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Antibacterial and Solubility Optimization of Thiomuracin A

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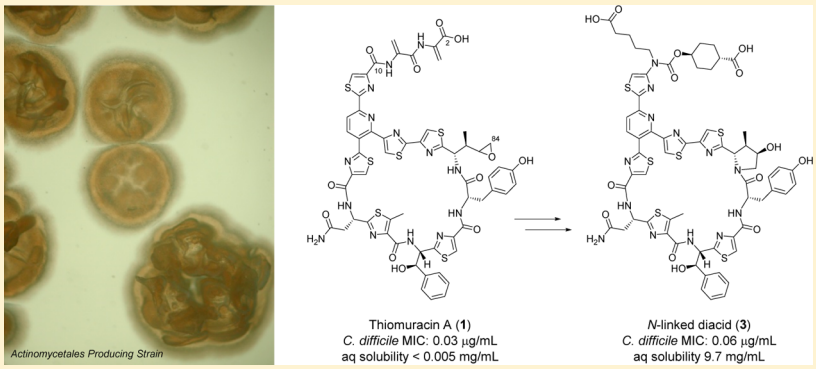
Supporting Information



ABSTRACT: Synthetic studies of the antimicrobial secondary metabolite thiomuracin A (**1**) provided access to analogues in the Northern region (C2−C10). Selective hydrolysis of the C10 amide of lead compound **2** and subsequent derivatization led to novel carbon- and nitrogen-linked analogues (e.g., **3**) which improved antibacterial potency across a panel of Gram-positive organisms. In addition, congeners with improved physicochemical properties were identified which proved efficacious in murine sepsis and hamster *C. difficile* models of disease. Optimal efficacy in the hamster model of *C. difficile* was achieved with compounds that possessed both potent antibacterial activity and high aqueous solubility.

INTRODUCTION

Novel classes of antibiotics with new modes of action and no cross-resistance with clinically used antibiotics remains a pressing need in infectious disease care because of the rising incidence of resistance against marketed antibiotics. In 2009 we reported the isolation, structural elucidation, antimicrobial activity, and biosynthetic gene cluster of a new class of thiazoyl actinomycetes metabolites, the thiomuracins (**1**, Figure 1).[1] These architecturally complex, secondary metabolites were fermented from the rare soil actinomycetes species *Nonomurae* and possess highly modified, sulfur-containing macrocyclic peptide structures. The thiomuracins are structurally related to the GE2270 A (**4**) class of antibiotics[2] (Figure 1), another actinomycetes fermentation product upon which we have based extensive drug discovery efforts.[3] Both the thiomuracins and **4** derive their antibiotic activity via binding to elongation factor Tu, thereby disrupting prokaryotic protein synthesis. Medicinal chemistry optimization of **4** resulted in the identification of LFF571 (**5**),[4] an investigational agent for *C. difficile* infection in humans.[5] General applications of these SAR findings to other classes of thiopeptides, such as the baringolins, have recently been reported by others.[6] In addition, a de novo biosynthesis of the thiomuracin core has also been reported,[7] as well as manipulation of thiopeptide biosynthetic gene clusters toward structural variation.[8] Indeed both semisynthetic and biosynthetic efforts have contributed to the evolving SAR of the thiopeptide class.[9]

Previously we reported initial synthetic studies on thiomuracin A, which chemically stabilized the C84 epoxide region via pyrrolidine cyclization, removed the C2−C10 side chain, and improved the isolation and material supply for continued medicinal chemistry optimization.[10] These studies culminated in the identification of lead compound pyrrolidine **2**, which retained potent antibacterial activity against five target Gram+ organisms with MIC values <1 μg/mL and also displayed potent in vivo activity in the mouse sepsis model of infection. Due to the structural

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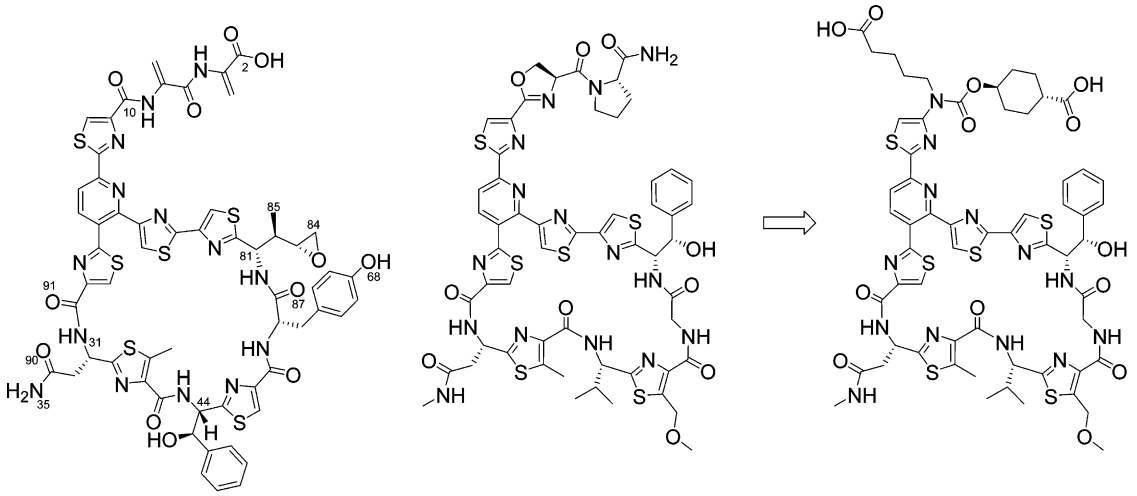

