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Solid-Phase Synthesis of Methyl Carboxymycobactin T 7 and Analogues as Potential Antimycobacterial Agents

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Abstract—A solid-phase approach for the total synthesis of methyl carboxymycobactins **1a**–**d**, with an on-resin cyclization leading to azopine **5** as the key step, was developed. The iron-affinity of these compounds was assessed by a competitive sulfoxine-binding assay, and antimycobacterial activity was tested against the growth of *Mycobacterium avium*.

Iron is a growth-limiting nutrient for microorganisms, and sequestration of ferric ion is an essential component for survival of many pathogens. These organisms have evolved ligands known as siderophores with extremely high affinity for ferric ion in order to sequester iron from their surroundings. An elaborate mechanism has co-evolved for pumping this siderophore–iron complex back into cells and releasing the iron by intracellular reduction. In mycobacteria, the three types of iron-binding compounds produced are generally classified as mycobactins, exochelins, and carboxymycobactins, based on their relative lipophilicities, overall structure, and location within or outside the cell.[1] Mycobactins are intracellular siderophores isolated from pathogenic strains, and are water insoluble with a lengthy alkyl component that confines them to the cell membrane. While the exochelins are water-soluble extracelluar siderophores synthesized by non-pathogenic strains, the carboxymycobactins are mycobactin-like extracellular siderophores from pathogenic strains. Prior to the structural elucidation of carboxymycobactins,[2] all extracelluar mycobacterial siderophores were referred to as exochelins. Even though the term exochelin is still used,[3] we prefer the term carboxymycobactins because of their structural similarity to mycobactins.

Methyl carboxymycobactin T 7 (**1a**) (Fig. 1), containing a saturated alkyl chain terminated with a methyl ester group, was isolated from *Mycobacterium tuberculosis*,[3a] and was later found effective in prevention of cardiac reperfusion injury,[4] ischemia/reperfusion injury,[5] and vascular injury by arresting smooth muscle cell proliferation.[6] Our interest was due, in part, to the hypothesized therapeutic potential[7] of modified mycobactins that might competitively bind iron and inhibit its assimilation by targeted forms of mycobacteria. Mycobactin analogues with moderate to high inhibition of the growth of *Mycobacterium tuberculosis* H37Rv were recently described by Miller's group.[8] In order to investigate these concepts, we developed a solid-phase approach for the synthesis of **1a** and its analogues, in addition to known solution methods.[8,9]

Retrosynthetically, disconnection at the bonds depicted (Fig. 1) gives the required synthons for stepwise construction on the solid support. Key intermediates to these synthons comprise commercially available acids **4** that include 3-hydroxybutyric- and suitably protected 2,3-diaminopropionic acids (Bachem, CA), the known oxazoline **2**,[8a] and the monoester **6**.[10] The choice of Bpoc [1-methyl-1-(4-biphenyl)ethoxycarbonyl] for temporary protection[11] of the resin-bound azopine **5** was compatible with the acid-labile linker of the Wang resin. Moreover, Bpoc protection for the ε-amino group in lysine derivative **3** was compatible with 2,4-dimethoxybenzyl as

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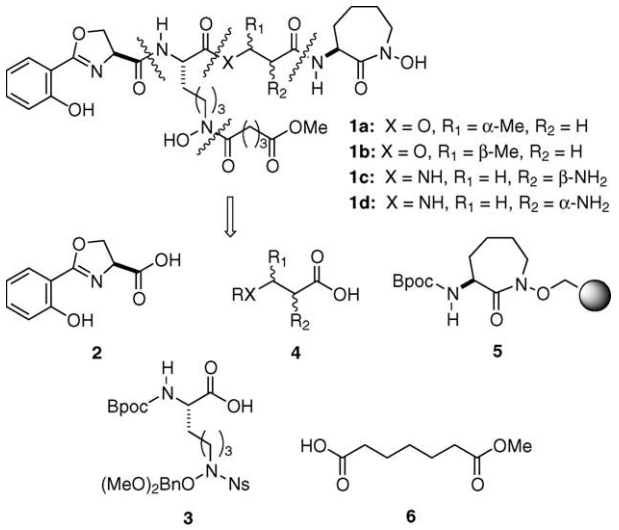
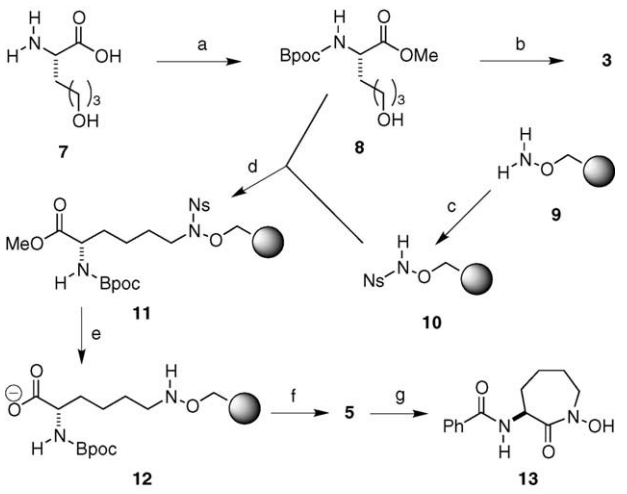


