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Article

Atypically Modified Carbapenem Antibiotics Display Improved Antimycobacterial Activity in the Absence of β-Lactamase Inhibitors

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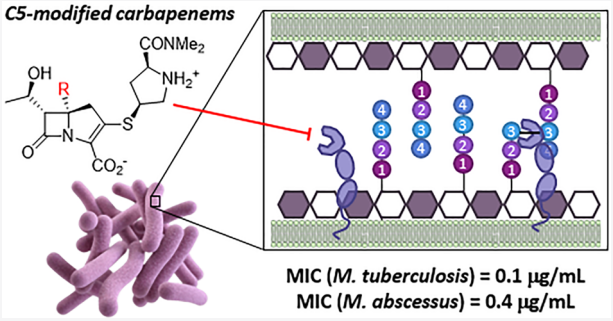
ABSTRACT: Commercial carbapenem antibiotics are being used to treat multidrug resistant (MDR) and extensively drug resistant (XDR) tuberculosis. Like other β-lactams, carbapenems are irreversible inhibitors of serine D,D-transpeptidases involved in peptidoglycan biosynthesis. In addition to D,D-transpeptidases, mycobacteria also utilize nonhomologous cysteine L,D-transpeptidases (Ldts) to cross-link the stem peptides of peptidoglycan, and carbapenems form long-lived acyl-enzymes with Ldts. Commercial carbapenems are C2 modifications of a common scaffold. This study describes the synthesis of a series of atypical, C5α modifications of the carbapenem scaffold, microbiological evaluation against *Mycobacterium tuberculosis* (*Mtb*) and the nontuberculous mycobacterial species, *Mycobacterium abscessus* (*Mab*), as well as acylation of an important mycobacterial target Ldt, Ldt_{Mt2}. *In vitro* evaluation of these C5α-modified carbapenems revealed compounds with standalone (*i.e.*, in the absence of a β-lactamase inhibitor) minimum inhibitory concentrations (MICs) superior to meropenem-clavulanate for *Mtb*, and meropenem-avibactam for *Mab*. Time-kill kinetics assays showed better killing (2−4 log decrease) of *Mtb* and *Mab* with lower concentrations of compound **10a** as compared to meropenem. Although susceptibility of clinical isolates to meropenem varied by nearly 100-fold, **10a** maintained excellent activity against all *Mtb* and *Mab* strains. High resolution mass spectrometry revealed that **10a** acylates Ldt_{Mt2} at a rate comparable to meropenem, but subsequently undergoes an unprecedented carbapenem fragmentation, leading to an acyl-enzyme with mass of Δm = +86 Da. Rationale for the divergence of the nonhydrolytic fragmentation of the Ldt_{Mt2} acyl-enzymes is proposed. The observed activity illustrates the potential of novel atypical carbapenems as prospective candidates for treatment of *Mtb* and *Mab* infections.



KEYWORDS: *Mycobacterium, antimicrobial activity, carbapenems, L,D-transpeptidase*

Tuberculosis (TB) is caused by the slow-growing intracellular pathogen *Mycobacterium tuberculosis* (*Mtb*), and currently infects an estimated 2 billion people, representing one-fourth of the world's population. TB causes 1.5 million deaths annually and requires a challenging treatment regimen involving the administration of four antitubercular agents for a period of at least six months. The emergence of multidrug resistant (MDR), extensively resistant (XDR), and totally resistant (TDR) TB necessitates development of new antitubercular agents.[1] *Mycobacterium abscessus* (*Mab*), by contrast, is a rapidly growing nontuberculous mycobacterial (NTM) species that typically causes pulmonary infections, but can also cause soft-tissue, burn, and wound infections.[2] Like *Mtb*, *Mab* requires long-term treatment with multiple antibiotics, frequently resulting in significant adverse side effects.[3] Due to the intrinsic antimicrobial resistance of *Mab*, cure rates are <50%, and recurrence rates are 50%, underscoring the urgent need for new therapeutic agents.[4,5]

β-Lactam antibiotics are not currently included among the first line antitubercular agents isoniazid, rifampin, ethambutol, and pyrazinamide. The earliest studies of β-lactam antibiotics indicated little antitubercular activity.[6−8] It was soon recognized that their apparent inactivity was due to the presence of mycobacterial β-lactamases.[9] Penicillinase-resistant penicillins, such as oxacillin, and third-generation cephalosporins, which are poor substrates for the mycobacterial β-lactamase, were observed to possess activity.[10,11] Later it was demonstrated that carbapenems and combinations of β-lactam antibiotics and β-lactamase inhibitors were also active

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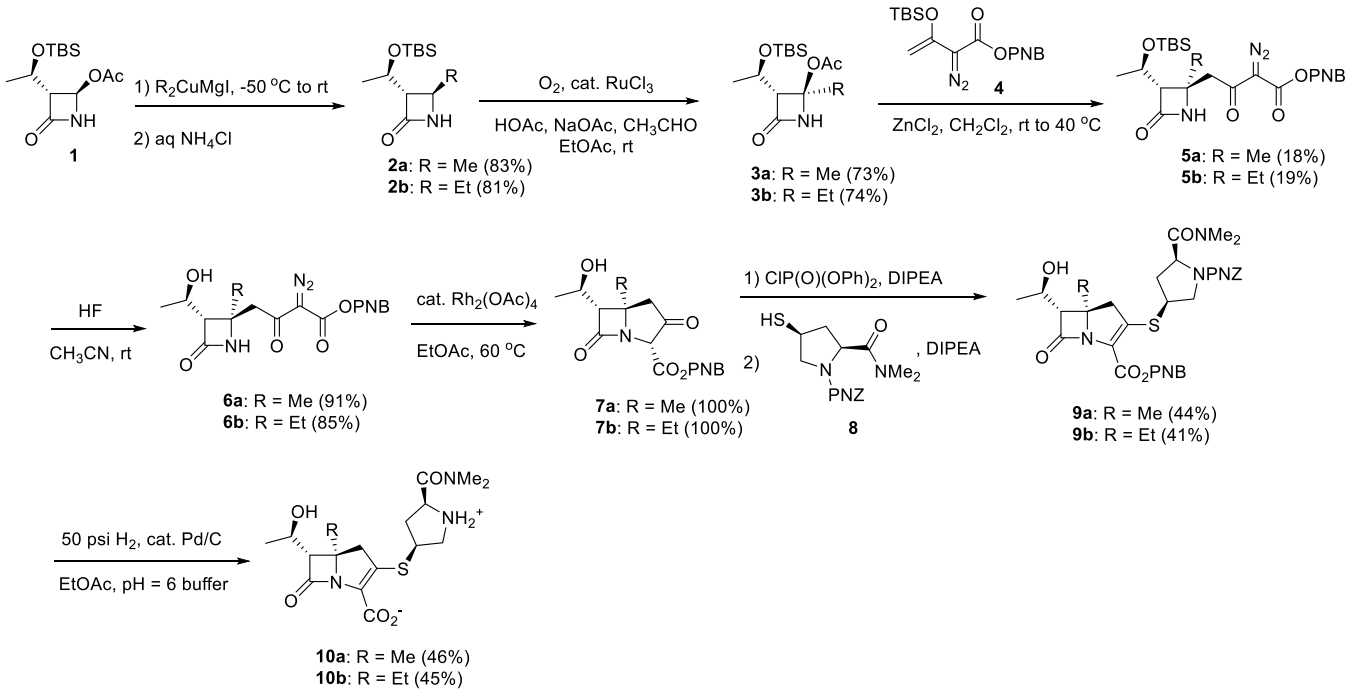
Scheme 1. Syntheses of C5α-Substituted Carbapenems 10a and 10b