Figure 1. Thiomuracin A (**1**), **4**, and **5**.

simplification, chemical stability, optimized material supply, and retention of potency, amide **2** was selected for continued medicinal chemistry optimization. Considering our recent SAR findings on *amino*thiazolyl analogues of **4**, the potential for Northern region analogues of thiomuracin A was of considerable interest.[3,4]

RESULTS

Synthesis. During the course of identifying lead amide **2**, an acid-mediated hydrolysis of the C10 didehydroalanine side chain and acetate protecting group strategy emerged (Scheme 1, **1** → **2**). Three hydroxyls of chlorohydrin **6** were protected as acetate esters using standard conditions *at room temperature* (Ac_2O, DMAP). Subsequent DBU cyclization formed the N81 pyrrolidine, and the esters were then removed to provide **2**. Acetylation *at elevated temperature* (60 °C), however, resulted in selective imide formation at C10 (**7**). The C10 primary amide is sterically more accessible than the macrocyclic secondary amides and perhaps more acidic than the C34 primary amide due to the northern thiazolyl ring (via pseudo-benzylic stabilization). Attempts at further derivatization of **7** at the C10 imide were initially unfruitful due to acid and base sensitivity of the imide, chlorohydrin, hydroxyphenylalanine, and acetate protecting groups. For example, NaOH treatment of **7** led to **2**, but retroaldol decomposition of the C44 hydroxyphenylalanine was also observed (not shown). In addition, HCl treatment of **7** led to unproductive imide hydrolysis, providing the C10 amide.

After significant experimentation, selective allylation of the imide was possible (NaH, DMF, allyl bromide), although the yields were variable. This provided the tertiary imide with concomitant N81 to C84 pyrrolidine cyclization (**8**). The tertiary imide was then hydrolyzed to the C10 carboxylic acid (**9**). Although we had achieved an initial synthesis of the carboxylic acid **9**, a key intermediate en route to Northern carbonyl-based analogues, the yields (<10%) were untenable. We hypothesized that selective C10, acid-mediated hydrolysis would be possible because the C10 amide in **2** was prone to selective imide formation and was the most sterically accessible (vide supra)[10] and pseudo-benzylic. We had previously demonstrated that thiomuracin A was quite stable to strong acid conditions (HCl, MeOH, 50 °C), aside from the C2−C10 hydrolysis and C84 epoxide opening.[10] Thus, treatment of pyrrolidine **2** with HCl, H_2O, and THF at elevated temperatures (RT → 100 °C) revealed that selective hydrolysis indeed occurred at 80−100 °C to provide **9** directly and in acceptable yield (80%). Incidentally, macrocyclic hydrolysis of the amides occurred at 110 °C and thus could be avoided.

The carboxylic acid (**9**, Scheme 2) was then used as a strategic intermediate for the synthesis of Northern region analogues via amide couplings (e.g., **9** → **10**−**12**) and via a Curtius rearrangement sequence (e.g., **9** → **16**, **3**). Accordingly, a variety of structural motifs were examined, partially guided by our previous studies of aminothiazolyl-based analogues of **4**.[3,4] For C-linked analogues, acyclic acids (e.g., **10**), cyclohexylcarboxylic acids (e.g., **11**), and diacid structures were prepared (e.g., **12**). Other polar functional groups (e.g., amines, alcohols) were not considered, as previous studies demonstrated these functional groups had little impact on the aqueous solubility of this class.[3,4] For N-linked analogues, carboxylate **9** was activated via ethyl chloroformate, and azide displacement furnished intermediate **13**. Interestingly, carbonate protection of the tyrosine residue occurred and proved chemically stable to isolation and further chemistry (vide supra). Curtius rearrangement using methyl *trans*-4-hydroxycyclohexane-1-carboxylate afforded urethane **14**. Exhaustive hydrolysis of the ester and carbonate moieties (LiOH) afforded acid **16**. To selectively alkylate the Northern urethane, the remaining hydroxyl functional groups of **14** were protected as acetates (e.g., **15**). Alkylation of the urethane and global deprotection then furnished diacid **3**, which structurally resembled **5**.

Antibacterial Activity. Thiomuracin A (**1**), lead amide (**2**), and novel analogues **9**−**12**, **16**, and **3** were then evaluated in MIC (minimum inhibitory concentration) assays for G+ bacterial growth inhibition (Table 1). Five species comprised our antibacterial screen and included *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and the anaerobic intestinal pathogen *Clostridium difficile*. Thiomuracin A (**1**) and amide **2** were exquisitely potent in all five organisms tested with MICs ranging from 0.03 to 1 μg/mL. Carboxylic acid (**9**) retained moderate antibacterial activity in all of the target organisms but was less potent as compared to thiomuracin A (**1**). Unfortunately, the aqueous solubility of **9** was poor (0.001 mg/mL

