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Water-soluble phosphate prodrugs of pleuromutilin analogues with potent in vivo antibacterial activity against Gram-positive pathogens

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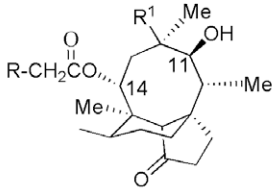
ABSTRACT

A phosphate prodrug strategy was investigated to address the problem of poor aqueous solubility of pleuromutilin analogues. Water-soluble phosphate prodrugs **6a**, **6b** and **6c** of pleuromutilin analogues were designed and synthesized. Three compounds all exhibited excellent aqueous solubility (>50 mg/mL) at near-neutral pH and sufficient stability in buffer solution. In particular, the phenol pleuromutilin prodrug **6c** displayed favourable pharmacokinetic profiles and comparable potency with vancomycin against MSSA and MRSA strains in vivo.

Due to rapid emergence of multi-drug-resistant Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae* (PRSP), and vancomycin-resistant *enterococci* (VRE),[1,2] there is an urgent need to identify and develop new antibacterial agents with novel mechanisms of action against drug-resistant bacterial strains. One strategy is to re-evaluate old generation of antibiotics that have not been widely used in clinical. The natural product pleuromutilin (**1**) belongs to this class of under-exploited antibiotics.[3]

Pleuromutilin (**1**) was first isolated in 1951 from basidiomycetes *Pleurotus* and *Pleurotus passeckerianus*. Further studies have shown that this class of antibiotics selectively inhibit bacterial protein synthesis by specifically targeting the 50s subunit of the bacterial ribosome and display modest activity against Gram-positive organisms in vitro and relatively weak activity in vivo.[4–6] Over the past years, the Sandoz group studied structure–activity relationships (SARs) of pleuromutilin analogues focused on variations in the C-14 glycolic acid side chain. As a result of these efforts, tiamulin (**2**) and valnemulin (**3**) were successfully developed as veterinary medicine to treat serious infections in swine and poultry.[7,8] In 2007, GlaxoSmithKline's novel pleuromutilin analogue retapamulin (**4**) (Fig. 1), with excellent activity in vitro, was first approved for human use as a topical antimicrobial agent to treat skin infections.[9] Although these semi-synthetic pleuromutilins with thioether substituents in the C-14 ester side chain are generally potent in vitro, they suffer from rapid and extensive metabolism in vivo because of their strong hydrophobic nature.[10]

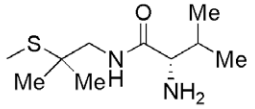
Another series of pleuromutilin analogues attracted great attention is the C-14 acyl-carbamates derivatives. Like thioether derivatives, these carbamates show broad-spectrum antibacterial activities, however, are much more metabolically stable.[3] SmithKline recently reported two potent pleuromutilin analogues with C-14 aroyl-carbamates side chain, **5a** (**SB-225586**) and **5b** (**SB-222734**) (Fig. 2), which had excellent antibacterial potency but fur-



Pleuromutilin (**1**); R = OH, R^1 = CH=CH_2

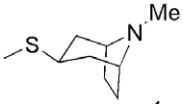
Tiamulin (**2**); R = $SCH_2CH_2NEt_2$, R^1 = CH=CH_2

Valnemulin (**3**); R =



R^1 = CH=CH_2

Retapamulin (**4**); R =



R^1 = CH=CH_2

Figure 1. Pleuromutilin and its derivatives.

