Bioorganic & Medicinal Chemistry Letters 20 (2010) 1355–1359

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and in vitro evaluation of bisphosphonated glycopeptide prodrugs for the treatment of osteomyelitis

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ARTICLE INFO

Article history:
Received 27 November 2009
Revised 29 December 2009
Accepted 4 January 2010
Available online 11 January 2010

Keywords:
Glycopeptides
Prodrugs
Osteomyelitis

ABSTRACT

As therapeutic agents of choice in the treatment of complicated infections, glycopeptide antibiotics are often preferentially used in cases of osteomyelitis, an infection located in bone and notoriously difficult to successfully manage. Yet frequent and heavy doses of these systemically administered antibiotics are conventionally prescribed to obtain higher antibiotic levels in the bone and reduce the high recurrence rates. Targeting antibiotics to the bone after systemic administration would present at least three potential advantages: (i) greater efficacy, by concentrating the therapeutic agent in bone; (ii) greater convenience, through a reduction in the frequency of administration; and (iii) greater safety, by reducing the levels of systemic drug exposure. We present here the design, synthesis and in vitro evaluation of eight prodrugs of the glycopeptide antibacterial agents vancomycin and oritavancin taking advantage of the affinity of the bisphosphonate group for bone for delivery to osseous tissues.

Osteomyelitis is an inflammatory process accompanied by bone necrosis which results from an underlying microbial infection,[1] primarily caused by gram-positive microorganisms.[2] It is a challenging disease, routinely treated by a combination of surgical debridement and a prolonged course of parenterally administered high doses of antibiotics. It is characterized by frequent relapses[3,4] and sometimes a need for amputations.[5] None of the antibiotics marketed in the United States have been approved for gram-positive osteomyelitis and treatments are devised through practice.

The difficulty in treating osteomyelitis is often attributed to the sheltered environment provided by necrotic bone and the likely quiescent state of bacteria found in such sequestra. Antibacterial agents are therefore administered in large doses to provide a sufficient level in the bone. In order to avoid the potential side effects associated with the systemic administration of such large doses, polymeric or mineral beads impregnated with antibiotics[6–10] have been proposed to concentrate the therapeutic agent at the site of infection. Unfortunately, surgical intervention is required to insert the beads, a significant hurdle in the context of a disease where recurrences are common and repeat treatments are often required.

Targeting antibacterial drugs to bone after systemic administration would clearly provide a more convenient form of treatment. Bisphosphonates,[11–13] pyrophosphate analogs with high, near-irreversible affinity for hydroxyapatite, the calcium phosphate bone mineral, have been used to deliver therapeutics,[14–29] including antibacterial agents,[26–29] to bone.

While efficient bone delivery through conjugation with bisphosphonates is expected, our previous work[26,28] has demonstrated the near irreversible binding of bisphosphonates to calcium mineral requires subsequent release of the free antibiotic in order for it to freely exert its activity. The bisphosphonated entity must therefore act as a prodrug.

Glycopeptide antibacterial agents are naturally occurring or semisynthetic compounds, several members of which are currently used clinically or are under clinical evaluation.[30,31]. They are active only against gram-positive microorganisms. The archetypical glycopeptide antibiotic is vancomycin (**1**, Fig. 1), the discovery of which triggered the isolation of a large number of related compounds, including teicoplanin.[30]

Given its low potential for the selection of resistant microorganisms, vancomycin is the primary agent for the treatment of methicillin resistant gram-positive bacterial infections.[32,33] As such, it is very frequently used for the treatment of osteomyelitis, administered by infusion twice daily.[34]

The clinical relevance of these naturally occurring glycopeptides and, to some extent, the emergence of vancomycin resistance, in particular in enterococci, spurred programs for the development of second generation semisynthetic glycopeptides. This effort resulted in the discovery of three drug candidates: dalbavancin, oritavancin (**2**, Fig. 1) and telavancin.[35] Oritavancin demonstrates substantial potency in vitro,[36] displays rapid bactericidal activity

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doi:10.1016/j.bmcl.2010.01.006