Figure 1. Retrosynthetic analysis of methyl carboxymycobactin T 7 (**1a**) and analogues.

the *O*-protecting group,[12] since the former can be selectively cleaved with the nosyl (2-nitrobenzenesulfonyl, Ns)[13] as the *N*-protecting group on the hydroxylamine moiety. The nosyl group was favored here based on our own experience with its utility in the synthesis of polyhydroxamate analogues.[14]

The *N*,*C*-bisprotected 6-hydroxynorleucine **8** was prepared by reacting a preformed carboxylate salt of L-6-hydroxynorleucine (**7**) with Bpoc reagent, followed by esterification with *N*,*N*′-diisopropyl-*O*-methylisourea.[15] Reaction of **8** with *O*-[(2,4-dimethoxyphenyl)methyl]-*N*-(2-nitrophenylsulfonyl)-hydroxylamine[14] under Mitsunobu conditions, followed by saponification afforded **3** (Scheme 1). The on-resin cyclization strategy leading to azopine **5**, constitutes the most important aspect of our synthesis. Thus, Wang resin (1.1 mmol/g)-bound hydroxylamine **9**[16] was activated with the nosyl group for alkylation with alcohol **8** under Mitsunobu conditions to give intermediate **11**. The nosyl group was deprotected using thiolate anion[17] and the methyl ester hydrolyzed with 0.5 N LiOH/THF to insure proper swelling of the resin. The resulting carboxylate salt **12** was activated with 7 - azabenzotriazol - 1 - yloxytris (pyrrolidino)phosphonium hexafluorophosphate (PyAOP) and cyclized to the azopine **5**, which was characterized as its *N*-benzoyl derivative **13**. The MS (ESI) analysis of **13** [*m*/*z* 271 $(M+Na)^+$] confirmed formation of the azopine **5** with no linear byproduct, indicating a high yield of cyclization (78%) based on HPLC.

Methyl carboxymycobactin T 7 (**1a**) and its analogues were assembled on the solid support as depicted in Scheme 2. Each intermediate was evaluated by RP-HPLC with UV detection and MS (ESI), after derivatization of an aliquot of the resin with benzoyl chloride in the presence of pyridine, followed by TFA cleavage. The resin-bound azopine derivative **14**, obtained from **5** by deprotection of the Bpoc-group and neutralization, was coupled with (*S*)-3-hydroxybutyric



Scheme 1. Synthesis of intermediates **3** and **5**: (a) (i) Triton B, MeOH, rt, 30 min; (ii) 2-(4-biphenylyl)-prop-2-yl 4′-methoxycarbonylphenyl carbonate, DMF, 50 °C, 4 h; (iii) *N*,*N*′-diisopropyl-*O*-methylisourea, CH_2Cl_2, reflux, overnight; 60%; (b) (i) 2,4-$(MeO)_2$BnO-NH-Ns, Ph_3P, DIAD, THF, 37 °C, overnight; (ii) 1 N LiOH, THF, rt, 6 h; 92%; (c) NsCl, 2,6-lutidine, 1,2-dichloroethane (DCE); (d) Ph_3P, DIAD, THF, 37 °C, overnight; (e) (i) $HS(CH_2)_2OH$, DBU, DMF, rt, 2×30 min; (ii) 0.5 N LiOH, THF, rt, 5 h; (f) PyAOP, DIPEA, NMP, rt, 2×3 h; (g) (i) 1% TFA/5% triethylsilane (TES) in DCE, rt, 2×15 min; (ii) 5% DIPEA in DCE; (iii) PhCOCl, pyridine, DCE, rt, 4 h; (iv) TFA–CH_2Cl_2 (4:1), rt, 30 min.