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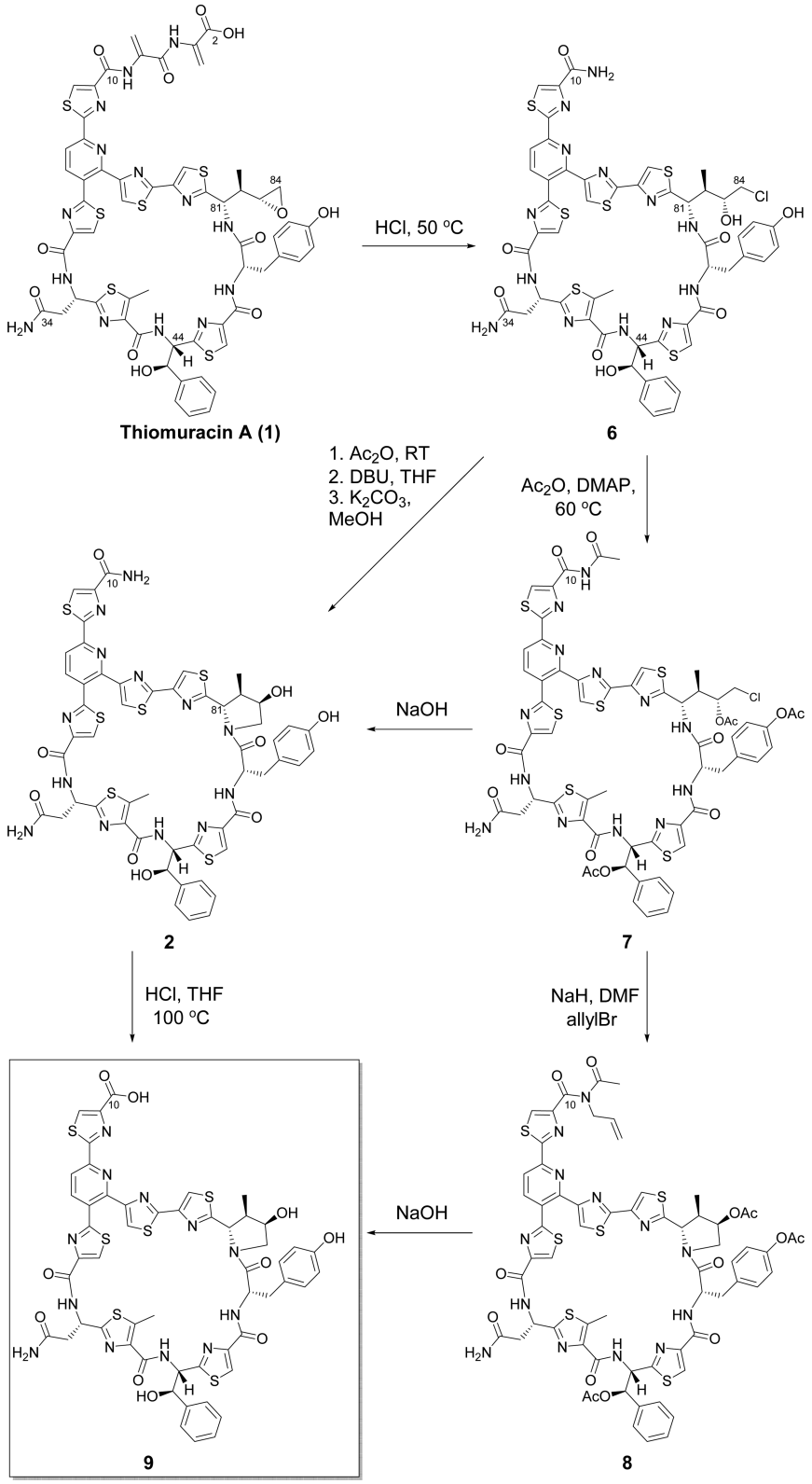
Scheme 1. Synthesis of Intermediate 9



in pH 7.4 buffer). Straight-chained and cycloalkanes terminating in carboxylic acids proved exquisitely potent and soluble in the aminothiazole series of **4**.[3,4] In the current C-linked studies of the thiomuracin scaffold, these functional groups also retained potent biological activity against the target organisms (e.g., **10**: MICs 0.06−0.5 μg/mL). Likewise, a cyclohexane-based carboxylic acid functional group was evaluated (**11**) and proved equipotent as the natural product thiomuracin A (**1**) and the acyclic analogue **10**. Importantly, the aqueous solubility was also improved (**11**: 0.75 mg/mL in pH 7.4 buffer). C-linked dicarboxylic acids were also evaluated (e.g., **12**) but significantly lowered antibacterial activity across organisms (MICs = 0.125−1 μg/mL).

