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Abstract.

The colibactins are hybrid polyketide-nonribosomal peptide natural products produced by certain strains of commensal and extraintestinal pathogenic *E. coli.* The metabolites are encoded by the *clb* gene cluster as pro-drugs termed precolibactins. *clb*$^+$ *E. coli* induce DNA double-strand breaks (DSBs) in mammalian cells *in vitro* and *in vivo* and are found in 55–67% of colorectal cancer patients, suggesting that mature colibactins could initiate tumorigenesis. However, elucidation of their structures has been an arduous task as the metabolites are obtained in vanishingly small quantities (μg/L) from bacterial cultures and are believed to be unstable. Herein we describe a flexible and convergent synthetic route to prepare advanced precolibactins and derivatives. The synthesis proceeds by late-stage union of two complex precursors (e.g., **28** + **17** → **29a**, 90%) followed by a base-induced double dehydrative cascade reaction to form two rings of the targets (e.g., **29a** → **30a**, 79%). The sequence has provided quantities of advanced candidate precolibactins that exceed those obtained by fermentation, and is envisioned to be readily-scaled. These studies have guided a structural revision of the predicted metabolite precolibactin A (from **5a** or **5b** to **7**) and have confirmed the structures of the isolated metabolites precolibactins B (**3**) and C (**6**). Synthetic precolibactin C (**6**) was converted to *N*-myristoyl-D-Asn and its corresponding colibactin by colibactin peptidase ClbP. The synthetic strategy outlined herein will facilitate mechanism of action and structure–function studies of these fascinating metabolites, and is envisioned to accommodate the synthesis of additional (pre)colibactins as they are isolated.

Introduction.

Bacteria residing in and on humans (the human microbiota) play an integral role in regulating physiology and disease.[1] The intestinal tract has been estimated to contain 500–1000 species of bacteria constituting ~1.5 kg of biomass.[2] Certain strains of gut commensal and extraintestinal pathogenic *E. coli* harbour a gene cluster (*clb or "pks"*) that encodes a group of molecules termed precolibactins.[3] Precolibactins are substrates for colibactin peptidase ClpP, a protease encoded within the *clb* gene cluster. ClbP is anchored within the inner periplasmic membrane of the bacteria[4] and removes an *N*-acyl-D-asparagine side chain from the precolibactins. This cleavage step converts precolibactins to cytotoxic colibactins and likely constitutes a prodrug resistance mechanism in the bacteria.[5] c*lb*$^+$ *E. coli* induce DNA double-strand breaks (DSBs) in mammalian cells *in vitro*[3a] and *in vivo*.[6] Host inflammation promotes proliferation of *E. coli*[7] and expression of *clb*,[8] the *clb* pathway promotes colorectal cancer in colitis-susceptible mice treated with azoxymethane,[7] and two studies revealed the presence of *clb*$^+$ *E. coli* in 55–67% of colorectal cancer patients.[7,9] Collectively, these data suggest that colibactins initiate tumorigenesis by a mechanism involving induction of DNA DSBs.

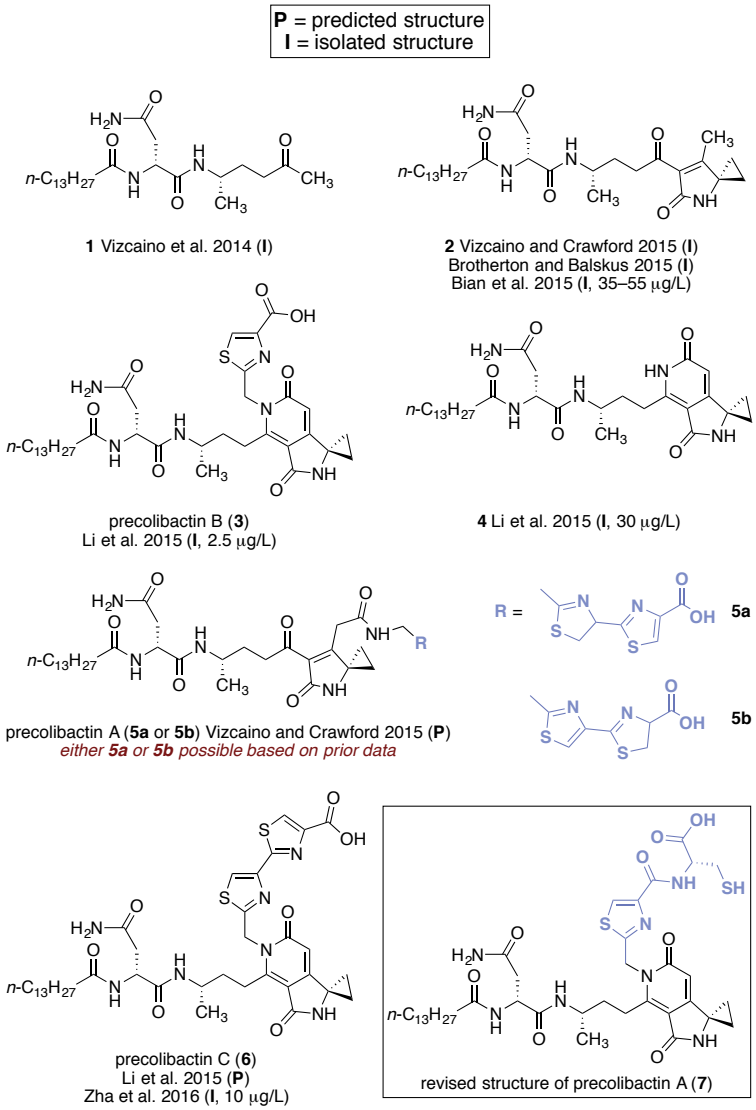


Figure 1. Structures of isolated, predicted, and synthesized candidate precolibactins. Fermentation yields (micrograms of product per liter of fermentation broth) are shown in parentheses, where available.

