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Bisphosphonated Benzoxazinorifamycin Prodrugs for the Prevention and Treatment of Osteomyelitis

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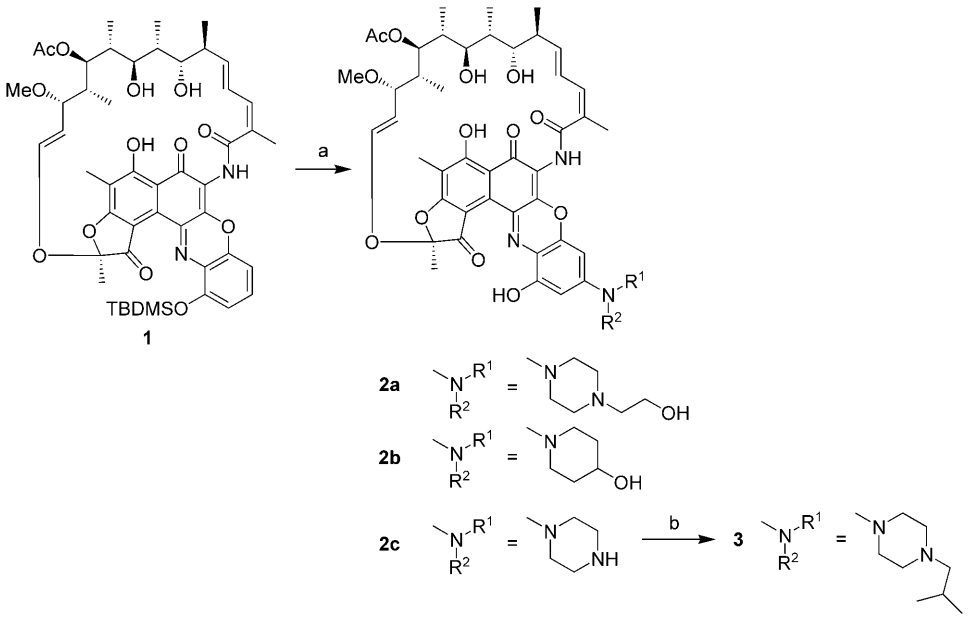
Dedicated to the memory of Professor Dmitry M. Rudkevich.

Osteomyelitis represents a challenge to modern medicine. This inflammatory process is accompanied by bone necrosis, and results from an underlying microbial infection[1] primarily caused by *Staphylococcus aureus*.[2] It is routinely treated by a combination of surgical debridement and a heavy and prolonged course of parenterally administered antibiotics. Frequent relapses are observed,[3] and sometimes amputations are required.[4] In general, osteomyelitis is established as a result of trauma, bone surgery, or joint replacement, and in cases of decreased vascularization, such as in diabetic and elderly patients. None of the antibiotics marketed in the United States have been approved for Gram-positive osteomyelitis; as such, it represents a clear medical need.

The sheltered environment provided by necrotic bone and the likely quiescent state of bacteria found in such sequestra are clear hurdles that require antibacterial agents to be administered in large doses to achieve a satisfactory therapeutic outcome. To avoid the systemic administration of large amounts of antibiotics, polymeric or mineral beads impregnated with antibiotics[5] have been proposed in order to concentrate the therapeutic agent at the site of infection. Unfortunately, these materials must be surgically inserted, resulting in significant inconveniences in the context of a disease for which recurrences are common and repeat treatments are often required.

Drug delivery to bone by way of systemic administration would present clear advantages in this case. Bisphosphonates,[6] pyrophosphate analogues with strong, near-irreversible affinity to hydroxyapatite, the calcium phosphate bone mineral, have been used to deliver small-molecule therapeutics,[7] ligands for radioisotope imaging,[8] and even proteins[9] to bone. Given their efficiency in this process, bisphosphonates would appear to be ideal targeting agents for the delivery of antibacterial agents to bone. Although a ciprofloxacin–bisphosphonate conjugate with demonstrated high affinity for bone has been synthesized,[10] the bisphosphonate moiety in this strategy is likely to remain tethered to the antibiotic. As such, it would predictably immobilize ciprofloxacin irreversibly to the bone, thereby preventing it from accessing its intracellular target, bacterial topoisomerase. In contrast, a prodrug strategy uses bisphosphonates to direct antibiotics to bone but allows for their release at the site of infection and access to their pharmacological target. Bisphosphonated prodrugs have been described for the delivery of small molecules to bone, such as diclofenac,[7a,b] prostaglandins,[7c] steroids,[7d] and carboxyfluorescein.[7e,f] As such, a drug-delivery strategy that involves prodrugs seems more judicious.