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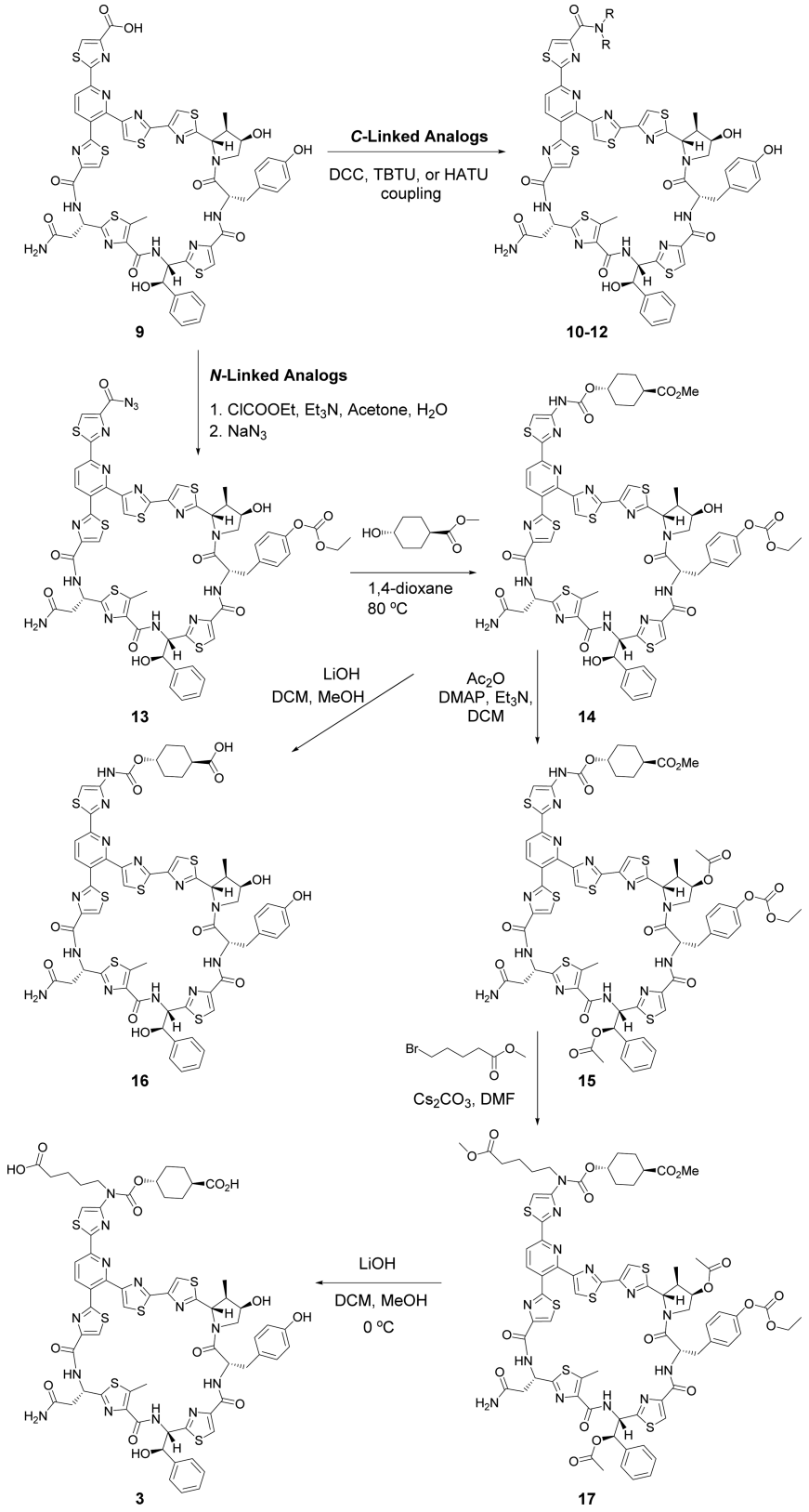
Scheme 2. Synthesis of C-Linked and N-Linked Northern Analogues



In the N-linked series of analogues, two congeners (**16**, **3**) were evaluated and were more potent than the C-linked diacid (**12**), and equipotent to the C-linked monoacids (**10**, **11**) across the panel of organisms. Overall, *Clostridium difficile* was very sensitive to all the analogues. N-linked dicarboxylic acid **3** was the only analogue which maintained potent antibacterial activity (MICs 0.03−0.25 μg/mL) while significantly improving aqueous solubility (9.7 mg/mL in pH 7.4 buffer).

Crystallography. Several compounds from these lead optimization studies were selected for cocrystallographic studies with

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Table 1. Antibacterial and Solubility Data for 1 and Analogues[a]

Compound	MIC assays: μg/mL *E. faecalis*	*E. faecium*	*S. aureus*	*S. pyogenes*	*C. difficile*	*aq solubility (mg/mL)*
1	0.5	0.5	0.5	0.5	0.03	BLD
2	0.25	0.5	0.25	1	<0.008	BLD
9	0.5	0.5	2	0.5	0.125	0.001
10	0.125	0.125	0.5	0.125	0.06	0.73
11	0.125	0.125	0.25	0.25	0.03	0.75
12	1	1	2	0.5	0.125	3.4
16	0.125	0.125	0.25	0.125	0.03	0.006
3	0.25	0.125	0.25	0.125	0.06	9.7

[a]BLD: below limit of detection.

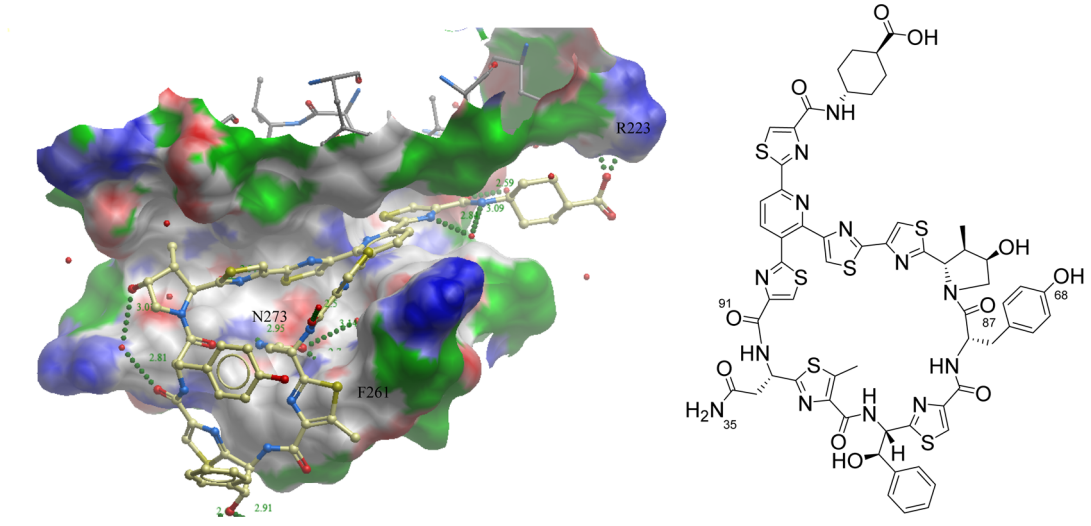

Figure 2. Cocrystal structure of *E. coli* EF-Tu and **11**.