Fully elaborated (pre)colibactins have been difficult to isolate in homogenous form, and the definitive structures of the most active metabolite(s) are not known. This has been attributed to the low levels of natural production of the metabolites, their instability under fermentation conditions, and the inflammation-dependant up-regulation of the native *clb* gene cluster. The metabolites **1**,[5c] **2**,[10] **3** (referred to hereafter as "precolibactin B"),[10] and **4**[10] were obtained in vanishingly small quantities (2.5–55 μg/L for **2**–**4**) from the fermentation broth of genetically-engineered *clb*$^+$ *E. coli* and implicated as shunt metabolites and/or degradation products in the colibactin biosynthetic pathway (Figure 1). Using the isolation of **2**, as well as HRMS analysis, isotope

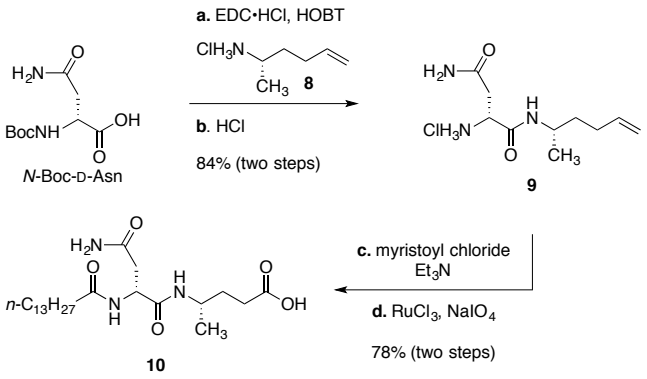
labelling, and bioinformatics based on established biosynthetic logic, the structure of precolibactin A was predicted as **5a** or **5b**.[10a] Key elements within the proposed structures include a hydrophobic *N*-terminal fragment, a spirocyclic aminocyclopropane, and (read from left to right) a thiazoline–thiazole chain. As the presence of the thiazoline–thiazole fragment was inferred by bioinformatic analysis,[10a] **5a** and **5b** could not be unequivocally distinguished at that time, and the absolute stereochemistry of the putative thiazoline ring was not determined. A compound with an exact mass corresponding to **5a** was observed in unpurified extracts, but all efforts to isolate this structure were hampered by its low levels of production and instability.[10a] The pyridone structure **6** (referred to hereafter as "precolibactin C") was recently proposed as a candidate precolibactin based on biosynthetic considerations, isolation of precolibactin B (**3**), and HRMS analysis,[11] and during the preparation of this manuscript, Balskus and co-workers reported the isolation of precolibactin C (**6**) from a mutant strain (0.5 mg of **6** was obtained from an optimized 48-L fermentation).[12] Although one can envision cyclodehydration of **5a** or **5b** to form pyridones resembling **6**, the biosynthetic relationship between these structures had not been established. **2** was shown to weakly cross-link DNA *in vitro*,[10a] suggesting that the colibactins may damage DNA by induction of replication-dependant DSBs.[13] Detailed structure–function analyses of the colibactins have been impossible to conduct owing to their low yields of natural production and the absence of a synthetic route to the targets. However, the aminocyclopropane fragments within **2**–**6** are reminiscent of yatakemycin, CC-1065, and the duocarmycins, which have been shown to alkylate DNA via nucleophilic ring-opening,[14] and the biheterocyclic fragment may serve as a DNA intercalation motif.[15]

In light of the immense difficulties associated with isolating natural precolibactins, chemical synthesis provides an attractive avenue to resolve the ambiguities surrounding the composition of the active metabolite(s) and to enable mechanism of action and structure–function studies. Studies indicate the presence of an aminomalonyl unit in the biosynthetic pathway,[12,16] suggesting additional colibactins are formed, but no evidence relevant to the structures of these metabolites exists, to our knowledge. Consequently, we initially focused on the synthesis of the predicted structures of precolibactin A (**5a** and **5b**) and precolibactin C (**6**), as these represent the most advanced precolibactins for which structural data had been presented. Herein we report a convergent and high-yielding synthesis of structures **5a** and **5b** by cyclization of a fully linear precursor, establish that these materials are distinct from natural precolibactin A, propose and validate by synthesis a revised structure for precolibactin A (as **7**), demonstrate that acyclic

precolibactins undergo cyclodehydration to form the pyridone residues found in precolibactin B (**3**), **4**, and precolibactin C (**6**) under mild conditions, and confirm the structures of precolibactins B (**3**) and C (**6**) by total synthesis. We anticipate that this route will be amenable to synthesis of more advanced structures, including those containing aminomalonyl units, as they are proposed.