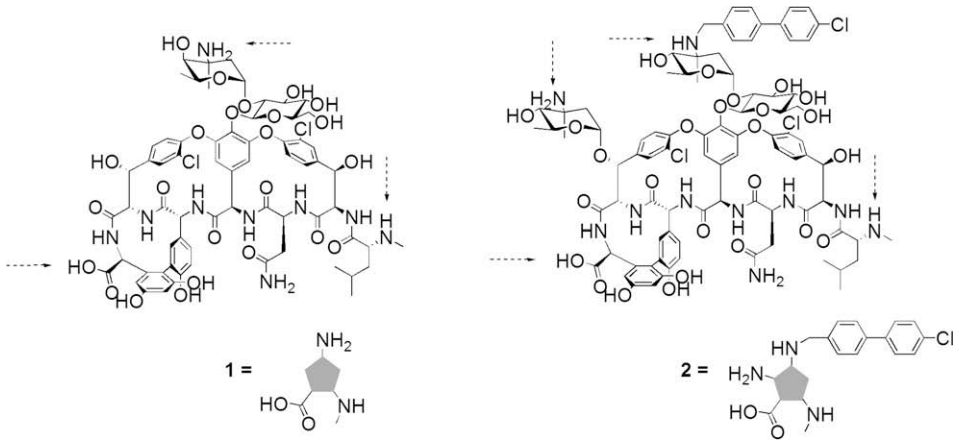


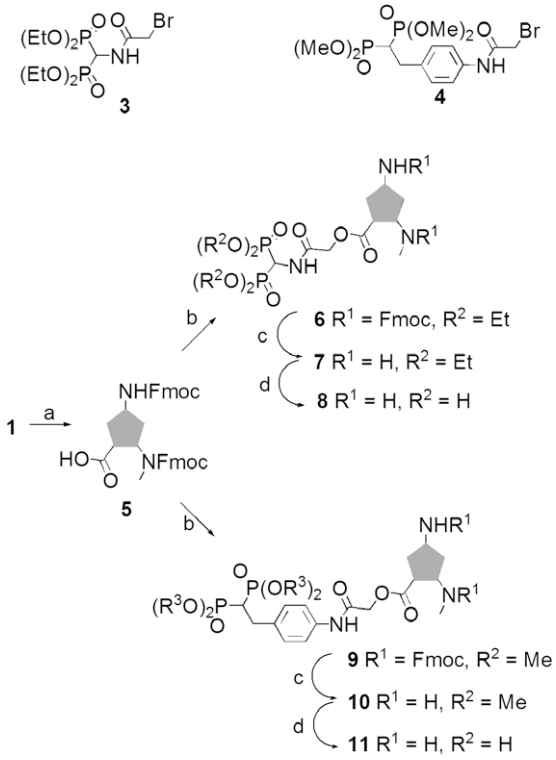
Figure 1. The structures and schematic representations for vancomycin (**1**) and oritavancin (**2**). The arrows indicate the relevant amino and carboxylate groups depicted schematically.

against planktonic bacteria,[37] and, unlike vancomycin, is active against cells both in stationary phase and in biofilms.[38] It therefore has the potential to be an attractive alternative to vancomycin for the treatment of osteomyelitis. From this perspective, we present herein the synthesis and in vitro evaluation of bisphosphonated derivatives of vancomycin and oritavancin.

The lability of glycolamide esters under physiological conditions,[39] probably as a result of cleavage by acetylcholinesterases and other esterases, provides a convenient strategy for the design of prodrugs based on drugs possessing free carboxylates. The concept has been successfully transposed to the use of bisphosphonates,[14,15] in particular with fluoroquinolone antibiotics.[27] Thus the preparation of bisphosphonated glycolamide esters of glycopeptides appeared to be particularly attractive. Bisphosphonated vancomycin glycolamides **8** and **11** were prepared by the treatment of Fmoc protected vancomycin **5** with bromoacetamides **3**[18] and **4**[27] under basic conditions followed by deprotection reactions (Scheme 1). The same procedure was not able to provide the parent oritavancin prodrugs after deprotection, and therefore the preparation was performed with allyl protected bromoacetamides **15** and **18** prepared from tetraallyl methylenebisphosphonate by a sequence of either electrophilic amination or alkylation and reduction, followed by acylation with bromoacetyl bromide (Scheme 2). The alkylation of allyloxycarbamate protected oritavancin **19** with either **15** or **18**, and subsequent global deprotection by palladium catalyzed deallylation, furnished bisphosphonated oritavancin glycolamides **21** and **23**, respectively (Scheme 3).