acid (**4a**, XR = OH, R_1 = β-Me, R_2 = H) sodium salt in the presence of HOBT/HBTU to give alcohol **15**. The acylation of the secondary alcohol group of **15** with lysine derivative **3** under Mitsunobu conditions gave the desired ester **16** (>50%) with inversion of configuration at the methyl group, along with the by-product formed by dehydration (presumably olefinic derivative, LC-MS data). In order to improve the yield, several other methods for activation of the carboxyl group were tested. EDC/HOAt in DMF (with or without DMAP) was the only reagent that gave small amounts of the desired ester (~30%, opposite stereochemistry at the methyl group). Since the Mitsunobu reaction gave better yield of the product and the by-product would not interfere in subsequent steps, the Mitsunobu method was used for generating **16**. Intermediate **16**, after Bpoc deprotection and neutralization, was coupled with oxazoline **2** in the presence of EDC/HOAt to give **17** (>50%). After deprotection of the nosyl group, the resulting hydroxylamine was then coupled to monoester **6** in the presence of EDC/HOAt. Subsequent on-resin deprotection of the 2,4-dimethoxybenzyl group, followed by cleavage from the resin provided crude methyl carboxymycobactin T 7 (**1a**) with ~50% purity. This material was purified by RP-HPLC [YMC CombiPrep ODS AQ C_{18} 20×50 mm, 218 nm, 25 mL/min, 0–100% B/10 min (A: water; B: acetonitrile)], and **1a** was obtained in 14% overall yield (87% pure).

Methyl carboxymycobactin **1b**, having an opposite stereochemistry at the methyl group (*S*), was synthesized in 19% overall yield (86% pure) by using (*R*)-3-hydroxybutyric acid (**4b**, XR = OH, R_1 = α-Me, R_2 = H) sodium salt early in the coupling with azopine **14** and repeating all subsequent reactions.

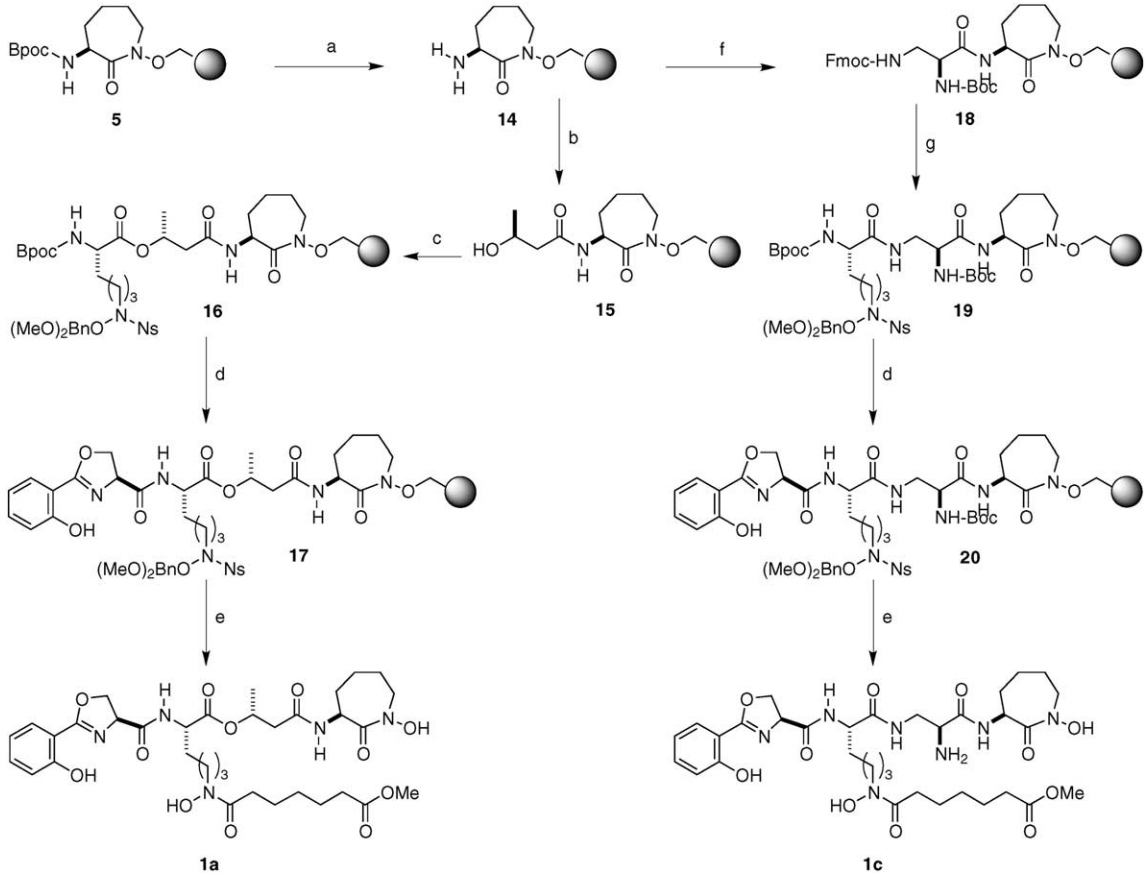
Both amide analogues **1c** and **1d** (amide linkage instead of central ester linkage) with pendant *S*- and *R*-amino groups were synthesized utilizing suitably protected 1,3-diaminopropionic acid derivatives, Boc-Dap(Fmoc)-OH (**4c**) and Boc-D-Dap(Fmoc)-OH (**4d**) respectively (only **1c** is shown in Scheme 2). Thus, resin-bound azopine **14** was coupled with **4c** in the presence of HATU to give **18**. After deprotection of the Fmoc group, the free amine was coupled with lysine derivative **3** in the presence of EDC/HOAt to give **19**, which was then transformed to **1c** (58%; ~2:1 diastereomeric mixture; LC-MS evidence) as described above in the preparation of **1a**. Similarly, **1d** (63%, ~2:1 diastereomeric mixture) was synthesized by initial coupling of **4d** with **14**, and subsequently repeating the later steps. The crude products were purified by preparative RP-HPLC as described earlier, to obtain **1c** and **1d** in overall yields of 19% and 24%, with purities of 93% and 91%, respectively.[18] The formation of diastereomers was not expected. We assume epimerization occurred during the coupling reaction leading to the intermediate **19** rather than the lysine derivative **3** being not optically pure, as this problem was not observed during the synthesis of **1a** and **1b**. The stated HPLC purities of the methyl carboxymycobactins **1a–d**, did not include 3–6% of compounds complexed with iron during isolation.