EF-Tu derived from *E. coli*, including cyclohexanecarboxylic acid **11**.[11] The cocrystal structure of **11** (Figure 2, 2.0 Å, PDB code 5JBQ) confirmed the location of binding within domain 2 of EF-Tu and also displayed the same macrocyclic conformation as previously reported for **4** and amide **2** in their respective bound conformations.[10,12] Additionally, similar interactions were observed between **11** and EF-Tu as compared to **4**, including H-bonding with EF-Tu at several locations (Asn273, Phe261). Intramolecular H-bonding across the macrocycle from the primary amide N35 (donor) to the tyrosine carbonyl O87 (acceptor) was observed (3.0 Å distance). Likewise, amide carbonyl O91 (acceptor) interacts with tyrosine OH68 (donor) in an intramolecular H-bond (3.4 Å distance). Presumably, both through-space interactions allow for conformational stabilization of the macrocyle. In the Northern region, the amide linker orients the cyclohexane carboxylic acid side chain toward solvent and Arg223. We speculate that both the cyclohexane functional motif and the appropriate linker distance orient the carboxylate favorably for optimal interaction with Arg223.

Pharmacokinetics and Efficacy Studies. The cyclohexane carboxylic acid **11** was investigated in rat pharmacokinetic and efficacy experiments. Accordingly, acid **11** was dosed intravenously to male Sprague–Dawley rats (1 mg/kg).[13] Acceptable exposure (AUC = 13187 nM·h), low clearance (0.98 mL/min/kg), low volume of distribution (V_{ss} = 0.032 L/kg), and a moderate half-life ($T_{1/2}$ = 1.8 h) were observed. Furthermore, in the mouse sepsis model of infection,[14] acid **11** displayed dose-dependent efficacy (*S. aureus* ED_{50} = 3.42 mg/kg, 95% confidence interval = 2.48–4.90 mg/kg; *E. faecalis* ED_{50} = 0.71 mg/kg, 95% confidence interval = 0.7–2.0 mg/kg). This compares favorably with the

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previously described efficacy of amide **2** (*S. aureus* ED_{50} = 8.0 mg/kg, 95% confidence interval = 4.8−53.1 mg/kg; *E. faecalis* ED_{50} = 1.5 mg/kg, 95% confidence interval = 0.31−5.0 mg/kg), presumably due to the improvement in MIC against these organisms.[8] These experiments were controlled with both daptomycin (*S. aureus* ED_{50} = 0.21 mg/kg, 95% confidence interval = 0.07−0.36 mg/kg; *E. faecalis* ED_{50} = 2.60 mg/kg, confidence interval not calculatable) and vancomycin (*S. aureus* ED_{50} = 1.1 mg/kg, 95% confidence interval = 0.77−1.47 mg/kg; *E. faecalis* ED_{50} = 7.74 mg/kg, 95% confidence interval = 6.7−20 mg/kg). Thus, acid **11** was commensurate with vancomycin in the *S. aureus* model of infection and less effective than daptomycin. But in the *E. faecalis* sepsis model, acid **11** proved superior to both vancomycin and daptomycin.

Several analogues were also evaluated in the *C. difficile* Golden Syrian hamster model of infection (Figures 3 and 4).[15] After oral

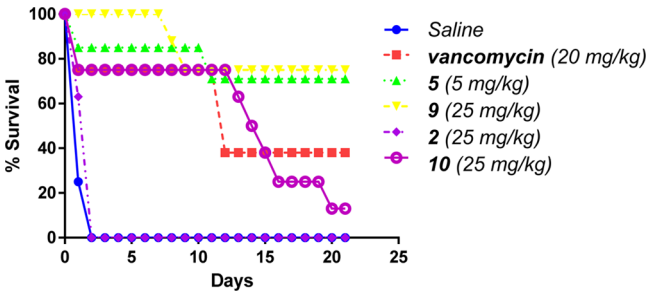

Figure 3. Evaluation of compounds **2**, **9**, and **10** in the *C. difficile* hamster model. **5** and vancomycin data are the compiled average of seven independent experiments;[4b] data for compounds **2**, **9**, and **10** are from discrete experiments.