Scheme 1. Reagents and conditions: a) R^1R^2NH, MnO_2, DMSO; b) isobutyraldehyde, $NaHB(OAc)_3$. DMSO = dimethyl sulfoxide.

The rifamycins are a class of semisynthetic antibacterial ansamycins, several members of which are currently used clinically

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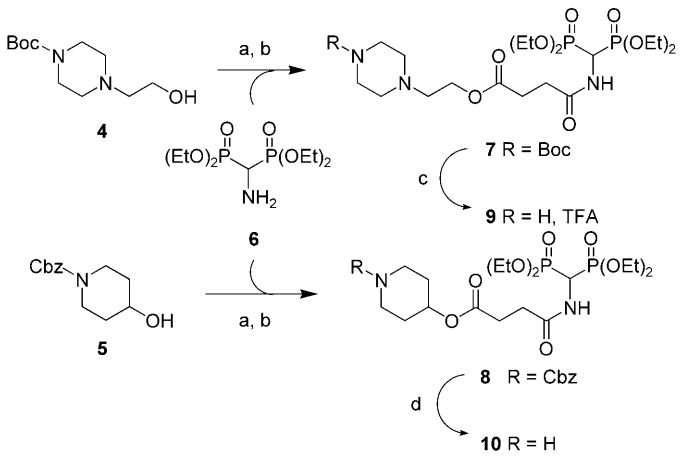
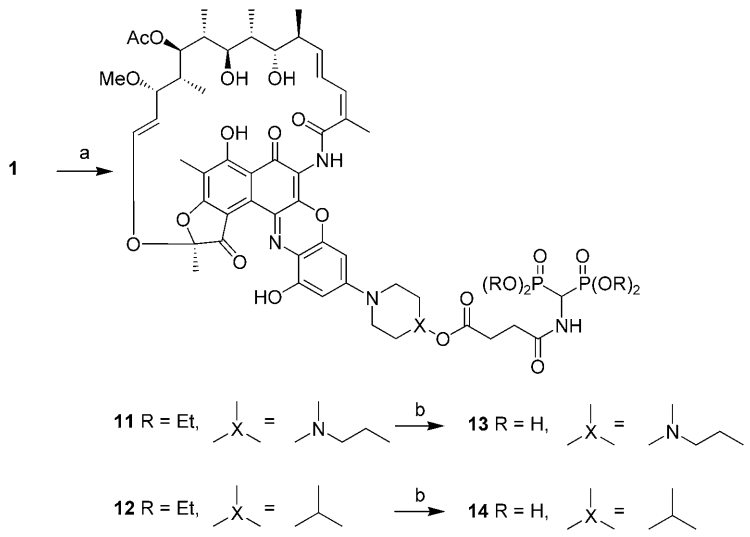
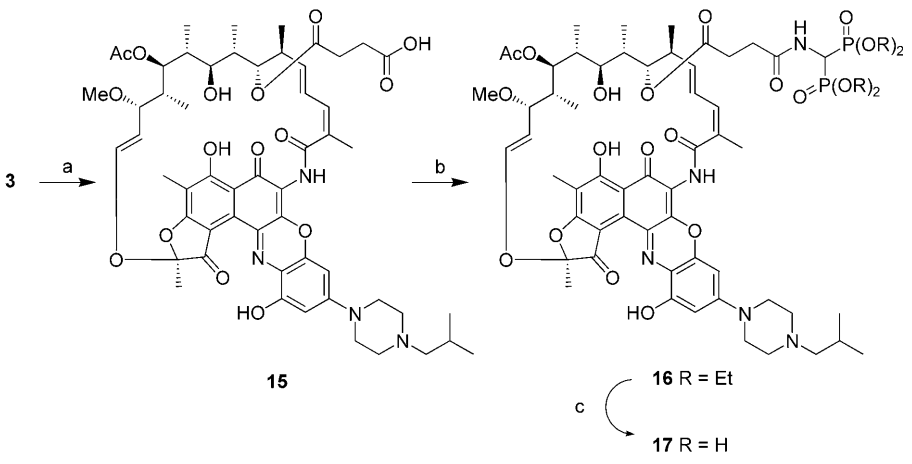


