXI inverse probe, or an Avance III 400 MHz spectrometer using a BBOF + ATM probe. ^{31}P NMR spectra were recorded on the Avance 600 MHz spectrometer referenced to inorganic phosphate (external). All assignments were confirmed with the aid of two-dimensional ^{1}H, ^{1}H (COSY) and ^{1}H, ^{13}C (HSQC) experiments. Processing of the spectra was performed with Mestrelab Research MestRecNova version 6.0.4-5850 software. The high-resolution mass spectra were recorded in positive ion-mode with an ESI ion source on an Agilent Time-of-Flight LC/MS mass spectrometer. Analytical thin-layer chromatography (TLC) was performed on aluminum plates pre-coated with silica gel 60F-254 as the adsorbent. The developed plates were air-dried, exposed to UV light and/or dipped in $KMnO_4$ solution and heated. Column chromatography was performed with silica gel 60 (230–400 mesh).

All animal experiments were carried out in compliance with animal care guidelines and policies of the Canadian Council on Animal Care and under protocols approved by the Simon Fraser University Animal Care Committee.

3.2. Ethyl 7-((*R*)-2-((*R*,*E*)-4,4-difluoro-3-((4-nitrophenoxy)-carbonyloxy)-4-phenylbut-1-enyl)-5-oxopyrrolidin-1-yl)-heptanoate (8)

Alcohol **7** (216 mg, 0.510 mmol, 1.0 equiv) was dissolved in dichloromethane (5 mL), cooled to 0 °C and treated with 4-nitrophenylchloroformate (113 mg, 0.561 mmol, 1.1 equiv) and triethylamine (140 μL, 1.00 mmol, 2.0 equiv). The reaction mixture was stirred while warming to room temperature over 2 h, then at room temperature for 14 h after which it was quenched with saturated aqueous NH_4Cl. The layers were separated and the aqueous phase was extracted with dichloromethane (3×). The organic layers were combined, dried over $MgSO_4$, filtered and concentrated and the product was isolated by flash chromatography (EtOAc/hexanes) to afford compound **8** (256 mg, 0.435 mmol, 85%, R_f = 0.40 in 70% EtOAc/hexanes) as a yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ = 8.26 (d, J = 9.2 Hz, 2H), 7.52–7.50 (m, 3H), 7.48–7.45 (m, 2H), 7.28 (d, J = 9.2 Hz, 2H), 5.73 (qd, J = 15.5, 7.1 Hz, 2H), 5.59 (td, J = 10.2 Hz, 6.7 Hz, 1H), 4.11 (q, J = 7.1 Hz, 2H), 4.07 (td, J = 7.8, 5.1 Hz, 1H), 3.47 (ddd, J = 13.8, 8.6, 7.2 Hz, 1H), 2.61 (ddd, J = 13.7, 8.6, 5.2 Hz, 1H), 2.41–2.29 (m, 2H), 2.27 (t, J = 7.5 Hz, 2H), 2.24–2.18 (m, 1H), 1.72–1.66 (m, 1H), 1.62–1.57 (m, 2H), 1.44–1.28 (m, 4H), 1.25–1.20 (m, 2H), 1.24 (t, J = 7.1 Hz, 3H).

^{13}C NMR (150 MHz, $CDCl_3$) δ = 174.7 (C_4), 173.8 (C_4), 155.2 (C_4), 151.5 (C_4), 145.7 (C_4), 139.4 (CH), 133.1 (t, $J_{C,F}$ = 25.3 Hz, C_4), 131.0 (CH), 128.8 (CH), 126.0 (CH, $J_{C,F}$ = 6.2 Hz, CH), 125.5 (CH), 122.8 (CH), 121.7 (CH), 119.1 (t, $J_{C,F}$ = 249.2 Hz, C_4), 79.2 (t, $J_{C,F}$ = 32.5 Hz, CH), 60.4 (CH_2), 59.7 (CH), 40.6 (CH_2), 34.3 (CH_2), 29.9 (CH_2), 28.9 (CH_2), 27.2 (CH_2), 26.6 (CH_2), 25.2 (CH_2), 24.9 (CH_2), 14.4 (CH_3). HRMS (ESI): Found $(M+H)^+$ at 589.2389 for $C_{30}H_{35}F_2N_2O_9$. Calculated at 589.2361.

3.3. Tetrabutylammonium alendronate synthesis

A suspension of alendronic acid (1.50 g, 6.02 mmol, 1.0 equiv) in deionized water (10 mL) was treated with tetrabutylammonium hydroxide ($NBu_4OH{\cdot}30\ H_2O$, 4.82 g, 6.03 mmol, 1.0 equiv). The resulting mixture was stirred at room temperature for 2 h, after which all the reagents had dissolved to give a clear colorless solution. The water was removed by lyophilization to afford tetrabutylammonium alendronate (NBu_4 alendronate·5.5 H_20) as a hygroscopic white solid (3.54 g, 100%). ^{1}H NMR (MeOH-d_4) δ = 3.28–3.24 (m, 8H), 3.01 (t, J = 6.3 Hz, 2H), 2.17–2.08 (m, 4H), 1.72–1.64 (m, 8H), 1.44 (quintuplet, J = 7.4 Hz, 8H), 1.05 (t, J = 7.4 Hz, 12H).

3.4. 4-(((*R*,*E*)-4-((*R*)-1-(7-ethoxy-7-oxoheptyl)-5-oxopyrrolidin-2-yl)-1,1-difluoro-1-phenylbut-3-en-2-yloxy)carbonylamino)-1-hydroxybutane-1,1-diphosphoric acid (9)