Results.

As shown in Figure 1, at the time we began our studies, the structure of precolibactin A had been predicted as **5a** or **5b**. Consequently, we designed our synthetic route to accommodate either heterocyclic sequence and to provide access to the bithiazole found in precolibactin C (**6**). The synthesis of the common left-hand fragment began with N_{α}-(*tert*-butoxycarbonyl)-D-asparagine, which was coupled with (*S*)-hex-5-en-2-amine (**8**, prepared in three steps, 96% yield, and 88% ee from pent-4-en-al)[17] using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC•HCl). Cleavage of the *tert*-butoxycarbonyl protective group (hydrochloride acid) provided the amine hydrochloride **9** (84%, two steps). Acylation of the amine **9** with myristoyl chloride, followed by oxidative cleavage of the alkene (ruthenium chloride, sodium periodate) generated the carboxylic acid **10** (78%, two steps).



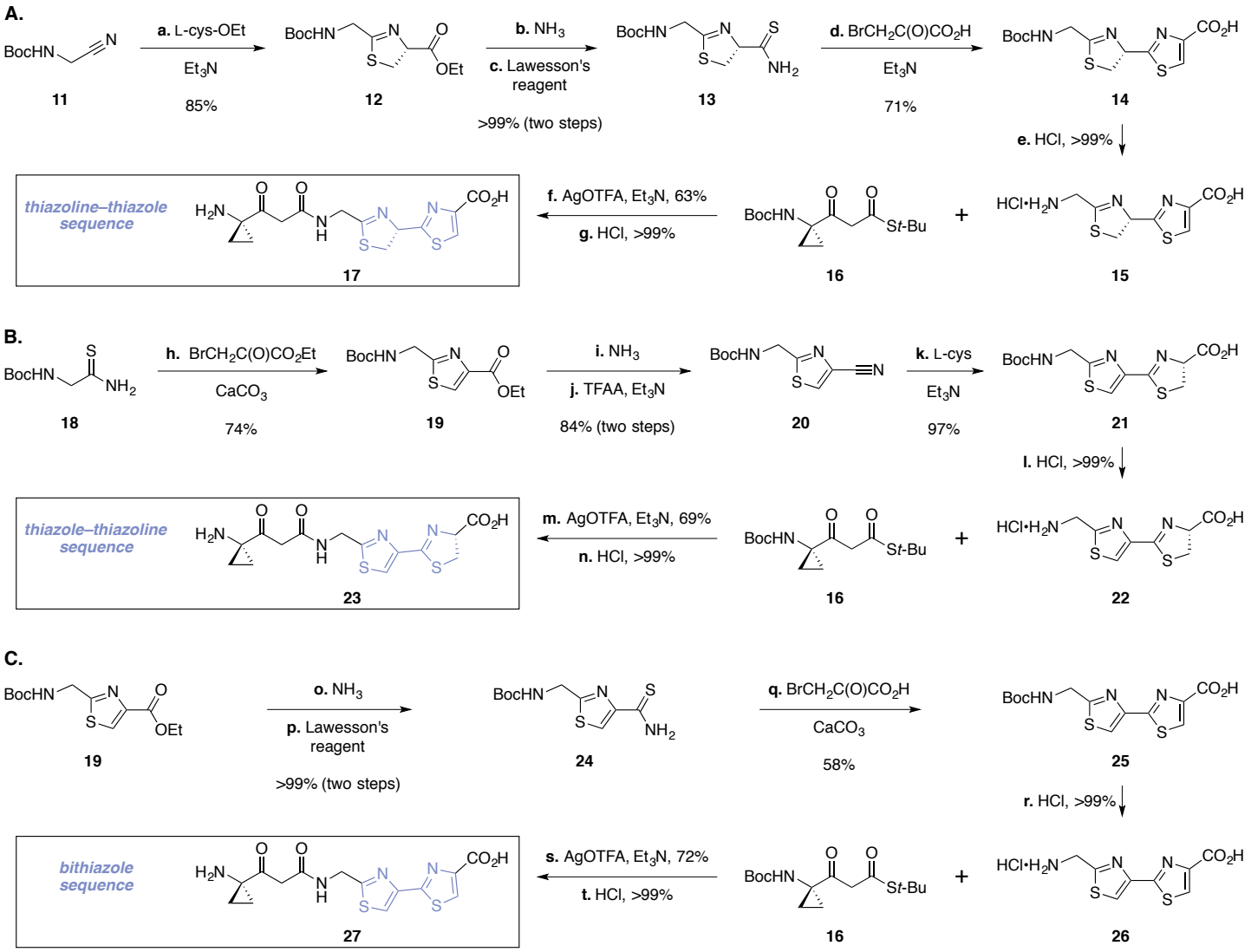
Scheme 1. Synthesis of the carboxylic acid **10**. Reagents and conditions: a. (*S*)-hex-5-en-2-amine hydrogen chloride (**8**), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC•HCl), hydroxybenzotriazole (HOBT), *N*,*N*-diisopropylethylamine (DIPEA), DMF, 23 °C, 84%; b. HCl, CH_2Cl_2–1,4-dioxane (7:1), 23 °C, >99%; c. Myristoyl chloride, triethylamine (Et_3N), DMF, 23 °C, 82%; d. $RuCl_3$, $NaIO_4$, H_2O–EtOAc–CH_3CN (3:2:2), 50 °C, 95%.

