Fluorescently Labeled Risedronate and Related Analogues: "Magic Linker" Synthesis

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Fluorescently Labeled Risedronate and Related Analogues: "Magic Linker" Synthesis

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We report synthesis of the first fluorescently labeled conjugates of risedronate (**1**), using an epoxide linker strategy enabling conjugation of **1** via its pyridyl nitrogen with the label (carboxyfluorescein). Unlike prior approaches to create fluorescent bisphosphonate probes, the new linking chemistry did not abolish the ability to inhibit protein prenylation in vitro, while significantly retaining hydroxyapatite affinity. The utility of a fluorescent **1** conjugate in visualizing osteoclast resorption in vitro was demonstrated.

Nitrogen-containing bisphosphonates (N-BPs) are therapeutic agents for the treatment of diseases associated with increased bone resorption, such as osteoporosis and Paget's disease (*1, 2*). N-BPs inhibit farnesyl diphosphate synthase (FPPS), preventing the prenylation of small GTPase proteins that are crucial for the function and survival of bone-resorbing osteoclasts (*3*−*6*). Phosphonocarboxylate (PC) analogues of BPs are also able to inhibit bone resorption, but inhibit Rab geranylgeranyl transferase (RGGT), thereby selectively preventing the prenylation of Rab proteins (*7*).

Some N-BPs and PC analogues also demonstrate antitumor effects in vitro and in vivo (*1, 2*). However, it is unknown whether the antitumor effects of these drugs result from direct effects on tumor cells or derive chiefly from inhibition of bone resorption (*1, 2, 8*). Therefore, specific imaging probes to elucidate the skeletal distribution and cellular uptake of these drugs in bone and other tissues are highly desirable.

Fluorescent analogues of bisphosphonates are of increasing interest as biological probes exploiting the high bone affinity of these compounds. Recently, the primary aminoalkyl N-BP alendronate was coupled to Alexa Fluor 488 via the drug's γ-amino functionality; however, the conjugated labeled drug was obtained in only 7% purity (*9*). In a separate study, ε-amino N-BP pamidronate conjugated to IRDye78 or IRDye800CW was observed to bind to hydroxyapatite (HA) (*10, 11*). In all these cases, the imaging dyes were attached via an amide linkage at the terminal amino group of the drug (*9*−*12*), resulting in a large decrease in nitrogen basicity expected to lower greatly the inhibitory potency of the resulting conjugate. Also, this approach is not applicable to heterocyclic N-BPs such as risedronate (RIS, **1**), which lack a primary amine susceptible to facile acylation by activated esters of fluorescent labels. Thus, no successes in fluorescent labeling of any member of this class of drugs have yet been reported (*9*−*13*).

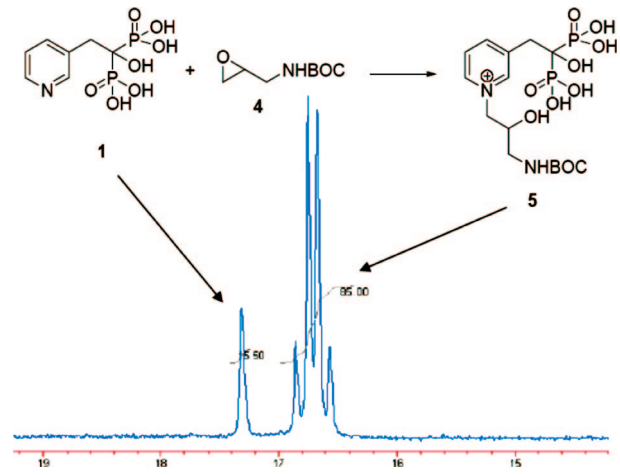

Figure 1. ^{31}P NMR spectrum of reaction mixture of **1** and **4** after 18 h at 40 °C.

We began our work with the concept of designing a universal linker, which, once attached to the drug, would permit conjugation with any fluorophore activated ester while, ideally, not adversely influencing bone affinity and retaining some ability to inhibit protein prenylation. On this basis, the phosphonate/carboxylate moieties (or the α-hydroxyl group) of the drug were considered to be unsuitable sites for attachment of a linker moiety. If a linker group could be connected via the pyridine nitrogen, a positively charged quaternary nitrogen atom would be generated, perhaps simulating a putative carbocation-like transition state analogue in the active site (*6*), thereby offering the possibility that the fluorescent conjugate could retain activity. Finally, this linking chemistry would need to be compatible with an incorporated primary amine group to allow subsequent conjugation of the drug−linker complex to an imaging molecule, and should address the low solubility of the drug in nonaqueous media.