Carbonate **8** (26.3 mg, 0.0447 mmol, 1.0 equiv) was dissolved in DMF (0.5 mL) and treated with a solution of NBu_4 alendronate· 5.5 H_20 (26.4 mg, 0.0447 mmol, 1.0 equiv) and iPr_2NEt (distilled over CaH_2, 38.9 μL, 0.224 mmol, 5.0 equiv) in DMF (0.5 mL). The reaction was stirred at room temperature for 2 h, after which the solvent was evaporated. The crude reaction product was taken up in to 1:1 EtOAc/H_2O and the pH of the aqueous phase was adjusted to 3. The layers were separated and the aqueous phase was extracted with EtOAc (4×). The remaining aqueous phase was passed through an ion exchange column (Amberlite IR-120 ion exchange resin, H^+ form) and the resulting aqueous solution was lyophilized to give a yellow solid. This solid was purified by reverse phase chromatography (C18 Sep-Pak cartridge, gradient elution from H_2O to MeOH), giving **9** (10.2 mg, 0.0146 mmol, 33%) as a white solid. ^{1}H NMR (600 MHz, MeOH-d_4) δ = 7.52–7.45 (m, 5H), 5.75–5.62 (m, 3H), 4.17 (dd, J = 13.8, 8.0 Hz, 1H), 4.11 (q, J = 7.1 Hz, 2H), 3.37–3.32 (m, 2H), 3.12–3.05 (m, 2H), 2.71 (ddd, J = 13.7, 8.4, 5.3 Hz, 1H), 2.36–2.33 (m, 2H), 2.30 (t, J = 7.5 Hz, 2H), 2.22 (ddd, J = 16.9, 14.1, 8.4 Hz, 1H), 2.06–1.98 (m, 2H), 1.91–1.82 (m, 2H), 1.73–1.64 (m, 1H), 1.60 (p, J = 7.6 Hz, 2H), 1.48–1.29 (m, 5H), 1.26–1.22 (m, 2H), 1.23 (t, J = 7.1 Hz, 3H). Phosphonate protons were not observed. ^{31}P NMR (243 MHz, D_2O) δ = 21.9 (s).

HRMS (ESI): Found $(M+Na)^+$ at 721.2104 for $C_{28}H_{42}F_2N_2O_{12}P_2$-Na. Calculated at 721.2079.

3.5. (5(6-((*R*)-2-((*R*,*E*)-4,4-difluoro-3-hydroxy-4-phenylbut-1-enyl)-5-oxopyrrolidin-1-yl)hexyl)-2H-tetrazol-2-yl)methyl 4-nitrophenylcarbonate (10a, 10b)

Tetrazole 6 (331 mg, 0.75 mmol, 1.0 equiv) and $Hg(OAc)_2$ (221 mg, 0.38 mmol, 0.5 equiv) were dissolved in acetonitrile (17 mL) under an atmosphere of argon and the mixture was stirred at room temperature for 2 h. The solvent was evaporated and the mixture was placed under high vacuum for 3 h. A further aliquot of acetonitrile (10 mL) was added followed by stirring (4 h), evaporation and pumping under high vacuum for 18 h. The resulting compound was taken up in 1,2-dichloroethane (25 mL) and treated with iodomethyl 4-nitrophenyl carbonate (244 mg, 0.75 mmol, 1.0 equiv). This mixture was heated at reflux for 24 h after which the reaction was cooled and the solvent was evaporated. Compound **10a** (305 mg, 66%) and its 1,5-regioisomer **10b** (107 mg, 23%) were isolated by gradient column chromatography using hexanes and ethyl acetate on silica in a total yield of 81%. Compound 10a characterization data: ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.29 - 8.31 (d, 2H), 7.43–7.46 (m, 5H), 7.41–7.43 (d, 2H), 6.56 (s, 2H), 5.61–5.70 (m, 2H), 4.51–4.62 (m, 1H), 4.00–4.06 (m, 1H), 3.35–3.44 (m, 1H), 2.90–2.97 (t, 2H), 2.70–2.76 (m, 1H), 2.25–2.40 (m, 2H), 2.16–2.21 (m, 1H), 1.76–1.84 (m, 2H), 1.57–1.68 (m, 2H), 1.35–1.45 (m, 4H), 1.15–1.32 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 174.8, 168.2, 154.8, 151.0, 145.8, 135.2, 133.7 (t, $J_{C,F}$ = 25.5 Hz), 130.3, 128.3,127.5, 125.9 (t, $J_{C,F}$ = 6 Hz), 125.4, 121.6 , 120.7 (t, $J_{C,F}$ = 246 Hz), 74.3(t, $J_{C,F}$ = 32 Hz), 74.2, 60.1, 40.5, 30.0, 28.5, 27.5, 27.0, 26.3, 25.4, 25.3.

Compound **10b** (more polar) characterization data:^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.27–8.31 (d, 2H), 7.4–7.5 (m, 5H), 7.35–7.38 (d, 2H), 6.35 (s, 2H), 5.60–5.75 (m, 2H), 4.53–4.62 (m, 1H), 4.00–4.05 (m, 1H), 2.96–3.00 (t, 2H), 2.75–2.85 (m, 2H), 2.3–2.8 (m, 2H), 2.2–2.25 (m, 1H), 2.15–2.24 (m, 1H), 1.83–1.90 (m, 2H), 1.58–1.70 (m, 1H), 1.35–1.50 (m, 4H), 1.15–1.35 (m, 2H). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 175.1, 156.4, 154.6, 151.2, 145.9, 135.0, 133.8 (t, $J_{C,F}$ = 25 Hz), 130.3, 128.3, 127.8, 125.9 (t, $J_{C,F}$ = 6 Hz), 125.5, 121.6, 120.7 (t, $J_{C,F}$ = 134 Hz), 74.3 (t, $J_{C,F}$ = 32 Hz), 69.8, 60.3, 40.4, 30.0, 28.3, 26.8, 26.6, 26.0, 25.4, 22.8.