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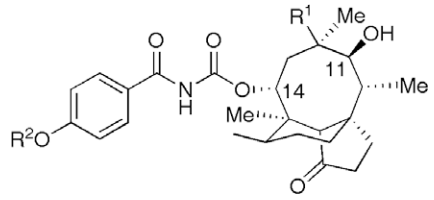


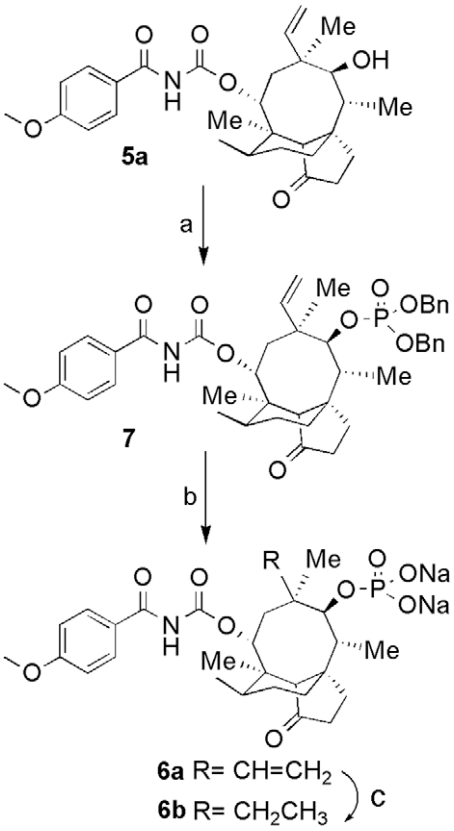
Figure 2. Pleuromutilin analogues with C-14 aroyl-carbamates side chain.

ther development was hindered by their atrocious solubility in water.[3,11] An effective approach to overcome poor solubility is to prepare water-soluble prodrugs.[12] Hydroxyl group and phenol presented in **5a** and **5b** provide ideal sites for modification to generate prodrugs. Phosphorylation of alcohols and phenols have been successfully employed to produce water-soluble prodrugs for poorly soluble drugs such as amprenavir, etoposide, fluconazole, propofol and combretastatin A-4.[13–16] The soluble phosphates can be cleaved by alkaline phosphatase (ALP) in vivo to release parent drugs. In this letter, we report our efforts in the development of novel pleuromutilin phosphate prodrugs with sufficient solubility in water, great stability in buffer and high efficacy in vivo.

Compounds **5a**–**d** (Fig. 2) were prepared in our lab following the method reported by Gerald Brooks.[10] The in vitro antibacterial activity of the compounds against a spectrum of resistant and susceptible Gram-positive bacteria was tested with retapamulin and linezolid as positive controls. Minimum inhibitory concentration (MIC) values were determined using agar dilution method according to NCCLS. The result is summarized in Table 1. It clearly showed that four compounds displayed excellent antibacterial activities and the reduced compounds **5c** and **5d** are more potent than the corresponding vinyl analogues **5a** and **5b**. To improve their water solubility, the most potent compounds **5a**, **5c** and **5d** were selected as the parent drugs to generate phosphate prodrugs.

The synthesis of prodrugs **6a**, **6b** and **6c** is outlined in Schemes 1 and 2. Reacting **5a** with di-benzyl *N*,*N*-diisopropyl-phosphoramidite in the presence of 1*H*-tetrazole followed by oxidation with mCPBA afforded dibenzyl-phosphate ester **7** in high yield (91.8%). Deprotection of the benzyl group was accomplished by use of trimethylbromosilane and methanol, and subsequent treatment with sodium carbonate gave the crude sodium salt **6a**, which was purified by C-18 reverse phase column in moderate isolate yield (73.2%).[17] Hydrogenolysis of the vinyl moieties of **6a** in methanol provided the bis-sodium salt **6b** in good yield (98.2%).

The regioselective phosphorylation of the phenolic hydroxyl group of **5b** at the C-14 side chain without protecting the C-11 skeleton hydroxyl group relied on the higher acidity of the phenolic proton and the steric hindrance of the C-11 hydroxyl group.[18] Treatment of **5b** with *N*,*N*-dimethylaminopyridine (DMAP), carbon tetrachloride, *N*,*N*-diisopropylethylamine (DIPEA) and dibenzyl