The isomeric thiazoline–thiazole and the related bithiazole fragments were prepared by the sequences shown in Scheme 2. Deuterated cysteine labelling experiments[18] supported preservation of the L-amino acid

configuration in precolibactin A[10a] so we selected L-cysteine as the building block for the thiazoline ring. Treatment of *N*-(*tert*-butoxycarbonyl)aminoacetonitrile (**11**) with L-cysteine ethyl ester provided the thiazoline **12** (85%, Scheme 2A). Aminolysis of the ester, followed by heating with Lawesson's reagent, generated the thioamide **13** (>99%, two steps). Exposure of **13** to bromopyruvic acid in the presence of triethylamine formed the thiazoline–thiazole **14** (71%). The thiazoline–thiazole and subsequent intermediates were found to be exceedingly unstable toward hydrolytic ring-opening and, to a lesser and variable extent, oxidation to a bithiazole. Accordingly, the identification of conditions to isolate and purify these intermediates without exposure to water was essential to the success of the route. Cleavage of the *tert*-butoxycarbonyl protective group (hydrochloric acid, >99%) generated the amine **15**. Coupling (silver trifluoroacetate, triethylamine) of the amine **15** with the β-ketothioester **16** (prepared in one step and 56% yield from *N*-(*tert*-butoxycarbonyl)-1-aminocyclopropane-1-carboxylate)[17] followed by carbamate cleavage (hydrochloric acid, >99%) furnished the thiazoline–thiazole fragment **17**.

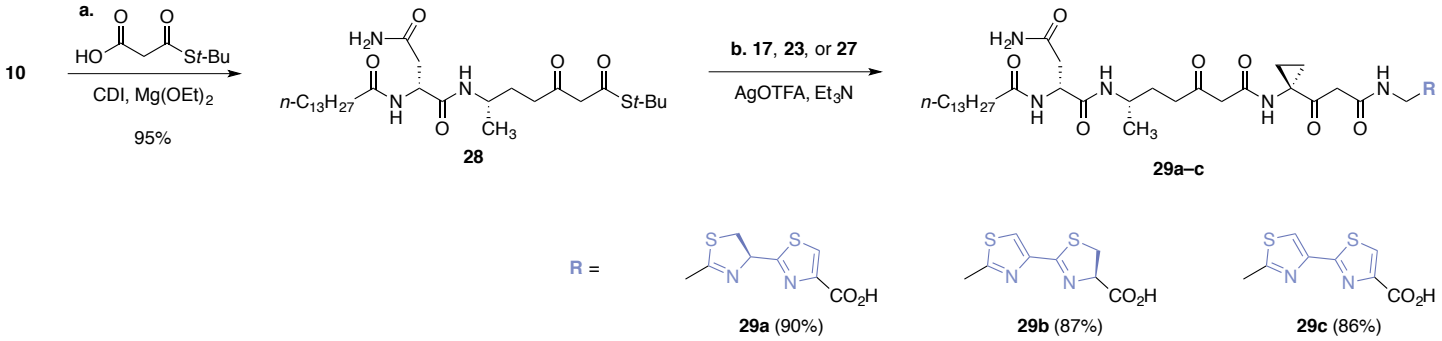
The isomeric thiazole–thiazoline fragment was prepared by a modified sequence (Scheme 2B). Treatment of *N*-(*tert*-butoxycarbonyl)-2-aminoethanthioamide (**18**) with ethyl bromopyruvate formed the thiazole **19** (74%). Aminolysis of **19** followed by dehydration of the resulting primary amide (trifluoroacetic anhydride, triethylamine) generated the nitrile **20** (84%, two steps). Coupling of **20** with L-cysteine formed the thiazole–thiazoline **21** (97%). In contrast to the isomeric intermediate **14**, **21** was found to be stable toward aqueous workup and atmospheric oxygen. A three-step sequence analogous to that described above provided the thiazole–thiazoline **23** (69% overall).

The bithiazole fragment was prepared by the sequence shown in Scheme 2C. Aminolysis of the ester **19** followed by heating with Lawesson's reagent formed the thioamide **24** (>99%, two steps). Treatment of the thioamide **24** with bromopyruvic acid in the presence of calcium carbonate formed the bithiazole **25** (58%). Prior efforts to prepare and isolate **25** were impeded by its instability;[19] we found that rigorous exclusion of water during work-up and purification facilitated the isolation of **25** and subsequent intermediates in homogenous form. A three-step sequence analogous to that used to prepare **17** and **23** then generated the bithiazole fragment **27** (72% overall).