In search of such a "magic" linker, we investigated the reaction of heterocyclic N-BPs with the epoxide **4** to generate N-alkylated analogues of the parent drugs **1**−**3** (Scheme 1). We find that epoxide **4** couples to the pyridine nitrogen of **1**−**3** under

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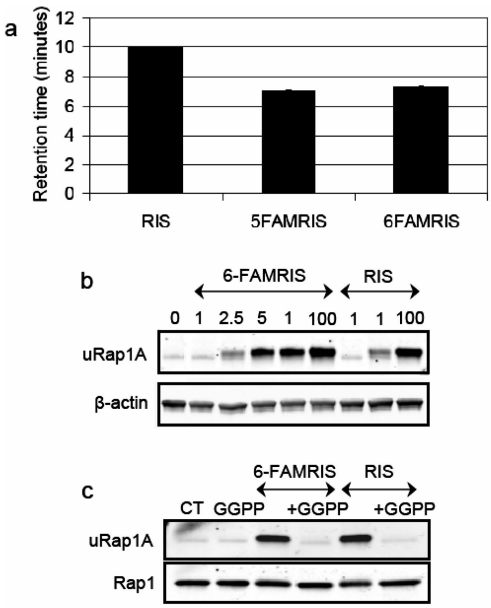
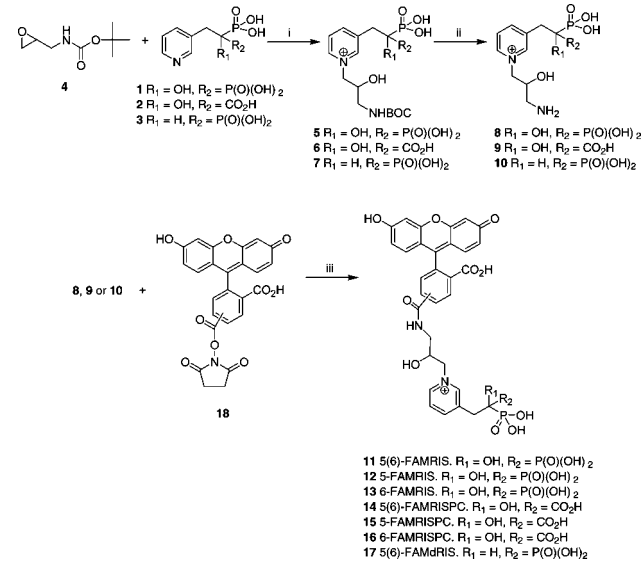


Figure 2. (a) Comparison of RIS (**1**) (**12**) and 6-FAMRIS (**13**) on hydroxyapatite column. (b, c) Western blot assays for unprenylated Rap1A (uRap1A). J774.2 macrophages were treated for 24 h with (b) 0−100 μM **13** or **1**; or (c) vehicle, 20 μM **13**, or 20 μM **1** in the presence or absence of 100 μM GGPP. Detection of β-actin (b) or total Rap1 (c) served as loading control.

Scheme 1[a]



[a] Reagents: (i) ~5% MeOH/H_2O, 40−50 °C. (ii) 1:1 TFA/H_2O, room temperature; (iii) $NaHCO_3$/DMF, pH 8.3, room temperature, in darkness.

mild (pH nearly neutral, 40−50 °C) aqueous conditions, with high yields and regioselectivity (*14*). This linker affords the key functionalities referred to above: (1) a primary amine (after suitable deprotection) for facile conjugation to activated esters of fluorescent or other labels; (2) a permanent positive charge on the pyridinium nitrogen; and (3) a hydroxyl group (from the epoxide ring-opening) to balance the hydrophobic alkyl chain.

At 40 °C, nearly quantitative yields are achieved, affording intermediates **5**−**7** with less than 1% O-alkylation according to ^{31}P NMR (Figure 1). Aminohydroxypropane esters of 1-hydroxyethylidene-1,1-bisphosphonic acids were previously synthesized in aqueous conditions near neutral pH at 60−70 °C (*15*). Since we aimed to avoid forming ester linkages between the phosphonate group and the linker, the regioselectivity of our reaction was a welcome discovery. This linking strategy may be applicable to N-alkylation of other nitrogen-containing heterocycles (*16*).

Treatment of the N-*t*BOC-protected linker−drug intermediates **5**−**7** with TFA yielded the corresponding free amino forms **8**−**10**, which were then readily coupled to 5(6)-carboxyfluorescein via its succinimidyl ester (5(6)-FAM, SE, **18**). Individual isomers (5- and 6-FAM) of the labeled products may be isolated by semipreparative HPLC, although compounds **12**, **15**, and **16** were also synthesized directly from their respective isomerically pure FAM, SE starting materials. All labeled products were characterized by high-resolution mass, ^{1}H and ^{31}P NMR, UV absorption, and fluorescence emission spectra (see Supporting Information). FAM-labeled compound **11** showed no decomposition by analytical TLC after 1 week at room temperature in phosphate buffer (pH 7.2), demonstrating the stability of the labeled product.

