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Synthesis and evaluation of inhibitors of bacterial drug efflux pumps of the major facilitator superfamily

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ABSTRACT

Inhibitors of drug efflux pumps have great potential as pharmacological agents that restore the drug susceptibility of multidrug resistant bacterial pathogens. Most attention has been focused on the discovery of small molecules that inhibit the resistance nodulation division (RND) family drug efflux pumps in Gram-negative bacteria. The prototypical inhibitor of RND-family efflux pumps in Gram-negative bacteria is MC-207,110 (Phe-Arg-β-naphthylamide), a C-capped dipeptide. Here, we report that C-capped dipeptides inhibit two chloramphenicol-specific efflux pumps in *Streptomyces coelicolor*, a Gram-positive bacterium that is a relative of the human pathogen *Mycobacterium tuberculosis*. Diversity-oriented synthesis of a library of structurally related C-capped dipeptides via an Ugi four component reaction and screening of the resulting compounds resulted in the discovery of a compound that is threefold more potent as a suppressor of chloramphenicol resistance in *S. coelicolor* than MC-207,110. Since chloramphenicol resistance in *S. coelicolor* is mediated by major facilitator superfamily drug efflux pumps, our findings provide the first evidence that C-capped dipeptides can inhibit drug efflux pumps outside of the RND superfamily.

1. Introduction

Membrane-bound efflux pumps often underlie drug resistance in pathogenic bacteria.[1–3] In many cases, drug-resistance in human pathogens is correlated with over-expression of genes encoding efflux pumps.[1] Nearly all classes of antibacterial drugs are known to be substrates of one or more efflux pumps.[4] Some efflux pumps exhibit high specificity for certain antimicrobial agents while others act upon drugs from unrelated structural classes and thereby confer multidrug-resistance.[1,3] There are five main families of efflux pumps in bacteria: the ATP binding cassette (ABC) family, the major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE) family, the resistance nodulation division (RND) family, and the small multidrug resistance (SMR) family.[5] While members of the ABC, MFS, MATE and SMR families of efflux pumps are commonly observed in Gram-positive bacteria, efflux pumps in the RND, MFS and ABC families are often found in Gram-negative bacteria.

Given the significance of efflux pumps in many multidrug-resistant phenotypes of pathogenic bacteria, there is much interest in either circumventing or directly inhibiting their activity. The former strategy focuses on developing derivatives of antibacterial drugs that are poor substrates of efflux pumps,[6–10] while the latter is focused on the identification of efflux pump inhibitors.[11,12] In a manner analogous to the use of clavalunic acid (a β-lactamase inhibitor) in combination with β-lactam drugs, inhibitors of drug efflux pumps could be used to restore the antibacterial susceptibility of drug-resistant bacteria.[3,13–15] Inhibition of drug efflux pumps enables the intracellular concentration of drugs to reach levels that are sufficient for antibacterial activity. In this context, the observation of increased drug activity is called potentiation.

Since RND-family drug efflux pumps are often associated with the recalcitrance of Gram-negative bacteria to antibacterial drugs, a great deal of effort has been made to discover small molecules that inhibit these pumps. The prototypical RND-family efflux pump inhibitor is MC-207,110 (also known as Phe-Arg-β-naphthylamide).[11] This compound was discovered by MPEX pharmaceuticals in a screen for potentiators of levofloxacin activity against a *Pseudomonas aeruginosa* strain that over-expressed a levofloxacin-specific RND-family efflux pump.[11] Through medicinal chemistry efforts aimed at improving the activity and pharmacological properties of MC-207,110, the group at MPEX Pharmaceuticals discovered MC-02,595 and MC-04,124 (Fig. 1).[4,16] While all three compounds potentiated the activity of levofloxacin to similar degrees, MC-02,595 was more stable than MC-207,110 and MC-04,124 exhibited greater stability and lower toxicity than the other two compounds. Collectively, these compounds typify a class of efflux pump inhibitors known as C-capped dipeptides. They act by competitively binding the substrate binding sites of RND-family efflux pumps; indeed, they are recognized and exported by RND pumps.[17] Other classes of efflux pumps that have been reported against RND pumps include arylpiperazines,[18] arylpiperidines and quinoline compounds.[19]

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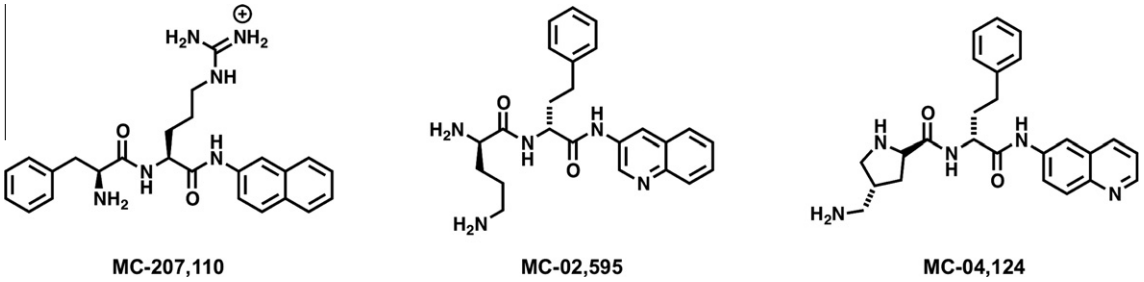
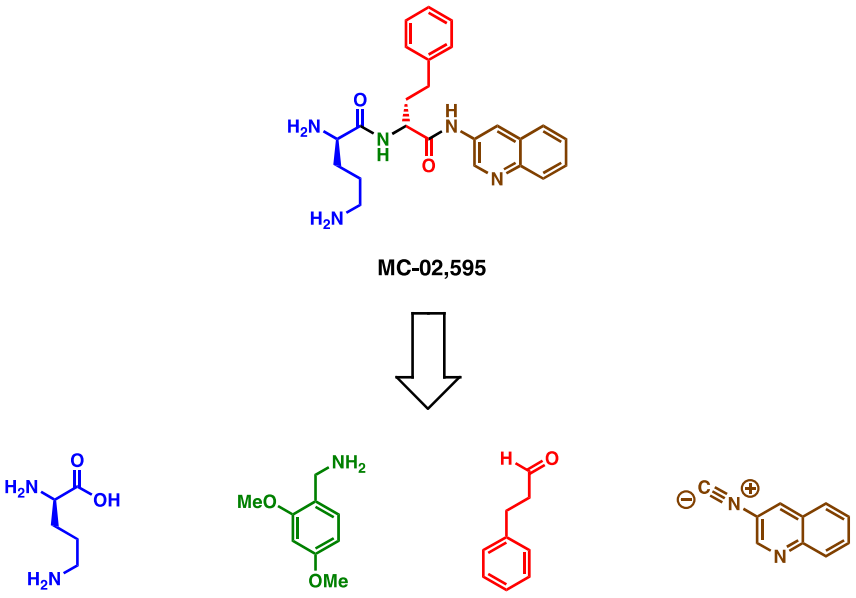


Figure 1. Broad-spectrum efflux pump inhibitors reported by MPEX pharmaceuticals. These basic dipeptide compounds are competitive inhibitors of multiple RND-transporters from Gram-negative bacteria.

We recently reported that MC-207,110 potentiated the activity of chloramphenicol (a clinically used antibacterial drug) against *Streptomyces coelicolor*.[20] This Gram-positive bacterium has two MFS drug efflux pumps that mediate chloramphenicol resistance (minimal inhibitory concentration is 65 μg/mL). Although *S. coelicolor* is not pathogenic, it is a close relative of pathogenic mycobacteria (e.g., *Mycobacterium tuberculosis*).[21,22] Since chloramphenicol resistance in *S. coelicolor* is mediated by two MFS efflux pumps, we were intrigued to determine how the prototypical inhibitor of RND-family efflux pumps in Gram-negative bacteria potentiated chloramphenicol activity in *S. coelicolor*, a gram positive bacterium. Genetic evidence suggested MC-207,110 potentiated the activity of chloramphenicol by inhibiting the two chloramphenicol-specific MFS pumps in *S. coelicolor*. Specifically, the capacity of MC-207,110 to potentiate chloramphenicol activity was significantly diminished in strains of *S. coelicolor* lacking either of the two chloramphenicol-specific MFS pumps. To learn more about the structural requirements for C-capped dipeptide mediated potentiation of chloramphenicol activity in a bacteria with MFS efflux pumps, we decided to synthesize a targeted library of C-capped dipeptides. Our initial synthetic efforts were guided by the structures of the pharmacologically enhanced C-capped dipeptide inhibitors, MC-02,595 and MC-04,124, reported by MPEX Pharmaceuticals. Our plan was to assess the relative potencies of these compounds by measuring the degree to which they potentiated chloramphenicol activity against *S. coelicolor*.

To facilitate the preparation of a diverse array of C-capped dipeptides, we sought an alternative to the laborious peptide chemistry that has been used to prepare this class of compounds. We recognized that the core structures of C-capped dipeptides are reminiscent of the characteristic products of the Ugi four component reaction. In this multicomponent reaction, an amine, an aldehyde, an isocyanide and either an aldehyde or ketone react in a single flask to yield α-acylaminocarboxamides in good to excellent yields. An added benefit of using the Ugi reaction in the preparation of the C-capped dipeptides is that it has a broad substrate scope. With the judicious selection of Ugi reaction substrates, we envisioned the preparation of a diverse array of compounds that share a common dipeptide scaffold. A retrosynthesis of MC-02,595, one of the efflux pump inhibitors reported by MPEX Pharmaceuticals, is depicted in Scheme 1. We hypothesized that this compound could be synthesized via an Ugi reaction using di-Boc-ornithine, hydrocinnamaldehye, 2-isocyanoquinoline and dimethoxybenzylamine (an ammonia equivalent) as substrates followed by acid-promoted removal of the Boc and dimethoxylbenzyl protecting groups of the Ugi product. While the synthesis of MC-02,595 using standard peptide coupling reactions requires five steps, it can be prepared in only two steps via the Ugi reaction. This synthetic strategy was used in the preparation of a collection of structurally diverse C-capped dipeptides that were evaluated for their capacity to potentiate chloramphenicol activity against *S. coelicolor*.



Scheme 1. Retrosynthesis of MC-02,595, an efflux pump inhibitor with broad spectrum activity against RND pumps in Gram-negative bacteria, from Ugi four component reaction substrates.

2. Results and discussion

2.1. Synthesis and evaluation of MC-02595

In light of the ability of MC-207,110 (Phe-Arg-β-naphthylamide) to potentiate the activity of chloramphenicol in *S. coelicolor*, we assessed the efficacy of MC-02,595, one of the pharmacologically enhanced analogs (Fig. 1), in potentiating chloramphenicol activity. MC-02,595 was synthesized as shown in Scheme 2. Because the use of ammonia as a substrate in Ugi reactions often yields undesired side products,[23–27] the ammonia equivalent 2,4-dimethoxybenzylamine was used as a substrate.[28] The protected α-acylaminocarboxamide was obtained from the reaction of di-Boc-ornithine, 2,4-dimethoxybenzylamine, hydrocinnamaldehye and 2-isocyanoquinoline. The desired dipeptide was obtained by using trifluoroacetic acid to simultaneously remove the Boc protecting groups and the dimethoxybenzyl protecting group (Scheme 2).

