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Natural Products Synthesis

Total Synthesis of Mutanobactins A, B from the Human Microbiome: Macrocyclization and Thiazepanone Assembly in a Single Step

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Abstract: We report the first total syntheses of tricyclic mutanobactins A and B, lipopeptides incorporating a thiazepanone, isolated from *Streptococcus mutans*, a member of the human oral microbiome. A rapid, solid-phase peptide synthesis (SPPS) based route delivers these natural products from a cascade of cyclization reactions. This versatile process was also employed in a streamlined synthesis of mutanobactin D. Additionally, we provide an independent synthesis of a truncated mutanobactin A analog, utilizing a novel thiazepanone amino acid building block.

The human microbiota constitute a complex consortium of hundreds of species, inhabiting external surfaces of our torus-shaped body.[1] The healthy microbiota collectively are believed to contribute positively to the well-being of the host.[2] *Streptococcus mutans* is a prominent member of the human oral consortium and the primary etiological agent of dental caries.[3] Investigations of these bacteria have led to the identification of 16 lipo-peptides named mutanobactins and mutanolins (Figures 1 and 2).[4–6] Only macrocyclic lipopeptides A–D have been properly characterized, while the structures for the remaining have merely been postulated by analogy based only on mass spectrometric analysis.[6] Mutanobactins A (**1**) and B (**2**) differ by one amino acid, with the former incorporating L-valine where the latter features L-isoleucine (Figure 1A). Both **1** and **2** contain an uncommon[7,8] 1,4-thiazepan-5-one ring embedded within a hexapeptide macrolactam, and mutanobactin D lacks the thiazepanone altogether. Mutanobactins A–C are structurally intriguing and the synthetically most challenging members of the lipopeptide family. Mutanobactins A, B, and D displayed activity in reducing pathogenic yeast-to-hyphae transition of the human commensal *Candida albicans* without inhibiting its cell growth; by contrast mutanobactin C was reported as inactive (>200 μM IC_{50}).[5] We have previously documented a synthesis of the simplest member of the family, mutanobactin D, that combined solid-phase peptide synthesis (12 steps) and solution-phase methods (9 steps). The latter proved critical for structural elucidation and configurational assignment.[9]