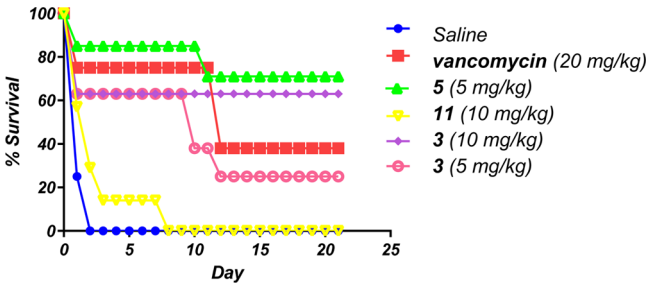

Figure 4. Evaluation of compounds **11** and **3** in the *C. difficile* hamster model. **5** and vancomycin data are the compiled average of seven independent experiments;[4b] data for compounds **3** and **11** are from discrete experiments.

gavage of *C. difficile* inoculum, hamsters were treated with **2**, **9**, or **10** (25 mg/kg), or controls which included saline, vancomycin (20 mg/kg), or **5** (5 mg/kg) for 5 days. The animals were then observed for an additional 16 days. While no animals survived after saline treatment, 38% survival was observed with vancomycin (20 mg/kg) and 71% survived with **5** (5 mg/kg). Considering the first three analogues, animal survival was the highest with acid **9** (75%) versus alkyl acid **10** (13%) and amide **2** (0%). This result was surprising considering the antibacterial activity of **2** (MIC, *C. difficile* = 0.008 μg/mL). We speculate that low solubility of **2** precluded growth inhibition in the gut. Particularly noteworthy was the high dose of **9** (25 mg/kg) which provided similar survival as **5** at a lower dose (5 mg/kg).

Two additional analogues were evaluated in the *C. difficile* hamster model of infection (Figure 4): C-linked cyclohexanecarboxylic acid **11** and N-linked dicarboxylic acid **3**. While there was 0% survival with C-linked monoacid **11**, N-linked diacarboxylic acid **3** provided improved results. At 5 mg/kg, 25% survival was observed, while at 10 mg/kg, compound **3** appeared superior (63% survival) to vancomycin (38% survival) and approximately equipotent to **5** (75% survival, 5 mg/kg), albeit at 2-fold higher dose.

DISCUSSION AND CONCLUSIONS

A selective, acid-mediated hydrolysis of the C10 amide of the thiomuracin scaffold has been achieved which enabled the synthesis and biological evaluation of Northern region analogues. Cyclic and acyclic C- and N-linked carboxylic acid analogues were evaluated for antibacterial activity and aqueous solubility. Congeners with improved physicochemical properties were identified and evaluated in murine sepsis and hamster *C. difficile* models of disease. C-linked cyclohexanecarboxylic acid **11** was evaluated in pharmacokinetic experiments and proved efficacious in the mouse sepsis model of infection. Observed EC_{50}s were either superior or comparable to the standards of care (e.g., vancomycin, daptomycin) and dependent on the model organism. Multiple analogues were evaluated in the Golden Syrian hamster model of *C. difficile*, and optimal efficacy was achieved with compounds that possessed both potent antibacterial activity and high aqueous solubility. These studies culminated in the identification of N-linked dicarboxylic acid **3**, which proved significantly more soluble than thiomuracin A and more potent than vancomycin in the hamster model of *C. difficile* infection.

The thiomuracins and associated analogues contained herein represent a novel class of antibiotics with a new mode of action and no cross-resistance with clinically used antibiotics,[1] a pressing need in infectious disease care because of rising incidence of resistance against marketed antibiotics. These interesting and architecturally complex structures have proven amenable to semisynthetic derivatization, gene cluster manipulation, and de novo biosynthesis. Optimization of physicochemical properties and antibacterial activity and translation of SAR from one series to another within the thiopeptide class have been successfully demonstrated. Novel analogues with improved properties and antibacterial activity have enabled efficacy studies in multiple animal models of infection, and a leading thiopeptide **5** has demonstrated clinical utility. Furthermore, this evolving knowledge and now considerable body of literature around the thiopeptide class perhaps serves as a roadmap for other novel classes of antibiotics yet to be discovered and characterized.

EXPERIMENTAL SECTION

Compound Synthesis and Characterization. Synthetic procedures and compound characterization data are found in the Supporting Information. Compound purity was assessed by HPLC to confirm >95% purity.

MIC Assays. MIC assays were conducted according to broth microdilution methods described by the Clinical and Laboratory Standards Institute with the exception that broth microdilution methods were utilized for all species tested: CLSI Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Eighth Edition. CLSI document M07-A8; Clinical and Laboratory Standards Institute: Wayne, PA, 2009. Methods for antimicrobial susceptibility testing of anaerobic bacteria: Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria; Approved Standard—Eighth Edition (M11-A8); Clinical and Laboratory Standards Institute: Wayne, PA, 2012. Bacterial strains included *E. faecalis* (ATCC 29212), *E. faecium* (Prof. Chopra, Univ. Leeds UK, strain 7130724), *S. aureus* (MRSA from Prof. Willinger, isolated from a

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pharyngeal smear, AKH Vienna A4.018), *S. pyogenes* (ATCC BAA-595), and *C. difficile* (ATCC 43255).

Rat Pharmacokinetic Studies. PK studies (N = 2, iv) were performed with male Sprague−Dawley rats, weighing 220−270 g and approximately 6−10 weeks old. They were obtained from Harlan Research Laboratories (South Easton, MA), each bearing dual implanted jugular vein cannula. The rats were fasted overnight before use and for 8 h after dosing. Blood samples were taken into K_2-EDTA-coated tubes and then centrifuged to yield plasma samples for analysis by LC-MSMS. Bioanalysis of rat plasma from in-life experiments were performed by LC-MSMS using a system with the following configuration: Agilent liquid chromatograph (Santa Clara, CA), LEAP Technologies CTC-PAL autosampler (Carrboro, NC), and Applied Biosystems API 4000 mass spectrometer (Framingham, MA). LC was performed in gradient mode with reversed phase C18 columns (2.1 mm × 30−50 mm × 3.5−5 μm particle size). Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. Gradients were run from 5% B to 95% B for ∼3.5 min. Plasma samples were protein-precipitated with acetonitrile containing glyburide as the internal standard (Sigma-Aldrich, St. Louis, MO).