After the successful synthesis of MC-02,595, we tested the efficacy of the compound as a potentiator of chloramphenicol activity against *S. coelicolor*. To carry out the potentiation assays, 1 μL of a 10^8 cfu/μL stock of wild-type *S. coelicolor* spores were patched on solid Difco nutrient agar plates that contained 25 μg/mL of MC-02,595 and varying concentrations of chloramphenicol. (A concentration of 25 μg/mL of MC-02,595 was selected because MC-207,110 potentiates chloramphenicol activity 2.6-fold at the same concentration.) The effect of MC-02,595 on the chloramphenicol MIC of *S. coelicolor* was assessed after a two-day incubation period at 30 °C. Surprisingly, we found that 25 μg/mL of MC-02,595 did not potentiate chloramphenicol activity against *S. coelicolor*.

2.2. Syntheses and evaluation of MC-02,595 analogs

Since it was reported that both MC-207,110 and MC-02,595 potentiated chloramphenicol activity against *P. aeruginosa* and that MC-207,110 strongly affected the chloramphenicol minimal inhibitory concentration (MIC) of *S. coelicolor*, the inability of MC-02,595 to affect the chloramphenicol MIC of *S. coelicolor* was intriguing. We sought to identify differences between the structures of the C-capped dipeptides to which the potentiation activity could be ascribed.

In a side by side comparison of both compounds (Fig. 2), we noted obvious difference in their structures: (I) The identity of the amino acid in position 1 of MC-207,110 is a phenylalanine while in MC-02,595 it is an ornithine, (II) the amino acid in position 2 of MC-207,110 is an arginine while in MC-02,595 it is a homophenylalanine, (III) a naphthalene group is used to cap the dipeptide in MC-207,110 while a quinoline group is used to cap the dipeptide in MC-02,595 and (IV) both amino acids in MC-207,110 are of 'L' configuration while those in MC-02,595 are of 'D' configuration. Using the Ugi reaction, we synthesized a set of compounds with variations of amino acid configuration and the capping group on MC-02,595. The results are summarized in Table 1. Although

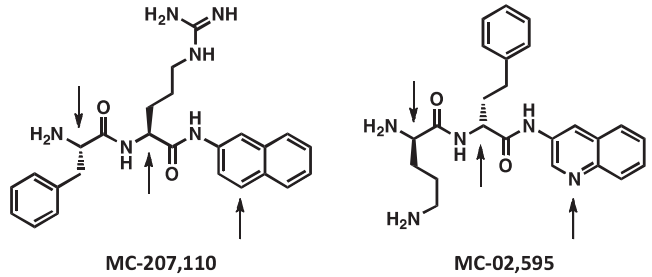
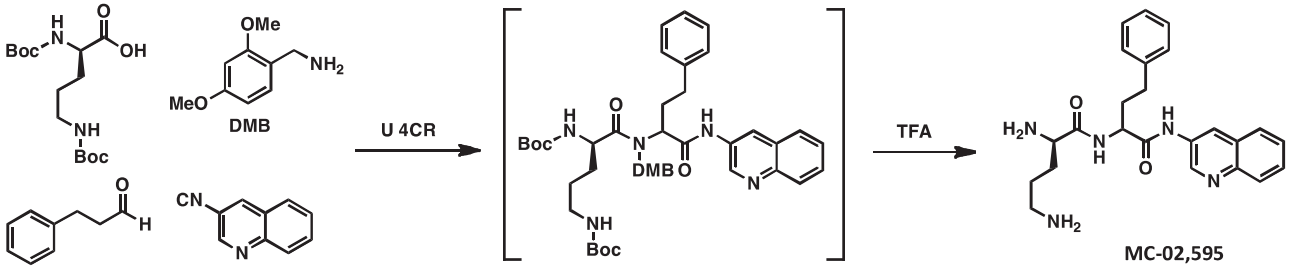


Figure 2. Side by side comparison of MC-207,110 (Phe-Arg-β-naphthylamide) and MC-02,595 highlighting differences in stereochemistry of the amino acids and differences in capping group

activity data for MC-207,110 (Phe-Arg-β-naphthylamide) is included in Table 1 (entry b) for the purpose of comparison, it was not synthesized in this study.

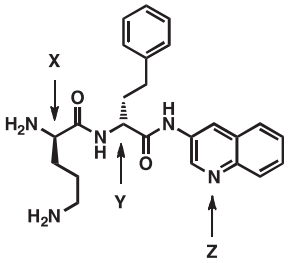
In the table, entry 'a' is a negative control (no compound was added). MC-207,110 (Phe-Arg-β-naphthylamide) is entry b, and the remaining entries are analogs of MC-02,595. From the table, the strongest potentiation activity of the compounds was achieved in compounds that had a naphthalene moiety (entries d and e) and not the quinoline moiety of MC-02,595 (entries c and f). In contrast, variation of the configuration of the first amino acid position (ornithine) had no significant effects on the ability of the compounds to potentiate chloramphenicol activity (entries d and e). Interestingly, the compound that was most active in potentiating the activity of chloramphenicol was a mixture of diastereomers-one diastereomer had *R*-homophenylalanine in the second position of the dipeptide and the other had *S*-homophenylalanine. (As the Ugi reaction is not stereoselective, reactions with chiral substrates will yield diastereomeric mixtures of products.) To assess the potentiation activity of each diastereomer, we separated them by column chromatography and tested them individually in chloramphenicol assays. The absolute configuration of the homophenylalanine moiety in position 2 of the dipeptide had little bearing on the compounds' potentiation activity (entries g and h).

From our preliminary structure–activity relationship studies, we found that a C-capped dipeptide with L-ornithine in the first position, a D-homophenylalanine in the second position and a naphthalene capping group (BU-005, entry g in Table 1) had much higher potency as a potentiator of chloramphenicol activity against *S. coelicolor* than MC-207,110. As a sevenfold potentiator of chloramphenicol activity at a concentration of 25 μg/mL, BU-005 is three times more active than MC-207,110. Perhaps what is most interesting in this analysis is that BU-005 has a very similar structure to MC-02,595, a compound with no chloramphenicol potentiation activity. The only differences between their structures are the configuration of the ornithine moiety and the identity of the C-cap (naphthalene vs quinoline). Since the configuration of ornithine residue at the first position is not critical for potentiation activity, it is obvious that the identity of the C-cap is most important with respect to potentiation.



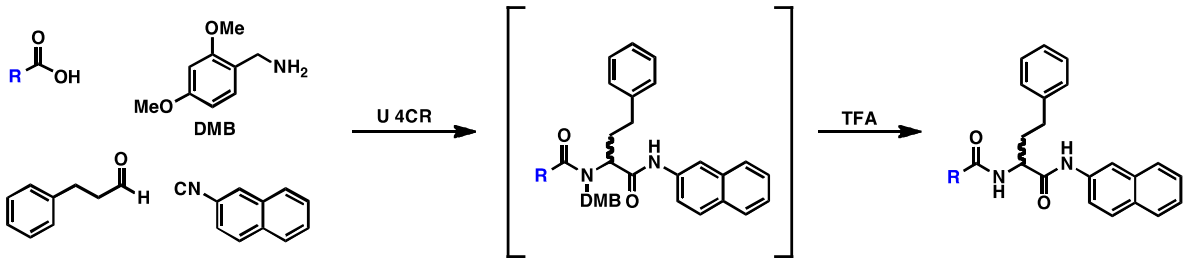
Scheme 2. Synthetic route to MC-02,595, a known efflux pump inhibitor, via the Ugi reaction.

Table 1
Systematic variation of the amino acid configuration, and the identity of the capping group on MC-02,595



Entry	X	Y	Z	CML MIC[a,b]	Fold potentiation
a	—	—	—	65	—
b (Phe-Arg)	L-Phenylalanine	L-Arginine	Naphthalene	25	2.6
c (MC-02,595)	D-Ornithine	D/L-Homophe	Quinoline	>40	—
d	D-Ornlthlne	D/L-Homophe	Naphthalene	10	6.5
e	L-Ornithine	D/L-Homophe	Naphthalene	10	6.5
f	L-Ornithine	D/L-Homophe	Quinoline	>40	—
g	L-Ornithine	D-Homophe	Naphthalene	9	7.2
h	L-Ornithine	L-Homophe	Naphthalene	11	5.9

Reported values are MIC of chloramphenicol in *S. coelicolor* at 25 μg/mL of the inhibitors. All compounds were purified by HPLC prior to testing.
[a] CML refers to chloramphenicol. Reported MICs have units of μg/mL.
[b] All compounds reported displayed no intrinsic toxic activity to *S. coelicolor* at the concentrations that they were tested (25 μg/mL).



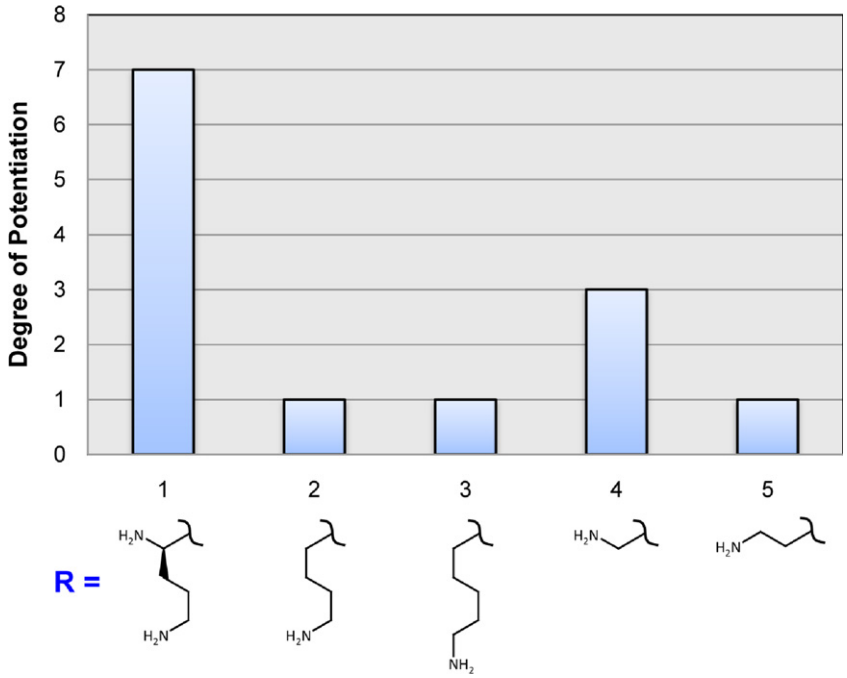


Figure 3. Analysis of chloramphenicol potentiation activity in wild-type *S. coelicolor* by *C*-capped dipeptides with various amino acids at the *N*-terminus. All compounds reported displayed no intrinsic toxic activity to *S. coelicolor* at the concentrations that they were tested (25 μg/mL).