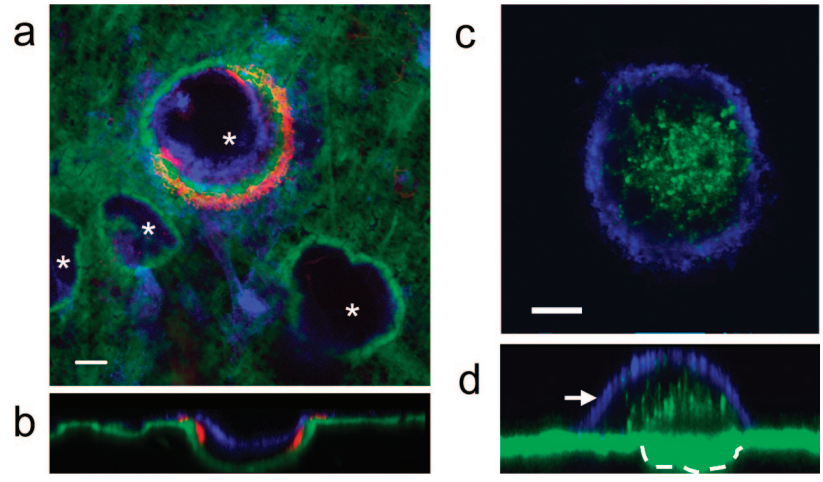


Figure 3. Binding and "recycling" of 5(6)-FAMRIS **11** to the surface of dentine (a, b) and uptake of **11** by rabbit osteoclast in vitro (c, d); bar = 10 μM. Rabbit osteoclasts were seeded onto dentine discs that had been precoated with **11** (green); osteoclasts were immunostained for VNR (blue), and actin rings of osteoclasts were visualized using TRITC-phalloidin (red; a, b only). (a) 1 μm *xy* image at the surface of the dentine; resorption pits identified by asterisks; (b) *zx* image of the same field-of-view. (c) 1 μm *xy* image 8 μm above the surface of the dentine and (d) *zx* image of the same osteoclast. *Z* position of (c) is denoted by arrow in (d). Dashed line in (d) shows the outline of the resorption pit. Note that detector gain in (c, d) was optimized to detect **11** intracellularly resulting in saturated signal from **11** at the dentine surface.

To investigate whether structural differences between **1** and labeled **12** and **13** could interfere with their role as imaging probes, a hydroxyapatite column was utilized as a model for their binding affinity to bone (*17*). The labeled compounds showed only a moderate decrease in retention time ($R_t = 7$ min) compared to **1** ($R_t = 10$ min) (Figure 2a). Probe **13** also inhibits the prenylation of the small GTPase Rap1A (at similar concentrations to RIS) (Figure 2b), an effect that could be reversed by the addition of exogenous GGPP (the isoprenoid required for prenylation of Rap1A; Figure 2c), indicating that the compound retains this activity of the parent N-BP. Fluorescent alendronate (F-ALN), where the primary amino nitrogen was converted to a neutral amide by the dye attachment chemistry, was inactive in this assay (*18*).

11, intensely labeled the surface of dentine, demonstrating its high affinity for a physiological mineralized substrate. Furthermore, following the culture of rabbit osteoclasts for 18 h on dentine discs precoated with **11** prior to seeding of the cells, **11** also labeled the newly exposed surface of resorption pits beneath actively resorbing osteoclasts (Figure 3a,b), indicating that **11** binds to newly exposed mineral surfaces after being released from the dentine surface during resorption (*18*). Other fluorescent bone-labeling agents with low bone affinity, such as calcein and xylenol orange, do not exhibit this recycling to resorption pits (*18*). In these cultures, **11** could also be detected within intracellular vesicles in resorbing osteoclasts (Figure 3c,d), in accordance with our previous studies demonstrating endocytic uptake of F-ALN by both macrophages and resorbing osteoclasts (*9, 18*).

In summary, we report the first synthesis of fluorescently labeled conjugates of drugs **1**−**3**, using a novel linking strategy. Advantages of the approach described include the exceptionally mild reaction conditions used to attach the linker to the drug (aqueous solution, pH near neutral, ∼room temperature), high yields, and regioselectivity. This approach has provided, for the first time, a fluorescent conjugate of an N-BP that inhibits protein prenylation.

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Supporting Information Available: Detailed experimental procedures and data. This material is available free of charge via the Internet at http://pubs.acs.org.

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