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New C25 carbamate rifamycin derivatives are resistant to inactivation by ADP-ribosyl transferases

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Abstract—A novel series of 3-morpholino rifamycins in which the C25 acetate group was replaced by a carbamate group were prepared and found to exhibit significantly improved antimicrobial activity than rifampin against *Mycobacterium smegmatis*. Further characterization of such compounds suggests that relatively large groups attached to the rifamycin core via a C25 carbamate linkage prevent inactivation via ribosylation of the C23 alcohol as catalyzed by the endogenous rifampin ADP-ribosyl transferase of *M. smegmatis*. SAR studies of the C25 carbamate rifamycin series against *M. smegmatis* and other bacteria are reported.
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Antibiotics of the rifamycin class, like rifampin (Fig. 1), are important agents in the treatment of infections caused by Mycobacteria and a variety of other bacterial pathogens.[1] The antibacterial activity of the rifamycins is due to inhibition of the initiation of transcription by bacterial DNA-dependent RNA polymerase, thus effectively terminating further RNA synthesis and hence protein synthesis.[2] Two major resistance mechanisms have been described for the rifamycins: (i) target-based mutations that arise in the *rpoB* gene encoding the β-subunit of RNA polymerase,[1a] and (ii) enzymatic inactivation due to a recently described class of rifamycin-specific ADP-ribosyl transferases. To date, a series of sequence-related rifampin ADP-ribosyl transferases have been described and/or characterized from soil microorganisms including Arr-1 of *Mycobacterium smegmatis*, Arr-2 of *Pseudomonas aeruginosa*, and Arr-3 of *Streptomyces coelicolor*.[3] In *M. smegmatis*, formation of 23-[*O*-(α-D-ribofuranosyl)] rifampin is thought to occur via an intermediate, 23-(*O*-ADP-ribosyl) rifampin, in a reaction catalyzed by the Arr-1 enzyme.[4]

The major metabolites of rifamycin class antibiotics are C25-deacetylated forms that primarily arise through the action of β-esterases in the liver.[5] In a program focused on exploring the potential for modifying the *ansa* side chain of the rifamycins, we set out to convert the C25 acetate group to a carbamate group that would be insensitive to β-esterases, acetyl migration,[5d] and should be more stable to non-enzymatic hydrolysis. In addition, we sought to understand how derivation of the rifamycin scaffold through this modification might alter susceptibility to rifamycin-specific ADP-ribosyl transferase enzymes.

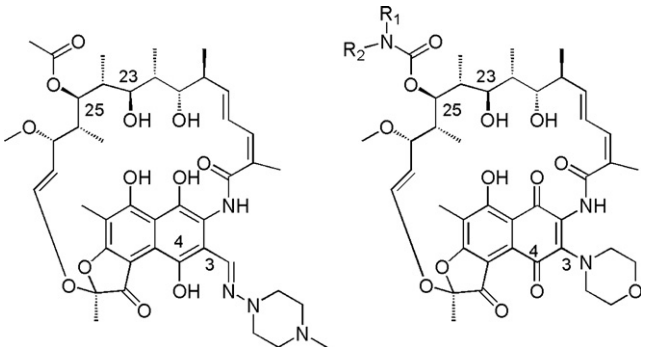

Figure 1. Rifampin, **1**, and a C25-modified carbamate derivative.

Keywords: Rifamycin; ADP-ribosyl transferase; Mycobacterium smegmatis; Inactivation.
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C25 carbamate derivatives of the 3-morpholino rifamycins were prepared starting from the known C21–C23 acetonide **2**.[6] Compound **2** was reacted with excess carbonyl diimidazole (CDI) (Fig. 2). Direct substitution of the carbamoyl imidazole with an amine at room temperature was slow and at elevated temperature gave a mixture of products including amine exchange at the C3 position of rifamycin. It has been reported that the C25 alcohol of compound **2** is very difficult to esterify due to the steric hindrance caused by the adjacent acetonide.[7] The C21–C23 acetonide probably also blocks addition of an amine to the carbamoyl imidazole group even though the reactive center is somewhat removed from the acetonide when compared to the C25 alcohol. Activation of the carbamoyl imidazole with benzyl bromide gave a benzyl imidazolium group that could be rapidly and cleanly displaced by a wide variety of amines within minutes at room temperature (Fig. 2 and Supplementary data). A methyl imidazolium reactive intermediate similar to **3** could be formed in a single step using a 1,1′-carbonylbis(3-methylimidazolium) triflate that has been reported in the literature[8] but we were unable to prepare the bis triflate salt. The product of treating CDI with two equivalents of methyl triflate as described in the literature procedure[8] gave cleanly the carbamoyl imidazole adduct identical to that obtained by direct treatment with CDI and did not provide any synthetic advantage.