3.6. 4-(((5-(6-((*R*)-2-((*R*,*E*)-4,4-difluoro-3-hydroxy-4-phenylbut-1-enyl)-5-oxopyrrolidin-1-yl)hexyl)-2*H*-tetrazol-2-yl)methoxy)-carbonylamino)-1-hydroxybutane-1,1-diyldiphosphoric acid (11a)

Compound **10a** (40 mg, 0.065 mmol, 1.0 equiv) and tetra-*n*-butylammonium alendronate (38 mg, 0.071 mmol, 1.1 equiv) were dissolved in DMF (0.5 mL) and treated with iPr_2NEt (55 μL, 0.32 mmol). The reaction was stirred at room temperature for 1.5 h, after which the DMF was removed by evaporation. The residue was taken up in to water (8 mL) and extracted with dichloromethane (8 × 10 mL). The remaining aqueous phase was lyophilized and the residue was taken up in to deionized water and passed through an ion-exchange column (Amberlite IR-120 ion exchange resin, H^+ from). The eluent was once again lyophilized giving **11a** (36 mg, 77% crude yield) as a white solid. An analytical sample of **11a** was obtained by further HPLC purification:

50% MeOH/ 50% ammonium formate (0.01 M, pH 6.5), 35 °C, 3.5 mL/min, Phenomenex reverse phase column (P/N 00G-4337-N0). Product retention time: variable from 8.0 to 9.8 min. Compound **11a** characterization data: ^{1}H NMR (600 MHz, D_2O): δ = 7.40–7.55 (m, 5H), 6.42 (s, 2H), 5.55–5.75 (m, 2H), 4.62–4.73 (m, 1H), 4.15–4.23 (m, 1H), 3.13–3.21 (m, 1H), 3.09–3.13 (m, 2H), 2.90–2.95 (t, 2H), 2.48–2.56 (m, 1H), 2.34–2.40 (t, 2H), 2.15–2.25 (m, 1H), 1.86–1.98 (m, 2H), 1.63–1.85 (m, 5H), 1.11–1.35 (m, 6H). ^{13}C NMR (150 MHz, D_2O): δ = 178.0,167.6, 155.6, 136.1, 133.5 (t, $J_{C,F}$ = 25 Hz), 130.6, 128.6, 127.6, 125.6, 121.3 (t, $J_{C,F}$ = 245 Hz), 74.0 (t, $J_{C,F}$ = 29 Hz), 73.8 (t, $J_{C,P}$ = 130 Hz), 72.4, 60.9, 41.2, 40.6, 30.8, 30.0, 27.4, 26.6, 25.9, 25.3, 24.4, 24.2, 23.7. ^{31}P NMR (243 MHz, D_2O) δ = 20.7 (s).

HRMS (ESI): Found $(M+H)^+$ at 725.2271 for $C_{27}H_{41}F_2N_6O_{11}P_2$. Calculated at 725.2277.

*Compound **11b** 1,5-regioisomer characterization data:* ^{1}H NMR (600 MHz, D_2O): δ = 7.40–7.55 (m, 5H), 6.26 (s, 2H), 5.55–5.75 (m, 2H), 4.62–4.73 (m, 1H), 4.15–4.23 (m,1H), 3.15–3.25 (m, 1H), 3.08–3.14 (t, 2H), 3.03–3.08 (t, 2H), 2.53–2.63 (m, 1H), 2.34–2.42 (t, 2H), 2.15–2.27 (m, 1H), 1.85–1.97 (m, 2H), 1.75–1.85 (m, 3H), 1.65–1.74 (m, 1H), 1.25–1.39 (m, 4H), 1.15–1.25 (m, 2H). ^{13}C NMR (150 MHz, D_2O): δ = 177.8, 170.8, 157.2, 155.4, 135.8, 133.3 (t, $J_{C,F}$ = 25 Hz) 130.3, 128.3, 127.4, 125.5, 121.1 (t, $J_{C,F}$ = 245 Hz), 74.1 (t, $J_{C,F}$ = 29 Hz), 73.83 (t, $J_{C,P}$ = 130 Hz), 67.6, 60.8, 41.2, 40.4, 31.0, 29.7, 27.4, 25.7, 25.2, 24.2, 23.7, 22.0. ^{31}P NMR (243 MHz, D_2O) δ = 19.6 (s).

HRMS (ESI): Found $(M+Na)^+$ at 747.2128 for $C_{27}H_{40}F_2N_6O_{11}P_2Na$. Calculated at 747.2096.

3.7. Preparation of [^{3}H]-11a and [^{3}H]-11b

[^{3}H]-Tetrazole **6** (a kind gift from Merck; tritium labelled in the *para* position of the phenyl moiety) (30 mg; 1.03 mCi; 15 mCi/mmol) was converted as above to a mixture of [^{3}H]-**10a** and **10b** (0.45 mCi) after purification by column chromatography on silica gel. The mixture was converted as above to yield a mixture of [^{3}H]-**11a** and **11b** (6 mg; 0.1 mCi; nominally 15 mCi/mmol).

3.8. Phenol intermediate en route to 17a: (*R*,*E*)-4-((*R*)-1-(7-ethoxy-7-oxoheptyl)-5-oxopyrrolidin-2-yl)-1,1-difluoro-1-phenylbut-3-en-2-yl 2-acetoxybenzoate (17)

A solution of alcohol 7 (214 mg, 0.505 mmol, 1.0 equiv) in DCM (5 mL) was treated with triethylamine (352 μL, 2.53 mmol, 5.0 equiv) and 2-acetylsalicyloyl chloride (251 mg, 1.26 mmol, 2.5 equiv) and stirred at room temperature for 18 h. The reaction was quenched with saturated aqueous NH_4Cl, the layers were separated and the aqueous phase was extracted with DCM (3 × 10 mL). The organic layers were combined, dried over $MgSO_4$, filtered and concentrated and the product was isolated by flash chromatography (EtOAc/hexanes) to afford 17 (264 mg, 0.451 mmol, 89%, R_f = 0.30 in 80% EtOAc/hexanes) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.94 (dd, *J* = 7.9, 1.7 Hz, 1H), 7.59 (ddd, *J* = 8.0, 8.0, 1.7 Hz, 1H), 7.53–7.49 (m, 2H), 7.45–7.40 (m, 3H), 7.32 (ddd, *J* = 7.8, 7.8, 1.2 Hz, 1H), 7.11 (dd, *J* = 8.1, 1.0 Hz, 1H), 5.94–5.87 (m, 1H), 5.69–5.67 (m, 2H), 4.11 (q, *J* = 7.1 Hz, 2H), 4.06–4.00 (m, 1H), 3.44 (ddd, *J* = 14.9, 7.1, 7.1 Hz, 1H), 2.62 (ddd, *J* = 13.6, 8.2, 5.3 Hz, 1H), 2.41–2.23 (m, 4H), 2.29 (s, 3H), 2.21–2.13 (m, 1H), 1.75–1.55 (m, 3H), 1.44–1.19 (m, 6H), 1.24 (t, *J* = 7.1 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ = 174.8 (C_4), 173.9 (C_4), 169.6 (C_4), 162.7 (C_4), 151.0 (C_4), 138.0 (CH), 134.6 (CH), 133.8 (C_4, t, $J_{C,F}$ = 99.8 Hz), 131.9 (CH), 130.7 (CH), 128.6 (CH), 126.3 (CH), 125.9 (CH, t, J_F = 25.2 Hz), 124.2 (CH), 123.8 (CH, t, $J_{C,F}$ = 11.5 Hz), 122.4 (C_4), 119.7 (C_4, t, $J_{C,F}$ = 245.6 Hz), 74.7 (CH, t, $J_{C,F}$ = 31.6 Hz), 60.3 (CH_2), 59.8 (CH), 40.6 (CH_2), 34.4 (CH_2), 30.0 (CH_2), 28.9 (CH_2), 27.3 (CH_2), 26.6 (CH_2), 25.3 (CH_2), 25.0 (CH_2), 21.1 (CH_3), 14.4 (CH_3). HRMS (ESI): Found $(M+H)^+$ at 586.2625 for $C_{32}H_{38}F_2NO_7$. Calculated at 586.2616.