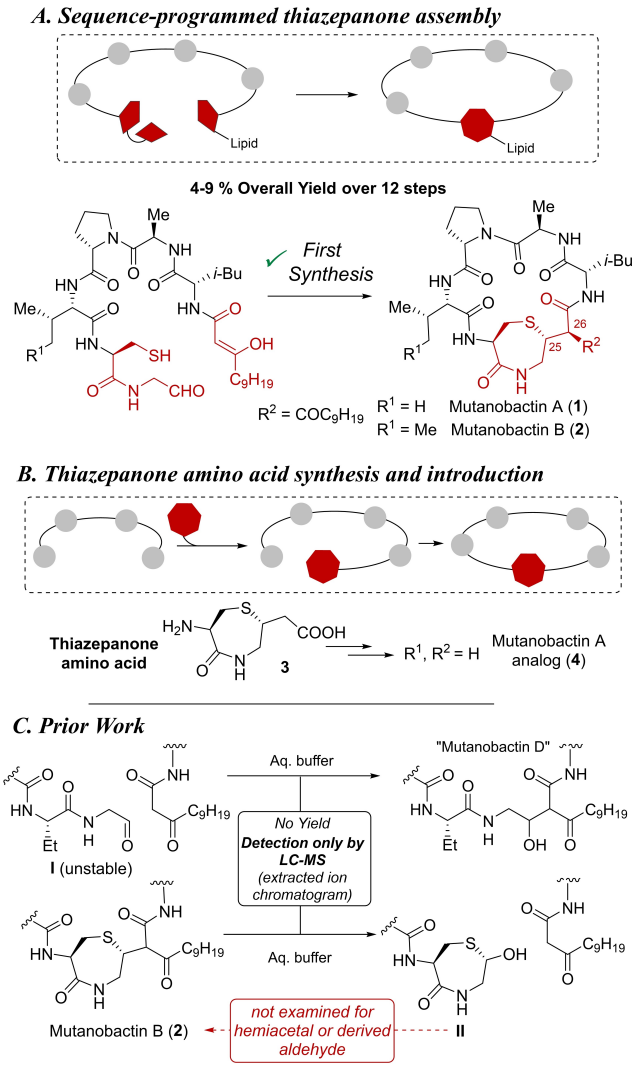

Figure 1. Synthetic approaches to Mutanobactins A, B, and D and peptide **4**.

Herein for the first time, we report two complementary approaches for the total synthesis of thiazepanone-containing peptides (Figure 1A and B). The first route was designed to examine whether a suitable linear peptide precursor might be identified inherently programmed to form the thiazepanone and macrocycle in one step (Figure 1A). These

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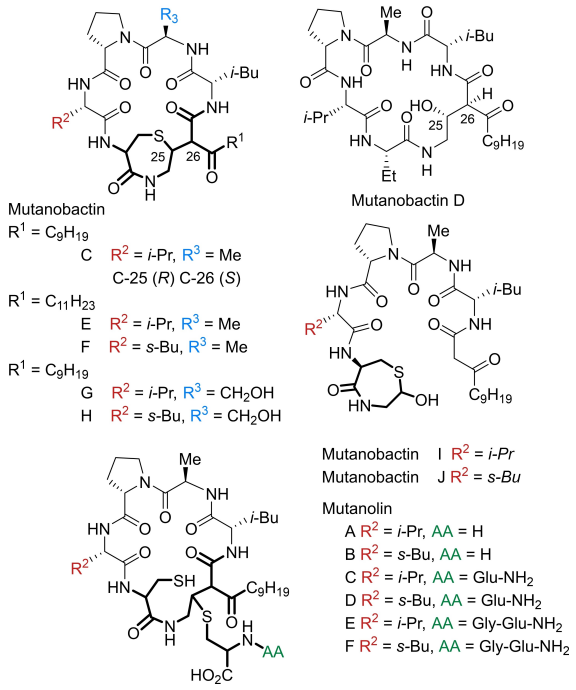


Figure 2. Mutanobactins E–J and mutanolins A–F identified by MS-MS in *S. mutans* fermentation broth extracts. We employ atom-numbering shown as C-25 and C-26 for mutanobactins C–D for the purposes of simplifying their description in the text. They are otherwise numbered differently in the isolation work.

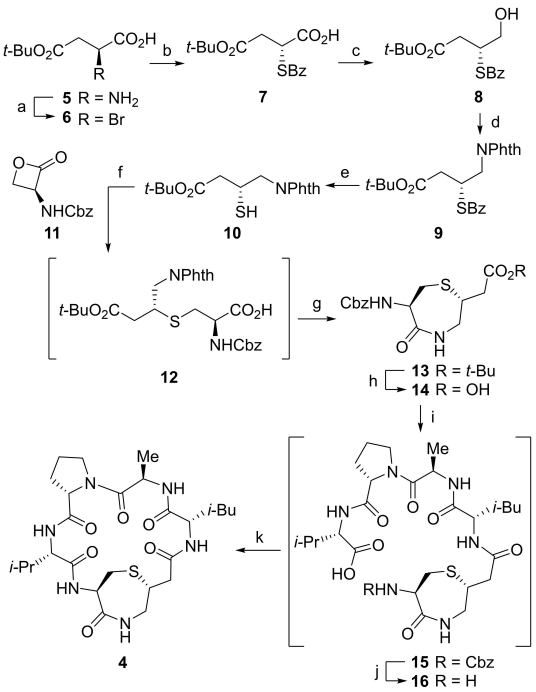
investigations were successful, leading to the first total synthesis of mutanobactins A and B (**1** and **2**) via an acetal-linker based SPPS approach (12 steps total and 4–9 % overall yield). The strategy also provides access to mutanobactin D via a route that is considerably more streamlined when compared to our earlier report. Over the course of our study, we became interested in assessing the significance of the lipid side chain for activity in connection with *Candida albicans*. Preparation of the abiological peptidic core **4** lacking the lipid side chain required a new approach (Figure 1B), involving synthesis of a protected derivative of a thiazepanone amino acid building block **3**. Investigation of **4** allowed us for the first time to ascertain that the lipid side chain is important for activity. Both synthesis approaches provide opportunities for further study of these fascinating molecules found in the human oral microbiome.