Simple PEGylated amides of vancomycin, formed by derivatization on the *N*-methyl-leucyl residue, were shown, albeit unexpectedly, to be labile under physiological conditions, and as such useful as prodrugs.[40] Therefore simple bisphosphonated amides of oritavancin were prepared by selective acylation on the *N*-methyl-leucyl nitrogen[41] with the *N*-hydroxysuccinimide esters of bisphosphonated acids **24**[28] and **25**[28] (Scheme 4).

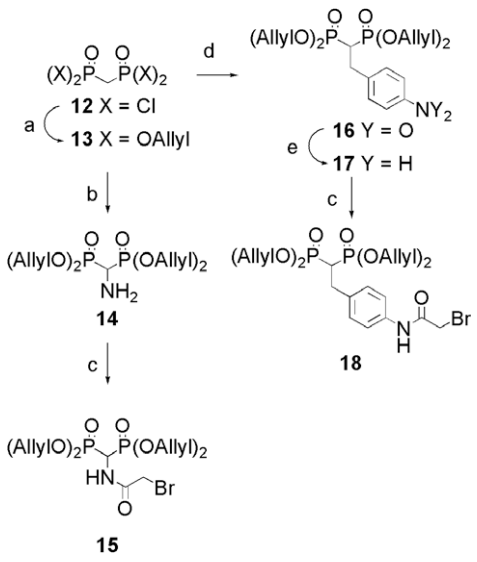
Substituted acyloxyalkyl esters and carbamates are frequently used as prodrugs,[42–44] and this strategy has been successfully combined with the use of bisphosphonates for delivery to bone.[28] Acyloxyalkyl carbamates are generally prepared by treatment of a chloromethyl carbamate with an acid. In this case, treatment of **2** with chloromethyl chloroformate or *N*-(chloromethoxyformyloxy) succinimide as a first step resulted in a mixture of formylated derivatives and no sign of product. The bisphosphonated derivatives were therefore prepared by assembling the bisphosphonated acyloxyalkyl formylating agent prior to acylation (Scheme 5). This was performed by treatment of acids **25** and **32**[28] with *S*-ethyl *O*-iodomethyl carbonothionate,[45] electrophilic substitution of the



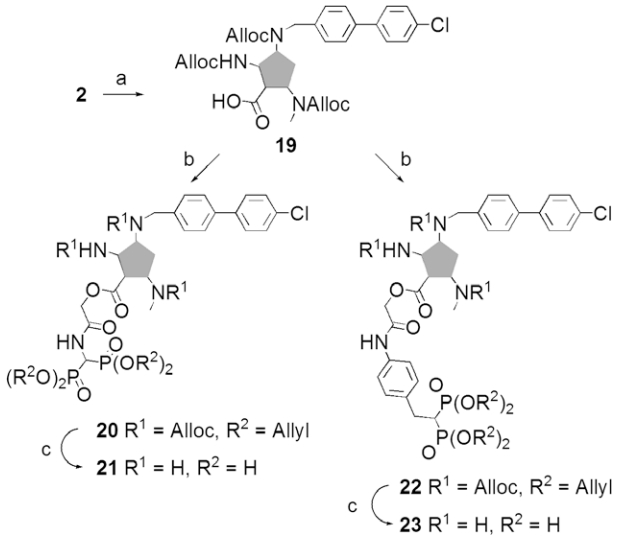
Scheme 1. (a) *N*-(9*H*-fluorenylmethoxycarbonyloxy) succinimide, $NaHCO_3$, Dioxane/H_2O (Quant.); (b) **3** (for **6**) or **4** (for **9**), $NaHCO_3$, DMF (56% for **6**, 61% for **9**); (c) Piperidine, DMF (82% for **7**, 95% for **10**); (d) TMSBr, 2,6-lutidine, DMF, then HF.pyridine, pyridine DMF (44% for **8**, 42% for **11**).

ethylmercapto group with chlorine and acylation of *N*-hydroxysuccinimide with the resulting chloroformates **35** and **36**. The activated carbonates **37** and **38** were then used with **2** in a sequence of acylation and deprotection to furnish the bisphosphonated oritavancin derivatives **41** and **42**. It should be noted that attempts to prepare the same type of prodrug with the shorter bisphosphonated acid **24** did not succeed, possibly as a result of its greater lability.