Scheme 2. Reagents and conditions: a) succinic anhydride, DMAP, $CHCl_3$; b) **6**, EDCI, DMAP, CH_2Cl_2; c) TFA, CH_2Cl_2; d) H_2, Pd/C. DMAP = 4-dimethylaminopyridine, EDCI = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, TFA = trifluoroacetic acid.



Scheme 3. Reagents and conditions: a) **9** (for **11**) or **10** (for **12**), MnO_2, DMSO; b) TMSBr, CH_2Cl_2, 2,6-lutidine, then NH_4OAc/AcOH (50 mM, pH 5). TMS = trimethylsilyl.



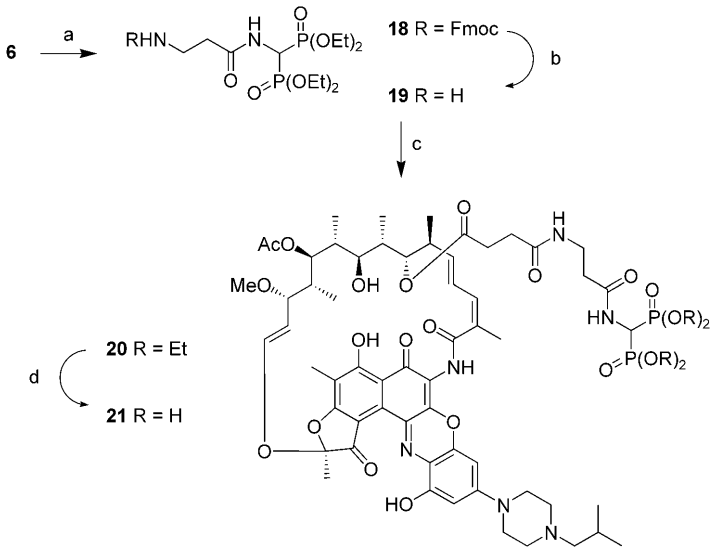
Scheme 4. Reagents and conditions: a) succinic anhydride, DMAP, THF, Δ; b) **6**, EDCI, Et_3N, DMAP, CH_2Cl_2; c) TMSBr, CH_2Cl_2, 2,6-lutidine, then NH_4OAc/AcOH (50 mM, pH 5).

or are under clinical evaluation.[11] Rifamycins target the bacterial DNA-dependent RNA polymerase with far greater selectivity (2–4 orders of magnitude) than the equivalent eukaryotic enzymes.[12] They are extremely potent against Gram-positive pathogens, less so against the Gram-negative microbes, and present the unique ability to kill bacteria in a quiescent state,[13] probably as a result of the need for short bursts of RNA synthesis even in the absence of growth. Rifamycins are therefore ideal candidates for the treatment of chronic infections. From this perspective, they present a very favorable profile for the treatment of osteomyelitis, and their efficacy, generally in combination with other antibacterial agents, has been demonstrated in animal models.[14]

Recent developments in the chemical derivatization of the rifamycin scaffold have afforded benzoxazinorifamycins,[15] a subclass of rifamycins with unrivalled potency, generally orders of magnitude more potent than other rifamycins in vitro. In particular, rifalazil (**3**) is under clinical development for the treatment of chlamydial infections.[16] These compounds would appear to be ideal warheads in a bisphosphonate prodrug strategy given the fact that they will be released from the bisphosphonated prodrugs at low concentrations over prolonged periods of time, a situation in which their high potency would be quite favorable.

Benzoxazinorifamycins **2a**–**c** were prepared as reported by the treatment of the silylated precursor **1** and a secondary amine under oxidative conditions (Scheme 1).[15] Compound **2c** can be readily converted into rifalazil (**3**) by reductive alkylation with isobutyraldehyde.