Mouse Systemic Infection Model. The studies were approved by the Institutional Animal Care and Use Committee of the Novartis Institutes for BioMedical Research Inc., Cambridge MA. Animals were maintained under controlled conditions with free access to food and water. Female CD1 mice (21−25 g, Charles River Laboratories, Wilmington, MA) were used for infections with *S. aureus* (ATCC 49951) and *E. faecalis* (NB04025, a clinical isolate from the Novartis bacterial collection that is resistant to erythromycin, tetracycline, and gentamicin, courtesy of Dr. B. Willinger, Vienna General Hospital, Austria). MICs of amide **2** were 1 μg/mL and 0.125 μg/mL for animal strains *S. aureus* (ATCC 49951) and *E. faecalis* (NB04025), respectively. Lethal infections were induced by intraperitoneal injection of a freshly prepared bacterial suspension of 1 × 10^8 CFU/mouse in either 50% sterile rat fecal extract (*E. faecalis*) or 0.9% NaCl (*S. aureus*). The injected bacterial dose corresponded to 10−100 times the minimal lethal dose as determined from previous lethal dose titration studies. Against *E. faecalis* infections the mice were treated once immediately following the bacterial inoculation, while against *S. aureus* infections mice were treated 1 and 5 h after inoculation. Compound **2** was formulated in 5% DMA, 30% PEG400, 10% cremophor EL, 5% 0.1 N NaOH, and 50% pH 7.4 buffer. Daptomycin was formulated in saline. Compounds were administered iv via tail vein bolus injection at several dose levels to groups of six mice each. Following bacterial inoculation, the mice were observed for 5 days. In addition, body temperature was monitored by electronic microtransponders (Bio Medic Data Systems, Inc. Seaford, Delaware) that were implanted in mice subcutaneously prior to infection. Mice that became moribund as indicated by a combined score based on clinical observations and drop in body temperature were preemptively euthanized. The 50% effective dose (ED_{50}), the dose providing protection to 50% of mice, and the 95% confidence limits (95% C.I.) were calculated from the survival data at day 5 by probit analysis using the program Systat (SPSS Inc.). All animals in the vehicle-treated control groups developed lethal infections.

Golden Syrian Hamster Model of *C. difficile* Infection. Hamsters were kept under controlled conditions with 12 h dark−12 h light cycles, 68−72 °F constant temperature, 50% relative humidity, and 10−15 exchanges of a fresh HEPA filter air per hour. Animals were kept in 7.5 in. wide by 6 in. deep cages (Alternative Design Manufacturing Silom Springs, AR) with sterilized Bed O-Cob bedding (corn cob) and free access to water and standard rodent chow (Harlan). Animal details: Hamster-Golden Syrian (*Mesocricetus auratus*), Michigan-206 Golden Syrian wild-type from Harlan, male, 90−110 g, 5−6 weeks old. Statement on Animal Welfare: Studies described were approved by the Institutional Animal Care and Use Committee (IACUC) of the Novartis Institutes for BioMedical Research Inc., Cambridge, under protocol number OS 20,061.

Experimental Conditions. Infection: *C. difficile* ATCC 43255 was received from American Type Culture Collection and stored at −80 °C in Brucella broth supplemented with 20% glycerol. On day −1, all animals received a single subcutaneous injection of clindamycin (10 mg/kg). At 24 h after clindamycin pretreatment, hamsters were infected by oral gavage with approximately 10^6 CFU of the *C. difficile* culture. All culture work was completed within 30 min, and all cultures were back in anaerobic conditions by this time. Briefly, strains were resuspended from overnight plates (in reinforced clostridial medium + 1% oxyrase (Oxyrase, Inc. Mansfield, OH) and diluted to 1 × 10^7 cfu/mL (OD_{600} nm = 1.1−1.3, dilute 1:100). Hamsters were immediately infected with 0.75 mL of inoculum (∼7.5 × 10^6 CFU/hamster final). An aliquot of inoculum was diluted 1:1000 and spiral-plated (100 μL, slow deposition) on TSAB or RCMA (×2) and incubated at 37 °C anaerobically for determination of infection titer. Treatment: hamsters were administered the test compound (eight animals per dose level) starting 24 h after infection. Single antibiotic doses were administered orally and formulated as described below. Antibiotics were administered 1 time per day and continued for up 5 days. The control group was administered the solution vehicle alone. Animals were observed two times a day for the duration of the experiment. General observations included signs for mortality and morbidity, for the presence of diarrhea ("wet tail"), overall appearance (activity, general response to handling, touch, ruffled fur), and recorded. Animals judged to be in a moribund state were euthanized. Criteria used to assign a moribund state are extended periods (5 days) of weight loss, progression to an emaciated state, prolonged lethargy (more than 3 days), signs of paralysis, skin erosions or trauma, hunched posture, and a distended abdomen. Observations continued, with any deaths or euthanasia recorded for a period up to 21 days postinfection (for relapse). Any animal that died during the observation period was necropsied and the contents of their cecums removed, diluted with an equal volume of PBS, and frozen at −80 °C until processing (efficacy study). All surviving animals were euthanized by CO_2 inhalation and sampled in a similar manner as above.