antimycobacterial agents.[12−14] The major *Mtb* β-lactamase, BlaC, is an endogenous Ambler class A serine penicillinase that has broad substrate specificity and is inhibited by clavulanic acid.[15−17] A combination of clavulanic acid and the carbapenem meropenem was shown to be active against XDR-*Mtb* and recent studies compared *in vitro* efficacy of several β-lactam/β-lactamase inhibitor combinations against MDR-TB.[18−20] *Mab* also expresses an endogenous serine class A β-lactamase, Bla_{Mab}, with broad substrate specificity that hydrolyzes the conventional β-lactamase inhibitors, clavulanate, sulbactam, and tazobactam, but is inhibited by the newer diazabicyclooctanes (DBO, e.g., avibactam, relebactam) and boronic acid (e.g., vaborbactam) inhibitors.[21−23] Carbapenems were found to be more effective against *Mab* in combination with other antibiotics, both β-lactam and non-β-lactam, rather than when used alone.[24−33] Newly designed penems are in development to treat *Mab*.[34]

β-Lactam antibiotics inhibit the transpeptidases involved in cell wall biosynthesis. The cell wall component peptidoglycan (PG) is a cross-linked polymeric structure comprised of glycan polymers cross-linked by short peptide strands which protrude from these carbohydrates. Glycan strands of most bacteria are comprised of alternating β(1 → 4)linked *N*-acetylglucosamine (GlcNAc) and modified *N*-acetylmuramic acid (MurNAc) residues, and in both *Mtb* and *Mab*, the MurNAc is extensively (∼70%) replaced by *N*-glycolylmuramic acid (MurN-Glyc).[35−37] The muramic acid residues are covalently attached to short (3 to 5 residue) peptide side chains, which are cross-linked to provide structural stability, protecting the microorganism from the effects of turgor pressure. The stem pentapeptides consist of glycan-linked-L-Ala-D-γGlu-*meso*-DAP-D-Ala-D-Ala, where DAP = diaminopimelate. Two types of mycobacterial cross-links are observed: (1) 4 → 3 cross-links, formed by D,D-transpeptidase-catalyzed cleavage between the D-Ala-D-Ala linkage and joining the resultant acyl-enzyme carbonyl to the γ-amino group of DAP, and (2) 3 → 3 cross-links, formed by L,D-transpeptidase (Ldt)-catalyzed cleavage of the DAP-D-Ala linkage and joining the carbonyl group of the resultant acyl-enzyme with the same γ-amino group of DAP. The 4 → 3 cross-links were historically commonly observed, and D,D-transpeptidases have become known as penicillin-binding proteins (PBPs). By contrast, the Ldt-catalyzed 3 → 3 cross-links are predominant (70−80%) in the PG of both *Mtb* and *Mab*.[38] These 3 → 3 cross-links have since been observed in numerous species, including *Enterococcus faecium*, *Corynebacterium jeikeium*, *Escherichia coli*, *Clostridium difficile*, *Acinetobacter baylyi*, and *Acinetobacter baumannii*.[39−44] The acceptor substrate for an Ldt is a *tetrapeptide* stem, which has the terminal D-Ala residue cleaved from the D-Ala-D-Ala terminus of the stem pentapeptide, a transformation performed by a D,D-carboxypeptidase.[39]

The carbapenem class of β-lactam antibiotics exhibits high potency, broad spectrum activity, and stability to 20th century β-lactamases (e.g., TEM-1). Carbapenems have also demonstrated in vitro efficacy against *Mtb*, the ability to inhibit *Mtb* PBPs, activity in combination with β-lactamase inhibitors against XDR *Mtb*, as well as ability to inhibit Ldt_{Mt1} and Ldt_{Mt2}.[14,20,45−47] Additionally, it was recently discovered that meropenem inhibits the D,D-carboxypeptidase responsible for cleaving the terminal D-Ala-D-Ala linkage of the stem pentapeptides to provide the tetrapeptide substrates for Ldts.[48] Thus, carbapenems interfere with *Mtb* cell wall biosynthesis at multiple steps, including PBP-catalyzed 4 → 3 cross-linking, D,D-carboxypeptidase-catalyzed cleavage of the terminal D-Ala residue, and Ldt-catalyzed 3 → 3 cross-linking.[49−52]

The carbapenem meropenem and the carbapenem/dehydropeptidase-I inhibitor combination, imipenem/cilastatin, have recently been included as add-ons in treatment of MDR-TB, when used in combination with the β-lactamase inhibitor clavulanic acid.[53] Studies of clinical use of carbapenems, including meropenem, imipenem, and ertapenem, in the treatment of MDR-TB documented their safety and tolerability and improvement of success rates.[54−57]