The 3-morpholino group is necessary to block nucleophilic substitution at the third position. Treatment of rifamycin S with CDI results in significant addition of imidazole to the third position of rifamycin S. Efforts to prepare a rifampin C21–C23 acetonide with a C3 hydrazone derivative similar to **3** were unsuccessful.

The chemical shift of the C25 methine is diagnostic for the transformations described in Figure 2. The ^{1}H NMR chemical shift for the C25 methine of compound **2** is 4.22 ppm. The carbamates **4** give a corresponding downfield shift to 4.82 ppm. The C25 acetate of **2** has a methine signal at 4.62 ppm.

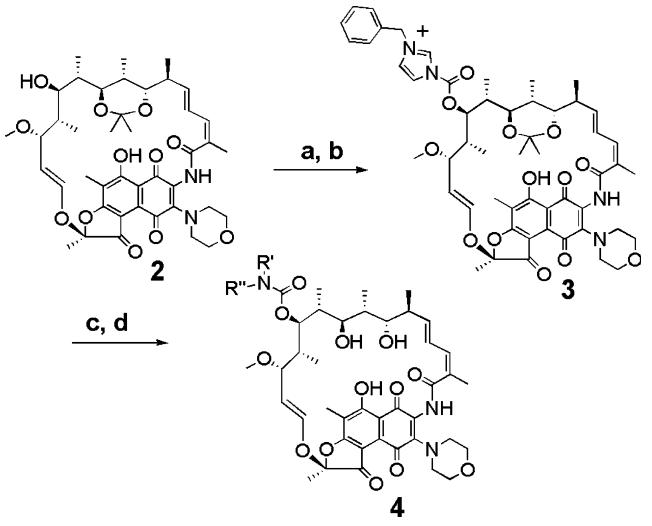


Figure 2. Synthetic scheme for the preparation of C25 carbamate derivatives of rifamycins. (a) CDI, CH_2Cl_2, 86%; (b) benzyl bromide, CH_3CN, 87%; (c) primary or secondary amine, CH_2Cl_2; (d) 10% aq HCl, THF, 8–58% (two steps).

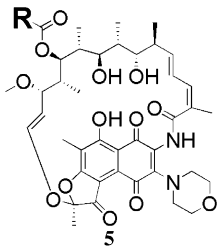
A series of 3-morpholino rifamycin derivatives with different functionalities linked to the C25-carbamate group (**5a**–**l** in Table 1) were screened initially against a rifamycin-sensitive strain of *Staphylococcus aureus* (ATCC# 29213), a derivative of ATCC# 29213 that bears a single point mutation in *rpoB* (His481Tyr) that confers high-level rifampin resistance, and a pair of otherwise isogenic *M. smegmatis* strains—which differ only by the presence (strain DSM 43756) or absence (strain 43756Km1) of a functional rifampin ADP-ribosyl transferase enzyme (Arr-1).[4]

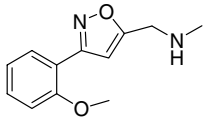
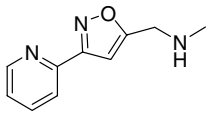
Most of the C25-carbamate rifamycin derivatives retained rifampin-like antimicrobial activity against *S. aureus* ATCC# 29213, but lost most or all detectable activity against the rifampin-resistant strain. These data suggest that the anti-staphylococcal activity of these compounds is mediated through 'on-target' activity through inhibition of RNA polymerase. As expected[4], rifampin was observed to be 64-fold more potent against *M. smegmatis* strain 43756Km1 in which the gene encoding the Arr-1 ADP-ribosyl transferase enzyme has been genetically inactivated. In contrast, several compounds bearing larger C25-carbamate-linked groups showed substantially improved antimicrobial activity against *M. smegmatis* DSM 43756 that was equivalent for strain 43756Km1 (**5g**, **5j**–**l** in Table 1). These data are consistent with the possibility that such compounds are insensitive to cellular inactivation by the Arr-1 ADP-ribosyl transferase enzyme.