Scheme 2. A. Synthesis of the thiazoline–thiazole **17**. **B.** Synthesis of the thiazole–thiazoline **23**. **C.** Synthesis of the bithiazole **27**. Reagents and conditions: a. L-(+)-cysteine ethyl ester hydrochloride, Et_3N, CH_3OH, 23 °C, 85%; b. NH_3, CH_3OH–H_2O (2:1), 23 °C, >99%; c. Lawesson's reagent, CH_2Cl_2, 23 °C, >99%; d. bromopyruvic acid, Et_3N, CH_3OH, reflux, 71%; e. HCl, CH_2Cl_2–1,4-dioxane (4:1), 23 °C, >99%; f. silver trifluoroacetate (AgOTFA), Et_3N, DMF, 0 °C, 63%; g. HCl, CH_2Cl_2–1,4-dioxane (4:1), 23 °C, >99%; h. ethyl bromopyruvate, $CaCO_3$, EtOH, 23 °C, 74%; i. NH_3, CH_3OH–H_2O (2:1), 23 °C, >99%; j. trifluoroacetic anhydride (TFAA), Et_3N, CH_2Cl_2, 0→23 °C, 84%; k. L-(+)-cysteine, Et_3N, CH_3OH, reflux, 97%; l. HCl, CH_2Cl_2–1,4-dioxane (8:1), 23 °C, >99%; m. AgOTFA, Et_3N, DMF, 0 °C, 69%; n. HCl, CH_2Cl_2–1,4-dioxane (4:1), 23 °C, >99%; o. NH_3, CH_3OH–H_2O (2:1), 23 °C, >99%; p. Lawesson's reagent, CH_2Cl_2, 23 °C, >99%; q. bromopyruvic acid, $CaCO_3$, EtOH, 23 °C, 58%; r. HCl, CH_2Cl_2–1,4-dioxane (4:1), 23 °C, >99%; s. AgOTFA, Et_3N, DMF, 0 °C, 72%; t. HCl, CH_2Cl_2–1,4-dioxane (4:1), 23 °C, >99%.

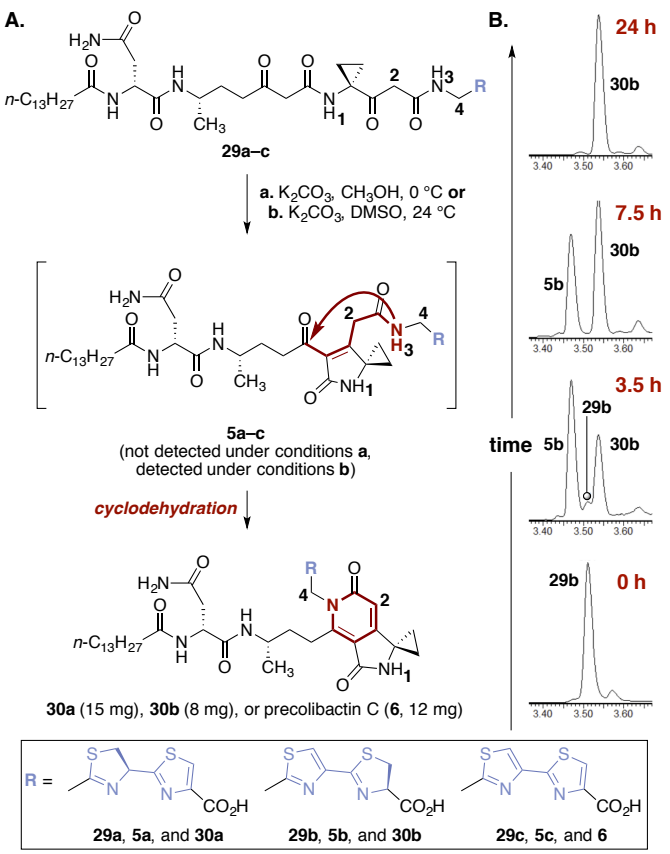
To complete the synthesis of the precolibactin A skeleton, the carboxylic acid **10** was first converted to the β-ketothioester **28** by activation with carbonyl diimidazole followed by the addition of 3-(*tert*-butylthio)-3-oxopropanoic acid and magnesium ethoxide (Scheme 3).[20] Silver-mediated coupling of **28** with the heterocyclic fragments **17**, **23**, or **27** then formed the penultimate intermediates **29a**–**c** (90%, 87%, and 86% for **29a**, **29b**, and **29c**, respectively). The stabilities of the fully linear precursors **29a**–**c** paralleled those of **17**, **23**, or **27**; the thiazole–thiazoline **29b** was stable toward aqueous work-up, while the thiazoline–thiazole **29a** and the bithiazole **29c** were unstable toward aqueous conditions.



Scheme 3. Synthesis of the acyclic advanced precursors **29a**–**c**. Reagents and conditions: a. Carbonyl diimidazole (CDI), 4 Å molecular sieves, DMF, then malonic acid half-thioester, magnesium ethoxide ($Mg(OEt)_2$), 23 °C, 95%; b. **17**, **23**, or **27**, AgOTFA, Et_3N, DMF, 0 °C, 90% (**29a**); 87% (**29b**); 86% (**29c**).