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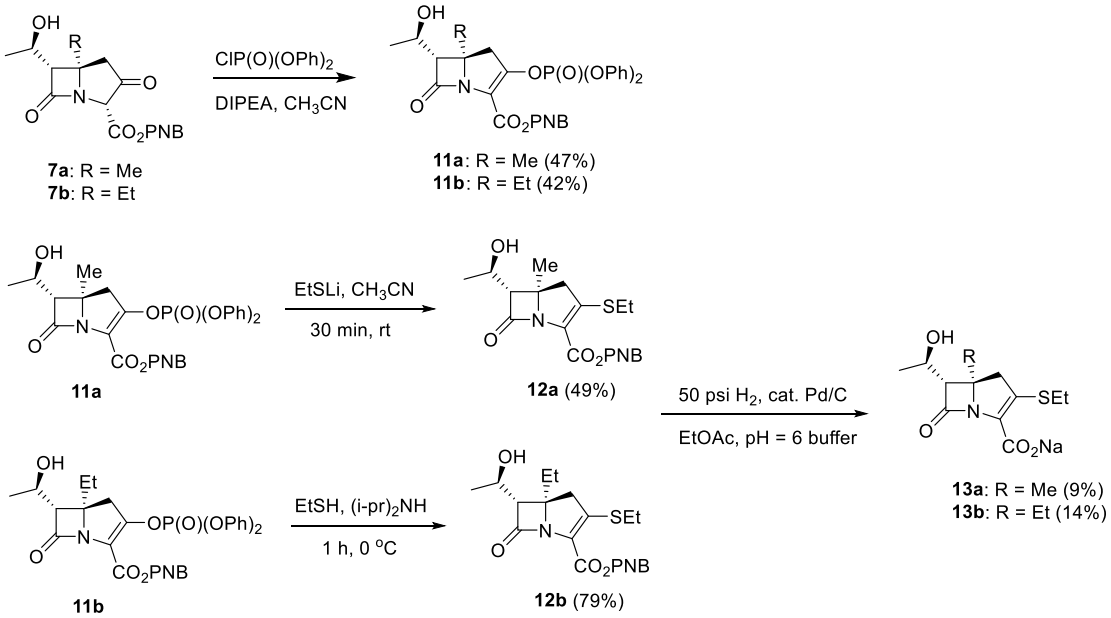
Scheme 2. Syntheses of C5α-Substituted Carbapenems 13a and 13b



Imipenem is included in British Thoracic Society guidelines as part of the regimen to treat NTM infections including *Mab*, and the US Cystic Fibrosis Foundation and European Cystic Fibrosis Society includes imipenem in the regimen for treating pulmonary NTM infections.[58,59] Analysis of outcomes indicate improved success rates with NTM patients receiving imipenem, along with other agents.[60]

Structural development of the carbapenem class in the past 30 years has been limited to modification of the C2 position on the scaffold, despite the fact that multiple pathogens have evolved carbapenemases capable of hydrolyzing all commercial carbapenems.[61−68] We recently began a program to reinvestigate atypical (i.e., substituted at positions other than C2) carbapenems with respect to their susceptibility to carbapenemase-mediated hydrolysis and antimicrobial efficacy against resistant pathogens. We reasoned that such atypical substitutions had potential to differentially affect interaction of the carbapenems to β-lactamases relative to PBPs, including noncovalent recognition, the rate of protein acylation, and, in the case of β-lactamases, the rate of acyl-enzyme hydrolysis. We realized that these differences had potential to render the new carbapenem antibiotics less susceptible to carbapenemase-mediated hydrolysis, while maintaining the ability to bind PBP and Ldt enzymes. Due to the synthetic challenges, the C5 position of the carbapenem scaffold is notably underexplored, although one early study, not including mycobacterial strains, of C5α substituted carbapenems indicated very modest activity against a few representative strains.[69] Our study describes the synthesis and evaluation of C5α-substituted (C5α-methyl, **10a** and **13a**, and C5α-ethyl, **10b** and **13b**, see Schemes 1 and 2) carbapenems as antibacterial agents targeting *Mtb* and *Mab*. The C5α position substituent is hydrogen in all bicyclic commercial β-lactam antibiotics. We also noted that the substituted pyrrolidine C2 side chains of commercial carbapenems incorporated into **10a** and **10b** may not be optimal for *Mtb*, and thus, in addition to incorporating the meropenem pyrrolidine C2 side chain, we decided to synthesize and evaluate simplified C2 thioethyl analogues (**13a** and **13b**) of the new atypical C5α-substituted carbapenems.[70] In reported studies, the carbapenem with the minimal C2 thioethyl group was able to acylate the Ldt_{Mt1} protein eight times more efficiently than meropenem.[70]

RESULTS

Chemistry. The syntheses of C5α-methyl carbapenems **10a** and **13a** as well as the C5α-ethyl carbapenems **10b** and **13b** are illustrated in Schemes 1 and 2. In summary, the C4-alkylazetidinone **2a** or **2b** was prepared by reaction of commercially available 4-acetoxyazetidinone **1** with iodomagnesium dimethylcuprate or iodomagnesium diethylcuprate, respectively. The unstable tertiary acetate, **3a** or **3b** was prepared utilizing a ruthenium(III) chloride catalyzed oxidation process as shown.[71] This compound was reacted with the highly functional TBS enol ether **4** in the presence of $ZnCl_2$ to generate **5a** or **5b** with appropriate stereochemistry.[72] The TBS protecting group was then removed from the side chain using aqueous HF in acetonitrile at room temperature and the cyclization of the (hydroxyethyl)diazodicarbonyl compound **6a** or **6b** performed using catalytic $Rh_2(OAc)_4$ in EtOAc to generate ketoester **7a** or **7b**. The meropenem pyrrolidine sulfide side chain was introduced by formation of the enol phosphate, and subsequent reaction with thiol **8** without isolation of the intermediate as shown in Scheme 1. Deprotection in a two-phase system using catalytic palladium under hydrogen pressure removed the two PNZ protecting groups and subsequent purification using a DiaionHP20 chromatography column and increasing ethanol/water concentration, followed by lyophilization produced antibiotic **10a** or **10b**.

As shown in Scheme 2, to introduce the C2 thioethyl side chain, we chose to first isolate enol phosphate **11a** or **11b**, and subsequently react with ethanethiol. In the case of **11a**, we utilized a THF solution of preformed lithium thioethoxide, and in the case of **11b** we utilized ethanethiol in the presence of DIPA to generate **12a** or **12b**, respectively, and then deprotected utilizing catalytic palladium under hydrogen pressure in a two-phase system, and subsequent purification as described above.