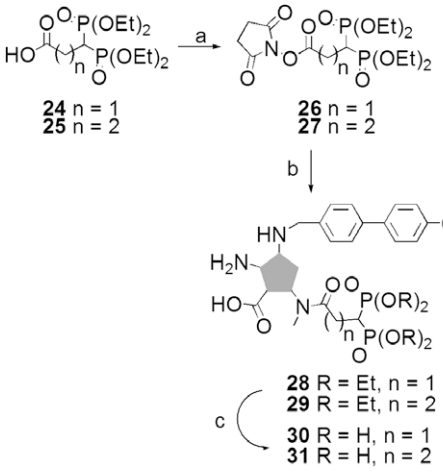
The antibacterial activities (as minimum inhibitory concentrations—MICs) for vancomycin **1**, oritavancin **2** and the bisphosphonated derivatives against *S.aureus* ATCC13709 were determined (Table 1). This bioactivity provides a useful if only indirect means by which to evaluate the behaviour of the prodrugs in solution. In-



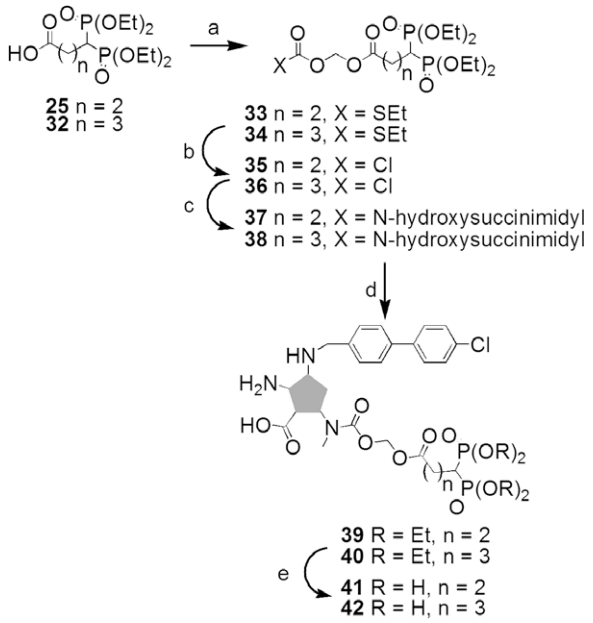
Scheme 2. (a) Allyl alcohol, pyridine, PhMe (69%); (b) NaH, DMF then $Ph_2P(O)ONH_2$, THF (59%); (c) bromoacetyl bromide, pyridine, CH_2Cl_2 (74% for **15**, 76% for **18**); (d) NaH, DMF, 4-nitrobenzyl bromide (39%). (e) Zn, NH_4Cl, MeCN (86%).



Scheme 3. (a) *N*-(allyloxycarbonyloxy)succinimide, $NaHCO_3$, DMF/H_2O (93%); (b) **15** (for **20**) or **18** (for **22**), $NaHCO_3$, DMF; (c) $Pd(PPh_3)_4$, DMF, morpholine (24% over two steps for **21**, 43% over two steps for **23**).



Scheme 4. (a) *N*-Hydroxysuccinimide, DCC, CH_3CN (quant for **26**, 93% for **26**); (b) **2**, $NaHCO_3$, H_2O/dioxane (12% for **28**, 22% for **29**); (c) TMSBr, 2,6-lutidine, DMF, then HF/pyridine, DMF (14% for **30**, 15% for **31**).