Solubility Experiments. One to two milligrams of compound was weighed into 1 mL glass tubes, and a fixed volume of each vehicle was added to yield approximately 20 mg/mL of compound. After initial mixing using brief vortexing and sonication (5−10 min), samples were equilibrated by shaking for 24 h at room temperature. After equilibration, the vials were visually examined. If a clear solution was obtained, solubility was reported as >X mg/mL (where X is the starting concentration in that sample) and the pH of the sample was recorded. Suspensions or solutions with visible particles were filtered through 0.22 μm PVDF membrane filters, their pH was recorded, and the dissolved drug concentration was analyzed using an RP-HPLC assay.

Dosing Solution Formulation. *Compound 11.* Concentration: 5 mg/mL; ingredients (amount for 1 mL (%)): 0.1 N NaOH [100 μL (10% v/v)], PEG-300 [100 μL (10% v/v)], purified water [770 μL (77% v/v)], 0.1 N HCl [30 μL (3% v/v)]. Procedure: the compound was weighed in a vial, PEG-300 was added, and the mixture was stirred/sonicated for 15 min at 50 °C until clear. NaOH (0.1 N) was added and stirred for 15 min. Water was added and mixed thoroughly, and the pH was adjusted to 7.0−7.5 with 0.1 N HCl. The final formulation was a clear solution with pH 7.3. The solution was physically stable for at least 24 h at room temperature.

Crystallization: Compound 11·EF-Tu Complex. To form the EF-Tu·compound **11** complex, 10 mg/mL (227 μM) *E. coli* EF-Tu protein in a buffer containing 50 mM Tris pH 8 and 50 mM NaCl was incubated with 1 mM compound **11** for 1 h at 4 °C. The sample was centrifuged at 20 000*g* to remove any resulting precipitate. Crystallization was carried out using 300 nL of the protein sample plus 300 nL of crystallization solution containing 0.1 mM Tris pH 8.2, 18% PEG3350, and 0.2 M $MgSO_4$, using a sitting drop format and equilibrated against a reservoir of the crystallization solution. The crystal was flash frozen with liquid nitrogen after being stabilized in a cryobuffer containing 0.1 mM Tris pH 8.2, 18% PEG3350, 0.2 M $MgSO_4$, and 20% ethylene glycol.

X-ray Data Collection and Structure Determination. Initial data from a single crystal of the EF-Tu·compound **11** complex were collected on a Rigaku Saturn 92 detector using Cu−Kα radiation (λ = 1.5418 Å) from a Rigaku FR-E rotating anode generator. The data were measured from a single crystal maintained at 100 K, and the reflections were indexed, integrated, and scaled using XDS.[11] Crystals were diffracted to 2.0 Å resolution in the space group $P2(1)2(1)2$. The structure of the

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EF-Tu·GDP·compound **11** complex was determined by molecular replacement as implemented in PHASER,[11] using *E. coli* EF-Tu protein as a search model (1D8T). The resulting molecular replacement solution contained one EF-Tu·GDP·compound **11** protein complex in the asymmetric unit. Structure determination was achieved through iterative rounds of positional refinement using BUSTER,[11] with model building using COOT.[11] Individual *B*-factors were refined using an overall anisotropic *B*-factor refinement along with bulk solvent correction. The solvent, GDP, and compound **11** were built into the density in later rounds of the refinement. Data collection and refinement statistics are shown in Table 2.

Table 2. | Crystallographic Data and Refinement Statistics

parameters	EF-Tu·compound **11** complex
space group	$P2_12_12$
unit cell (Å)	a = 80.35, b = 125.35, c = 45.40
resolution range (Å)	42.69–2.006 (2.012–2.006)
total observations	144646
unique reflections	31079
completeness (%)[a]	98.5 (66.2)
multiplicity	4.7 (2.6)
$\langle I/\sigma(I)\rangle$[a]	15.7 (4.2)
R_{merge}[a,b]	0.065 (0.250)
R_{cryst}/R_{free}[c]	0.205/0.253 (0.224/0.235)
protein atoms	3027
heterogen atoms	124
solvent molecules	224
average *B*-factor (Å^2)	27.4
rmsd bond lengths (Å)	0.01
rmsd bond angle (deg)	1.16

[a]Numbers in parentheses are for the highest resolution shell. [b]$R_{merge} = \sum|I_h - \langle I_h\rangle|/\sum I_h$ over all h, where I_h is the intensity of reflection h. [c]R_{cryst} and $R_{free} = \sum||F_o| - |F_c||/\sum|F_o|$, where F_o and F_c are observed and calculated amplitudes, respectively. R_{free} was calculated using 5% of data excluded from the refinement.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.6b00726.

Analytical methods, experimental synthetic procedures, and compound characterization data (PDF)
SMILES and MIC data (CSV)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

MIC, minimum inhibitory concentration; EC_{50}, 50% effective dose; G+, Gram positive; MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant enterococci; *S. aureus*, *Staphylococcus aureus*; *E. faecalis*, *Enterococcus faecalis*; *E. faecium*, *Enterococcus faecium*; *S. pyogenes*, *Streptococcus pyogenes*; EF-Tu, elongation factor-Tu; BLD, below the limit of detection

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