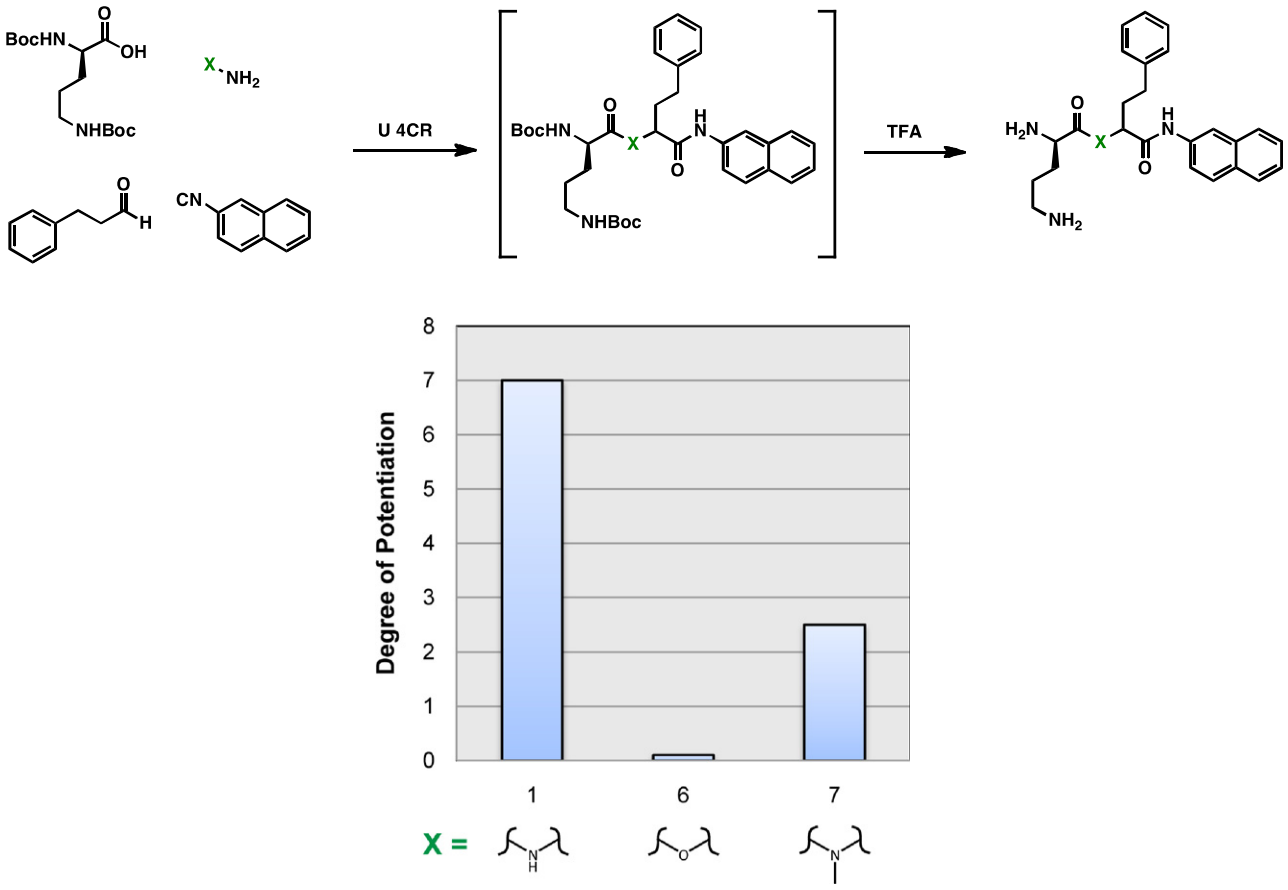


Figure 4. Analysis of chloramphenicol potentiation activity in wild-type *S. coelicolor* by substituted *C*-capped dipeptides or *C*-capped depsipeptide. All compounds reported displayed no intrinsic toxic activity to *S. coelicolor* at the concentrations that they were tested (25 μg/mL).

2.3. Structure–activity studies of BU-005

To identify the structural features of BU-005 that were critical for its ability to potentiate chloramphenicol activity, we performed systematic structure–activity relationship studies that were enabled by our use of the Ugi four-component reaction. Given the number of compounds that we intended to test, we deprotected the C-capped dipeptides and used them in chloramphenicol potentiation assays without further purification. Our finding that a crude, diastereomeric mixture of BU-005 (**1**) had the same potentiation activity as the column-purified material gave us confidence in this screening approach. The results are summarized in the graphs of Figures 3 through 6.

The identity of the first amino acid in BU-005 is an ornithine. The ornithine moiety has two primary amino groups at the α and δ carbons. We were interested in determining if either or both of the amino groups were important for the potentiation activity. To prepare these compounds, we used a variety of Boc-protected amino acids as carboxylic acid reactants in the Ugi reaction with hydrocinnamaldehye, dimethoxybenzylamine and 2-napthyl isocyanide. The structures prepared and the results of this analysis are presented in Figure 3. Compounds that lacked the α-amino group—the compounds derived from amino valeric acid (**2**) and amino caproic acid (**3**)—were significantly less active than BU-005. Likewise, a compound that lacked the δ amino group (**4**) also exhibited loss of activity, though not to the same extent as those without the α amino group. This result indicated that the α amino group was more important for the activity of BU-005 than the δ amino group. To gain further insights, we prepared and tested an analog with a β-amino group (derived from β-alanine). This compound (**5**) had a diminished activity, further demonstrating the necessity of an α-amino group for potentiation of chloramphenicol activity.

BU-005 has two secondary amide linkages. It is possible that they contribute to the activity of the molecule by acting as H-bond acceptors and/or donors. To investigate the significance of the first secondary amide bond in BU-005, we used multicomponent reactions to synthesize compounds with other linkages between the first and second amino acids. We used Ugi reactions with various primary amines instead of dimethyl benzylamine to prepare compounds with a tertiary amide in place of the secondary amide. A compound with an ester in place of the first secondary amide (i.e., a depsipeptide) could be prepared via a Passerini three component reaction of di-Boc-ornithine, hydrocinnamaldehyde, and 2-naphthyl isocyanide. The structures of three compounds that were prepared and their ability to potentiate chloramphenicol activity are presented in Figure 4.

First, we prepared a compound with an ester in place of the secondary amide of BU-005 using the Passerini reaction (**6**). This compound was completely inactive. It is not clear whether inactivity was due to instability of the compound or lack of binding to the chloramphenicol-specific efflux pumps. Next, we prepared compounds with tertiary amides in place of the first secondary amide in BU-005. Interestingly, most of these compounds (e.g., those with *N*-butyl or *N*-benzyl moieties) were toxic to *S. coelicolor*, even in the absence of chloramphenicol. Only a compound with a *N*-methyl tertiary amide linkage linking ornithine and homophenylalanine was non-toxic (**7**). Though this compound retained some activity, it was not as active as the BU-005. This observation can be explained by the limited conformational flexibility of a second-

ary amide or by the ability of a secondary amide to act as a hydrogen-bond donor.

Next, the significance of the homophenylalanine moiety in the second amino acid position of BU-005 was investigated. In using the Ugi reaction for C-capped dipeptide synthesis, we could access a much wider variety of structures in the second amino acid position. BU-005 has a hydrophobic aromatic ring separated from the backbone of the dipeptide by a chain of two methylene groups. Using Ugi reactions with a variety of aldehydes as substrates in place of hydrocinnamaldehyde, we prepared a wide variety of C-capped dipeptides with various substituted aromatic rings, and an aliphatic group at the second amino acid position of the dipeptides. The set of compounds that were prepared are depicted in Figure 5.

Although all the compounds had diminished activities compared to the BU-005, most of the compounds did have chloramphenicol potentiation activity. With the exception of a compound with a furan moiety (**11**), any compound with an aromatic functionality in place of homophenylalanine had activity. Further, even compound **12** with an aliphatic moiety in place of the homophenylalanine moiety of BU-005 had activity. In any case, the homophenylalnine of BU-005 appears to be a significant contributor to its chloramphenicol potentiation activity.

Finally, we investigated the significance of the identity of the moiety at the C-terminus of the dipeptide. BU-005 has a 2-amino naphthyl group at the C-terminus of the dipeptide. To assess its importance, we prepared compounds in which the 2-amino naphthyl group was replaced by other aromatic moieties as well as aliphatic moieties. These compounds were prepared using aromatic and aliphatic isocyanides as substrates with hydrocinnamaldehyde, dimethoxybenzylamine, and di-Boc-ornithine in Ugi reactions (Fig. 6). First, we prepared compound **13** which had a benzyl group in place of the 2-aminonapthyl moiety of BU-005 at the C-terminus; it had slightly less potentiation activity than BU-005. The increased flexibility and lower hydrophobicity of the benzyl moiety could explain this observation. Next, we prepared compounds with aliphatic groups at the C-terminus of the dipeptide (compounds **14**–**16**). Compared to BU-005, these compounds had negligible chloramphenicol potentiation activity. In summary, there was a direct correlation between the size of the capping group and the activity of the corresponding molecule. Specifically, as the size of the moiety used to cap the dipeptide was reduced, the ability of the compound to potentiate chloramphenicol activity was also reduced.

2.4. Mechanistic studies of chloramphenicol potentiation in *S. coelicolor* by *C*-capped dipeptides

The capacity of MC-207,110 (Phe-Arg-β-naphthylamide) and BU-005 to potentiate chloramphenicol activity in *S. coelicolor* was highly suggestive of inhibition of the major facilitator superfamily efflux pumps, CmlR1 and CmlR2.[20] This phenomenon was of particular interest because C-capped dipeptides were not known to be inhibitors of major facilitator superfamily efflux pumps. Given