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***In Vitro* Antimycobacterial Activity.** *Mtb* and *Mab* produce extended spectrum β-lactamases BlaC and Bla_{Mab}, which are inhibited by clavulanate and avibactam, respectively.[73] Since these enzymes are capable of hydrolyzing commercial carbapenems, the four atypical C5α-substituted carbapenems (**10a**, **10b**, **13a**, and **13b**) were evaluated against these two mycobacterial species both alone and in the presence of the appropriate β-lactamase inhibitors as shown in Table 1,

Table 1. MIC Values (μg/mL) of C5α-Substituted Carbapenem Antibiotics against *Mtb* and *Mab*[a]

	Mtb		*Mab*	
carbapenem	alone	with clavulanate (5 μg/mL)	alone	with avibactam[#] (5 μg/mL)
meropenem*	1.1	0.5	2.6	3.1
10a	0.1	0.1	0.4	0.5
10b	2.1	1.6	22.3	11.6
13a	0.8	0.6	7.5	5.8
13b	7.9	5.6	NA	NA

[a]NA: No activity at the highest conc. (30.3 μg/mL). *$P < 0.05$, comparison of +/- clavulanate. [#]No statistical difference with +/- avibactam.

using commercial meropenem as reference. All C5α-substituted carbapenems retained activity against *Mtb*, with **10a** exhibiting ~10-fold enhanced potency compared to meropenem (Table 1). Replacement of the C2 substituted pyrrolidine group with a simplified thioethyl moiety in **13a** and **13b** resulted in decreased activity toward *Mtb*. Only meropenem showed a statistically significant decrease in MIC when combined with clavulanate.

Similarly, when evaluated against *Mab*, **10a** was the most potent C5-substituted analogue, while others showed little (**10b**, **13a**) to no (**13b**) inhibition of *Mab* growth (Table 1). Inhibition of Bla_{Mab} with avibactam did not significantly alter the MIC of any tested compounds.

The activity of carbapenem **10a** was further characterized in time-kill assays as shown in Figure 1. Concentration dependent killing of both *Mtb* and *Mab* by **10a**, superior to that of meropenem, was observed at 2, 4, and 8 times the MIC.

To confirm the selectivity of C5α-substituted carbapenems, **10a** was tested for cytotoxicity against murine macrophage (J774) and hepatic (HepG2) cell lines using 2-fold dilution series of the compound. No toxicity was noted even at the highest concentration, indicating a selectivity index (SI = IC_{50}/MIC) of >90.

We determined MIC values for **10a** against five clinical strains of *Mtb* and *Mab* (Table 2) in comparison to reference

Table 2. MIC Values (μg/mL) against *Mtb* and *Mab* Clinical Strains

	carbapenem	CI-1	CI-2	CI-3	CI-4	CI-5	reference
Mtb	meropenem	4.6	12.5	5.1	0.7	5.9	2.6
	10a	0.7	0.7	0.8	0.2	1.0	0.7
Mab	meropenem	5.2	9.7	5.4	16.0	96.1	4.4
	10a	1.1	0.8	1.1	0.9	1.3	0.8

strains CDC1551 (*Mtb*) and 390S (*Mab*). The slightly different MIC values for reference strains noted here likely stems from the use of different *Mtb* assay readouts (Table 2: resazurin assay vs Table 1: luciferase assay) and slight loss of compound activity during storage.

Clinical isolates exhibited variable susceptibility to meropenem, most notably the enhanced sensitivity of *Mtb* CI-4 belonging to the Indo-Oceanic lineage 6 and dramatically

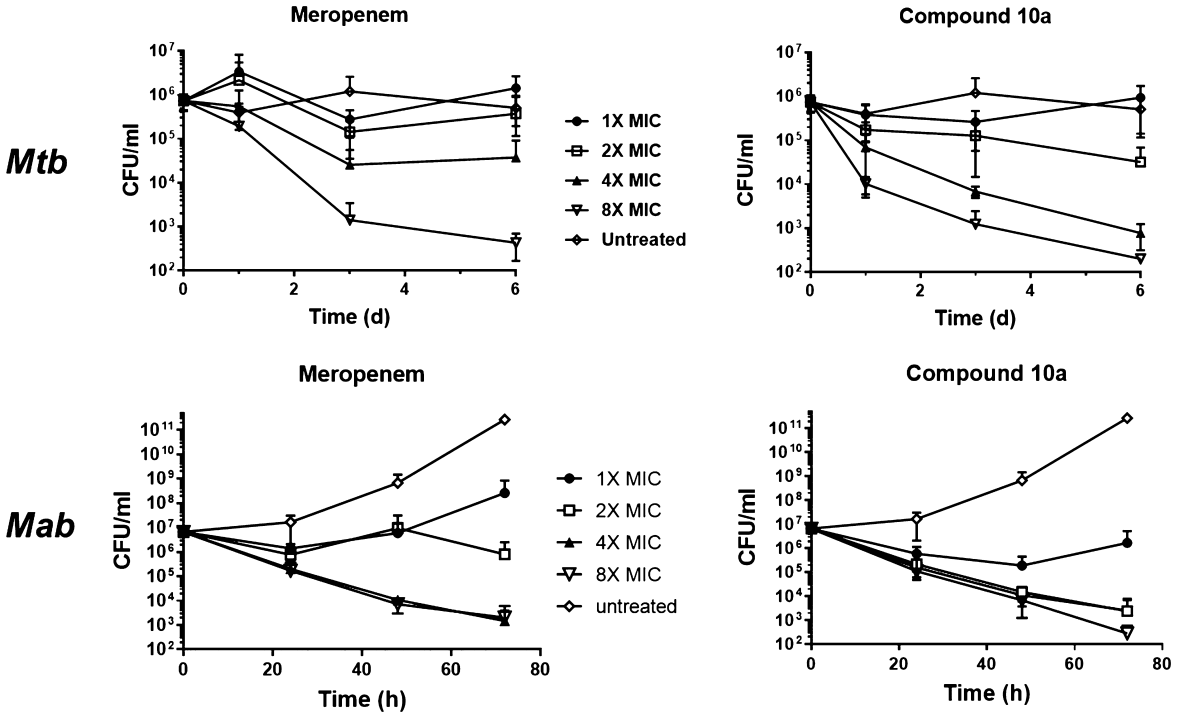

Figure 1. Concentration- and time-dependent bactericidal activity of **10a** and meropenem against *Mtb* and *Mab*. The bacterial cultures were treated with different drug concentrations for 6 days with *Mtb* and for 72 h in the case of *Mab* and plated for CFU at indicated time points. Data is an average of four independent experiments with a total of 12 technical replicates with standard deviation error bars.