The iron-binding affinity of methyl carboxymycobactin T 7 (**1a**) and its analogues was assessed using a competitive spectrophotometric assay with 8-hydroxyquinoline-5-sulfonic acid (sulfoxine).[18b] The measurement reflects the relative affinities of the ligands, compared with the sulfoxine-iron complex. The screening results are summarized in Table 1 with desferrioxamine B (DFO)[18b] as a control. Methyl carboxymycobactin T 7 (**1a**) shows better affinity than the corresponding *S*-isomer **1b** though somewhat inferior to DFO itself. The selectivity of one isomer over the other is worth noting, considering similar profiles obtained for isomers from

Table 1. Relative Fe(III) affinity of methyl carboxymycobactin T 7 (**1a**) and its analogues in sulfoxine binding assay

Compd	% Fe removed from pfS[a]	% Fe removed from pfL[b]
DFO	75	75
1a	54	54
1b	21	23
1c	49	49
1d	52	52

[a]Preformation of the sulfoxine–iron complex with subsequent addition of the test ligand.
[b]Preformation of the ligand–iron complex with subsequent addition of sulfoxine.



Scheme 2. Synthesis of methyl carboxymycobactin T 7 (**1a**) and analogues: (a) (i) 1% TFA/5% TES in DCE, rt, 2×15 min; (ii) 5% DIPEA in DCE; (b) (i) (*S*)-3-hydroxybutyric acid (**4a**) sodium salt, HOBT, HBTU, DMA, rt, 2 h; (c) **3**, Ph_3P, DEAD, THF, rt, 20 h; (d) (i) 1% TFA/5% TES in DCE, rt, 2×15 min; (ii) 5% DIPEA in DCE; (iii) **2**, EDC, HOAt, DMF, rt, 7 h; (e) (i) $HS(CH_2)_2OH$, DBU, DMF, rt, 2×30 min; (ii) **6**, EDC, HOAt, DMF, rt, 8 h; (iii) 1% TFA/5% TES in DCE, rt, 2×30 min; (iv) TFA–CH_2Cl_2 (4:1), rt, 30 min; (f) Boc-Dap(Fmoc)-OH (**4c**), HATU, DIPEA, DMA, rt, 6 h; (g) (i) 25% piperidine in DMF, rt, 15 min; (ii) **3**, EDC, HOAt, DMF, rt, 8 h.

competition experiments within the carboxymycobactin family.[9] Both isomers of amide analogues **1c** and **1d** displayed similar iron affinities, comparable to that of **1a**.

The methyl carboxymycobactins **1a–d** were screened for anti-mycobacterial activity against *Mycobacterium avium*, one of the most common opportunistic pathogens associated with AIDS. Each compound was tested at four different concentrations ranging from 2–20 μg/mL and added at the time of inoculation to *Mycobacterium avium*. Growth was followed visually, and after one week, all compounds showed strong inhibition of growth (10–15% of the controls). However, growth was about 75–80% of the controls after 2 weeks, and no differences were seen after 3 and 4 weeks. At lower concentrations (0.1–1 μg/mL), no inhibition was seen with any of the compounds after one week. All compounds were then tried in multiple doses with 20 μg/mL each being added at time zero, and 7 and 14 days later. While all compounds retarded growth after one week, there was no discernible difference after 2 weeks, and there was no inhibition after 3 weeks. This could be explained by an initial slow degradation of the drug by bacteria, which is sufficient to decrease the effective concentration of the drug, eventually leading to the bacterial growth returning to the level of controls.

In conclusion, a solid-phase method for the synthesis of methyl carboxymycobactin T 7 (**1a**) and its analogues was developed. These compounds were found to be very effective iron-chelators, but the inhibitory activity against *Mycobacterium avium* growth was found to diminish after the first week of culture.

Acknowledgements

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