To determine whether this apparent effect is enzyme specific, otherwise-isogenic pairs of *Escherichia coli* strains were employed that either bear plasmid (pCTF104) that expresses the *P. aeruginosa* derived Arr-2 rifampin ADP-ribosyl transferase, or a control plasmid (pCTF104P). In these experiments, an *lpxC*101 mutant strain of *E. coli* (CGSC# 5163) was also employed as it exhibits a 'leaky' outer membrane phenotype which renders the cell more susceptible to large hydrophobic antimicrobials like rifampin. Comparison of MIC values determined against these strain pairs for several of the compounds bearing larger C25-carbamate-linked groups such as **5d-f** and **5h**–**l** suggests they may be similarly insensitive to cellular inactivation by the related Arr-2 enzyme.

Finally, a purified recombinant form of the *M. smegmatis* Arr-1 enzyme was employed in an in vitro assay to provide more direct evidence that compounds like **5l** were insensitive to inactivation by rifamycin-specific ADP-ribosyl transferases.[9] In these studies, increasing concentrations of the recombinant Arr-1 protein and test compounds were co-incubated for 30 min at 37 °C and aliquots of the reactions then spotted onto paper disks placed on a lawn of *S. aureus* ATCC# 29213. Decreases in the apparent zone of inhibition with increasing Arr-1 concentrations are interpreted to indicate that the test rifamycin has been inactivated. As

Table 1. MIC data for rifampin and compounds **5a**–**l** for *S. aureus* and *M. smegmatis* with and without Arr-1 and *E. coli* with and without Arr-2



Compound	Compound or R group substitution tested	*S. aureus*		MIC in μg/ml or ratio of MICs (Ribosylase$^+$/Ribosylase$^-$) for:								
				Mycobacterium smegmatis			*Escherichia coli* wild-type (DH5α)			*E. coli*—CGSC# 5163 *lpxC101* 'leaky' strain		
		ATCC# 29213 WT[a] (Rif-sensitive)	ATCC# 29213 *rpoB*^{H481Y} (Rif-resistant)	DSM 43756 (Arr-1$^+$)	43756Km1 (Arr-1$^-$)	Ratio[a] of MICs	pCTF104 (Arr-2$^+$)	pCTF104P (Arr-2$^-$)	Ratio[a] of MICs	pCTF104 (Arr-2$^+$)	pCTF104P (Arr-2$^-$)	Ratio[a] of MICs
	Rifampin	0.008	>63	32	0.5	64	>250	8	>32	8	0.24	32
5a	MeNH–[c]	0.004	4	4	0.5	8	>250	8	>32	16	0.5	32
5b	CH═CHCH$_2$NH–	0.004	NT[b]	2	0.06	>32	>250	8	>32	4	0.24	16
5c	HOCH$_2$CH$_2$NH–	0.016	16	8	2	4	>250	8	>32	8	0.5	16
5d	Me$_2$NCH$_2$CH$_2$NH–	0.006	>63	0.5	0.12	4	8	4	2	0.24	0.24	1
5e	Morpholine	0.03	NT[b]	2	1	2	63	31	2	1	1	1
5f	4-(MeO)–PhCH$_2$NH–	0.016	4	0.06	0.03	2	>250	16	>16	0.5	0.5	1
5g	4-(Me$_2$N)–PhCH$_2$NH–	0.06	>63	0.06	0.06	1	>250	63	>4	16	1	16
5h	3-Cl–PhCH$_2$NH–	0.008	>63	0.12	⩽0.03	>4	>250	>250	na[d]	0.5	0.24	2
5i	4-CF$_3$–PhCH$_2$NH–	0.03	NT[b]	0.12	⩽0.03	>4	>250	>250	na[d]	1	0.5	2
5j	4-(Benzylamino)-1-piperidine	0.03	>63	0.12	0.12	1	16	8	2	0.24	0.24	1
5k		0.06	NT[b]	0.12	0.12	1	>250	>250	na[d]	2	1	2
5l		0.008	>63	0.5	0.5	1	4	4	1	2	2	1

[a] Ratio of MICs for Ribosylase $^+$/Ribosylase$^-$ strain pairs.
[b] Not tested.
[c] For synthetic procedures and analytical data see Supplementary data.
[d] Not applicable.