3.9. (*R*,*E*)-4-((*R*)-1-(7-ethoxy-7-oxoheptyl)-5-oxopyrrolidin-2-yl)-1,1-difluoro-1-phenylbut-3-en-2-yl 2-hydroxybenzoate (17a)

A solution of **17** (264 mg, 0.451 mmol, 1.0 equiv) in absolute ethanol (4.5 mL, 0.1 M) was cooled to 0 °C and bubbled with dry HCl gas for 10 min. The mixture was slowly allowed to warm to room temperature for 3.5 h after which the reaction was bubbled with argon for 20 min. The solvent was removed by evaporation at temperatures that do not exceed 30 °C. Purification by flash chromatography (EtOAc/hexanes) afforded phenol **17a** (177 mg, 0.325 mmol, 72%, R_f = 0.35 in 80% EtOAc/hexanes) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 10.32 (s, 1H), 7.82 (dd, *J* = 8.0, 1.7 Hz, 1H), 7.52–7.49 (m, 3H), 7.47–7.41 (m, 3H), 6.97 (d, *J* = 8.4 Hz, 1H), 6.91 (dd, *J* = 7.1, 7.1 Hz, 1H), 5.94 (td, *J* = 10.3, 6.0 Hz, 1H), 5.79–5.65 (m, 2H), 4.11 (q, *J* = 7.1 Hz, 2H), 4.07–4.02 (m, 1H), 3.42 (ddd, *J* = 13.8, 7.0, 7.0 Hz, 1H), 2.65 (ddd, *J* = 13.7, 8.4, 5.3 Hz, 1H), 2.42–2.13 (m, 3H), 2.27 (t, *J* = 7.5 Hz, 2H), 1.71–1.64 (m, 1H), 1.59 (dt, *J* = 15.2, 7.5 Hz, 2H), 1.45–1.19 (m, 6H), 1.24 (t, *J* = 7.2 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ = 174.8 (C_4), 173.8 (C_4), 168.3 (C_4), 162.0 (C_4), 138.5 (CH), 136.6 (CH), 133.6 (C_4, t, $J_{C,F}$ = 25.3 Hz), 130.8 (CH), 130.0 (CH), 128.6 (CH), 125.9 (CH, t, $J_{C,F}$ = 6.3 Hz), 123.8 (CH, t, $J_{C,F}$ = 2.8 Hz), 119.6 (CH), 119.5 (C_4, t, $J_{C,F}$ = 247.1 Hz), 117.9 (CH), 111.7 (C_4), 74.9 (CH, t, J_F = 32.4 Hz), 60.3 (CH_2), 59.9 (CH), 40.7 (CH), 34.3 (CH), 30.0 (CH), 28.9 (CH), 27.3 (CH), 26.6 (CH), 25.3 (CH), 24.9 (CH), 14.4 (CH_3). HRMS (ESI): Found $(M+H)^+$ at 544.2479 for $C_{30}H_{36}F_2NO_6$. Calculated at 544.2511.

3.10. (*R*,*E*)-4-((*R*)-1-(7-ethoxy-7-oxoheptyl)-5-oxopyrrolidin-2-yl)-1,1-difluoro-1-phenylbut-3-en-2-yl-((4-nitrophenoxy)-carbonyloxy)benzoate (18)

A solution of phenol **17a** (117 mg, 0.326 mmol, 1.0 equiv) in dichloromethane (5 mL) was treated with triethylamine (136 μL, 0.978 mmol, 3.0 equiv) and 4-nitrophenylchloroformate (99 mg, 0.489 mmol, 1.5 equiv). This mixture was stirred at room temperature for 5 h after which the reaction is quenched with aqueous NH_4Cl. The layers were separated and the aqueous phase was extracted with dichloromethane (3×). The organic layers were combined, dried over $MgSO_4$, filtered and concentrated and the product was isolated by flash chromatography (EtOAc/hexanes) to afford compound **18** (223 mg, 0.315 mmol, 97%, R_f = 0.35 in 80% EtOAc/hexanes) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.25–8.21 (m, 2H), 7.98 (dd, *J* = 7.0, 1.6 Hz, 1H), 7.60 (td, *J* = 7.9, 1.7 Hz, 1H), 7.28–7.16 (m, 8H), 7.24 (dd, *J* = 8.1, 8.1 Hz, 1H), 5.94–5.88 (m, 1H), 5.69–5.60 (m, 2H), 4.04 (q, *J* = 7.1 Hz, 2H), 3.99–3.93 (m, 1H), 3.35 (dt, *J* = 13.9, 7.8 Hz, 1H), 2.54 (ddd, *J* = 13.7, 8.2, 5.4 Hz, 1H), 2.29–2.15 (m, 2H), 2.19 (t, *J* = 7.4 Hz, 2H), 2.12–2.03 (m, 1H), 1.59–1.47 (m, 3H), 1.35–1.10 (m, 6H), 1.17 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 174.7 (C_4), 173.7 (C_4), 162.3 (C_4), 155.5 (C_4), 150.64 (C_4), 150.62 (C_4), 145.7 (C_4), 138.1 (CH), 135.0 (CH), 133.5 (C_4, t, $J_{C,F}$ = 25.2 Hz), 132.1 (CH), 130.7 (CH), 128.6 (CH), 127.2 (CH), 126.1 (C_4), 125.8 (CH, t, $J_{C,F}$ = 6.2 Hz), 125.3 (CH), 123.6 (CH, t, $J_{C,F}$ = 2.4 Hz), 123.5 (CH), 121.9 (CH), 119.5 (C_4, t, $J_{C,F}$ = 247.5 Hz), 74.8 (CH, t, $J_{C,F}$ = 31.5 Hz), 60.2 (CH_2), 59.6 (CH), 40.5 (CH), 34.2 (CH), 29.8 (CH), 28.8 (CH), 27.1 (CH), 26.5 (CH), 25.1 (CH), 24.8 (CH), 14.3 (CH_3). HRMS (ESI): Found $(M+H)^+$ at 709.2525 for $C_{36}H_{37}F_2N_2O_{10}$. Calculated at 709.2573.