Cichewicz was first to describe in 2012 the isolation of mutanobactins A–D from *S. mutans*.[4,5] These cyclic peptides were identified and characterized in the course of a study of a hybrid gene cluster that encodes for polyketide synthases-nonribosomal peptide synthetases (PKS-NRPS). Experiments in which *S. mutans* and a mutant strain (Δmub) were separately cultured in the presence of *C. albicans* showed the presence of wild-type *S. mutans* maintained *C. albicans* perpetually in the yeast morphological state. However, in co-cultures of *C. albicans* with Δmub, lacking mutanobactin producing abilities, the yeast was observed to undergo a shift to a mycelial pattern. This morphological transition is linked to the invasive form of the fungus, leading to pathologies such as oral thrush.[4] Subsequent mass spectrometric studies suggested that these commensal bacteria also biosynthesize a large collection of closely related structures, mutanobactins E–J and mutanolins A–F[6] (Figure 2).

Prior work on the mutanobactins has been rather limited (Figure 1C). Although we have reported the total synthesis of mutanobactin D, no synthetic work has been done for mutanobactins A (**1**) and B (**2**). Wang and co-workers conducted investigations into the putative biosynthetic pathway focused on the production of the simplest member of this class, namely, mutanobactin D.[10] They suggested that a (thio-)reductase converts a thioester precursor to aldehyde **I**, noted as unstable. In turn, conversion of **I** to mutanobactin D was observed in analytical-scale experiments. Thus, characterization of the latter was carried out merely by HRMS, no yield was specified, and the configuration at C-25 and C-26 in the products was not established. In the only set of investigations involving mutanobactin B (**2**), analytical-scale experiments suggest it undergoes fragmentation to hemithioacetal **II** (LC-MS characterization only) in unspecified yields. Based on these findings, they hypothesized that the biosynthesis of **1** and **2** might involve an intermediate incorporating an aldehyde, ketoamide, and thiol. However, it is important to note that no experimental evidence is provided for this hypothetical pathway, and the reverse reaction in which **II** could lead to mutanobactins A (**1**) or B (**2**) was not examined.

The lipid components in lipopeptide antifungal and antibacterial agents are generally central to biological activity.[11] Due to the biological significance of these targets, in investigations of mutanobactins A and B, a crucial control experiment is to establish the importance of the decanoyl sidechain. We commenced with the synthesis of **4** (Figure 1B), an analog of mutanobactin A that lacks the lipid. The approach focused on the preparation of the unusual thiazepanone amino acid whose synthesis had not been previously reported.[12] In this strategy, the thiazepanone would be synthesized as a building block independently from the rest of the peptide. This amino acid would be coupled to a tetrapeptide precursor by standard peptide coupling techniques.

Bromide **6** was generated from (*S*)-*tert*-butyl aspartate **5**, using the procedure described by Ellman (Scheme 1).[13] A sequence of steps involving substitution of the bromide with potassium thiobenzoate, borane reduction of carboxylic acid **7**, and Mitsunobu displacement of the resulting primary alcohol **8** with phthalimide led to **9** in 54 % yield over three steps. Thioester **9** was subjected to methanolysis by treatment with NaOMe affording thiol **10**. Slow addition of Cbz-serine β-lactone **11**[14] to the cesium salt of **10** in DMF gave thioether **12**, which was used without purification. Phthalimide deprotection with hydrazine and final ring closure by PyAOP-mediated amide bond formation gave protected thiazepanone amino acid **13** in 64 % over three steps. After its deprotection with TFA, **14** was converted to the corresponding pentafluorophenyl ester by treatment with $F_3CCO_2C_6F_5$. The active ester in turn was coupled to H-L-Leu-D-Ala-L-Pro-L-Val-OH, and the resulting Cbz-hexapep-