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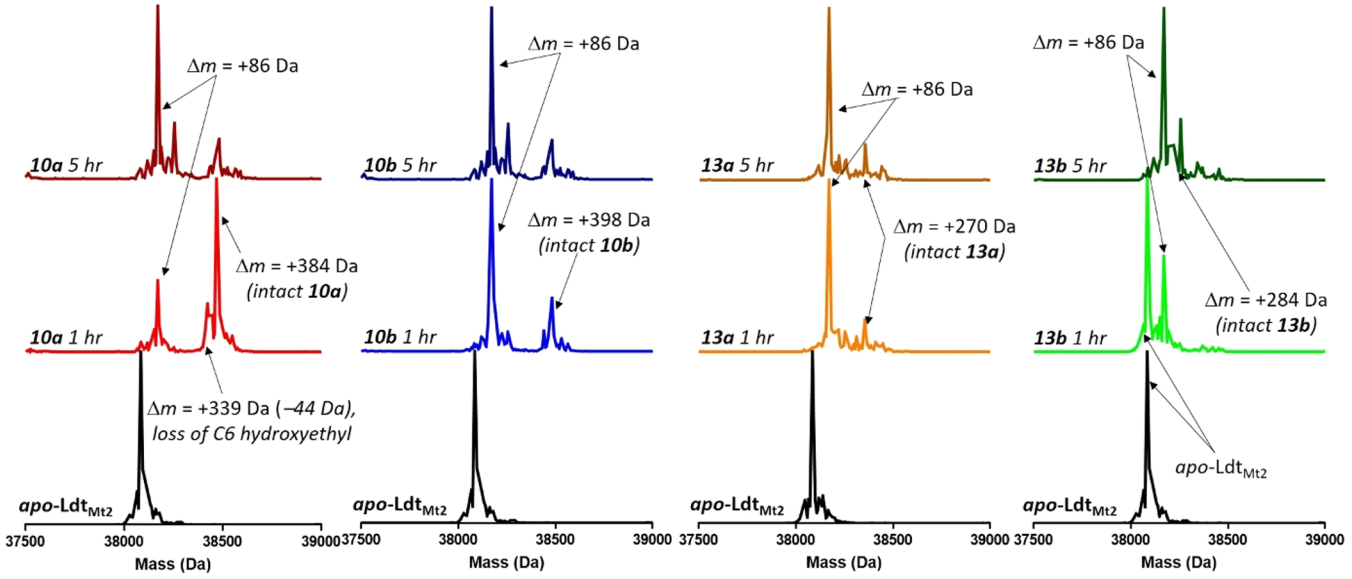
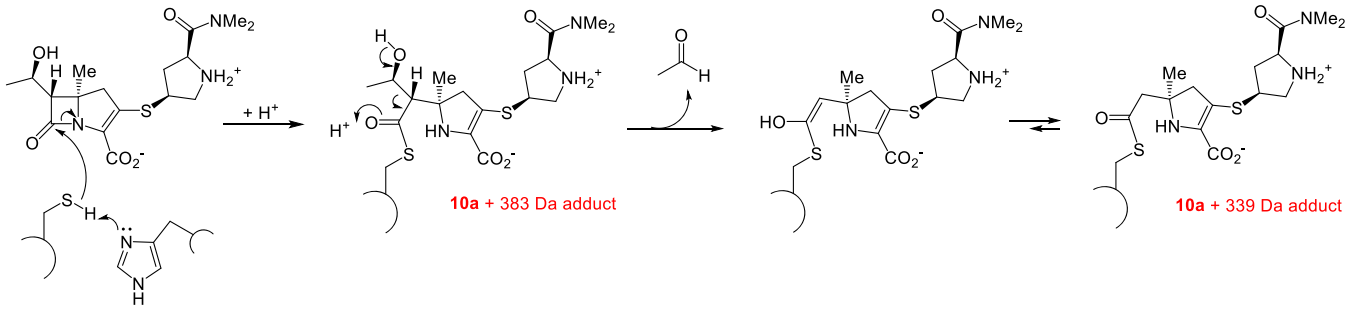
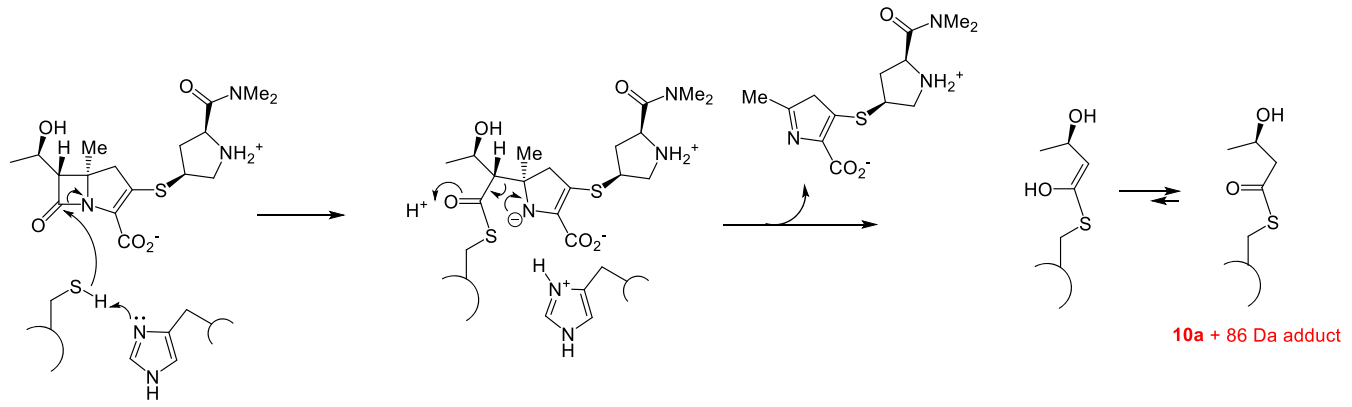


Figure 2. Ldt$_{Mt2}$ time course showing that C5α-substituted carbapenems **10a** (red traces), **10b** (blue traces), and **13a** (orange traces) fully acylate Ldt$_{Mt2}$ after 1 h, and **13b** (green traces) fully acylates Ldt$_{Mt2}$ after 5 h as indicated by change in mass (Δm). All C5α-substituted carbapenems degrade to the Δm = +86 Da adduct on the enzyme.

Scheme 3. Mechanism of Ldt$_{Mt2}$ Inactivation by 10a and Subsequent Loss of the C6 Hydroxyethyl Substituent



Scheme 4. Mechanism of C5α-Substituted Carbapenem Degradation to the Observed Δm = +86 Da Adduct



decreased sensitivity of *Mab* CI-5 (MIC 21× higher than reference). However, **10a** was largely unaffected by strain-specific differences in susceptibility.