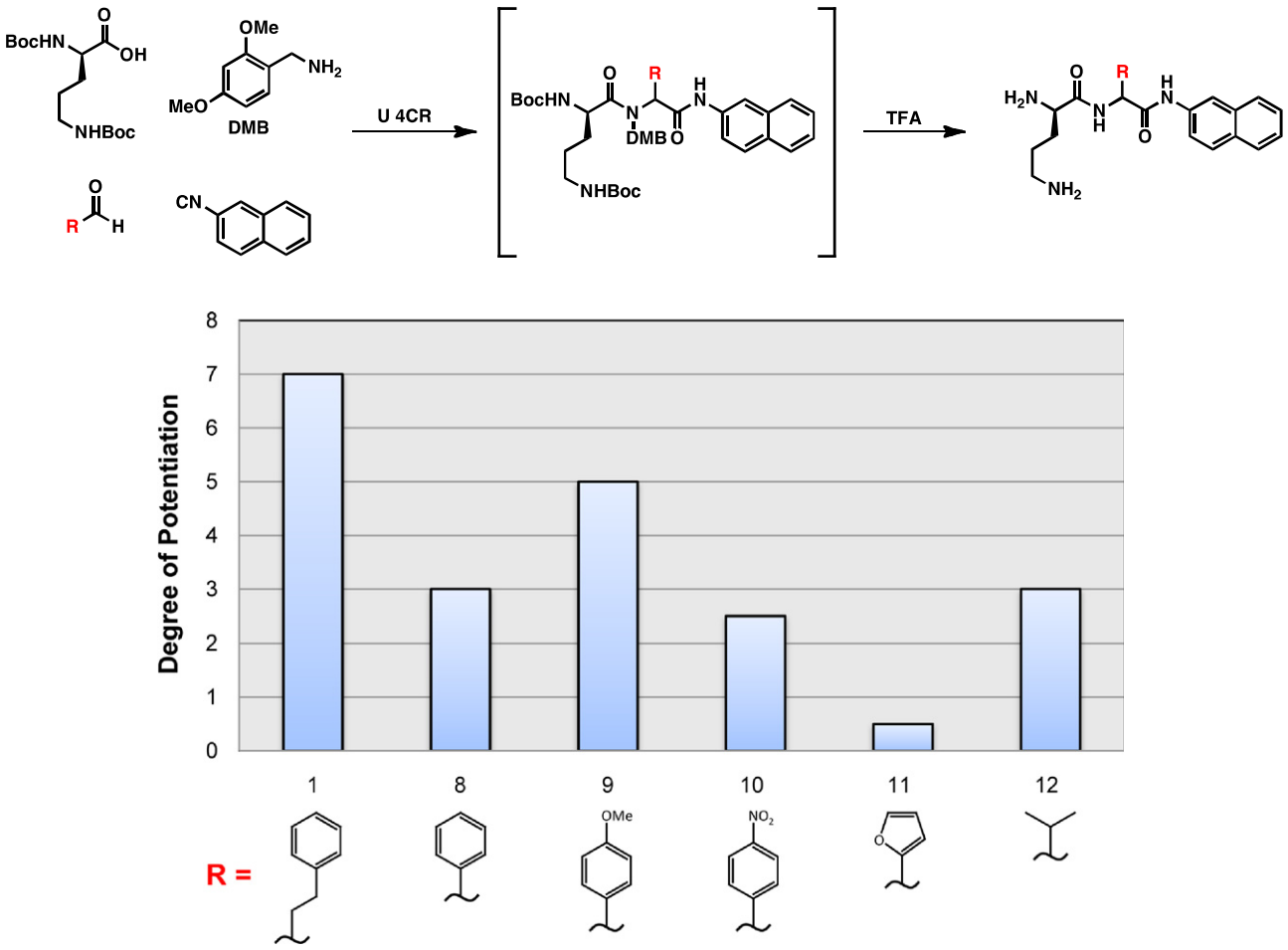


Figure 5. Analysis of chloramphenicol potentiation activity in wild-type *S. coelicolor* by *C*-capped dipeptides with various amino acids in the second position. All compounds reported displayed no intrinsic toxic activity to *S. coelicolor* at the concentrations that they were tested (25 μg/mL).

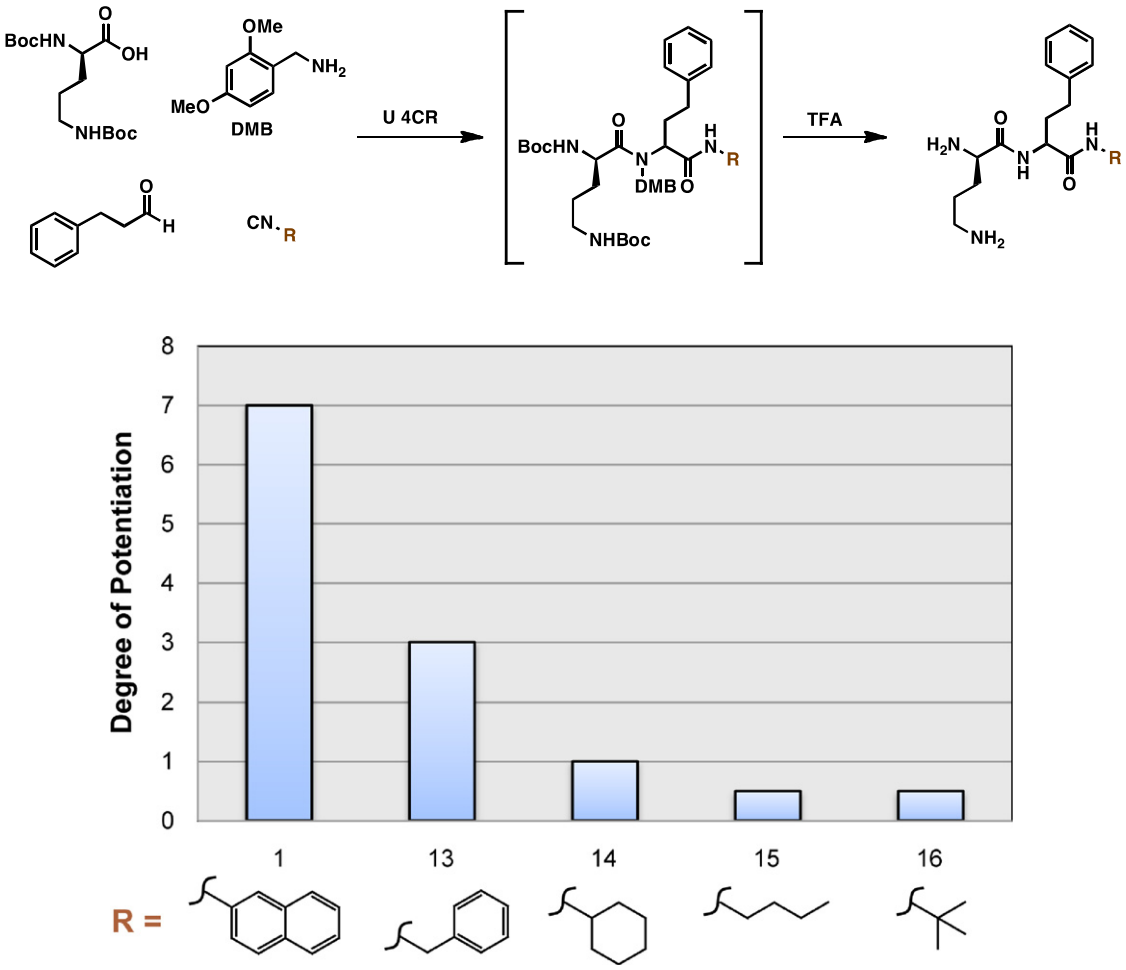
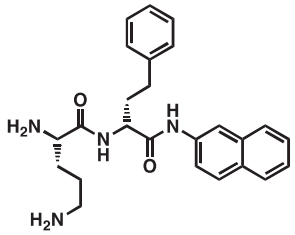


Figure 6. Analysis of chloramphenicol potentiation activity in wild-type *S. coelicolor* by *C*-capped dipeptides with various *C*-caps. All compounds reported displayed no intrinsic toxic activity to *S. coelicolor* at the concentrations that they were tested (25 μg/mL).

the novelty and potency of BU-005, we carried out a series of experiments to elucidate the mechanism by which it potentiated chloramphenicol activity against *S. coelicolor*. To rule out the possibility that BU-005 potentiated chloramphenicol activity in an efflux pump-independent fashion, we tested its ability to affect chloramphenicol resistance in *S. coelicolor* strains lacking the genes encoding the chloramphenicol-specific efflux pumps. We used a PCR-targeting procedure to construct *S. coelicolor* strains in which either *cmlR1* or *cmlR2* was deleted or in which both *cmlR1* and *cmlR2* were deleted. Deletion of the *cmlR1* gene reduces chloramphenicol MIC of *S. coelicolor* to 25 μg/mL while deletion of the *cmlR2* gene reduces chloramphenicol MIC to 6 μg/mL. The *S. coelicolor* strain lacking both *cmlR1* and *cmlR2* had a chloramphenicol MIC of 4.5 μg/mL. We tested the ability of BU-005 at 25 μg/mL to potentiate chloramphenicol activity in the strains lacking each efflux pump or both efflux pumps. The degree to which BU-005 potentiated chloramphenicol activity (sevenfold) was highest in the wild-type strain and lowest (1.8-fold) in the strain lacking both *cmlR1* and *cmlR2* (see Table 2 entries a–d). The correlation between the presence of the pumps and the degree to which chloramphenicol activity was potentiated by BU-005 is highly suggestive of competitive inhibition. The apparent potentiation activity in the strain lacking both efflux pumps can be explained by the presence of two putative chloramphenicol efflux pump genes (SCO2254 and SCO3915) in *S. coelicolor* that have yet to be characterized.

To further test the hypothesis that BU-005 acted by interfering with chloramphenicol efflux, we constructed strains that over-expressed either *cmlR1* or *cmlR2*. (The strains over-expressing each efflux pump were derived from the *cmlR1*/*cmlR2* null strain and in each case the gene was expressed under the con-

Table 2
Effects of BU-005 on chloramphenicol susceptibility in various strains of *S. coelicolor*[a,b,c]



Entry	*S. coelicolor* strain	MIC	MIC with EPI	Fold MIC reduction
a	Wild-type	65	9	7.2
b	*cmlR1* null	25	7.5	3.3
c	*cmlR2* null	6	2.5	2.4
d	*cmlR1*/*cmlR2* null	4.5	2.5	1.8
e	*cmlR1* over expressed	7	2	3.5
f	*cmlR2* over expressed	55	7.5	7.3

[a] MIC values have units of μg/mL.
[b] EPI in the table refers to BU-005.
[c] BU-005 displayed no intrinsic toxic activity to any of the strains tested at the concentration that it was used (25 μg/mL).

trol of a strong constitutive promoter). For both of the over-expression strains, the degree of potentiation by BU-005 was higher than in the strain lacking both *cmlR1* and *cmlR2*, further suggesting that the potentiation activity of the compound was efflux pump-dependent (entries d, e, and f). Most importantly, the degree of potentiation was significantly higher in the over-expression strain than in a strain that expressed the pump from its native promoter. Specifically, the degree to which BU-005 potentiated chloramphenicol activity was 3.3-fold is the *cmlR1* null strain (*cmlR2* expressed from its native promoter) (entry b), but it potentiated chloramphenicol activity 7.3-fold in the strain over-expressing *cmlR2* (entry f). Likewise, the degree to which BU-005 potentiated chloramphenicol activity was 2.4-fold in the *cmlR2* null strain (*cmlR1* expressed from its native promoter) (entry c), but it potentiated chloramphenicol activity 3.5-fold in the strain over-expressing *cmlR1* (entry e). Taken together, these results suggest that BU-005 potentiates chloramphenicol activity against *S. coelicolor* by inhibition of both CmlR1 and CmlR2.