3.11. 3-benzyl-*2H*-benzo[*e*][1,3]oxazine-2,4(*3H*)-dione (20)

Carbonate **18** (20.7 mg, 0.0292 mmol, 1.0 equiv) was dissolved in DMF (1 mL) and treated with iPr_2Et (25.4 μL, 0.146 mmol, 5.0 equiv) and benzylamine (3.2 μL, 0.029 mmol, 1.0 equiv). After stirring at room temperature for 18 h, the solvent was evaporated and the product was isolated by flash chromatography (EtOAc/hexanes) to afford known compound **7** (11.0 mg, 0.0260 mmol, 89%, R_f = 0.20 in 80% yield and compound **20** (6.8 mg, 0.027 mmol, 92%, R_f = 0.50 in 80% EtOAc/hexanes) as a colorless liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.10 (dd, J = 7.9, 1.4 Hz, 1H), 7.69 (ddd, J = 7.8, 7.8, 1.7 Hz, 1H), 7.54–7.51 (m, 2H), 7.39–7.26 (m, 5H), 5.21 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 160.7 (C_4), 152.7 (C_4), 148.3 (C_4), 136.3 (CH), 135.9 (C_4), 129.4 (CH), 128.8 (CH), 128.4 (CH), 128.3 (CH), 125.6 (CH), 116.6 (CH), 114.4 (C_4), 45.9 (CH_2). HRMS (ESI): Found $(M+Na)^+$ at 276.0642 for $C_{15}H_{11}NO_3Na$. Calculated at 276.0637.

3.12. Perfluorophenyl 2-(4-(*tert*-butyldimethylsilyloxy)-phenyl)acetate (22)

A solution of carboxylic acid **21** (1.10 g, 4.13 mmol, 1.0 equiv) in dry, vacuum pumped DMF (25 mL) was treated with DCC (0.94 g, 4.56 mmol, 1.1 equiv). After dissolution, pentafluorophenol (0.91 g, 4.94 mmol, 1.2 equiv) was added and the mixture was stirred at room temperature for 18 h. The resulting white precipitate was removed by filtration and mixture was concentrated to dryness under reduced pressure. The resulting oil was purified via flash chromatography (EtOAc/hexanes) to afford compound **22** (1.54 g, 3.56 mmol, 86%, R_f = 0.70 in 5% EtOAc/hexanes) as a colorless liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.21 (d, J = 6.8 Hz, 2H), 6.84 (d, J = 6.8 Hz, 2H), 3.89 (s, 2H), 0.98 (s, 9H), 0.20 (s, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ = 167.9 (C_4), 155.5 (C_4), 142.1 (m, C_4), 140.5 (m, C_4), 138.9 (m, C_4), 137.2 (m, C_4), 130.4 (CH), 124.8 (C_4), 120.6 (CH), 39.6 (CH_2), 25.8 (CH_3), 18.4 (C_4), −4.3 (CH_3). HRMS (ESI): Found $(M+H)^+$ at 433.1257 for $C_{20}H_{22}F_5O_3Si$. Calculated at 433.1258.

3.13. Ethyl 7-((*R*)-2-((*R,E*)-3-(2-(4-(*tert*-butyldimethylsilyloxy)-phenyl)acetoxy)-4,4-difluoro-4-phenylbut-1-enyl)-5-oxopyrrolidin-1-yl)heptanoate (23)

A solution of alcohol **7** (366 mg, 0.864 mmol, 1.0 equiv) in THF (20 mL) was treated with NaH (60% in oil, 35 mg, 0.875 mmol, 1.0 equiv) and the mixture was stirred for 5 min. PFP ester **22** (374 mg, 0.865 mmol, 1.0 equiv) was then added to the mixture as a solution in THF (5 mL) and the reaction was stirred at room temperature for 18 h. The reaction was quenched by the addition of saturated NH_4Cl (aq), the layers were separated and the aqueous phase was extracted with Et_2O (3 × 20 mL). The organic layers were combined, dried over $MgSO_4$, filtered and concentrated and the product was isolated by flash chromatography (EtOAc/hexanes) to afford **23** (386 mg, 0.574 mmol, 66%, R_f = 0.40 in 50% EtOAc/hexanes) as a colorless oil (along with returned starting material **7** (88 mg, 0.208 mmol, 24%)). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.47–7.37 (m, 5H), 7.02 (d, J = 8.5 Hz, 2H), 6.77 (d, J = 8.5 Hz, 2H), 5.72 (ddd, J = 11.6, 9.3, 6.8 Hz, 1H), 5.63 (dd, J = 15.4, 6.6 Hz, 1H), 5.51 (dd, J = 15.3, 8.3 Hz, 1H), 4.13 (q, J = 7.3 Hz, 2H), 4.00 (td, J = 8.0, 5.3 Hz, 1H), 3.54 (d, J = 1.9 Hz, 2H), 3.51–3.41 (m, 1H), 2.99 (d, J = 5.2 Hz, 1H), 2.63 (ddd, J = 13.7, 8.4, 5.3 Hz, 1H), 2.37–2.27 (m, 4H), 2.20–2.11 (m, 1H), 1.66–1.57 (m, 3H), 1.45–1.19 (m, 5H), 1.26 (t, J = 7.2 Hz, 3H), 1.00 (s, 9H), 0.20 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 174.8 (C_4), 173.8 (C_4), 170.0 (C_4), 155.1 (C_4), 137.4 (CH), 133.7 (C_4, t, $J_{C,F}$ = 25.4 Hz), 130.5 (CH), 130.3 (CH), 128.4 (CH), 125.9 (CH, t $J_{C,F}$ = 6.4 Hz), 125.9 (C_4), 124.2 (CH, t, $J_{C,F}$ = 2.7 Hz), 120.3 (CH), 119.5 (C_4, t, $J_{C,F}$ = 246.1 Hz), 74.2 (CH, t, $J_{C,F}$ = 33.7 Hz), 60.3 (CH_2), 59.8 (CH), 40.6 (CH_2), 40.4 (CH_2), 34.3 (CH_2), 29.9 (CH_2), 28.9 (CH_2), 27.2 (CH_2), 26.6 (CH_2), 25.8 (CH_3), 25.2 (CH_2), 24.9 (CH_2), 18.3 (C_4), 14.4 (CH_3), −4.3 (CH_3). HRMS (ESI): Found $(M+Na)^+$ at 694.3401 for $C_{37}H_{51}F_2NO_6SiNa$. Calculated at 694.3351.