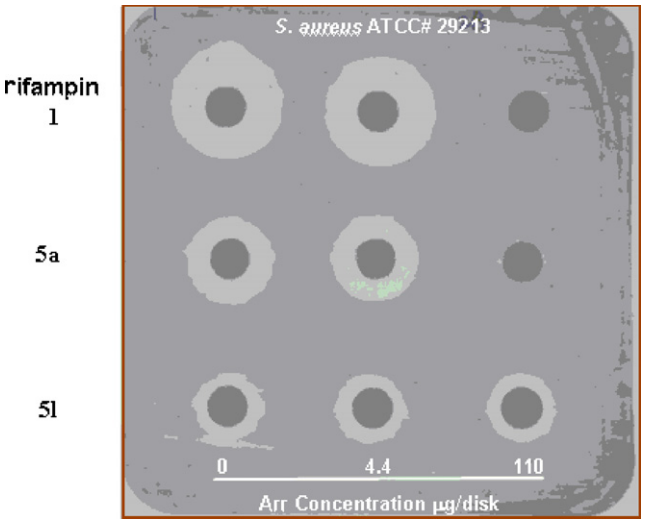


Figure 3. Zone of inhibition studies with increasing amounts of Arr-1.

shown in Figure 3, rifampin is inactivated by the addition of Arr-1 resulting in a decrease in the zone of inhibition with increasing Arr-1 concentration. Analysis of the rifampin reaction products by HPLC-MS resulted in the identification of a compound with a molecular mass (1362–1366 Da) consistent with that of ADP-ribosylated rifampin (data not shown). Consistent with the MIC data in Table 1, compound **5a**, that has a small methyl carbamate at C25, is similar to rifampin in its apparent sensitivity to inactivation by the recombinant Arr-1 enzyme in vitro. In contrast, compound **5l**, which exhibits equivalent antimicrobial potency against otherwise-isogenic Arr^+/Arr^- strain pairs, shows no apparent change in the zone of inhibition with increasing amounts of Arr-1 protein suggesting that it is insensitive to enzymatic inactivation in vitro. The smaller zones of inhibition observed with the C25 carbamate compounds in these disk diffusion assays probably result from poor aqueous solubility and/or reduced agar diffusion compared to rifampin.

A wide variety of functional groups appended at the C25 position via the carbamate linkage appear to be well-tolerated and retain antibacterial activity against *S. aureus*. The antimicrobial activity of the C25 carbamate derivatives against *M. smegmatis* is superior to rifampin for all compounds tested and compounds with a secondary amine (**5d** and **5j**) exhibited the best overall activity. Wild-type *M. smegmatis* strains (like DSM 4375) inactivate rifampin through enzyme-catalyzed addition of an ADP-ribose group to the C23 alcohol. The larger C25-carbamate-linked groups, like those in entries **5f–l**, may sterically block the ADP-ribosyl transferase enzyme and hence show little difference in MICs between strains with and without rifamycin ADP-ribosyl transferases. In contrast, compounds with smaller C25-carbamate-linked groups such as **5a** and **b** appear to remain sensitive to the rifamycin ADP-ribosyl transferase activity. A basic amine (**5d**, **5j**, and **5l**) also enhances the potency of the C25-carbamate derivatives and these compounds also appear to be more effective at penetrating the outer membrane of *E. coli*. A derivative of rifamycin in which the C25 acetate is replaced by a malonate ester that terminates in a basic amine has been reported to have good activity against several strains of *E. coli*.[10]

Tested C25-carbamate rifamycin derivatives maintained the excellent Gram-positive activity of the rifamycin class of antibiotics. A few derivatives with a basic amine attached at the C25 position had weak but improved activity against an *E. coli* strain. All but the two smallest (**5a** and **b**) of the C25-carbamate derivatives appear to show reduced susceptibility to inactivation by the rifamycin ADP-ribosyl transferases that are a major contributor of innate or acquired rifampin resistance in opportunistic human pathogens like *M. smegmatis*, *P. aeruginosa*, and certain enteric pathogens. Future efforts to improve the in vivo activity of the series will focus on heterocyclic derivatives such as **5l** which have activity in an in vivo infection model. Functional groups at the C25 position that maintain anti-bacterial efficacy in the presence of *rpoB* mutations are currently being explored.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.10.016.

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9. *Methods*. The *arr-1* gene of *Mycobacterium smegmatis* strain DSM 43756 was cloned in pET28 and a recombinant form of the Arr-1 enzyme, bearing an amino-terminal oligohistidine tag, purified to essential homogeneity by immobilized metal affinity chromatography (IMAC) following expression in *Escherichia coli*. Reactions with test agents were performed for 30 min at 37 °C in a buffer containing 25 mM Tris–HCl (pH 7.5), 0.5 mM $MgCl_2$, and 100 μg/mL nicotine adenine dinucleotide (NAD). Aliquots of the reactions were then spotted on 6 mm paper disks (Whatmann AA Grade 17CHR) pre-placed on lawns of *S. aureus* ATCC# 29213 seeded on cation-adjusted MHII medium. Plates were incubated for 18 h before the zone sizes were determined by standard methods.
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