3. Conclusion

In conclusion, we demonstrated the utility of the Ugi reaction in the synthesis of a targeted library of C-capped dipeptide efflux pump inhibitors. The compounds were tested for their ability to potentiate chloramphenicol in *S. coelicolor*, a relative of *Mycobacterium tuberculosis*. The use of the Ugi reaction afforded access to structural moieties that would otherwise be difficult to assess using standard peptide coupling reactions. From the synthesis and screening of the focused library of C-capped dipeptides, we discovered a compound three times more active than MC-207,110 (Phe-Arg-β-naphthylamide), the other C-capped dipeptide that has been demonstrated to potentiate chloramphenicol activity in *S. coelicolor*.[20] By assessing the ability of the compound to potentiate chloramphenicol activity in strains of *S.coelicolor* lacking each efflux pump and in strains over expressing each efflux pump, we discovered compelling evidence that BU-005 acts by inhibiting the activity of the two MFS pumps in *S. coelicolor*. Our findings suggest that C-capped dipeptide efflux pump inhibitors can be useful against multidrug-resistant, Gram-positive bacteria.

4. Experimental

4.1. General

All reactions were monitored by thin layer chromatography using precoated silica gel 60 plates (particle size 0.040–0.063 mm). Column chromatography was performed using 60 Å (230–400 mesh ASTM) silica gel. NMR analyses were performed on a Bruker Avance Ultrashield Spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C). The proton and carbon-13 chemical shifts are reported relative to residual solvent peaks (methanol or chloroform). Chemical shifts are reported on the δ scale and coupling constants J are in Hz. Multiplicities are described as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), or m (multiplet). Low-resolution analytical LC–MS was performed in the positive ion mode on a Thermo LCQ Deca XP MAX high sensitivity MSn ion trap mass spectrometer with a Shimadzu HPLC system using a Waters X-Terra MS C_{18} column (2.5 μm, 2.1 × 50 mm). High-resolution mass analyses were performed on a JOEL JMS-600H double focusing magnetic sector mass spectrometer using FAB ionization. HPLC separation was performed using a Phenomenex semi preparative reverse phase C_{18} column (10 μm, 10 × 150 mm). The solvents for the separation were 0.1% TFA in H_2O for the aqueous solvent (A) and acetonitrile as the organic solvent (B) and were run at a flow rate of 5 ml/min. Purity of isolated compounds was verified by LS-ESI-MS.

4.2. Synthesis

4.2.1. General procedure for isocyanide synthesis

Naphthyl isocyanides and 3-isocyanoquinoline were prepared following literature reported procedures.[29] To a well stirred solution of the amine (2.6 mmol) in CH_2Cl_2 (1.5 mL) and $CHCl_3$ containing n-Bu_4NHSO_4 (90 mg; 0.26 mmol) was added in one portion 50% aqueous NaOH (1.5 mL). The reaction mixture was stirred for 12 h, then diluted with water and extracted with $CHCl_3$. The combined extracts were dried over $MgSO_4$ and concentrated in vacuo. Products were purified by column chromatography with DCM as the mobile phase.

4.2.2. Spectral data for 3-isocyanoquinoline

^{1}H NMR (400 MHz; $CDCl_3$): δ 8.89 (d, J = 2.3, 1H), 8.18–8.14 (m, 2H), 7.85–7.80 (m, 2H), 7.68–7.64 (m, 1H). ^{13}C NMR(100 MHz; $CDCl_3$): δ 169.1, 147.52, 147.46, 133.1, 131.7, 130.1, 128.8, 128.2, 127.1. LRMS: calcd 154.1 for $C_{10}H_6N_2$, observed 154 $[M]^+$.

4.2.3. General procedure for synthesis of inhibitors

The amine (0.25 mmol, 1 equiv), and aldehyde (0.25 mmol, 1 equiv) were added to 400 ml of methanol in a ½ dram glass vial and mixed for 30 min by rotation. The isocyanide (0.2375 mmol, 0.95 equiv) and Boc protected amino acid (0.25 mmol, 1 equiv) were added and the resulting solution (0.5 M) was allowed to rotate for 15 h. The solvent was removed in vacuo after which the reaction products were purified by flash column chromatography. The α-acylaminocarboxamides were treated with trifluoroacetic acid (0.1 M solutions) for 16 h to remove the dimethoxybenzyl and Boc protecting groups. After deprotection, the TFA was evaporated and stock solutions in DMSO were made from the crude deprotection products (for compounds in Table 1, a subsequent flash column purification, followed by a HPLC purification step was performed before making stock solutions in DMSO for testing).

4.2.4. Spectral data for compounds in Table 1

4.2.4.1. Entry C (Table 1). Post deprotection flash column chromatography conditions: 4:1 (Isopropanol/NH_4OH). HPLC conditions 0–35% B over 18 mins (t_R = 13 min). ^{1}H NMR (400 MHz; CD_3OD): δ 9.49–9.45 (m, 1H), 9.08–8.99 (m, 1H), 8.18–8.15 (m, 2H), 7.98 (m, 1H), 7.89–7.85 (m, 1H), 7.24 (m, 4H), 7.15–7.12 (m, 1H), 4.61 (m, 1H), 4.10 (q, J = 6.7, 1H), 3.04–2.99 (m, 2H), 2.93–2.82 (m, 1H), 2.82–2.72 (m, 1H), 2.34–2.24 (m, 1H), 2.23–2.12 (m, 1H), 2.05–1.96 (m, 2H), 1.92–1.81 (m, 2H). ^{13}C (100 MHz; CD_3OD): δ 171.8, 171.7, 168.9, 168.8, 140.6, 140.5, 133.1, 131.7, 131.6, 131.5, 130.41, 130.39, 130.02, 129.99, 129.2, 129.1, 128.2, 128.1, 125.9, 122.8, 122.64, 122.62, 122.60, 54.5, 54.3, 52.5, 52.1, 38.7, 38.6, 33.4, 33,3, 31.9, 31.8, 28.3, 28.2, 22.9, 22.4.

LRMS: calcd 420.24 for $C_{24}H_{30}N_5O_2$, observed 420.49 $[M+H]^+$.

4.2.4.2. Entry D (Table 1). Post deprotection flash column chromatography conditions: 4:1 (Isopropanol/NH_4OH). HPLC conditions 0–40% B over 18 min (t_R = 16.5 min ^{1}H NMR (400 MHz; CD_3OD): δ 8.20 (m, 1H), 7.86–7.77 (m, 3H), 7.58 (m, 1H), 7.49–7.40 (m, 2H), 7.31–7.25 (m, 4H), 7.19 (m, 1H), 4.62 (m, 1H), 4.08 (m, 1H), 3.05–3.00 (m, 2H), 2.93–2.72 (m, 2H), 2.29–2.19 (m, 1H), 2.19–2.11 (m, 1H), 2.06–1.97 (m, 2H), 1.92–1.82 (m, 2H). ^{13}C (100 MHz; CD_3OD): δ 171.3, 171.0, 168.6, 168.5, 140.7, 135.5, 135.4, 133.7, 130.89, 130.86, 128.3, 128.2, 128.1, 127.3, 127.23, 127.15, 127.10, 126.23, 126.17, 125.9, 124.89, 124.82, 120.0, 119.9, 117.0, 116.8, 54.5, 54.3, 52.5, 52.2, 38.7, 38.6, 34.0, 33.8, 32.0, 31.9, 28.3, 28.2, 22.9, 22.4. HRMS: calcd 441.2266 for $C_{25}H_{30}N_4NaO_2$, observed 441.2280 $[M+Na]^+$.

4.2.4.3. Entry F (Table 1). Post deprotection flash column chromatography conditions: 4:1 (Isopropanol/NH_4OH). HPLC conditions 0–35% B over 18 min (t_R = 12.5 min). ^1H NMR (400 MHz; CD_3OD): δ 9.41–9.38 (m, 1H), 8.97 (m, 1H), 8.14 (m, 2H), 7.96–7.92 (m, 1H), 7.82 (t, *J* = 7.7, 1H), 7.28–7.26 (m, 4H), 7.16 (m, 1H), 4.64 (m, 1H), 4.13 (q, *J* = 6.4, 1H), 3.07–3.02 (m, 2H), 2.95–2.86 (m, 1H), 2.84–2.76 (m, 1H), 2.35–2.26 (m, 1H), 2.25–2.15 (m, 1H), 2.10–1.98 (m, 2H), 1.95–1.84 (m, 2H). ^{13}C (100 MHz; CD_3OD): δ 171.8, 171.7, 168.9, 168.8, 140.6, 140.5, 133.1, 131.7, 131.6, 131.5, 130.41, 130.39, 130.02, 129.99, 129.2, 129.1, 128.2, 128.1, 125.9, 122.8, 122.64, 122.62, 122.60, 54.5, 54.3, 52.5, 52.1, 38.7, 38.6, 33.4, 33,3, 31.9, 31.8, 28.3, 28.2, 22.9, 22.4. LRMS: calcd 420.24 for $C_{24}H_{30}N_5O_2$, observed 420.39 $[M+H]^+$.

4.2.4.4. Entry G (Table 1). Post deprotection flash column chromatography conditions: 4:1 (isopropanol/NH_4OH). HPLC conditions 0–40% B over 18 min (rt = 16.5 min). ^1H NMR (400 MHz; CD_3OD): δ 8.21 (s, 1H), 7.79 (m, 3H), 7.60–7.56 (m, 1H), 7.43 (m, 2H), 7.26 (m, 4H), 7.18 (t, *J* = 6.9, 1H), 4.58 (dd, *J* = 8.5, 5.8, 1H), 4.04 (t, *J* = 6.5, 1H), 3.02 (t, *J* = 7.6, 2H), 2.86–2.69 (m, 2H), 2.28–2.19 (m, 1H), 2.18–2.08 (m, 1H), 2.06–1.95 (m, 2H), 1.88–1.80 (m, 2H). ^{13}C (100 MHz; CD_3OD): δ 170.9, 167.7, 140.7, 135.5, 133.8, 130.9, 128.2, 128.1, 127.2, 127.1, 126.2, 125.9, 124.8, 119.9, 116.8, 54.5, 52.5, 52.5, 38.7, 33.9, 32.0, 28.3, 22.9. HRMS: calcd 441.2266 for $C_{25}H_{30}N_4NaO_2$, observed 441.2280 $[M+Na]^+$.

4.2.4.5. Entry H (Table 1). Post deprotection flash column chromatography conditions: 4:1 (isopropanol/NH_4OH). HPLC conditions 0–40% B over 18 min (t_R = 16.5 min). ^1H NMR (400 MHz; CD_3OD): δ 8.15 (s, 1H), 7.84–7.76 (m, 3H), 7.54 (dd, *J* = 8.8, 2.2, 1H), 7.48–7.39 (m, 2H), 7.30–7.23 (m, 4H), 7.20–7.16 (m, 1H), 4.62 (dd, *J* = 9.0, 5.1, 1H), 4.07 (t, *J* = 6.3, 1H), 3.03–2.96 (m, 2H), 2.90–2.84 (m, 1H), 2.80–2.73 (m, 1H), 2.26–2.19 (m, 1H), 2.17–2.11 (m, 1H), 2.03–1.98 (m, 2H), 1.90–1.80 (m, 2H). ^{13}C (100 MHz; CD_3OD): δ 171.2, 168.4, 140.7, 135.4, 133.7, 130.9, 130.4, 130.2, 128.3, 128.2, 128.0, 127.3, 127.1, 126.2, 125.9, 124.9, 120.0, 117.0, 54.3, 52.2, 38.7, 34.0, 31.9, 28.2, 22.4. HRMS: calcd 441.2266 for $C_{25}H_{30}N_4NaO_2$, observed 441.2271 $[M+Na]^+$.