Scheme 1. Reagents and conditions: (a) (1) $(BnO)_2PN(i\text{-}Pr)_2$, 1*H*-tetrazole, CH_2Cl_2, rt, 2 h; (2) mCPBA, −40 °C, 1 h, 91.8%; (b) (1) TMSBr, CH_2Cl_2, 0 °C to rt, 1 h; (2) MeOH, 2 h; (3) Na_2CO_3, 30 min, 73.2%; (c) Pd/C, H_2, MeOH, 98.2%.

phosphite successfully produced the phosphoric acid dibenzyl ester compound **8** in high yield (81.8%). Hydrogenolysis of the benzyl and vinyl moieties of **8** followed by addition of sodium carbonate furnished the desired product **6c** (91.7% yield).[19]

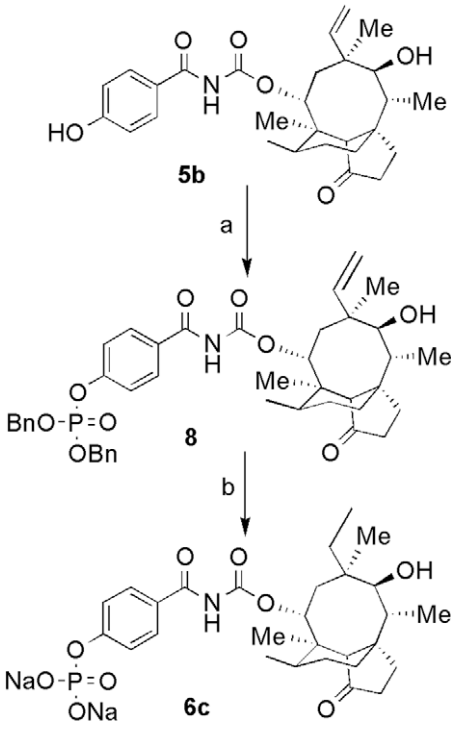
Not surprisingly, all of the di-sodium salts of phosphate derivatives were found to have significantly improved aqueous solubility (>50 mg/mL) at near-neutral pH (Table 2). Compound **6c** was shown to be more soluble than the rest of two compounds. Stabilities of **6a**–**c** were also investigated in buffer solution at pH 7.4 with concentration of 10 mg/mL. No degradation was observed at room temperature in a period of 8 h, and less than 0.5% of parent drugs were detected in buffer solution after 14 days standing at room temperature.

The pharmacokinetic profiles of prodrug **6a**–**c** were evaluated in male Sprauge-Dawley rats after oral and intravenous (iv) administration of the drugs (10 mg/kg bodyweight). Following iv admin-

Table 1
MIC ranges (μg/mL) of **5a**–**d** against Gram-positive clinical isolates

Compounds	MSSA[a] $n = 5$	MRSA[b] $n = 6$	MSSE[c] $n = 5$	MRSE[d] $n = 5$	S.p.[e] $n = 3$
5a	0.0625	0.0625	0.0625	0.0625	0.125
5b	0.125	0.125	0.0625	0.125	0.25
5c	0.031	0.031	0.031	0.031	0.125
5d	0.031	0.031	0.031	0.031	0.0625
Retapamulin	0.031	0.031	0.031	0.031	0.031
Linezolid	0.5	1	0.5	1	1

[a] MSSA = methicillin-susceptible *Staphylococcus aureus*.
[b] MRSA = methicillin-resistant *Staphylococcus aureus*.
[c] MSSE = methicillin-susceptible *Staphylococcus epidermidis*.
[d] MRSE = methicillin-resistant *Staphylococcus epidermidis*.
[e] S.p. = *Streptococcus pneumoniae*.



Scheme 2. Reagents and conditions: (a) (1) DMAP, CCl_4, DIPEA, CH_3CN, −10 °C; (2) dibenzylphosphite, −10 °C to rt, 3 h, 81.8%; (b) (1) Pd/C, H_2, MeOH, 40 psi, rt, 8 h; (2) Na_2CO_3, 30 min, 91.7%.