Succinamic and glutaramic esters undergo slow cyclization to the parent succinimides and glutarimides, simultaneously releasing an alcohol molecule.[17] This process provides a convenient form of prodrugs for alcohols. To this end, N-protected amino alcohols **4** and **5** were treated with succinic anhydride to produce the succinic acid monoesters, which were coupled with the aminomethylenebisphosphonate **6**[18] under standard peptide-coupling conditions to provide succinamic esters **7** and **8** (Scheme 2). N-deprotection under standard conditions and condensation with **1** provided protected bisphosphonated prodrugs **11** and **12** (Scheme 3). Deprotection with TMSBr, carried out in the presence of a base to avoid acid-mediated decomposition of the rifamycin moiety, and protolytic desilylation of the crude material furnished bisphosphonated rifamycin prodrugs **13** and **14**.



Scheme 5. Reagents and conditions: a) *N*-Fmoc-β-alanine, EDCI, Et_3N, DMAP, $CHCl_3$; b) piperidine, DMF; c) **15**, EDCI, Et_3N, DMAP, $CHCl_3$; d) TMSBr, CH_2Cl_2, 2,6-lutidine, then 0.1 N HCl, MeCN. DMF = *N*,*N*-dimethylformamide.

A similar protected bisphosphonated succinamic ester of rifalazil can be produced by a sequence of condensation with succinic anhydride followed by coupling with amine **6** (Scheme 4). Although this process is feasible, the subsequent deprotection furnished an extremely water-insoluble material. The NMR spectrum of the DMSO- and DMF-soluble material was inconclusive and could not be assigned to prodrug **17**. To bypass this problem, a spacer can be introduced either between the amide portion of the succinamate and the aminomethylenebisphosphonate moiety, or its ester portion and rifalazil.

The first approach is exemplified by the extension of amine **6** to amine **19** by coupling with *N*-Fmoc-β-alanine and subsequent deprotection (Scheme 5). Amine **19** was then coupled to rifalazil succinate **15**, and the subsequent deprotection of the bisphosphonate group provided bisphosphonated rifalazil prodrug **21**.

The second approach—inserting a spacer between the ester portion of the succinamate and rifalazil—needs to be considered more carefully, as cyclization to the succinimide would leave this spacer on rifalazil, and a second step would be required to regenerate the active antibacterial. Two such spacers were explored: a glycolate and a 4-hydroxybutyrate (Figure 1). In the first case, after formation of the succinimide, the glycolate would rely on an enzymatic process to regenerate rifalazil. In the second case, spontaneous cyclization of the 4-hydroxybutyrate to γ-butyrolactone would result in the free drug.

Acylation of rifalazil **3** with either bromoacetyl bromide or bromobutyryl bromide results in esters **22** and **23**. Treatment of amine **6** with succinic anhydride results in succinamic acid **24**, the alkylation of which with either **22** or **23** and subsequent deprotection of the bisphosphonates provides rifalazil prodrugs **27** and **28** (Scheme 6).

A β-aminoketone prodrug of ciprofloxacin was recently proposed, whereby the drug is freed by elimination.[19] A similar approach was envisaged for benzoxazinorifamycin **2c** by the preparation of prodrug **35** (Scheme 7). A sequence of alkylation of the sodium salt of tetraethyl methylenebisphosphonate with the protected bromopropanol **29**, deprotection, and iodination furnished iodide **32**. This later underwent a substitution reaction with hydroxyphenylpropenone to provide vinyl ketone **33**. The conjugate addition of **2c** onto the enone, followed by deprotection of the phosphonate esters, provided the desired prodrug **35**.

The benzoxazinorifamycins **2a**–**c** and **3** display similarly potent antibacterial activities (minimum inhibitory concentrations (MIC) of 0.00025, 0.0005, 0.0005, and 0.001 $\mu g\,mL^{-1}$ against *S. aureus* ATCC 13709, respectively). This bioactivity provides a useful means to study the behavior of the prodrugs. An estimation of the affinity of the prodrugs for osseous tissues can be obtained by measuring the amount of prodrug bound to bone powder in phosphate-buffered saline (PBS) at 37 °C over 1 h. This was ascertained by measuring antibacterial activity remaining in the supernatant to determine the unbound fraction (Table 1). The release of the parent rifamycin from these prodrugs immobilized on bone powder can similarly be determined by measuring the appearance of antibacterial activity in the supernatant over time. This was done in PBS and in 50% rat and human sera in PBS, to evaluate the potential for enzymatic cleavage (Table 1).