4.2.5. Spectral data for compounds in Figures 3–6

4.2.5.1. Compound 1, Figure 3. *Flash Chromatography conditions:* 8:1 (DCM/EtOAc)—49% yield. ^1H NMR (400 MHz; $CDCl_3$): δ 7.89 (s, 1H), 7.77–7.72 (m, 3H), 7.47–7.31 (m, 5H), 7.27–7.23 (m, 4H), 7.07–7.05 (m, 1H), 6.41–6.39 (m, 1H), 6.16–6.10 (m, 1H), 5.53–5.41 (m, 1H), 5.06–5.01 (m, 1H), 4.67–4.49 (m, 2H), 3.77 (d, *J* = 7.7, 3H), 3.61 (m, 3H), 3.18–3.13 (m, 2H), 2.77–2.67 (m, 2H), 2.42–2.38 (m, 2H), 1.80–1.78 (m, 1H), 1.67–1.58 (m, 3H), 1.48–1.43 (m, 18H). ^{13}C NMR (100 MHz; $CDCl_3$): δ 155.7, 141.0, 135.5, 133.80, 133.77, 130.4, 128.71, 128.66, 128.55, 128.51, 127.58, 127.47, 126.35, 126.31, 126.27, 124.7, 120.00, 119,97, 116.6, 116.3, 104.6, 104.4, 98.5, 79.9, 55.4, 55.3, 51.3, 40.3, 32.7, 32.6, 31.2, 30.8, 28.43, 28.39, 28.36, 28.32, 26.0. HRMS: calcd 791.3996 for $C_{44}H_{56}N_4NaO_8$, observed 791.3981 $[M+Na]^+$.

4.2.5.2. Compound 2, Figure 3. *Flash Chromatography conditions:* 7:1 (DCM/EtOAc)—38% yield. ^1H NMR (400 MHz; $CDCl_3$): δ 8.93 (s, 1H), 8.04 (s, 1H), 7.77 (m, 3H), 7.46 (t, *J* = 8.02, 1H), 7.40 (t, *J* = 7.5, 1H), 7.35–7.30 (m, 3H), 7.22 (m, 3H), 6.93 (d, *J* = 8.3, 1H), 6.38 (dd, *J* = 8.4, 2.3, 1H), 6.30 (d, *J* = 2.2, 1H), 4.85 (br, 1H), 4.51 (q, *J* = 13.7, 2H), 3.72 (s, 3H), 3.68 (s, 3H), 3.18–3.11 (m, 2H), 2.76–2.65 (m, 2H), 2.54 (t, *J* = 7.3, 2H), 2.16–2.12 (m, 1H), 1.85 (m, 1H), 1.77 (m, 2H), 1.55 (m, 2H), 1.45 (s, 9H). ^{13}C NMR (100 MHz; $CDCl_3$): δ 175.9, 169.1, 160.8, 158.0, 156.0, 141.0, 135.6, 133.8, 130.5, 129.1, 128.53, 128.51, 127.6, 127.5, 126.4, 126.2, 124.8, 120.0, 116.34, 116.32, 104.2, 98.6, 79.1, 55.3, 55.2, 40.3, 33.4, 32.8, 29.8, 29.4, 28.5, 22.5. HRMS: calcd 676.3363 for $C_{39}H_{47}N_3NaO_6$, observed 676.3355 $[M+Na]^+$.

4.2.5.3. Compound 3, Figure 3. *Flash Chromatography conditions:* 15:1 (DCM/EtoAc). ^1H NMR (400 MHz; $CDCl_3$): δ 8.96 (s, 1H), 8.04 (d, *J* = 0.71, 1H), 7.79–7.75 (m, 3H), 7.46 (t, *J* = 7.4, 1H), 7.40 (t, *J* = 7.3, 1H), 7.35–7.29 (m, 3H), 7.24–7.20 (m, 3H), 6.93 (d, *J* = 8.3, 1H), 6.37 (dd, *J* = 8.3, 2.0, 1H), 6.30–6.29 (m, 1H), 4.86 (br, 1H), 4.51 (m, 2H), 3.72 (s, 3H), 3.68 (s, 3H), 3.13–3.09 (m, 2H), 2.75–2.63 (m, 2H), 2.53–2.49 (m, 2H), 1.77–1.72 (m, 4H), 1.53–1.49 (m, 2H), 1.45 (s, 9H), 1.39–1.37 (m, 2H). ^{13}C NMR (100 MHz; $CDCl_3$): δ 176.2, 169.1, 160.7, 158.0, 141.0, 135.6, 133.8, 130.4, 128.5, 127.6, 127.5, 126.4, 126.2, 124.8, 120.0, 116.4, 116.3, 104.2, 98.5, 55.3, 55.2, 33.8, 32.8, 29.9, 29.4, 28.4, 26.6, 25.0. HRMS: calcd 690.3519 for $C_{40}H_{49}N_3NaO_6$, observed 690.3541 $[M+Na]^+$.

4.2.5.4. Compound 4, Figure 3. *Flash Chromatography conditions:* 20:1 (DCM/EtOAc)—48% yield. ^1H NMR (400 MHz; $CDCl_3$): δ 8.55 (s, 1H), 7.97 (d, *J* = 0.51, 1H), 7.78–7.73 (m, 3H), 7.46 (t, *J* = 7.3, 1H), 7.40 (t, *J* = 7.32, 1H), 7.33 (t, *J* = 7.3, 2H), 7.27 (dd, *J* = 2.4, 1.9, 1H), 7.23 (t, *J* = 6.4, 3H), 6.91 (d, *J* = 8.1, 1H), 6.39 (dd, *J* = 8.2, 2.0, 1H), 6.26 (d, *J* = 1.8, 1H), 4.65 (br, 1H), 4.41 (m, 2H), 4.23 (m, 2H), 3.71 (s, 3H), 3.67 (s, 3H), 2.72 (m, 2H), 2.60 (m, 1H), 2.25–2.18 (m, 1H), 1.50 (s, 9H). ^{13}C NMR (100 MHz; $CDCl_3$): δ 171.4, 168.6, 161.2, 158.4, 140.9, 135.3, 133.8, 130.5, 128.6, 128.4, 127.6, 127.5, 126.4, 126.3, 124.8, 120.0, 116.5, 115.0, 104.5, 98.7, 79.8, 55.4, 53.5, 43.0, 32.7, 29.2, 28.4. HRMS: calcd 634.2893 for $C_{36}H_{41}N_3NaO_6$, observed 634.2875 $[M+Na]^+$.

4.2.5.5. Compound 5, Figure 3. *Flash Chromatography conditions:* 15:1 (DCM/EtOAc)—22% yield. ^1H NMR (400 MHz; $CDCl_3$): δ 8.71 (d, *J* = 0.31, 1H), 8.00 (d, *J* = 1.7, 1H), 7.76 (t, *J* = 8.7, 3H), 7.46 (m, 1H), 7.40 (m, 1H), 7.34 (t, *J* = 7.3, 2H), 7.26–7.21 (m, 3H), 6.90 (d, *J* = 8.4, 1H), 6.38 (dd, *J* = 8.3, 2.4, 1H), 6.26 (d, *J* = 2.3, 1H), 4.70 (br, 1H), 4.46 (s, 2H), 3.71 (s, 3H), 3.65 (s, 3H), 3.54–3.50 (m, 2H), 2.81–2.77 (m, 2H), 2.74–2.69 (m, 2H), 2.54 (m, 1H), 2.21 (m, 1H), 1.45 (s, 9H). ^{13}C NMR (100 MHz; $CDCl_3$): δ 174.5, 169.0, 161.0, 158.3, 156.0, 141.0, 135.5, 133.8, 130.4, 130.0, 128.65, 128.57, 128.48, 127.6, 127.5, 126.4, 126.2, 124.8, 119.9, 116.4, 115.7, 104.4, 98.6, 79.3, 55.3, 55.3, 36.5, 34.4, 32.7, 29.3, 28.5. HRMS: calcd 648.3050 for $C_{37}H_{43}N_3NaO_6$, observed 648.3062 $[M+Na]^+$.

4.2.5.6. Compound 6, Figure 4. *Flash Chromatography conditions:* 10:1 (DCM/EtOAc)—10% yield. ^1H NMR (400 MHz; $CDCl_3$): δ 9.11 (m, 1H), 8.43 (d, *J* = 15.2, 1H), 7.87–7.79 (m, 4H), 7.49–7.40 (m, 2H), 7.32 (m, 2H), 7.26–7.21 (m, 3H), 5.43 (m, 1H), 4.30–4.14 (m, 1H), 3.22–3.19 (m, 2H), 2.85–2.73 (m, 2H), 2.42–2.31 (m, 2H), 2.01–1.81 (m, 2H), 1.68–1.63 (m, 2H), 1.51–1.42 (m, 18H). ^{13}C NMR (100 MHz; $CDCl_3$): δ 172.5, 171.0, 168.3, 168.0, 157.1, 156.34, 156.28, 156.18, 140.8, 140.7, 135.4, 135.2, 133.80, 133.75, 130.82, 130.78, 128.60, 128.56, 128.41, 128.35, 127.8, 127.7, 127.5, 126.34, 126.29, 126.22, 126.19, 125.9, 125.0, 124.99, 120.7, 120.2, 117.4, 117.1, 80.9, 80.8, 79.5, 74.8, 74.2, 62.2, 54.4, 54.2, 40.0, 39.8, 34.3, 33.4, 32.1, 31.5, 31.4, 28.2, 28.2, 28.0, 26.72, 26.66. HRMS: calcd 642.3155 for $C_{35}H_{45}N_3NaO_7$, observed 674.3218 $[M+Na]^+$.

4.2.5.7. Compound 7, Figure 4. *Flash Chromatography conditions:* 7:1 (DCM/EtOAc)—18% yield.

HRMS: calcd 655.3472 for $C_{36}H_{48}N_4NaO_6$, observed 655.3480 $[M+Na]^+$.

4.2.5.8. Compound 8, Figure 5. *Flash Chromatography conditions:* 8:1 (DCM/EtOAc)—39% yield. ^1H NMR (400 MHz; $CDCl_3$): δ

8.42 (br, 1H), 8.19–8.08 (m, 1H), 7.78–7.69 (m, 3H), 7.45–7.30 (m, 8H), 7.15–6.94 (m, 1H), 6.44–6.29 (m, 2H), 4.95–4.48 (m, 4H), 3.79–3.70 (m, 6H), 3.15–2.97 (m, 2H), 1.94–1.83 (m, 1H), 1.63–1.51(m, 3H), 1.47–1.43 (m, 18H). ^{13}C NMR (100 MHz; $CDCl_3$): δ 160.9, 156.02, 155.98, 155.95, 135.3, 135.0, 133.8, 130.7, 130.6, 129.2, 128.8, 128.6, 128.5, 128.4, 127.7, 127.6, 127.50, 127.47, 126.3, 124.9, 124.8, 120.3, 120.0, 116.9, 116.6, 116.2, 104.3, 104.0, 98.6, 98.5, 79.9, 79.6, 79.11, 79.09, 79.08, 79.06, 55.4, 55.2, 51.1, 51.0, 40.14, 40.11, 40.09, 28.4, 28.3, 25.8, 25.6. HRMS: calcd 763.3683 for $C_{42}H_{52}N_4NaO_8$, observed 763.3691 $[M+Na]^+$.