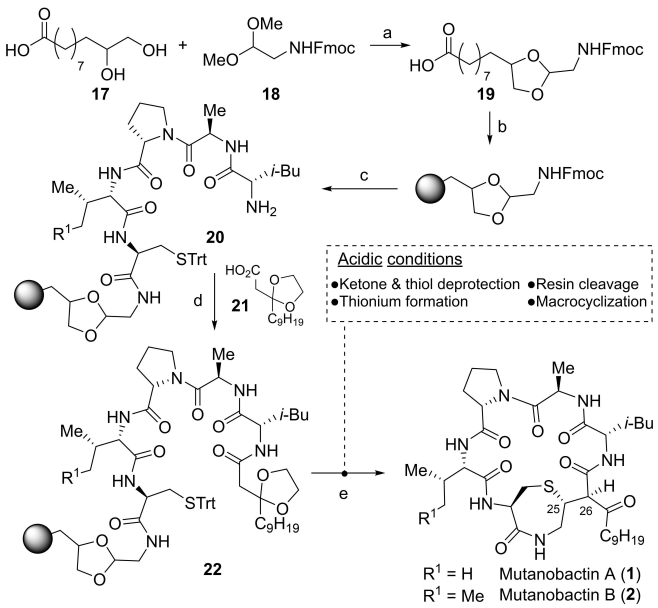


Scheme 1. Synthesis of abiological peptide core sans lipid. Reagents and conditions: a) $NaNO_2$, KBr, 0.75 M HBr, H_2O, −6 °C, 87%; b) KSBz, DMF, quant; c) $BH_3{\cdot}SMe_2$, THF, −20 °C to r.t., 65%; d) diisopropylazodicarboxylate, PPh_3, HNPhth, THF, −5 to 0 °C, 83%; e) NaOMe, MeOH-THF, 0 °C, quant.; f) Cs_2CO_3, DMF; g) i. N_2H_4, EtOH, reflux ii. PyAOP, HOAt, *sym*-collidine, DMF, 64% over three steps; h) TFA, TIPS, CH_2Cl_2, 93% i) i. F_5C_6OTFA, Hünig's base, 0 °C, ii. H-L-Leu-D-Ala-L-Pro-L-Val-OH, Et_3N, MeCN; j) HBr, AcOH; k) PyAOP, HOAt, *sym*-collidine, DMF, 9% over 3 steps and preparative HPLC.; PyAOP = ((7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate); HOAt = 1-hydroxy-7-azabenzotriazole; TIPS = triisopropylsilane.

tide **15** was deprotected with HBr in acetic acid. Macrocyclization was accomplished by slow addition of peptide **16** to PyAOP, HOAt, and *sym*-collidine in DMF. Purification of the isolated concentrate by preparative HPLC gave **4** in 9% overall yield from **14** (3 steps and preparative HPLC purification). We then proceeded to examine the activity of **4** on *C. albicans* in an assay devised to assess hyphae formation,[9] in which we observed for **4** $IC_{50} > 200\ \mu M$, leading to the conclusion that **4**, like mutanobactin C, is inactive.[5]

In our approach to the mutanobactins, we envisioned a complementary strategy that would explore intrinsically programmed formation of the thiazepanone from a linear peptide precursor incorporating a C-terminal, electrophilic glycinaldehyde subunit and an *N*-terminal, nucleophilic β-ketoamide (Figure 1A). The key challenge would be whether the conditions necessary for the final deprotection steps would concomitantly lead to thiazepanone formation and thereby **1** and **2**. This strategy called for the unmasking of an aldehyde upon cleavage from the solid support, a consideration that led us to utilize the dioxolane linker reported by Xu.[15,16]