The results from these in vitro assays show several trends. Firstly, these prodrugs are very efficient at binding bone powder, being taken up at >95% over 1 h, when the parent drugs are at best negligibly bound (results not shown). In fact,

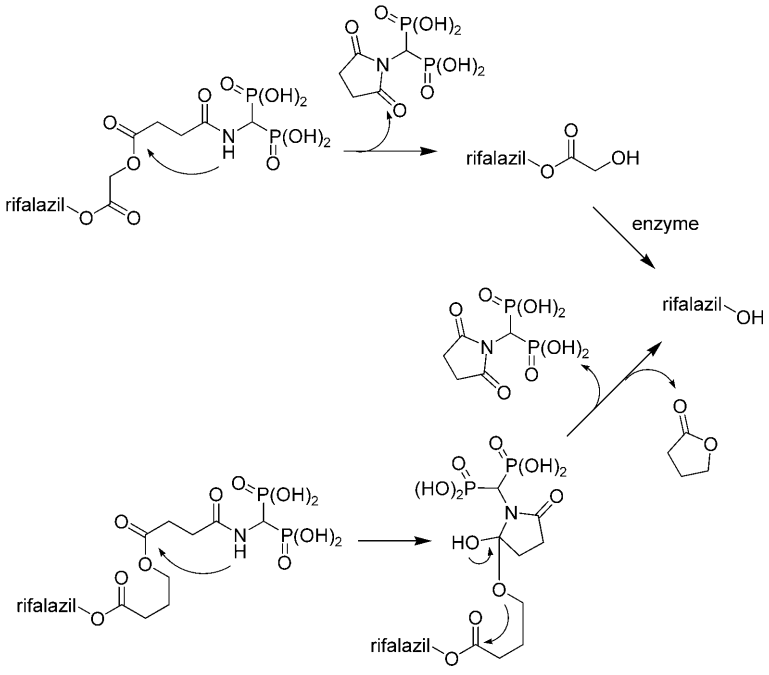
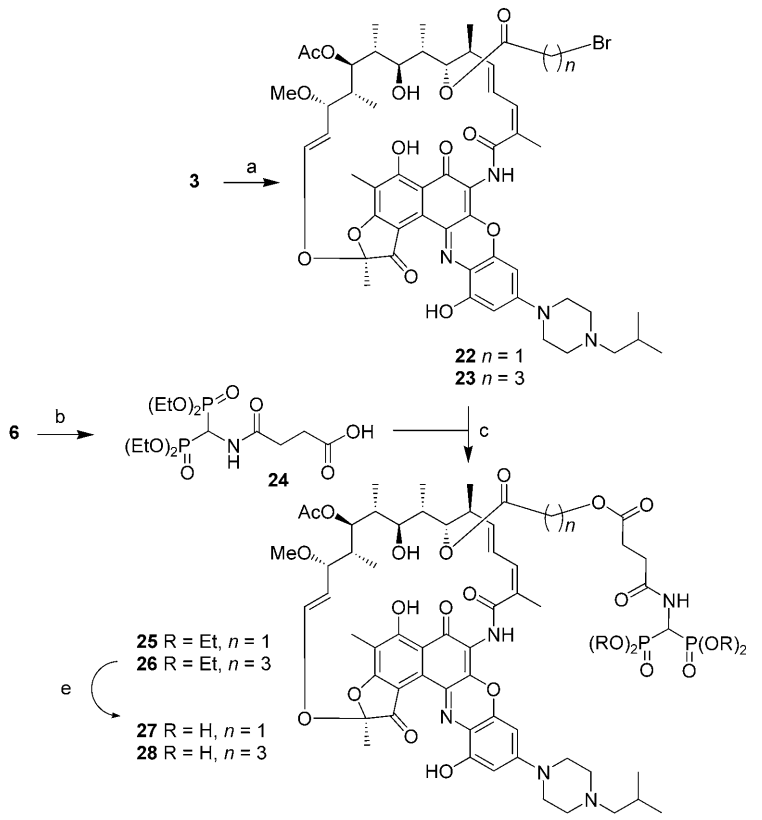
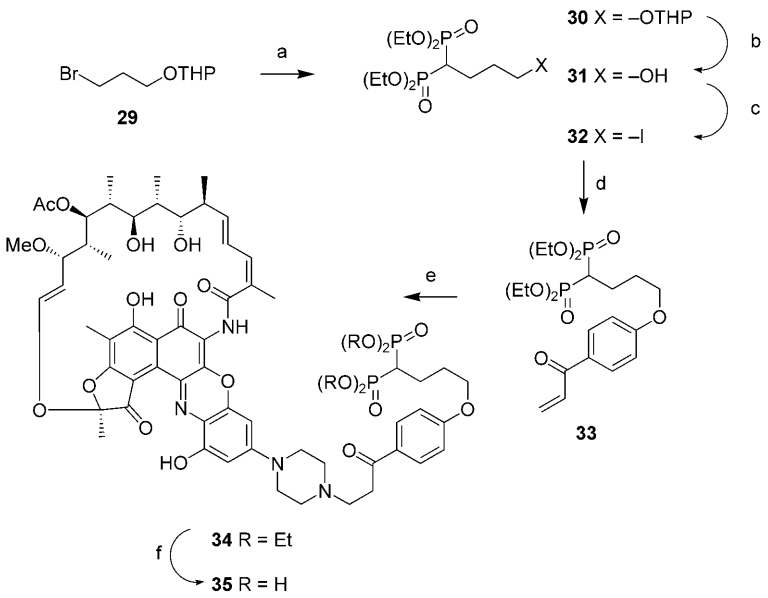