3.14. Phenol intermediate en route to carbonate 24: Ethyl 7-((*R*)-2-((*R,E*)-4,4-difluoro-3-(2-(4-hydroxyphenyl)acetoxy)-4-phenylbut-1-enyl)-5-oxopyrrolidin-1-yl)heptanoate (23a)

A solution of **23** (210 mg, 0.313 mmol, 1.0 equiv) in THF (10 mL) was treated with TBAF (1.0 M in THF, 0.63 mL, 0.63 mmol, 2.0 equiv) and stirred at room temperature for 1 h. The reaction was quenched by the addition of saturated NH_4Cl (aq), the layers were separated and the aqueous phase was extracted with Et_2O (3 × 15 mL). The organic layers were combined, dried over $MgSO_4$, filtered and concentrated and the product was isolated by flash chromatography (EtOAc/hexanes) to afford phenol intermediate **23a** (130 mg, 0.233 mmol, 74%, R_f = 0.40 in 80% EtOAc/hexanes) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.47–7.39 (m, 5H), 7.01 (d, J = 8.0 Hz, 2H), 6.76 (d, J = 8.0 Hz, 2H), 5.70–5.65 (m, 1H), 5.58 (dd, J = 15.4, 5.3 Hz, 1H), 4.97 (dd, J = 15.4, 9.0 Hz, 1H), 4.12 (q, J = 7.1 Hz, 2H), 3.94–3.90 (m, 1H), 3.50 (d, J = 4.5 Hz, 2H), 3.42–3.37 (m, 1H), 2.48–2.43 (m, 1H), 2.30–2.24 (m, 4H), 2.13–2.06 (m, 1H), 1.61–1.55 (m, 2H), 1.49–1.43 (m, 1H), 1.39–1.16 (m, 10H). No phenol OH observed. ^{13}C NMR (100 MHz, $CDCl_3$) δ = 175.4 (C_4), 174.1 (C_4), 169.9 (C_4), 156.0 (C_4), 135.4 (CH), 133.6 (C_4, t, $J_{C,F}$ = 20.3 Hz), 130.6 (CH), 130.4 (CH), 128.4 (CH), 126.0 (C_4, t, $J_{C,F}$ = 4.9 Hz), 124.8 (CH), 124.3 (CH), 119.5 (C_4, t, $J_{C,F}$ = 197.1 Hz), 116.2 (CH), 73.5 (CH, t, $J_{C,F}$ = 26.4 Hz), 60.4 (CH_2), 60.0 (CH), 40.8 (CH_2), 40.5 (CH_2), 34.3 (CH_2), 29.9 (CH_2), 28.8 (CH_2), 26.9 (CH_2), 26.5 (CH_2), 25.0 (CH_2), 24.8 (CH_2), 14.3 (CH_3). HRMS (ESI): Found $(M+H)^+$ at 558.2653 for $C_{31}H_{38}F_2NO_6$. Calculated at 558.2667.

3.15. Ethyl 7-((*R*)-2-((*R,E*)-4,4-difluoro-3-(2-(4-((4-nitrophenoxy)carbonyloxy)phenyl)acetoxy)-4-phenylbut-1-enyl)-5-oxopyrrolidin-1-yl)heptanoate (24)

A solution of phenol **23a** (130 mg, 0.233 mmol, 1.0 equiv) in DCM (5 mL) was treated with 4-nitrophenylchloroformate (52 mg, 0.258 mmol, 1.1 equiv) and triethylamine (98 μL, 0.703 mmol, 3.0 equiv). The mixture was stirred at room temperature for 2 h, after which it was quenched with saturated aqueous NH_4Cl. The layers were separated and the aqueous phase was extracted with DCM (3 × 10 mL). The organic layers were combined, dried over $MgSO_4$, filtered and concentrated and the product was isolated by flash chromatography (EtOAc/hexanes) to afford **24** (154 mg, 0.213 mmol, 91%, R_f = 0.40 in 80% EtOAc/hexanes) as a yellow oil ^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.31 (d, J = 9.2 Hz, 2H), 7.49 (d, J = 9.2 Hz, 2H), 7.44–7.35 (m, 5H), 7.21 (s, 4H), 5.75–5.68 (m, 1H), 5.62 (dd, J = 15.4, 6.7 Hz, 1H), 5.47 (dd, J = 15.3, 8.5 Hz, 1H), 4.10 (q, J = 7.1 Hz, 2H), 3.99 (td, J = 8.1, 5.2 Hz, 1H), 3.62 (s, 2H), 3.43 (ddd, J= 13.8, 8.6, 7.2 Hz, 1H), 2.57 (ddd, J = 13.7, 8.4, 5.2 Hz, 1H), 2.34–2.24 (m, 4H), 2.14 (ddd, J = 16.0, 13.0, 7.7 Hz, 1H), 1.64–1.55 (m, 3H), 1.43–1.19 (m, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 174.7 (C_4), 173.8 (C_4), 169.2 (C_4), 155.4 (C_4), 151.0 (C_4), 150.1 (C_4), 145.8 (C_4), 137.6 (CH), 133.6 (C_4, t, $J_{C,F}$ = 25 Hz), 131.8 (C_4), 130.7 (CH), 130.6 (CH), 128.5 (CH), 125.8 (CH, t, $J_{C,F}$ = 6.2 Hz), 125.5 (CH), 124.0 (CH, t, $J_{C,F}$ = 2.5 Hz), 121.9 (CH), 121.1 (CH), 119.4 (C_4, t, $J_{C,F}$ = 246.4 Hz), 74.4 (CH, dd, $J_{C,F}$ = 33.6, 30.7 Hz) 60.3 (CH_2), 59.7 (CH), 40.6 (CH_2), 40.5 (CH_2), 34.3 (CH_2), 30.0 (CH_2), 28.9 (CH_2), 27.2 (CH_2), 26.6 (CH_2), 25.2 (CH_2), 24.9 (CH_2), 14.4 (CH_3). HRMS (ESI): Found $(M+H)^+$ at 723.2751 for $C_{38}H_{41}F_2N_2O_{10}$. Calculated at 723.2729.