Given the higher stability of the thiazole–thiazoline **29b**, this compound was used to develop conditions to effect the key cyclization reaction (to **5b**). Surprisingly, we found that in preparative experiments treatment of **29b** with potassium carbonate in methanol at 0 °C resulted in formation of the pyridone **30b** (80%, Scheme 4A). Similar outcomes were obtained on exposure of **29b** to ammonium carbonate in ethanol or aqueous sodium hydroxide. Under these conditions, accumulation of the putative monocyclized intermediate **5b** was not observed (LC/MS analysis). The pyridone **30b** was fully characterized and spectroscopic data for this compound were in good agreement with the isolated metabolite precolibactin B (**3**; Table 1). In particular, H-2 and H-4 of **30b** resonated at 6.16 and 5.59/5.50 ppm, respectively; these values are nearly identical to those recorded for natural precolibactin B (**3**; 6.16 and 5.61/5.48 ppm, respectively).[11] A plausible mechanism for the formation of the pyridone **30b** involves cyclodehydration to **5b**, 1,2-addition of the primary amide to the

adjacent carbonyl, and aromatization. The facile formation of **30b** from **29b** provides evidence that the putative and isolated colibactin metabolites **3**, **4**, and **6** may derive from related acyclic precursors, although the timing of cyclizations in the modular biosynthetic pathway remains unknown. The cyclization of the thiazoline–thiazole derivative **29a** and the bithiazole derivative **29c** proceeded in a strictly analogous manner to provide the fully cyclized derivatives **30a** or precolibactin C (**6**), respectively.[17] The mass spectroscopic fragmentation data and ^{1}H NMR data for synthetic precolibactin C (**6**), as well as LC/MS co-injection with metabolite extracts, matched those of natural material (Figure S1, Tables S1 and S5).[10,12] In addition, synthetic precolibactin C (**6**) was converted to *N*-myristoyl-D-Asn and its corresponding colibactin in a ClbP-dependent manner, indicating that precolibactin C (**6**) represents a suitable substrate for ClbP (Figure S3). This sequence provided multimilligram quantities of **30a**, **30b**, and precolibactin C (**6**), and is envisioned to be easily scalable.



Scheme 4. **A.** Cyclodehydration of the linear precursors **29a–c**. Reagents and conditions: a. K_2CO_3, CH_3OH, 0 °C, 79% (**30a**); 80% (**30b**); 83% (**30c**) or b. K_2CO_3, dimethyl sulfoxide, 24 °C (for **29b**). **B.** UV trace (254 nm) of the cyclization of **29b** using potassium carbonate in dimethyl sulfoxide at 24 °C.

We ultimately found that treatment of **29b** with potassium carbonate (~3.0 equiv) in dimethyl sulfoxide at 24 °C proceeded more slowly and allowed for detection of **5b** in the reaction mixture (LC/MS analysis, Scheme 4B). By conducting the reaction in dimethyl sulfoxide-d_6, signals consistent with the monocyclized intermediate **5b** could also be observed by ^{1}H NMR analysis (Table 1).

Table 1. Selected ^{1}H chemical shifts and LC/MS data for **29b**, **5b**, **30b**, and precolibactin B (**3**).[a]

Position	**1**	**2**	**3**	**4**	$\mathbf{t_r}$ (min)
29b	8.83	3.60	8.94	4.56	3.52
5b	8.78	—[b]	8.65	—[b]	3.48
30b	8.46	6.16		5.59, 5.50	3.55
precolibactin B (**3**)[10]	8.44	6.16		5.61, 5.48	

[a] ^{1}H spectroscopic data recorded in DMSO-d_6 (500 MHz, 23 °C). Complete ^{1}H NMR spectra (Figure S2) and LC/MS conditions are presented in the Supporting Information. [b] Not resolved.

Mass-selective LC/HRMS-QTOF analysis was conducted to determine if either **5a** or **5b** corresponded to the structure of natural precolibactin A. As shown in Figure 2, the concentrated ethyl acetate extracts of *clb*$^+$ *E. coli* Δ*clbP*[10a] displayed a single prominent peak of *m*/*z* = 816.3788, which corresponds to $[M+H]^+$ for the proposed structure of precolibactin A. However, the retention times of synthetic **5a** and **5b** were distinct and the signals did not coalesce upon co-injection with the natural sample (Figures 2A and 2B, respectively). The retention times of **5a** and **5b** were nearly identical (t_r = 15.70 and 15.80 min, respectively), as expected, and their differences with respect to natural precolibactin A (t_r = 16.56 min) suggested a significant discrepancy in structure.