Table 2
In vitro stabilities and aqueous solubilities of phosphate derivatives **6a–c**

Compound	Aqueous solubility (mg/mL)	Buffer, pH 7.4[a] (% remaining at 8 h)	Buffer, pH 7.4[a] (% remaining at 14 d)
6a	57.6	100	99.8
6b	52.3	100	99.5
6c	96.8	100	99.6

[a] in vitro stabilities as determined by UV/HPLC.

istration, high level of the C-11 phosphate prodrug **6a** was detected in plasma (C_{max} = 30650 ng/mL) with a slow conversion to the parent drug **5a** (C_{max} < 5 ng/mL). The parent drug **5a** was not detectable after oral administration of **6a**.The similar results were observed with compound **6b** (data not shown). As a possible explanation for this observation, the phosphate moieties attached to the C-11 hydroxyl group may not be accessible for the alkaline phosphatase in a highly hindered steric environment. In contrast, the phosphate **6c** displayed satisfactory in vivo pharmacokinetic profiles after iv administration. As shown in Table 3, the maximum observed plasma concentration of the parent drug **5d** was achieved in less than 5 min (T_{max} < 5 min) and the prodrug **6c** could not be detected at the earliest time point, indicating a rapid conversion of the prodrug **6c** to **5d**. Within three dosages tested, C_{max} and AUC increased dose-dependently and approximate twofold of increase were observed at the dose of 100 mg/kg.

Table 3
Pharmacokinetic parameters of **5d** derived from **6c** after iv administration at the dose of 10 mg/kg, 30 mg/kg and 100 mg/kg in male Sprague-Dawley rats (n = 3)

Dose (mg/kg)	T_{max}[a] (h)	C_{max}[b] (μg/mL)	$AUC_{0-\infty}$[c] (μg h/mL)	$t_{1/2}$[d] (h)
10	0.083	3.39 ± 0.4	1.29 ± 0.13	0.51 ± 0.07
30	0.083	11.3 ± 4.8	5.04 ± 0.88	0.75 ± 0.14
100	0.083	67.5 ± 11.0	32.6 ± 1.2	0.63 ± 0.01

[a] Time at which C_{max} achieved.
[b] Maximum plasma concentration.
[c] Area under the concentration–time curve.
[d] Elimination half-life.

Table 4
Efficacy of intravenous **6c** in a systemic *S. aureus* infection model in mice

Organism	ED_{50}[a] (mg/kg, iv)	
	6c	Vancomycin
MSSA 232-51	17.38 (10.29–29.36)[b]	13.49 (8.87–20.52)
MRSA 236-29	20.89 (11.11–39.30)	15.85 (10.30–24.39)

[a] The efficacy criterion, ED_{50}, was calculated as the dose at which mice survival rate was 50%. Mice were inoculated intraperitoneally. Medication was given intravenously twice, 1 h and 4 h after infection.
[b] Numbers in parentheses are 95% confidence ranges.

The in vivo efficacy (ED_{50}) of the water-soluble phosphate prodrug **6c** was evaluated along with the positive control vancomycin in lethal systemic *S. aureus* (MSSA and MRSA) infection model in mice following iv administration. The results are summarized in Table 4. The iv administered prodrug **6c** exhibited potent protective effects against MSSA and MRSA strains with ED_{50} of 17.38 mg/kg and 20.89 mg/kg, respectively, which are comparable to the potent antibiotic vancomycin. In addition, the acute toxicity studies of **6c** after iv administration in SPF level ICR mice (n = 50) showed a LD_{50} of 1009 mg/kg, which was much greater than its ED_{50}.

In conclusion, a new serial of phosphate derivatives of pleuromutilin analogues were synthesized and evaluated as potential water-soluble prodrugs. All compounds were found to be sufficiently soluble and stable in water or buffer solution. More importantly, the phenolic phosphate prodrug **6c** was able to efficiently metabolize to the biologically active parent drug (**5d**) in vivo, and showed great efficacy comparable to vancomycin (VCM) in mice systemic infection model. These pre-clinical results indicate that the prodrug **6c** has potential as an promising injectable agent for chemotherapy of systemic bacterial infections. A more comprehensive study of **6c** will be reported in due course.