Figure 1. Spacer strategies on rifalazil.



Scheme 6. Reagents and conditions: a) bromoacetyl bromide (for **22**) or 4-bromobutyryl chloride (for **23**), DMAP, pyridine, CH_2Cl_2; b) **6**, succinic anhydride, $CHCl_3$; c) **24**, Cs_2CO_3, DMF; d) TMSBr, CH_2Cl_2, 2,6-lutidine, then NH_4OAc/AcOH (50 mM, pH 5).



Scheme 7. Reagents and conditions: a) NaH, tetraethyl methylenebisphosphonate, THF, Δ; b) pTsOH, MeOH; c) I_2, PPh_3, imidazole, CH_2Cl_2; d) 1-(4-hydroxyphenyl)prop-2-en-1-one, K_2CO_3, acetone; e) **2c**, DBU, PhMe; f) TMSBr, CH_2Cl_2, 2,6-lutidine, then 0.1 N HCl, MeCN. DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene.

it is reasonable to assume that the unbound fraction is at least partially the result of cleavage of the prodrug during the time course of the assay, thereby under-representing the true efficiency of the process. Secondly, as expected, prodrugs that rely on succinamate cyclization are heavily affected by steric bulk at the ester functional group. Thus **13** (ester of a primary alcohol) readily provided **2a**, whereas the more hindered secondary alcohol on **2b** resulted in a prodrug **14** with much slower release kinetics, and the more hindered **21** was completely ineffective in generating any rifalazil.

The introduction of a spacer between rifalazil and the succinamate linker did not result in a favorable outcome for **27**, either as a result of negligibly slow cyclization to the succinimide, or more likely as a result of a lack of subsequent hydrolysis of the glycolate spacer. This stands in contrast with prodrug **28**, which efficiently regenerated rifalazil, and emphasizes the role of the γ-hydroxybutyrate spacer.

These linkers that rely on succinamate cyclization were clearly sensitive to the presence of serum, with significantly better levels of regeneration. Per se, this is not an indication of the involvement of hydrolytic enzymes in the process, but it certainly suggests it to be a possibility.

Compound **35**, which relies on β-elimination to provide rifamycin **2c**, is similarly efficient in regenerating the parent drug. Interestingly, the presence of serum also markedly accelerates this ability.

Notably there is a marked decrease between the rates of regeneration in solution and the rates of regeneration once bound to bone. Thus compound **28** is rapidly converted into rifalazil (**3**) in PBS (31.10% ±1.63 over 24 h) and in rat plasma (50.6% ±6.50 over 24 h) as shown by the same bioassay. Based on the MIC values, the proportion of cleavage in solution for **13** is 8.4% over 24 h in either cation-adjusted Mueller Hinton broth (CAMHB) or 50% mouse serum in CAMHB, while compounds **14**, **21**, **27**, and **35** are all <1% converted under the same conditions. These rates provide a favorable profile given the fact that bisphosphonates are generally taken up rapidly (<1 h) in vivo.