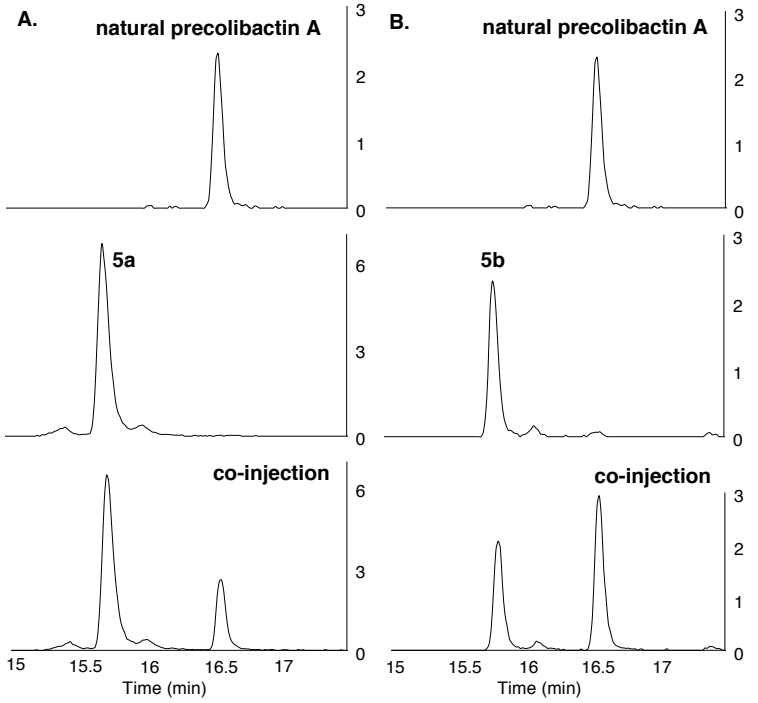
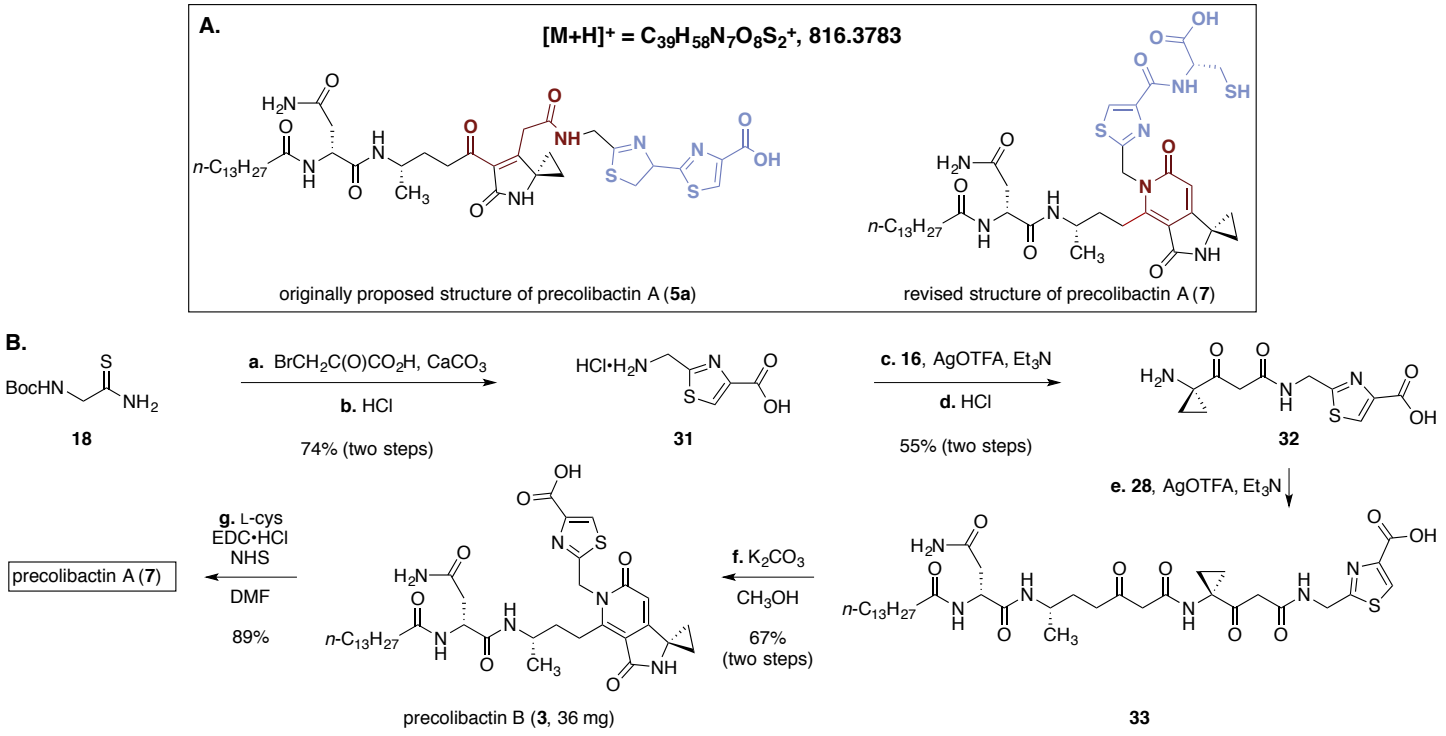


Figure 2. A. Mass-selective (*m*/*z* = 816.3788) LC/HRMS-QTOF analysis of the ethyl acetate extracts of *clb*$^+$ *E. coli* Δ*clbP* (top), synthetic **5a** (middle), and co-injection (bottom). **B.** Mass-selective (*m*/*z* = 816.3788) LC/HRMS-QTOF analysis of the ethyl acetate extracts of *clb*$^+$ *E. coli* Δ*clbP* (top), synthetic **5b** (middle), and co-injection (bottom). Y axis corresponds to relative ion intensity ($\times 10^4$).