3.16. (4-(((4-(2-(((*R*,*E*)-4-((*R*)-1-(7-ethoxy-7-oxoheptyl)-5-oxopyrrolidin-2-yl)-1,1-difluoro-1-phenylbut-3-en-2-yl)oxy)-2-oxoethyl)phenoxy)carbonyl)amino)-1-hydroxybutane-1,1-diyl)diphosphonic acid (25)

A solution of **24** (154 mg, 0.213 mmol, 1.0 equiv) in dioxane (2 mL) was treated with a solution of alendronic acid (66 mg, 0.265 mmol, 1.25 equiv) and triethylamine (149 μL, 1.06 mmol, 5.0 equiv) dissolved in H_2O (2 mL). The resulting yellow mixture was stirred at room temperature for 2 h, after which it was diluted with EtOAc/H2O (1:1, 10 mL). The layers were separated and the aqueous phase was extracted with EtOAc (2 × 5 mL). The remaining aqueous phase was lyophilized to afford a yellow solid which was purified by reverse phase chromatography (C18 Sep-Pak cartridge, gradient elution from H_2O to MeOH), ultimately giving **25** (111 mg, 0.112 mmol, 53%) as a white solid. The fractions containing the product were identified by MS analysis. ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.45–7.42 (m, 1H), 7.39–7.32 (m, 4H), 7.08 (d, *J* = 8.5 Hz, 2H), 7.02 (d, *J* = 8.5 Hz, 2H), 6.64 (br s, 1H), 5.72–5.66 (m, 1H), 5.61 (dd, *J* = 15.3, 6.9 Hz, 1H), 5.50 (dd, *J* = 15.3, 8.4 Hz, 1H), 4.70 (br s, 4H), 4.11 (q, *J* = 7.1 Hz, 2H), 4.01–3.97 (m, 1H), 3.55 (s, 2H), 3.46–3.40 (m, 1H), 3.31–3.26 (m, 2H), 3.04 (q, *J* = 7.1 Hz, 9.2H – protons from 1.52·Et_3N salt), 2.63–2.57 (m, 1H), 2.37–2.26 (m,4H), 2.19–2.01 (m, 5H), 1.64–1.56 (m, 3H), 1.43–1.19 (m, 10H), 1.25 (t, *J* = 7.1 Hz, 13.8H – protons from 1.52·Et_3N salt). ^{13}C NMR (150 MHz, $CDCl_3$) δ = 174.8 (C_4), 173.9 (C_4), 169.7 (C_4), 154.9 (C_4), 150.9 (C_4), 137.8 (CH), 133.6 (t, $J_{C,F}$ = 25.5 Hz, C_4), 130.7 (CH), 130.1 (CH), 129.6 (C_4), 128.6 (CH), 125.8 (t, $J_{C,F}$ = 6.1 Hz, CH), 124.2 (CH), 122.0 (CH), 119.5 (t, $J_{C,F}$ = 248.2 Hz, C_4), 74.4 (t, $J_{C,F}$ = 32.8 Hz, CH), 73.8 (t, $J_{C,P}$ = 134.8 Hz, C_4), 60.3 (CH_2), 59.8 (CH), 45.3 (CH_2), 42.1 (CH_2), 40.6 (CH_2), 31.5 (CH_2), 30.0 (CH_2), 29.8 (CH_2), 28.9 (CH_2), 27.2 (CH_2), 26.6 (CH_2), 25.3 (CH_2), 25.0 (CH_2), 24.1 (CH_2), 14.4 (CH_3), 8.7 (CH_3). ^{31}P NMR (243 MHz, D_2O as Na salt) δ = 19.0 (s).

HRMS (ESI): Found $(M+H)^+$ at 833.2602 for $C_{36}H_{49}F_2N_2O_{14}P_2$. Calculated at 833.2627. Does not fly as Et_3N salt in TOF-MS.

3.16.1. Stability of 7 in rat plasma

A 100 μg/mL solution of **7** in fresh rat plasma incubated at 37 °C and followed by HPLC analysis. The compound was quantitatively converted to **5** after 30 min.

3.16.2. Stability of 9, 11a and 11b in rat plasma

Conjugate **9** and a mixture of conjugates **11a** and **11b** (2.5:1) were each evaluated for stability at 100 μg/ml in rat plasma at 1, 3, 5, 7, 24 and 96 h incubation at 37 °C by monitoring the liberation of compounds 5 and 6 respectively by HPLC-MS-MS. Essentially no liberation of **5** from conjugate **9** was observed over 96 h while the mixture of conjugate **11a** and **11b** liberated 5.1 μg/mL **6** at 96 h, indicating 5.1% hydrolysis.