SPPS-linker **19** was synthesized in 89% by trans acetalization of **17**[17] and Fmoc-aminoacetaldehyde dimethyl acetal **18**.[18] Immobilization onto (aminomethyl)polystyrene resin was accomplished by the action of HATU and Hünig's base in DMF. The linker loading of the resin (0.6–0.7 $mmol\,g^{-1}$) was determined by photometric dibenzofulvene quantification after Fmoc deprotection by DBU.[19] Standard Fmoc-SPPS techniques (see Supporting Information for details) were used to couple sequentially the required amino acids L-Cys(Trt), L-Val, L-Pro, D-Ala, L-Leu, leading to **1**, and L-Cys(Trt), L-Ile, L-Pro, D-Ala, L-Leu, leading to **2**. Under a variety of conditions, direct coupling of 3-oxododecanoic acid to the N-terminus of immobilized peptide **20** was unsuccessful. However, dioxolane protected carboxylic acid **21** readily coupled with the N-terminus of resin bound peptide, mediated by HATU. Subsequent cleavage of immobilized peptide **22** using 95:4:1 TFA/water/thioanisole led to the formation of an oil, from which **1** (or **2**) were isolated after preparative reverse phase HPLC. Thus, mutanobactins A was routinely prepared in 5% overall yield based on linker loading over 12 steps. Mutanobactin B was obtained in 4% yield over 12 steps using the same sequence. The physical data for synthetic natural products **1** and **2** matched that reported by Cichewicz and co-workers in all regards (^{1}H NMR, ^{13}C NMR, IR, HRMS, and $[\alpha]_D$). When the synthesis route was repeated employing an L-Abu instead of L-Cys building block for SPPS (See Supporting Information), cleavage with 95:5 TFA/water delivered in 9% yield after purification by



Scheme 2. Synthesis of **1** and **2**. Reagents and conditions: a) *p*-TsOH (7 mol%), PhMe, reflux, 89%; b) HATU, Hünig's base, (aminomethyl)polystyrene, DMF, 0.6–0.7 $mmol\,g^{-1}$; c) SPPS: Fmoc-deprotection: 20% piperidine in DMF, amino acid couplings: HATU, Hünig's base, DMF; d) **21**, HATU, Hünig's base, DMF; e) 95:4:1 TFA/water/thioanisole then preparative-HPLC, 5% overall based on linker loading for **1**, 4% for **2**. HATU = O-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate.

preparative HPLC mutanobactin D, whose physical data also matched that reported previously in all regards (^{1}H NMR, ^{13}C NMR, IR, HRMS, and $[\alpha]_D$).

Illustrated in Figure 3 are proposed pathways for the formation of mutanobactins A, B and D from the linear peptide precursor produced in the final resin cleavage step. Following dioxolane hydrolysis, aldol addition reaction affords mutanobactin D (Figure 3), which is consistent with the hypothesis that had been previously proposed by Wang et al. for the biosynthesis of mutanobactin D.[10] On the basis of our successful synthesis, we postulate that for mutanobactins A and B dehydration of **23** would lead to acceptor **24**, which could undergo intramolecular conjugate addition by the thiol to furnish the natural products. An alternative sequence to these can be envisioned involving thioacetalization to transiently produce mutanobactins I and J, which would serve as precursors to **25**. Intramolecular trapping of the ensuing thionium ion[20,21] by the enol form of the ketoamide might ultimately lead to mutanobactins A and B. It is important to note that mutanobactins I and J have only been detected in mass spectrometry experiments of isolates from *S. mutans* cultures.[6] It remains to be determined whether they are naturally occurring. In this respect, our attempts at isolating and characterizing intermediates corresponding to mutanobactins I and J have been unsuccessful thus far.