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17. A solution of **5a** (200 mg, 0.4 mmol), di-benzyl *N*,*N*-diisopropylphosphoramidite (695 mg, 2.0 mmol), and 1*H*-tetrazole (140 mg, 2.0 mmol) in dichloromethane (10 mL) was stirred at room temperature for 2 h. The mixture was cooled to −40 °C, followed by dropwise addition of a solution of *m*-chloroperoxybenzoic acid (500 mg, 2.0 mmol) in dichloromethane (10 mL). The mixture was warmed to room temperature and stirred for 1 h. A 10% solution of $Na_2S_2O_3$ (15 mL) was added, and the mixture was stirred for 30 min. The reaction mixture was extracted with dichloromethane and washed with 10% $Na_2S_2O_3$ and then saturated $NaHCO_3$. The organic phase was dried over Na_2SO_4, filtered, and evaporated. The residue was purified by chromatography

on silica gel, to give **7** (280 mg, 91.8% yield). mp: 107–108 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ ppm 0.81 (3H, d, J = 6.6 Hz), 0.88 (3H, d, J = 7.0 Hz), 1.10–1.81 (9H, m), 1.15 (3H, s), 1.51 (3H, s), 2.09–2.26 (2H, m), 2.12 (1H, br s), 2.45 (1H, quintet J = 6.9 Hz), 3.86 (3H, s), 4.36 (1H, m), 4.90–5.05 (4H, m), 5.22 (1H, dd, J = 17.3, 1.5 Hz), 5.37 (1H, dd, J = 11.0, 1.5 Hz), 5.83 (1H, d, J = 8.5 Hz), 6.55 (1H, dd, J = 11.0, 1.5 Hz), 6.95 (2H, d, J = 8.8 Hz), 7.28–7.40 (10H, m), 7.92 (2H, d, J = 8.8 Hz), 7.88 (1H, br s); MS (ESI) m/z 780.4 $(M+Na)^+$, 756.2 $(M-H)^-$; ^{31}P NMR (121.5 MHz, $CDCl_3$, 85% phosphoric acid standard) δ –1.571 (s, 1P); Anal. Calcd for $C_{43}H_{52}NO_9P$: C, 68.15; H, 9.92; N, 1.85. Found: C, 68.20; H, 6.85; N, 1.79. To a solution of **7** (250 mg, 0.33 mmol) in dichloromethane (20 mL) at 0 °C was added trimethylsilyl bromide (111.2 mg, 0.73 mmol) via syringe, and the mixture was stirred at 0 °C for 1 h. The solvent was evaporated, and the residue was triturated with methanol (5 mL), stirred at rt for 2 h, then water (10 mL) were added. The pH was adjusted to 9 by addition of 10% Na_2CO_3 solution, gave the crude sodium salts **6a**. The residue was purified by chromatography (C18), eluting with a gradient of 0–70% methanol in water to give **6a** (150 mg, 73.2%). mp: 197–199 °C; ^{1}H NMR (CD_3OD, 300 MHz) δ ppm 0.81 (3H, d, J = 6.6 Hz), 0.88 (3H, d, J = 7.0 Hz), 1.10–1.81 (9H, m), 1.15 (3H, s), 1.51 (3H, s), 2.09–2.26 (2H, m), 2.12 (1H, br s), 2.50 (1H, quintet J = 6.9 Hz), 3.82 (3H, s), 4.25 (1H, m), 4.90–5.05 (4H, m), 5.22 (1H, dd, J = 17.3, 1.5 Hz), 5.37 (1H, dd, J = 11.0, 1.5 Hz), 5.83 (1H, d, J = 8.5 Hz), 6.55 (1H, dd, J = 11.0, 1.5 Hz), 7.05 (2H, d, J = 8.8 Hz), 7.28–7.40 (10H, m), 7.85 (2H, d, J = 8.8 Hz); ^{31}P NMR (121.5 MHz, $CDCl_3$, 85% phosphoric acid standard) δ 3.908 (s, 1P); the pure acid of **6a**: MS (ESI) m/z 602.0 $(M+Na)^+$; HRMS (ESI) calcd for $C_{29}H_{39}NO_9$ P $(M-H)^-$ 576.2362, found 576.2343.