3.16.3. Stability of 25 in rat plasma

A stock solution of **25** (6.5 mg/mL in PBS) was used to prepare 100 μL solutions of **25** (100 μg/mL) in fresh and boiled rat plasma. The samples were incubated in a 37 °C water bath for 24 h. Samples were analyzed at time points 0, 0.5, 1, 2, 4, 8, and 24 h. At each time point acetonitrile (100 μL) was added, the resulting mixture was centrifuged and the supernatant solution was analyzed by LC–MS to assess the concentration of EP4 acid **5**. After a 24 h incubation in fresh rat plasma, it was determined the concentration of **5** was 6.2 μg/mL, signifying 6.2% hydrolysis of **25**. After a 24 h incubation in boiled rat plasma, it was determined the concentration of **5** was 3.1 μg/mL, signifying 3.1% hydrolysis of **25.**

3.16.4. In vitro binding of EP4 agonists 5, 6 and 7 to rat bone

0.1 mg/mL solutions of **5**, **6** and **7** in H_2O were prepared. 400 μL samples of each compound were stirred with dried bone powder (100 mg) at room temperature for 30 min. The concentration of each EP4 agonist was measured before and after stirring with bone powder using an LC–MS, and it was found in each case that there was no change in detector response after exposing the compounds to the bone powder, indicating the free EP4 agonists **5**, **6** and **7**are not adsorbed by bone.

3.16.5. In vitro binding of conjugates 9, 11a and 11b to rat bone

A dilute sample of [^{3}H]-**11a** and [^{3}H]-**11b** (0.12 μCi; 15 mCi/mmol) in 400 μL water was stirred with dried powdered rat bone (100 mg) at room temperature. Samples (100 μL) were removed after various times, centrifuged and the supernatant counted for radioactivity. Free radioactivity equivalent to 12.9 and 7.5% was observed at 10 and 50 min respectively indicating 87 and 93% was bound to the bone. An analogous experiment using [^{3}H]-**9** resulted in 96% uptake after 5 min.

3.16.6. In vivo uptake and release of conjugate 9 in rat tibiae and femora

A solution of [^{3}H]-**9** (10 mg/mL, 15.2 mCi/mmol, 219 μCi/mL) was prepared in water and administered (100 μL) IV via the tail vein to female Sprague–Dawley rats as a single dose of 1 mg (ca. 5 mg/kg) of radiolabelled compound, equivalent to 21.9 μCi/animal. A total of 11 rats were dosed and were observed by animal technicians over the first hour and when taking subsequent blood samples. Dosed animals showed no unusual behavior or reaction to the drug treatment. Serial blood samples were removed from two of the rats at 0.5, 1, 2, 4 and 24 h. after dosing. These samples were diluted with an equivalent volume of acetonitrile, centrifuged and the supernatant was counted to determine the concentration of radioactivity in the blood. The elimination $t_{½}$ was determined to be <0.5 h. After 1, 7, 14 and 28 days animals were weighed, sacrificed by CO_2 and the tibiae and femora were dissected and cleaned free of any loose tissue. The amount of radioactivity incorporated into the bone was determined by incineration in a R. J. Harvey OX300 Biological Oxidizer after first drying the bone for 3 days in a vacuum desiccator. The percent of the compound retained in the skeleton at each time point was calculated by measuring activity per gram (μCi/g) in the collected samples and extrapolating to total activity retained in the skeleton, expressed as percent administered, by assuming the skeleton represents 8% of the body weight. The recovered label indicated 9.4% uptake after 1 day, 8.0% after 7 days, 6.2% after 14 days and 5.6% after 28 days.

3.16.7. In vivo uptake and release of conjugates 11a and 11b in rat tibiae and femora

A solution of [^{3}H]-**11a** and [^{3}H]-**11b**(specific activity 15 mCi/mmol) was prepared in water and administered (200 μL) IV via the tail vein to female Sprague-Dawley rats as a single dose of 1.1 μmol (ca. 2.5 mg/kg) of radiolabelled compound, equivalent to 17 μCi/animal. A total of 12 rats were dosed and were observed by animal technicians over the first hour and when taking subsequent blood samples. Dosed animals showed no unusual behavior or reaction to the drug treatment. Serial blood samples were removed from three of the rats at 0.5, 1, 2, 4 and 24 h. after dosing. These samples were diluted with an equivalent volume of acetonitrile, centrifuged and the supernatant was counted to determine the concentration of radioactivity in the blood. Elimination $t_{½}$ was determined to be <0.5 h. After 6 h, 1, 7, and 14 days animals were weighed, sacrificed by CO_2 and the tibiae and femora were dissected and cleaned free of any loose tissue. The amount of radioactivity incorporated into the bone was determined by incineration in a R. J. Harvey OX300 Biological Oxidizer after first drying the bone for 3 days in a vacuum desiccator. The recovered label indicated 9.0% uptake after 6 h and this remained essentially unchanged in bone samples after 1, 7 and 14 days.

3.16.8. In vivo uptake and release of conjugate 25 in rat tibiae and femora

A solution of [^{3}H]-**25** (20 mg/mL, 6.1 mCi/mmol, 147 μCi/mL) was prepared in PBS and administered (100 μL) IV via the tail vein to female Sprague-Dawley rats as a single dose of 2 mg (ca. 10 mg/kg) of radiolabelled compound, equivalent to 14.7 μCi/animal. A total of 11 rats were dosed and were observed by animal technicians over the first hour and when taking subsequent blood samples. Dosed animals showed no unusual behavior or reaction to the drug treatment. Serial blood samples were removed from two of the rats at 0.5, 1, 2, and 5 h after dosing. These samples were diluted with an equivalent volume of acetonitrile, centrifuged and the supernatant was counted to determine the concentration of radioactivity in the blood. $T_{½}$ of elimination was determined to be <0.5 h. After 6 h, 2, 7 and 14 days two animals were weighed, sacrificed by CO_2 and the tibiae and femora were dissected and cleaned free of any loose tissue. The amount of radioactivity incorporated into the bone was determined by incineration in a R. J. Harvey OX300 Biological Oxidizer after first drying the bone for 3 days in a vacuum desiccator. The percent of the compound retained in the skeleton at each time point was calculated by measuring activity per gram (μCi/g) in the collected samples and extrapolating to total activity retained in the skeleton, expressed as percent administered, by assuming the skeleton represents 8% of the body weight. The recovered label indicated 5.9% uptake after 6 h, 4.9% after 2 days, 2.2% after 7 days and 1.4% after 14 days.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.01.024.

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