Scheme 5. (a) Tetrabutylammonium hydrogensulfate, $NaHCO_3$, *S*-ethyl *O*-iodomethyl carbonothionate, H_2O, CH_2Cl_2 (76% for **33**, 83% for **34**); (b) Neat SO_2Cl_2; (c) *N*-hydroxysuccinimide, $NaHCO_3$, H_2O/dioxane (quant over two steps for **37**, 70% over two steps for **38**); (d) **2**, $NaHCO_3$, H_2O/dioxane (44% for **39**, 32% for **40**); (e) TMSBr, 2,6-lutidine, DMF, then HF/pyridine, DMF (54% for **41**, 44% for **42**).

deed, while the potency of the parent glycopeptides in the absence of serum is evident, the bisphosphonated conjugates were 16–64-fold less active in this assay. This suggests that the addition of the bisphosphonate moiety is detrimental to the antibacterial activity associated with the glycopeptide scaffold. It confirms that a strategy requiring the bisphosphonated group to be released under physiological conditions is necessary for the compounds to bring about a satisfactory therapeutic outcome.

The activity of the bisphosphonated conjugates of oritavancin was greatly enhanced in the presence of mouse and rat serum, which infers that under these conditions, these compounds are converted back to oritavancin. This conclusion is supported by the rapid bactericidal activity of oritavancin,[37] which is able to exert its activity immediately upon reaching a satisfactory concentration in the medium. If the same effect is not observed with the bisphosphonated vancomycin conjugates, it is likely the result of the slower kinetics of bacterial killing associated with this antibiotic. Thus, one can compare compounds **8** and **21** or **11** and **23**, which would be expected to release the parent drugs to a similar extent in plasma, but yet yield different outcomes in this assay.

The exceptions amongst the oritavancin derivatives are compounds **31** and **42** which did not display any significant antibacterial activity even in the presence of sera, and which are therefore expected to be stable in the presence of serum hydrolytic enzymes and unable to release oritavancin under these conditions.

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 1h. This was ascertained by measuring antibacterial activity remaining in the supernatant to determine the unbound fraction (Table 1). The release of the parent glycopeptide from these prodrugs immo-

Table 1
Susceptibility of *S.aureus* to selected compounds (minimum inhibitory concentrations [MICs] in μg/mL)

Compds	*S.aureus* ATCC 13709			
	CAMHB[a]	CAMHB[a] + 50% MS[b]	CAMHB[a] + 50% HS[c]	CAHMB[a] + 50% RS[d]
Vancomycin **(1)**	1	2	1	2
8	16	16	8	32
11	16	16	16	32
Oritavancin **(2)**[e]	0.5	2	1	0.25
21	16	2	32	2
23	>32	8	>32	8
30	32	4	32	4
31	>32	>32	>32	>32
41	8	0.5	4	1
42	32	32	>32	32

[a] Cation adjusted Mueller–Hinton broth.
[b] Mouse serum.
[c] Human serum.
[d] Rat serum.
[e] Measured in the absence of polysorbate-80.[46]

bilized on bone powder can similarly be determined by measuring the appearance of antibacterial activity in the supernatant over time, as determined by an agar diffusion bioassay using, as the indicator strains, *Bacillus subtilis* 1A754 for vancomycin derivatives (vancomycin MIC 0.5 μg/mL) and *Streptococcus pneumoniae* ATCC 700902 for oritavancin derivatives (oritavancin MIC 0.001 μg/mL). To evaluate the potential for enzymatic cleavage, conversion was assessed both in PBS and in 50% rat and human sera in PBS after 24 h (Table 1). Compound **23** was not soluble under the conditions of the assay and was therefore not investigated further.

As shown in this assay, bisphosphonated glycopeptide prodrugs displayed very high affinity for bone powder, with >95% binding over an hour. In fact, since the determination of binding affinity relies on a bioassay monitoring antibacterial activity, it is quite likely that at least a portion of the unbound material is the free parent drug, thereby underestimating the true affinity of the prodrugs for bone mineral.