Mutanobactin C is reported to differ from mutanobactin A in the configuration at C-26, namely, (*S*) and (*R*) for mutanobactin C and A, respectively. Mutanobactin C was reported to have no effect on *C. albicans* ($IC_{50} > 200$ μM),[5] underscoring the significance of ketoamide configuration on biological activity. This is consistent with NMR spectroscopic data reported by Cichewicz that indicate that the overall 3D structure depends heavily on the configuration at C-26, and in this respect the positioning of the thiazepanone is quite different for **1** and **3**.[5] Our analysis by ^{1}H NMR spectroscopy of the concentrate resulting from resin cleavage step (Scheme 2, **22**→**1**) clearly reveals resonances for mutanobactin A. However, characteristic resonances expected for mutanobactin C were absent. Thus, the final step (TFA) we describe for the synthesis of mutanobactin A (**1**) does not produce diastereomeric mutanobactin C. This would suggest that **1** and **2** are the thermodynamically preferred products while mutanobactin C, which differs only from **1** configurationally at C-26, might correspond to the kinetic product. However, proper study of this scenario awaits a new synthesis route that provides mutanobactin C, which enables assessment of whether it isomerizes to **1** under the conditions we employ in the final step. Solutions of mutanobactin A **1** (DMSO-d_6) over a three-month period remain unchanged, consistent with its configurational stability.

In conclusion, we have developed for the first time a short SPPS-based total synthesis route towards mutanobactins A, B, and D. The synthesis is executed on solid phase and is accomplished in 12 steps and 4–9 % overall yield with only one purification step. A salient feature of the strategy is that it provides rapid access to members of the mutanobactins that are known to date to have an effect on *C. albicans*. Additionally, we report a synthesis of a novel thiazepanone amino acid building block and its incorporation into a truncated mutanobactin A, lacking the lipid side chain. As this analog displayed $IC_{50} > 200$ μM, we demonstrate for the first time the importance of the lipid domain in these lipopeptides. The two complementary approaches should greatly facilitate access to the larger family of mutanobactin/mutanolin lipopeptides and their derivatives. We are in the process of generating the entire palette of mutanobactins/mutanolins using the approach described to study their function individually and collectively. More broadly, the strategies enable investigations of the exciting interface of chemistry and human oral microbiome-biology.

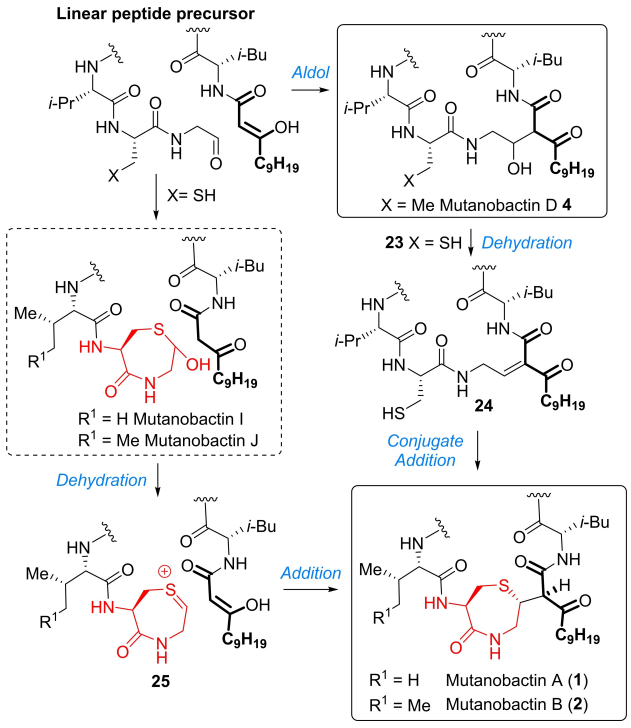


Figure 3. Mechanistic hypotheses for the formation of **1** and **2**.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

Keywords: Cyclization · Macrocycles · Peptide Synthesis · Total Synthesis

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