A pharmacokinetic study of the behavior of prodrug **13** is presented in Figure 2. For the purpose of this study, the tibiae of rats administered with **13** at 13 mg kg^{-1} i.v. bolus were ground, washed with methanol to remove any free **2a**, and incubated at 70 °C in 100 mM sodium phosphate adjusted to pH 10 to decompose **13** into **2a**, the concentration of which was determined by LC–MS.

This study demonstrates that the bisphosphonated prodrug accumulates in bone and releases the parent drug over time. This is to be contrasted with the parent antibiotics, which are not detectable after 48 h (results not shown). The release of the parent drug for **13** has a half-life of 3.2 days and therefore is predicted to result in a continuous exposure of the site of infection to the antibiotic. Interestingly, the rate of disappearance of the prodrug from bone is much higher than would

Table 1. Bone binding and conversion of bisphosphonated rifamycin prodrugs to parent drugs after binding to bone.[a]

Compound	Parent	Bone binding [%]	Medium	Conversion [%]
			PBS[b]	0.21
13	**2a**	99.8	50% HS[c]	0.86
			50% RS[d]	0.91
			PBS	0.02
14	**2b**	99.9	50% HS	n.d.[e]
			50% RS	0.24
			PBS	< l.o.d.[f]
21	Rifalazil (**3**)	99.9	50% HS	n.d.
			50% RS	0.01
			PBS	< l.o.d.
27	Rifalazil (**3**)	99.8	50% HS	n.d.
			50% RS	0.12
			PBS	2.0
28	Rifalazil (**3**)	94.8	50% HS	n.d.
			50% RS	2.5
			PBS	0.1
35	**2c**	99.9	50% HS	0.46
			50% RS	0.38

[a] Binding and conversion values expressed as percent prodrug converted after 24 h incubation. [b] PBS: phosphate-buffered saline. [c] 50% human serum in PBS. [d] 50% rat serum in PBS. [e] Not determined. [f] Below the limit of detection (0.01%).

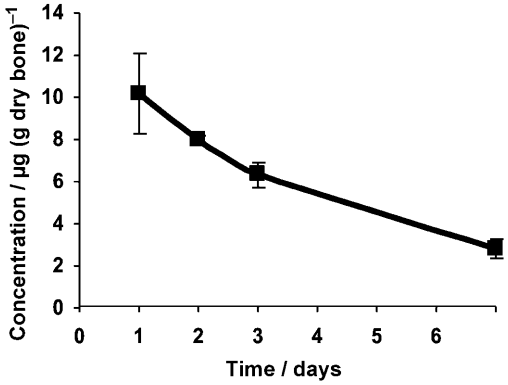


Figure 2. The concentration of prodrug **13** in rat femur after i.v. administration at 13 mg kg^{-1} over time.

have been predicted from in vitro results, a matter that may imply the involvement of hydrolytic enzymes.

The ability of the prodrugs to release the parent drugs over a long period of time would imply that they may be able to prevent the establishment of infection when administered prior to bacterial challenge. This notion was examined by adaptation of the rat model of osteomyelitis caused by *S. aureus*.[20] Bisphosphonated rifamycin prodrugs **13**, **14**, **28**, and **35** were administered intravenously to rats in a single dose either two or three days prior to the injection of bacteria into their tibiae. 24 h after bacterial challenge, the bacterial load in these bones was measured to determine efficacy (Table 2).

These experiments show that bisphosphonated rifamycin prodrugs are able to prevent the occurrence of infection when used as a prophylactic treatment. The result obtained with compound **13** ($p < 0.005$) clearly shows that bisphosphonated prodrugs are efficacious even when the parent drug has ceased to demonstrate efficacy. The comparison of prodrugs **13** and **14** in this animal model also reveals the importance of regenerating the parent drug at a sufficient rate. The inactivity of **14** in this in vivo model and its low rate of regeneration in vitro suggest that it is not able to release **2b** at a rate sufficient to reach therapeutically useful antibacterial concentrations.