4.2.5.9. Compound 9, Figure 5. *Flash Chromatography conditions:* 5:1 (DCM/EtOAc)—55% yield. ^{1}H NMR (400 MHz; $CDCl_3$): δ 8.29–8.09 (m, 2H), 7.78–7.71 (m, 3H), 7.45–7.36 (m, 3H), 7.36–7.30 (m, 2H), 7.15–7.00 (m, 1H), 6.85 (m, 2H), 6.45–6.33 (m, 2H), 4.91–4.44 (m, 4H), 3.82–3.74 (m, 9H), 3.13–2.99 (m, 2H), 1.85 (br, 1H), 1.63–1.53 (m, 3H), 1.46–1.43 (m, 18H). ^{13}C NMR (100 MHz; $CDCl_3$): δ 160.8, 156.0, 135.3, 133.8, 130.8, 130.7, 128.49, 128.46, 127.7, 127.6, 127.5, 126.3, 124.9, 120.2, 120.0, 116.8, 116.4, 114.2, 114.0, 104.0, 98.4, 79.9, 79.1, 65.37, 65.35, 65.34, 55.4, 55.32, 55.29, 55.24, 51.02, 51.00, 47.0, 40.1, 30.1, 28.2, 25.73, 25.71. HRMS: calcd 793.3789 for $C_{43}H_{54}N_4NaO_9$, observed 793.3768 $[M+Na]^+$.

4.2.5.10. Compound 10, Figure 5. *Flash Chromatography conditions:* 10:1 (DCM/EtOAc)—65% yield. ^{1}H NMR (400 MHz; $CDCl_3$): δ 8.22–8.05 (m, 1H), 8.01–7.98 (m, 1H), 7.93–7.91 (m, 1H), 7.76–7.71 (m, 3H), 7.62–7.53 (m, 2H), 7.49–7.36 (m, 3H), 7.26–7.22 (m, 1H), 7.10–7.08 (m, 1H), 6.50–6.26 (m, 2H), 5.16–4.90 (m, 2H), 4.74–4.34 (m, 2H), 3.85–3.70 (m, 6H), 3.21–2.95 (m, 2H), 1.68–1.71 (m, 2H), 1.60–1.56 (m, 2H), 1.49–1.42 (m, 18H). HRMS: calcd 808.3534 for $C_{42}H_{51}N_5NaO_{10}$, observed 808.3510 $[M+Na]^+$.

4.2.5.11. Compound 11, Figure 5. *Flash Chromatography conditions:* 8:1 (DCM/EtOAc)—46% yield. ^{1}H NMR (400 MHz; $CDCl_3$): δ 8.74–8.53 (m, 1H), 8.34–8.07 (m, 1H), 7.74 (m, 4H), 7.55–7.31 (m, 5H), 7.20–6.98 (m, 1H), 6.75–6.56 (m, 1H), 6.42–6.26 (m, 3H), 5.72–5.52 (m, 1H), 4.85–4.63 (m, 3H), 3.70 (m, 6H), 3.12–2.93 (m, 2H), 1.56–1.42 (m, 22H). ^{13}C NMR (100 MHz; $CDCl_3$): δ 174.9, 165.8, 160.7, 156.1, 147.9, 142.8, 135.3, 133.74, 133.72, 130.7, 130.6, 128.5, 128.3, 127.71, 127.66, 127.63, 127.54, 127.49, 126.4, 126.3, 124.9, 120.4, 120.0, 117.1, 116.8, 116.24, 116.22, 112.3, 110.8, 110.7, 104.3, 104.2, 98.2, 80.1, 79.6, 79.2, 57.2, 55.41, 55.35, 55.31, 55.27, 51.12, 51.08, 45.67, 45.65, 40.1, 29.9, 28.4, 28.3, 25.9, 25.6. HRMS: calcd 753.3476 for $C_{40}H_{50}N_4NaO_9$, observed 753.3461 $[M+Na]^+$.

4.2.5.12. Compound 12, Figure 5. *Flash Chromatography conditions:* 10:1 (DCM/EtOAc)—96% yield. ^{1}H NMR (400 MHz; $CDCl_3$): δ 9.58–9.33 (m, 1H), 8.02–7.85 (m, 1H), 7.77–7.65 (m, 4H), 7.44 (t, J = 7.5, 1H), 7.38 (t, J = 7.1, 1H), 7.30 (m, 1H), 7.12–7.09 (m, 1H), 6.40–6.30 (m, 2H), 4.78–4.55 (m, 3H), 3.11 (br, 2H), 2.78 (br, 1H), 2.03 (br, 1H), 1.82–1.71 (br, 1H), 1.60 (m, 2H), 1.47–1.41 (m, 18H), 1.00 (m, 6H). ^{13}C NMR (100 MHz; $CDCl_3$): δ 175.3, 168.6, 161.2, 155.97, 155.94, 155.29, 135.6, 135.5, 133.83, 133.80, 130.5, 130.4, 128.42, 128.37, 127.60, 127.56, 127.47, 126.34, 126.31, 124.7, 120.1, 119.9, 116.5, 116.3, 115.1, 98.7, 98.5, 79.8, 79.7, 55.4, 55.24, 55.20, 55.0, 51.3, 40.2, 31.4, 31.0, 28.4, 28.3, 27.1, 26.1, 25.7, 20.5, 20.3, 19.5, 19.0. HRMS: calcd 729.3839 for $C_{39}H_{54}N_4NaO_8$, observed 729.3820 $[M+Na]^+$.

4.2.5.13. Compound 13, Figure 6. *Flash Chromatography conditions:* 7:1 (DCM/EtOAc)—23% yield. ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.36–7.09 (m, 11H), 6.81 (m, 1H), 6.49–6.30 (m, 2H), 6.18–6.12 (m, 1H), 4.98–4.57 (m, 3H), 4.39–4.08 (m, 3H), 3.79 (d, J = 9.6, 4H), 3.58 (s, 1H), 3.34 (s, 1H), 3.16 (br, 1H), 3.05 (br, 1H), 2.78–2.55 (m, 2H), 2.19–2.06 (m, 1H), 1.85–1.65 (m, 1H), 1.62–1.54 (m, 3H), 1.47–1.42 (m, 18H). ^{13}C NMR (100 MHz; $CDCl_3$): δ 174.2, 172.4, 170.4, 170.2, 161.3, 161.1, 158.40, 158.37, 156.0, 155.9, 155.6, 155.5, 141.2, 141.1, 138.2, 128.64, 128.56, 128.53, 128.47, 127.9, 127.8, 127.3, 127.2, 126.2, 126.1, 104.2, 104.0, 99.0, 98.9, 79.9, 79.5, 79.2, 58.8, 55.42, 55.38, 55.0, 54.8, 50.7, 43.5, 32.8, 32.7, 31.3, 30.7, 30.6, 29.7, 29.27, 29.25, 28.40, 28.37, 28.3, 25.7. HRMS: calcd 755.3996 for $C_{41}H_{56}N_4NaO_8$, observed 755.3982 $[M+Na]^+$.

4.2.5.14. Compound 14, Figure 6. *Flash Chromatography conditions:* 8:1 (DCM/EtOAc)—60% yield. ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.31 (m, 1H), 7.28–7.26 (m, 1H), 7.23–7.13 (m, 3H), 7.08–6.93 (m, 1H), 6.45–6.35 (m, 2H), 4.92–4.76 (m, 1H), 4.66 (m, 2H), 4.40 (m, 1H), 3.84–3.76 (m, 6H), 3.64–3.57 (m, 1H), 3.15–3.08 (m, 2H), 2.66 (t, J = 7.9, 1H), 2.53 (t, J = 7.3, 1H), 2.04 (m, 1H), 1.78 (br, 2H), 1.68–1.57 (m, 6H), 1.44 (m, 18H), 1.38–1.27 (m, 3H), 1.20–0.86 (m, 3H). HRMS: calcd 747.4309 for $C_{40}H_{60}N_4NaO_8$, observed 747.4326 $[M+Na]^+$.

4.2.5.15. Compound 15, Figure 6. *Flash Chromatography conditions:* 8:1 (DCM/EtOAc)—62% yield. ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.33–7.14 (m, 5H), 7.11–6.95 (m, 1H), 6.43–6.35 (m, 2H), 5.46–5.31 (m, 1H), 4.69–4.41 (m, 3H), 3.82–3.77 (m, 6H), 3.20–2.98 (m, 4H), 2.66 (t, J = 7.3, 1H), 2.55 (t, J = 7.2, 1H), 2.07 (m, 1H), 1.90 (br, 1H), 1.72–1.65 (m, 1H), 1.62–1.55 (m, 3H), 1.44 (d, J = 13.8, 18H), 1.29 (t, J = 7.7, 3H), 1.21 (m, 1H), 0.92–0.86 (m, 3H). ^{13}C NMR (100 MHz; $CDCl_3$): δ 174.6, 170.2, 161.3, 160.0, 155.9, 155.5, 141.2, 141.1, 128.6, 128.55, 128.48, 128.42, 126.1, 126.0, 104.2, 103.8, 98.9, 98.7, 79.8, 79.5, 79.2, 59.2, 55.4, 55.32, 55.25, 51.0, 50.9, 50.8, 39.2, 39.1, 32.8, 31.5, 31.17, 31.15, 29.9, 29.5, 28.34, 28.27, 26.0, 25.8, 20.1, 19.9, 13.79, 13.77. HRMS: calcd 721.4152 for $C_{38}H_{58}N_4NaO_8$, observed 721.4175 $[M+Na]^+$.

4.2.5.16. Compound 16, Figure 6. *Flash Chromatography conditions:* 10:1 (DCM/EtOAc)—70% yield. ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.32–7.26 (m, 2H), 7.21 (m, 1H), 7.14 (m, 2H), 6.96–6.73 (m, 1H), 6.45–6.37 (m, 2H), 5.52–5.31 (m, 1H), 4.65–4.43 (m, 3H), 3.83–3.76 (m, 6H), 3.12 (br, 2H), 2.63 (t, J = 8.0, 1H), 2.51 (t, J = 7.3, 1H), 2.07–1.97 (m, 1H), 1.84 (br, 1H), 1.79–1.67 (m, 1H), 1.64–1.53 (m, 3H), 1.44 (m, 18H), 1.21 (m, 9H). HRMS: calcd 721.4152 for $C_{38}H_{58}N_4NaO_8$, observed 721.4135 $[M+Na]^+$.