Acylation of Ldt$_{Mt2}$. *Mtb* cross-links PG using four PBPs and the five Ldts, Ldt$_{Mt1}$–Ldt$_{Mt5}$. Ldt$_{Mt2}$ is essential for virulence and has been proposed as a potential antituberculosis target.[51] Carbapenems, penems, and cephalosporins are known to acylate class 2 Ldts, and thus we decided to evaluate the time-dependent interaction of our new C5α-alkylated carbapenems with Ldt$_{Mt2}$ using high resolution mass spectrometry (HRMS).[45] As shown in Figure 2, all four of the new carbapenems form stable adducts with Ldt$_{Mt2}$, and all degrade to a Δm = +86 Da adduct over the course of the 5-h reaction time. Like other C1-unsubstituted carbapenems, it was possible to observe the intact carbapenem antibiotic bound to the enzyme after 1 h of incubation in the case of **10a** and **10b**, but fragmentation of the acyl-enzyme to the Δm = +86 Da adduct was more rapid for **13a** and **13b** than for **10a** and **10b**

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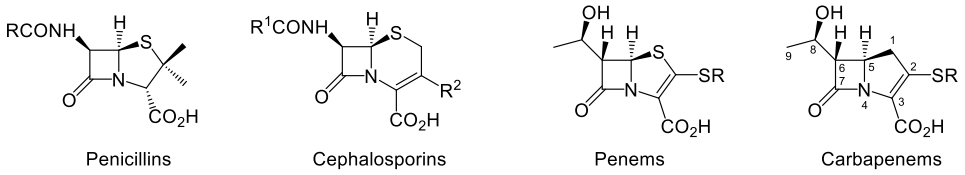


Figure 3. Structural classes of bicyclic β-lactam antibiotics

(Figure 2). Commercial carbapenems are observed to lose the C6-hydroxyethyl group, fragmenting to a Δm = +Antibiotic −44 Da adduct.[74] This typical carbapenem-Ldt adduct was only transiently observed in the case of **10a** as shown in Figure 2 and Scheme 3. With the exception of **13b**, the antibiotics rapidly formed covalent adducts with Ldt_{Mt2}, as witnessed by the absence of *apo*-enzyme at t = 1 h in the case of **10a**, **10b**, and **13a**. In the case of carbapenem **13b**, however, substantial *apo*-enzyme was observed at t = 1 h, and this species was completely replaced by the Δm = +86 Da adduct after 5 h of incubation. The mechanism for formation of the +86 Da adduct following acylation is proposed in Scheme 4.

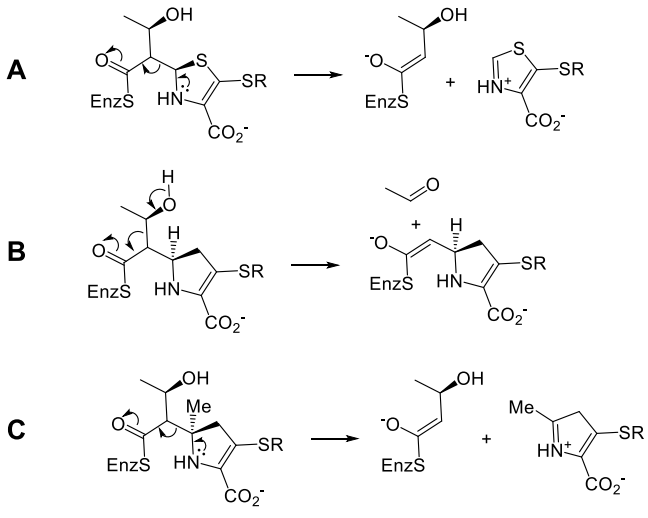
DISCUSSION

The in vitro activity data of the atypical carbapenems in Table 1 indicates that the C5α-methyl analogues **10a** and **13a** are superior to the C5α-ethyl analogues **10b** and **13b**, and that the meropenem-like C2 pyrrolidine side chain analogues **10a** and **10b** are superior to the less structurally complex C2 thioethyl analogues **13a** and **13b**, with respect to inhibition of growth of both *Mtb* and *Mab*. The effect of structure on activity was more pronounced in the case of *Mab* than with *Mtb*. As shown by the data in Table 1, the C5α-methylcarbapenem **10a** has standalone activity superior to that of either meropenem alone or meropenem combined with β-lactamase inhibitor against either *Mtb* or *Mab*. Of interest is the lack of synergy with clavulanate of all the novel atypical carbapenems, as indicated in Table 1, indicating improved stability of these C5α-substituted carbapenem analogues toward BlaC-mediated hydrolysis. Meropenem, by contrast, is reported to be an extremely slow BlaC substrate as also confirmed by our data, Table 1, demonstrating meropenem/clavulanate synergy.[75] Table 1 data suggests that incorporation of the C5α-alkyl group further slows the BlaC-mediated hydrolysis of these new atypical carbapenems. Structure can dramatically effect the rate β-lactamase mediated hydrolysis, as indicated by the generation of BlaC-specific fluorogenic cephalosporins for detection of *Mtb*.[76]

HRMS analysis of the acylation of Ldt_{Mt2} by the atypical carbapenems indicates that all four of the novel atypical carbapenems acylate the transpeptidase and subsequently degrade by nonhydrolytic fragmentation of the acyl-enzyme to the smaller hydroxybutyryl cysteine derivative (Δm = +86 Da), rather than the larger Δm = +carbapenem −44 Da analogue uniformly observed for commercial carbapenem antibiotics, including meropenem.[77] This hydroxybutyryl acyl-enzyme was also observed in the case of the interaction of Ldt_{Mt2} with the penem, faropenem, which has an identical C6α-hydroxyethyl side chain to meropenem (Figure 3), but has not previously been observed in the carbapenem series.[46,78] A previous crystallographic study indicated that, in the case of the faropenem-Ldt_{Mt2} complex, the Δm = +86 Da intermediate exists as a stable thioester with the active site Cys354 covalently linked to the carbonyl of the β-lactam.[78] In that study, the β-hairpin lid of the protein (residues 300−323) adopted a closed conformation and the carbon of the acylating carbonyl group was buried and inaccessible by solvent. Additionally, it has been reported that this hydroxybutyryl-Ldt_{Mt2} adduct is unreactive toward externally added amine receptors, including *meso*-DAP, further characterizing it as a highly stable acyl-enzyme, unable to liberate itself from the covalently attached moiety.[78]