Given this result, compounds **13**, **28**, and **35** were selected to be tested as treatments in the rat osteomyelitis model.[20] Briefly, the animals were surgically infected with *S. aureus* in one tibia and left untreated for 14 days to establish a chronic bone infection. Compounds were then administered for four sequential days and then every four days for a total of 28 days (10 doses administered in total). The animals were sacrificed 24 h after the last dose, and the bacterial loads in their tibiae were measured (Table 3).

The bisphosphonated prodrugs displayed statistically significant ($p < 0.005$) efficacy in this animal model. This is in contrast to the parent drugs, which do not show any impact on the infection. This experiment clearly demonstrates the beneficial role of the bisphosphonate group in delivering the benzoxazinorifamycins to the bone at the site of infection.

Given the high spontaneous rate of resistance associated with rifamycins, the number of animals possessing bacteria resistant to the parent drug was also assessed. The level of resistance was ascertained by extracting the ground bone with PBS and plating the extracts in the presence of parent drug at MIC to detect growth. At the end of treatment, it appears that 10% of the animals treated with either **13** or its parent **2a** had resistant bacteria in their bones, and that proportion was 30% with either **28** or its parent rifalazil (**3**) and 0% with either **35** or its parent **2c**. The altered pharmacokinetics associated with the slow release from the bisphosphonate clearly do not impact the proportion of bacteria developing resistance in a significant manner.

Benzoxazinorifamycins are extremely potent bactericidal antibacterial agents, and the combination of this activity with the

Table 2. Activity of bisphosphonated prodrugs and their non-bisphosphonated parent antibiotics as prophylactic treatments.[a]

Compound	Dose [mg kg^{-1}]	Days prior to infection	Bacterial load in bone [log CFU (g bone)$^{-1}$][b]	Bacterial load from parent [log CFU (g bone)$^{-1}$][c]
13	26	2	2.0 ± 0.06	3.6 ± 1.6
14	26	3	4.9 ± 0.66	2.8 ± 0.93
28	14	3	2.1 ± 0.06	2.2 ± 0.34
35	28	3	2.8 ± 1.0	n.d.

[a] Five animals per group. [b] CFU: colony-forming units. [c] Administered at an equivalent dose.

Table 3. Activity of selected bisphosphonated prodrugs and their non-bisphosphonated parent antibiotics in the rat model of chronic bone infection.[a]

Compound	Dose [mg kg^{-1}]	Bacterial load [log CFU (g bone)$^{-1}$]		
		Untreated group	Prodrug	Parent[b]
13	26	6.3 ± 0.35	4.9 ± 0.42	6.0 ± 0.30
28	28	5.8 ± 0.88	4.7 ± 0.25	5.7 ± 0.28
35[c]	28	5.6 ± 0.43	4.1 ± 0.28	5.2 ± 0.33

[a] Ten animals per group. [b] Administered at equivalent doses. [c] Eight animals per group were used.

generally accepted activity of rifamycins on biofilms suggests that this compound class may provide relief from a chronic and difficult to treat infection such as osteomyelitis. This study demonstrates that the use of bisphosphonates can bias the pharmacokinetic behavior of benzoxazinorifamycins, allowing them to exert both pre-challenge prophylactic and post-challenge therapeutic activity against *S. aureus* in in vivo models of bone infection, whereas the parent antibiotics were inactive in these settings. It also highlights that a prodrug strategy is required and that the rate of release must be sufficient to afford antibacterial activity. With judiciously chosen linkers, bisphosphonated benzoxazinorifamycin prodrugs **13**, **28**, and **35** demonstrate the potential of this approach in providing a therapeutic path for the treatment of osteomyelitis. Recent issues have been raised with the use of bisphosphonates in treating osteonecrosis of the jaw,[21] with particular respect to parenterally administered bisphosphonates. Certainly, the relative innocuousness of the bisphosphonate moiety in any of these prodrugs remains to be evaluated, and any impact on bone physiology should be limited. It should be noted from this respect, that the nature of infectious diseases suggests that any treatment for osteomyelitis would be limited to a matter of days or weeks, and the exposure to the bisphosphonated prodrug would be expected to be a fraction of the exposure used with clinically relevant bisphosphonates, but this has to be demonstrated.

Keywords: bisphosphonates · osteomyelitis · prodrugs · rifalazil · rifamycins

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