4.3. Bacterial strains and culture conditions

S. coelicolor strains were grown at 30 °C on mannitol soya flour medium (SFM), Difco Nutrient Agar medium (DNA), yeast extract–malt extract medium (YEME), or minimal liquid medium (NMMP). SFM was used for conjugations between *S. coelicolor* and *Escherichia coli* and for generating spore stocks. *E. coli* strains DH5α and ET12567/pUZ8002 were grown on Luria–Bertani medium at 37 °C for routine subcloning,[30] and *E. coli* strain BW25113/pIJ790 was grown on Luria–Bertani medium at 30 °C when maintaining selection for pIJ790. For selecting *E. coli*, ampicillin, apramycin, chloramphenicol, hygromycin, and kanamycin were employed at 100, 50, 25, 80, and 50 μg/mL, respectively. Nalidixic acid was used at 20 μg/mL to counter-select *E. coli* in conjugations with *S. coelicolor*. Apramycin and hygromycin were used at 50 μg/mL for selecting *S. coelicolor*.

4.3.1. Plasmids and primers

Standard cloning procedures were employed in generating the plasmids used. pIJ10257, derived from pMS81, was the vector used for over-expression of efflux pump genes in *S. coelicolor*.[31] DNA sequencing was performed by Davis Sequencing (Davis, California).

All primers used in the work were synthesized by Invitrogen. PCR was performed with *Taq* (Invitrogen) and *Pfu* (Stratagene, Agilent Technologies). All PCR reactions were performed with 5% (vol/vol) DMSO.[32]

4.3.2. Construction of *cmlR1* and *cmlR2* null mutants

PCR-targeted mutagenesis was used to replace the *S. coelicolor* SCO7662 (*cmlR2*) gene with the spectinomycin/streptomycin resistance marker, *aad*, in the *S. coelicolor cmlR1* null strain (SCO7552::*apr*).[33] The requisite PCR product was amplified from the spectinomycin/streptomycin-resistance gene insert of pIJ778 using primers: *cmlR2* KO F 5′-CCTTTCTCGTGCTTCTCCTCGCCTCG GAGCCACTCGATGACTAGTATTCCGGGATCCGTCGACC-3′ and *cmlR2* KO R 5′-GGGACACGACGTGGTCACG CCAGTCGGGTGGCGGTTTCAA CTAGTTGTAGGCTGGAGCTGCTTC-3′. The PCR product was introduced into *E. coli* BW25113/pIJ790 harboring cosmid St10F4 and expressing the λRED recombinase. The resultant recombinant cosmid, St10F4 Δ*cmlR2*::*aad*, was introduced into *E. coli* strain ET12567/pUZ8002 and then into *S. coelicolor* Δ*cmlR1*::*apr* by way of conjugation. Exconjugants lacking the *cmlR2* gene were identified by selection for spectinomycin-resistance and kanamycin-sensitivity. Gene replacements were confirmed by PCR analysis of genomic DNA isolated from the null mutants using primers: *cmlR2* KD FOR 5′-GCCGTGGTGACGAGATG-3′ and *cmlR2* KD REV 5′-GTGC GCGGGACACGAC-3′.

4.3.3. Over-expression of *cmlR1* and *cmlR2* genes

The *cmlR1* and *cmlR2* open reading frames were amplified from wild-type *S. coelicolor* genomic DNA using the following primers: *cmlR1* ORF F 5′-CATATGCCGCTCGCCGTCTAC-3′ and *cmlR1* ORF R 5′-CTCGAGTCACACCCGCTGGGCC-3′; *cmlR2* ORF F 5′-CATATGCCT CTTCCGCTGTAC-3′ and *cmlR2* ORF R 5′-CTCGAGTCACCGGGT CGCGTC-3′. The PCR products were cloned into pBluescript II KS+ and sequenced to confirm their identities. The genes were transferred into the integrative, constitutive expression vector pIJ10257[31] yielding pJS333 (*cmlR1*) and pJS334 (*cmlR2*). (pIJ10257 has the strong constitutive *ermE** promoter). The resultant plasmids were introduced into *E. coli* strain ET12567/pUZ8002 and then into the chloramphenicol-sensitive *S. coelicolor cmlR1*/*cmlR2* null strain by way of conjugation as previously described.[28] Exconjugants with either pJS333 or pJS334 were identified by selection for hygromycin resistance. The empty vector pIJ10257 was also introduced into the double knockout as a control.

4.3.4. Antimicrobial agents

Chloramphenicol and MC-207,110 (Phe-Arg-β-naphthylamide) were purchased from Sigma–Aldrich.

4.4. MIC assays

All MIC assays were performed on Difco nutrient agar medium supplemented with the indicated concentration of chloramphenicol and 25 μg/mL of the synthesized inhibitors. Growths were assessed after incubation at 30 °C for 48 h.

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.10.011.

References and notes

1. Webber, M. A.; Piddock, L. J. V. *J. Antimicrob. Chemother.* **2003**, *51*, 9.
2. Poole, K. *J Antimicrob. Chemother.* **2005**, *56*, 20.
3. Lomovskaya, O.; Zgurskaya, H. I.; Totrov, M.; Watkins, W. J. *Nat. Rev. Drug Disc.* **2007**, *6*, 56.
4. Renau, T. E.; Leger, R.; Flamme, E. M.; She, M. W.; Gannon, C. L.; Mathias, K. M.; Lomovskaya, O.; Chamberland, S.; Lee, V. J.; Ohta, T.; Nakayama, K.; Ishida, Y. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 663.
5. Piddock, L. J. *Nat. Rev. Microbiol.* **2006**, *4*, 629.
6. Testa, R. T.; Petersen, P. J.; Jacobus, N. V.; Sum, P. E.; Lee, V. J.; Tally, F. P. *Antimicrob. Agents Chemother.* **1993**, *37*, 2270.
7. Harding, I.; Simpson, I. *J. Chemother.* **2000**, *12*(Suppl 4), 7.
8. Ince, D.; Hooper, D. C. *Antimicrob. Agents Chemother.* **2001**, *45*, 2755.
9. Ince, D.; Zhang, X.; Silver, L. C.; Hooper, D. C. *Antimicrob. Agents Chemother.* **2002**, *46*, 3370.
10. Ince, D.; Zhang, X.; Silver, L. C.; Hooper, D. C. *Antimicrob. Agents Chemother.* **2003**, *47*, 274.
11. Renau, T. E.; Leger, R.; Flamme, E. M.; Sangalang, J.; She, M. W.; Yen, R.; Gannon, C. L.; Griffith, D.; Chamberland, S.; Lomovskaya, O.; Hecker, S. J.; Lee, V. J.; Ohta, T.; Nakayama, K. *J. Med. Chem.* **1999**, *42*, 4928.
12. Nelson, M. L.; Park, P. H.; Levy, S. B. *J. Med. Chem.* **1994**, *37*, 1355.
13. Lomovskaya, O.; Bostian, K. A. *Biochem. Pharmacol.* **2006**, *71*, 910.
14. Rouveix, B. *J. Antimicrob. Chemother.* **2007**, *59*, 1208.
15. Pages, J. M.; Amaral, L. *Bba-Proteins Proteom.* **2009**, *1794*, 826.
16. Renau, T. E.; Leger, R.; Filonova, L.; Flamme, E. M.; Wang, M.; Yen, R.; Madsen, D.; Griffith, D.; Chamberland, S.; Dudley, M. N.; Lee, V. J.; Lomovskaya, O.; Watkins, W. J.; Ohta, T.; Nakayama, K.; Ishida, Y. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2755.
17. Lomovskaya, O.; Warren, M. S.; Lee, A.; Galazzo, J.; Fronko, R.; Lee, M.; Blais, J.; Cho, D.; Chamberland, S.; Renau, T.; Leger, R.; Hecker, S.; Watkins, W.; Hoshino, K.; Ishida, H.; Lee, V. J. *Antimicrob. Agents. Chemother.* **2001**, *45*, 105.
18. Bohnert, J. A.; Kern, W. V. *Antimicrob. Agents Chemother.* **2005**, *49*, 849.
19. Pages, J. M.; Masi, M.; Barbe, J. *Trends Mol. Med.* **2005**, *11*, 382.
20. Vecchione, J. J.; Alexander, B.; Sello, J. K. *Antimicrob. Agents Chemother.* **2009**, *53*, 4673.
21. Bentley, S. D.; Chater, K. F.; Cerdeno-Tarraga, A. M.; Challis, G. L.; Thomson, N. R.; James, K. D.; Harris, D. E.; Quail, M. A.; Kieser, H.; Harper, D.; Bateman, A.; Brown, S.; Chandra, G.; Chen, C. W.; Collins, M.; Cronin, A.; Fraser, A.; Goble, A.; Hidalgo, J.; Hornsby, T.; Howarth, S.; Huang, C. H.; Kieser, T.; Larke, L.; Murphy, L.; Oliver, K.; O'Neil, S.; Rabbinowitsch, E.; Rajandream, M. A.; Rutherford, K.; Rutter, S.; Seeger, K.; Saunders, D.; Sharp, S.; Squares, R.; Squares, S.; Taylor, K.; Warren, T.; Wietzorrek, A.; Woodward, J.; Barrell, B. G.; Parkhill, J.; Hopwood, D. A. *Nature* **2002**, *417*, 141.
22. D'Costa, V. M.; McGrann, K. M.; Hughes, D. W.; Wright, G. D. *Science* **2006**, *311*, 374.
23. Ugi, I. *Intra-Sci. Chem. Rep.* **1971**, *5*, 229.
24. Waki, M.; Meienhofer, J. *J. Am. Chem. Soc.* **1977**, *99*, 6075.
25. Just, G.; Chung, B. Y.; Grozinger, K. *Can. J. Chem.* **1977**, *55*, 274.
26. Cao, X.; Moran, E. J.; Siev, D.; Lio, A.; Ohashi, C.; Majalli, A. M. M. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2953.
27. Kazmaier, U.; Hebach, C. *Synlett* **2003**, 1591.
28. Sheehan, S. M.; Masters, J. J.; Wiley, M. R.; Young, S. C.; Liebeschuetz, J. W.; Jones, S. D.; Murray, C. W.; Franciskovich, J. B.; Engel, D. B.; Weber, W. W.; Marimuthu, J.; Kyle, J. A.; Smallwood, J. K.; Farmen, M. W.; Smith, G. F. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2255.
29. Barton, D. H. R.; Ozbalik, N.; Vacher, B. *Tetrahedron* **1988**, *44*, 3501.
30. Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989.
31. Hong, H. J.; Hutchings, M. I.; Hill, L. M.; Buttner, M. J. *J. Biol. Chem.* **2005**, *280*, 13055.
32. Kieser, T. B.; Buttner, M. J.; Chater, K. F.; Hopwood, D. A. *Practical Streptomyces Genetics*; John Innes Foundation: Norwich, 2000.
33. Gust, B.; Challis, G. L.; Fowler, K.; Kieser, T.; Chater, K. F. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 1541.