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19. Compound **5b** (500 mg, 1.0 mmol) and dimethylaminopyridine (12.2 mg, 0.1 mmol) were dissolved in anhydrous acetonitrile (20 mL) under argon. The reaction mixture was cooled to −10 °C, carbon tetrachloride (0.5 mL, 5.0 mmol) and diisopropylethylamine (0.37 mL, 2.1 mmol) were added via syringe. After 10 min, dibenzyl phosphite (0.33 mL, 1.5 mmol) was added, and the reaction mixture was allowed to warm to room temperature and stirred for 3 h. The reaction mixture was poured into a 0.5 M monobasic sodium phosphate solution (10 mL), and the aqueous mixture was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were condensed in vacuo and purified via flash chromatography (silica gel, hexane/ethyl acetate 3:1–1:1 gradient) to provide **8** (630 mg, 81.8%) as a white solid, mp: 122–124 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ ppm 0.81 (3H, d, J = 6.6 Hz), 0.88 (3H, d, J = 7.0 Hz), 1.10–1.81(9H, m), 1.15 (3H, s), 1.51 (3H, s) 2.09–2.26 (2H, m), 2.12 (1H, br s), 2.35 (1H, quintet J = 6.9 Hz), 3.36 (1H, d), 5.10–5.16 (4H, d, J = 8.8 Hz), 5.22 (1H, dd, J = 17.3, 1.5 Hz), 5.37 (1H, dd, J = 11.0, 1.5 Hz), 5.80 (1H, d, J = 8.5 Hz), 6.60 (1H, dd, J = 11.0, 1.5 Hz), 7.20 (2H, d, J = 8.8 Hz), 7.30–7.40 (10H, m), 7.70 (2H, d, J = 8.8 Hz), 8.0 (1H, br s); MS (ESI) m/z 766.80 $(M+Na)^+$; ^{31}P NMR (121.5 MHz, $CDCl_3$, 85% phosphoric acid standard) δ −6.826 (s, 1P); Anal. Calcd for $C_{42}H_{50}NO_9P$: C, 67.82; H, 6.78; N, 1.88. Found: C, 67.94; H, 6.77; N, 1.81. To a solution of **8** (400 mg, 0.54 mmol) in methanol (50 mL) was added Pd on carbon (60 mg, 10% by wt Pd), and the mixture was shaken in a Parr hydrogenator under a 40 psi atmosphere of hydrogen for 8 h. The mixture was filtered through Celite, and the solvent was evaporated. Methanol and water were added, and the pH was adjusted to 9 by addition of 10% Na_2CO_3 solution. The sample was purified by chromatography (C18), eluting with a gradient of 0–70% methanol in water to give **6c** (300 mg, 91.7% yield). mp: 200–202 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ ppm 0.81 (3H, d, J = 6.6 Hz), 0.88 (3H, d, J = 7.0 Hz), 1.10–1.81 (12H, m), 1.15 (3H, s), 1.51 (3H, s), 2.09–2.26 (2H, m), 2.38 (1H, br s), 2.40 (1H, quintet J = 6.9 Hz), 3.45 (1H, d), 5.78 (1H, d, J = 8.5 Hz), 7.30 (2H, d, J = 8.8 Hz), 7.90 (2H, d, J = 8.8 Hz); ^{31}P NMR (121.5 MHz, $CDCl_3$, 85% phosphoric acid standard) δ -1.747 (s, 1P); the pure acid of **6c**: MS (ESI) m/z 565.2 $(M-H)^-$; HRMS (ESI) calcd for $C_{28}H_{39}NNaO_9$ P $(M+Na)^+$ 588.2316, found 588.2338.