Why does the C5α structural modification of these atypical carbapenems alter the fate of the Ldt acyl-enzyme, relative to that of commercial carbapenems? Why do the Ldt acyl-enzymes undergo these retroaldol-type fragmentations? A recently published study sought to understand the mechanistic reasons for the varying nonhydrolytic fragmentation pathways of the varying classes of β-lactam antibiotics (Figure 3) on the Ldt_{Mt2} cysteine transpeptidase through comparisons of analogous interactions of these antibiotics with the serine carbapenemase mutants OXA-48 (S70C) and KPC-2 (S70C).[74] That study observed that the retroaldol-type cleavages of the acyl-enzymes across C5−C6 (penicillin and penem) and C6−C7 (cephalosporin) occurred in the case of Ldt_{Mt2}, and in the case of the two S70C carbapenemase mutants, but not in the corresponding wild type serine carbapenemases. These data led to the hypothesis that the differing outcome of the Ldt or carbapenemase interactions with the antibiotics was due to the differing pK_a values of the corresponding, hypothetical product oxoester enolates (ester pK_a ≈ 25) and observed product thioester enolates (thioester pK_a ≈ 20). The acyl-enzyme fragments to form the less basic thioester enolate (Scheme 5A), whereas the oxoester does not (in the case of the wild type carbapenemases) fragment to the

Scheme 5. Observed Ldt_{Mt2} Acyl-Enzyme Nonhydrolytic Fragmentation Pattern for (A) Penems, (B) Commercial Carbapenems, and (C) Atypical C5α-Substituted Carbapenems



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more basic oxoester enolate. The fact that the carbapenem thioester acyl-enzymes degrade via an alternate C8−C6 retroaldol fragmentation route (Scheme 5B) leading to a similar (less basic) thioester enolate suggests that the S1 position sulfur atom (and sulfur electron pairs) in the penicillins, cephalosporins, and penems plays a crucial role in directing the C5−C6 fragmentation of the thioester Ldt acyl-enzyme, which is absent in the case of the carbapenems. The current observation that the fragmentation of the atypical carbapenem Ldt acyl-enzyme can now be redirected to follow the C5−C6 bond cleavage suggests that the transition state has carbocation character as shown in Scheme 5C. This carbocation character is stabilized by the tertiary nature of the C5 carbon in the atypical analogues and is also stabilized by the sulfur electrons in the case of penems, penicillins, and cephalosporins (Scheme 5A). This carbocation stabilization is missing in the case of the commercial carbapenem antibiotics, which have a hydrogen atom at C5 and also lack a second adjacent heteroatom, thus directing the fragmentation to occur at the C8−C6 bond of the heteroatom-containing side chain (Scheme 5B).

What factors are responsible for the improved antimycobacterial activity of these structurally modified antibiotics? Efficacy of a β-lactam antibiotic is a complex function of transpeptidase target recognition and inhibition, together with ability to avoid β-lactamase-catalyzed hydrolysis, penetrate the cell envelope, and avoid efflux. Our data indicate that these modified carbapenems target Ldt_{Mt2} and avoid the BlaC *Mtb* carbapenemase. It is likely that these novel carbapenems are also interacting with mycobacterial PBPs. *Mtb* and *Mab* produce ten transpeptidases, including representatives of five of the six mycobacterial Ldt classes (Ldt_{Mt1-5}), and at least five PBPs (PonA1, PonA2, PBPA, PBPB, and PBP-lipo), as well as a number of D,D-carboxypeptidases. The potentially nonredundant functions of these transpeptidases are under current investigation. A recently published study compared the efficacy of carbapenems with that of representative penicillins, cephalosporins, and β-lactamase inhibitors at binding to five *Mab* PBPs.[79] The authors concluded that the carbapenems imipenem and meropenem inactivated the widest range of PBPs at low concentration, indicating it is likely that the most efficacious antimycobacterial β-lactam antibiotic or combination of antibiotics will inactivate a series of mycobacterial transpeptidases. For example, it has been determined that loss of the PBP PonA2, either by mutation or treatment with cephalosporins, increases sensitivity to meropenem, potentially involving increasing reliance on Ldt_{Mt2}, a known meropenem target.[80] One theory is that β-lactam mediated cell death occurs through an uncoupling of the transpeptidase and transglycosylase activity of the high molecular weight PBPs.

With the previously reported improvement in Ldt acylation efficacy of the minimal C2 thioethyl side chain, it is challenging to rationalize the higher MIC values of compounds **13a** and **13b**, relative to the meropenem-like C2 pyrollidine analogues **10a** and **10b**.[70] In the case of Gram-negative pathogens, the basic C2 side chains of the commercial carbapenems are well-documented to enable porin-mediated transfer across the outer membrane, due to their overall charge similarity to the basic amino acid substrates of the porins, lysine and arginine.[81,82] The mycobacterial outer membrane can serve as a permeability barrier, containing substrate specific water-filled porin channels to facilitate antibiotic uptake. MspA, an outer membrane porin of the rapidly growing *Mycobacterium smegmatis* has been observed to be selective for positively charged compounds and was found to facilitate the transport of zwitterionic β-lactams, like cephaloridine, as opposed to negatively charged cephalosporins, like cephalothin.[83−85] While it is not yet clear that similar porins exist in slow-growing mycobacteria, such as *Mtb*, it has been observed that expression of the MspA porin in *Mtb* leads to decreased MIC values for zwitterionic β-lactam antibiotics as well as other antibiotics.[86] This has relevance to the present series of compounds which contain both zwitterionic (**10a** and **10b**) as well as negatively charged (**13a** and **13b**) carbapenems. The decrease in *in vitro* potency observed in Table 1 of the **13** series, relative to the **10** series, is potentially due to a lessening of efficiency in penetrating the mycobacterial cell envelope, particularly in the case of the rapidly growing mycobacterium *Mab*, which may possess zwitterionic importing porins, to reach the transpeptidase targets on the surface of the cytoplasmic membrane.

Taken together, the data presented here highlight the potential of atypical carbapenems as drugs to treat mycobacterial infections. Atypical carbapenem **10a** exhibited a surprising ability to maintain excellent *in vitro* antimycobacterial activity, even against strains which show high levels of resistance to meropenem. Further studies in determining the Ldt binding and acylation kinetics, stability to carbapenemases, and optimization of the atypical scaffold of these compounds are warranted and being explored.

METHODS

Chemical Compounds. Carbapenems were stereospecifically synthesized as described in the Supporting Information.