As was demonstrated with other classes of compounds,[14,15,27] bisphosphonated glycolamide prodrugs of vancomycin and oritavancin were readily converted to the parent drugs. In addition, while there is a definite acceleration of the process in the presence of serum, presumably as a result of the presence of serum hydrolytic enzymes, the conversion still occurs to a significant extent in phosphate buffered saline. Therefore it at least does not require the participation of any hydrolytic enzymes.

Simple bisphosphonated amides of oritavancin (compounds **30** and **31**) failed to significantly regenerate the parent drug in buffered saline. This was in agreement with our observations regarding bisphosphonated fluoroquinolone amides.[28] However, while the conversion of compound **30** to oritavancin remains insignificant in the presence of serum, there is a marked acceleration for compound **31** (0.01% over 24 h in the absence of serum as opposed to 0.9% over 24 h in the presence of serum, Table 2). This unexpected result somewhat mirrors the lability of simple PEGylated vancomycin amides.[40] The role of serum in this rate acceleration implies the possibility of enzymatic cleavage, and certainly the structural dependence suggested by the contrast between **30** and **31** indicates a selectivity generally associated with enzymes.

A similar comparison can be performed with compounds **41** and **42**. Indeed, while compound **42** is not converted back to oritavancin to any significant level either in PBS or in the presence of serum, compound **41** significantly decomposes to the parent drug in the presence of serum. For this latter compound, the rate acceleration in going from PBS to serum is spectacular. This stands in contrast with the parent gatifloxacin prodrug,[28] which involved the same prodrug strategy, but which was converted to the parent drug to a significant extent even in the absence of serum (13.4% conversion over 24 h in PBS, 25.4% in 50% rat serum). This contrast between the oritavancin prodrug and the gatifloxacin prodrug parallels the differences between the bisphosphonated vancomycin prodrugs **8** and **11**, for which conversion is relatively independent of the presence of serum, and the parent oritavancin prodrug **21**, for which the presence of serum results in significant rate acceleration. It is in fact quite likely that the greater dependence of the oritavancin prodrugs on serum for conversion is an experimental artifact due to the poor solubility of oritavancin in saline and its greater solubility in plasma,[46] and therefore poorer recovery in the former medium. This would result in a lower concentration of oritavancin in the supernatant in PBS and a greater concentration in 50% serum. Indeed spiking naïve rabbit bone powder and extracting with PBS containing 0.002% polysorbate 80 at 0.25, 1 and 4 μg/mL assuming full recovery after extraction resulted in <1% recovery as measured by MIC using *Enterococcus faecalis* ATCC 29212 as the indicator strain.

Table 2
Bone binding and conversion of bisphosphonated glycopeptide prodrugs to parent drugs after binding to bone (expressed as % prodrug converted after 24 h incubation)

Compounds	% Bone binding	% Conversion	
		PBS[a]	50% RS[b]
Vancomycin (**1**) *derivatives*			
8	96.5	2.1	2.6
11	96.7	3.5	4.1
Oritavancin (**2**) *derivatives*			
21	97.6	0.18	4.09
30	99.9	0.01	0.05
31	99.8	0.01	0.9
41	96.9	0.2	26.4
42	99.9	<lod[c]	0.05

[a] Phosphate buffered saline.
[b] Rat serum in phosphate buffered saline.
[c] Below the limit of detection.

Glycopeptides are important antibacterial agents in the treatment of complicated gram-positive infections such as osteomyelitis and the main recourse for infections caused by resistant gram-positive pathogens. This study demonstrates that the use of bisphosphonates as a means to impart bone affinity can be extended to the glycopeptide class. It also highlights the development of prodrug molecules around the glycopeptide scaffold and in particular presents protection/deprotection strategies which are compatible with both their complex molecular architecture and the chemically labile bisphosphonated linkers. As was already highlighted with other classes of molecules, the evaluation of these prodrugs

in vitro confirms the potential of glycolamides[14,15,27] and acyloxymethyl carbamates[28] as efficient linkers for bisphosphonate based bone delivery. But the results observed with prodrug **41** also suggest that simple bisphosphonated amides are sufficiently labile under physiological conditions to function as glycopeptide prodrugs. Further investigation of the efficacy and safety of these compounds in accepted animal models is necessary to fully evaluate their potential for the treatment of osteomyelitis.

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