Amikacin (AMK), rifampicin (RIF), and isoniazid (INH), meropenem, and clavulanic acid were purchased from Sigma-Aldrich. Avibactam was purchased from Med Chem Express.

Solution Preparations. Stock solutions of amikacin (AMK), rifampicin (RIF), and isoniazid (INH) were prepared according to the manufacturer's instructions. Carbapenem compounds stocks were prepared in water at 10 mM concentration and then diluted to appropriate assay concentrations. Stocks of clavulanic acid and avibactam were made fresh every time and used at final concentrations of 5 μg/mL. All the stocks were stored at −80 °C.

Bacterial Strains and Culture Conditions. Bacterial strains *Mtb* CDC1551, 5 *Mtb* clinical isolates, *Mab* 390S, *Mab* subsp. *abscessus* clinical isolates (obtained from National Jewish Health, Colorado), were used in this study (Table S1). *Mtb* and *Mab* strains were cultured in Middlebrook 7H9 supplemented with 0.05% Tween80 and 10% oleic acid/albumin/dextrose/catalase (OADC) and incubated at 37 °C and 5% CO_2.[87−89] Kanamycin 50 μg/mL (KAN), cycloheximide 100 μg/mL, and amikacin 32 μg/mL (AMK) were added when appropriate. Kanamycin was included during routine culturing of luciferase reporter strains to ensure maintenance of this construct. Cycloheximide was included in all solid agar media to prevent fungal contamination.

Minimum Inhibitory Concentration (MIC) Assay. MICs of synthesized carbapenem compounds were determined using bioluminescent strains of *Mab* and *Mtb*[90,91] in solid white 384-well microtiter plates (Corning). A 16-point 2-fold serial dilution series of the compounds (starting at 40 μg/mL) was used to conduct MIC determination as described previously.[90,92] MIC is defined as the lowest drug concentration at which more than 99% decrease in *lux* signal was observed as compared to the untreated control. MIC values reported are

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the average of 3 independent replicates with duplicate dose response curves on each plate (total of 6 replicates per sample).

Cytotoxicity Assay. Cytotoxicity was assessed using J774A.1 (murine macrophage-like) and HepG2 (human liver carcinoma) cell lines using 2-fold dilution of the compounds (starting at 40 μg/mL) as described previously.[90,92]

Time-Kill Kinetic Assay. We conducted a time-kill kinetic assay with the top C5α-substituted carbapenem (**10a**) and commercially available compound, meropenem. The assay is described in detail previously.[90,92] Briefly, *Mtb*, CDC1551 cultures of OD_{600} of 0.01 were added to a solid-white 384-well plate containing compounds at final concentrations of 0, 1×, 4×, and 8× MIC in a total volume of 30 μL. The plate was incubated for 6 days for *Mtb* and an aliquot was taken at 0, 24 (Day 1), 72 (Day 3), and 144 h (Day 6) postinoculation, serially diluted and plated onto 7H10 quad-plates supplemented with OADC. Colonies were counted after 3−4 weeks of incubation at 37 °C and CFU/mL was calculated. Similarly, a time-kill assay was performed with *Mab* 390S culture where an aliquot was taken out at 0, 24, 48, and 72 h for plating. Colonies were counted after 5 days of incubation following plating. Our limit of detection by CFU/mL is 10^2 assuming at least 1 colony with the lowest dilution plated (10^1). The data in Figure 1 is an average from 4 independent experiments each with 3 technical replicates.

In Vitro Activity against *Mtb* and *Mab* Clinical Strains. Activity of compounds were evaluated against 5 clinical strains of *Mtb* from 5 different phylogenetic lineages.[88] MIC determination was carried out in a 16 step 2-fold dilution series (starting at 40 μg/mL) as described previously.[90,92] The screening plate was incubated for 5 days at 37 °C, thereafter resazurin dye was added at 1/10th of the total volume and incubated for 24 h. Fluorescence was measured at Ex/Em 530/590 with auto gain settings.

MIC of the compounds was also determined against *Mab* clinical strains using five bioluminescent clinical strains of *Mab* subsp. *abscessus* (Table S1) similarly to the MIC assay mentioned earlier and as described.[90,92]

Ldt_{Mt2}-Adduct HRMS Analyses. A truncated version of Ldt_{Mt2} lacking amino acids 1−26 was purified as reported previously.[77] Carbapenems were prepared as 20 mM or 40 mM stock solutions in ddH_2O. Ldt_{Mt2} (10 μM) was incubated in the presence or absence of 200 μM **10a**, **10b**, **13a**, or **13b** in 50 mM HEPES, pH 7.5, for 1 or 5 h at 37 °C. Reactions were quenched with formic acid (0.1% final v/v). Samples were desalted following passage through a polyacrylamide spin column (Pierce). Desalted samples were analyzed by high-resolution mass spectrometry (HRMS) as previously reported.[77] Briefly, UPLC-high resolution MS samples were analyzed on a Waters Acquity H-Class ultraperformance liquid chromatography (LC) system equipped with a multiwavelength UV-violet diode array detector (200−500 nm) in conjunction with a Waters Acquity BEH-300 ultraperformance LC column packed with a C_4 stationary phase (2.1 × 50 mm; 1.7 μm) in tandem with high resolution MS analysis by a Waters Xevo-G2 quadrupole-TOF electrospray ionization mass spectrometer. Enzyme samples were resolved at 60 °C in order to improve peak resolution. The samples were resolved with a flow rate of 0.3 mL/min and the following mobile phase: 0−1 min 90% water, 10% ACN, 0.1% formic acid (FA); 1−7.5 min gradient up to 20% water, 80% ACN, 0.1% FA; 7.5−8.4 min 20% water, 80% ACN, 0.1% FA; 8.4−8.5 min gradient up to 90% water, 10% ACN, 0.1% FA; 8.5−10 min 90% water + 10% ACN 0.1% FA. Mass Spectra were deconvoluted using the maxEnt1 algorithm running as part of the Waters BioPharmaLynx data processing software package. Mass/intensity data were extracted from the processed data for further processing as necessary. Data were normalized and MS images were created using GraphPad Prism 7.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.1c00185.

Description of synthetic procedures; Table of the strains (PDF)

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JDB, KHR, and LABB wrote the manuscript. JDB conceived the project and designed and synthesized the new

carbapenems. LABB prepared samples for HRMS and interpreted the data. PM ran HRMS samples. RG and BSS conducted all antimicrobial assays.

Notes

The authors declare no competing financial interest.

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