In light of the facile cyclization of our synthetic intermediates to the pyridone residues found in precolibactins B (**3**) and C (**6**), we reasoned that precolibactin A may also incorporate this substructure. The original isotope labeling, HRMS, and MSMS data for natural precolibactin A[10a] could not exclude this assignment. In addition, careful inspection of the initial report revealed that precolibactin A production was optimized by increasing the concentration of L-cysteine in the media 5-fold (to 1 g/L). Walsh and co-workers have previously reported the formation of cysteine derailment products in the biosynthesis of yersiniabactin.[21] Accordingly, we hypothesized that the structure of natural precolibactin A may comprise the pyridone found in precolibactins B (**3**) and C (**6**) and a terminal cysteine residue appended to a single thiazole ring (Scheme 5A). This structure (**7**) possesses an exact mass that is identical to the originally predicted structures **5a** or **5b** and would similarly match the reported amino acid isotope labeling studies. Such a change in the terminal

heterocyclic fragment would also be consistent with the large differences in retention times between **5a** or **5b** and natural precolibactin A. The synthesis of the revised precolibactin A structure **7** was readily-accomplished using our synthetic strategy (Scheme 5B). Treatment of *N*-(*tert*-butoxycarbonyl)-2-aminoethanthioamide (**18**) with bromopyruvic acid[22] followed by removal of the *tert*-butoxycarbonyl protective group generated the thiazole **31** (74%, two steps). Silver trifluoroacetate-mediated coupling of **31** and the thioester **16**, followed by carbamate cleavage, formed the amine **32** (55%, two steps). Coupling of the amine **32** with the thioester **28** (Scheme 3; silver trifluoroacetate, triethylamine), followed by double-cyclization (potassium carbonate, methanol) generated precolibactin B (**3**; 67% over two steps, 36 mg). NMR spectroscopic data for synthetic precolibactin B (**3**) and LC/MS co-injection with metabolite extracts matched those of natural material (Figure S1, Tables S3, S4),[11,12] thereby confirming the structure of the natural product.[17] Finally, coupling of precolibactin B (**3**) with L-cysteine mediated by *N*-hydroxysuccinimide (NHS) and EDC•HCl generated **7** (89%). Mass-selective LC/HRMS-QTOF analysis against the concentrated ethyl acetate extracts of *clb*$^+$ *E. coli* Δ*clbP*[10a] revealed that **7** corresponded exactly to natural material (Figure 3). In addition, both synthetic **7** and natural precolibactin A displayed identical mass spectral fragmentation patterns, providing further confirmation of structure (Table S2). As natural precolibactin A is not isolable in amounts sufficient for NMR analysis,[10a] a direct comparison of the NMR spectra of synthetic and natural precolibactin A is not possible at this time.[23]



Scheme 5. A. The originally predicted (**5a**) and revised (**7**) structures of precolibactin A. **B**. Synthesis of the revised structure of precolibactin A (**7**). Reagents and conditions: a. bromopyruvic acid, $CaCO_3$, EtOH, 23 °C, 74%; b. HCl, CH_2Cl_2–dioxane (3:1), 23 °C, >99%; c. AgOTFA, Et_3N, DMF, 0 °C, 55%; d. HCl, CH_2Cl_2–1,4-dioxane (3:1), 23 °C, >99%; e. AgOTFA, Et_3N, DMF, 0 °C; f. K_2CO_3, CH_3OH, 0 °C, 67% (two steps); g. L-cysteine, *N*-hydroxysuccinimide (NHS), EDC•HCl, Et_3N, DMF, 0→23 °C, 89%.

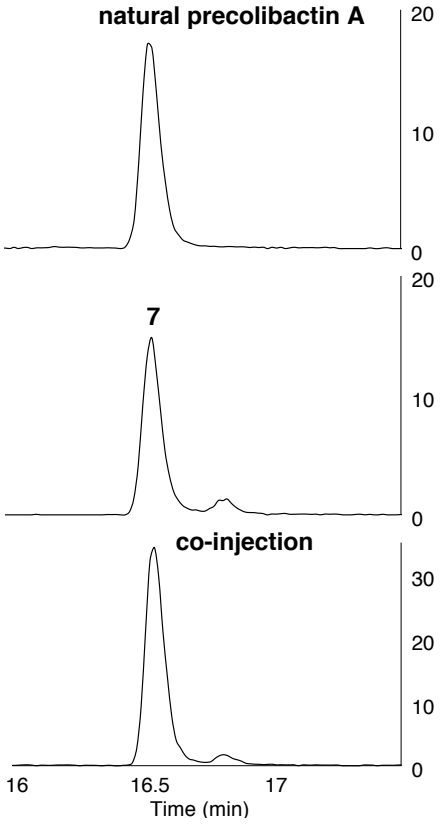


Figure 3. Mass-selective (*m*/*z* = 816.3788) LC/HRMS-QTOF analysis of the ethyl acetate extracts of *clb*$^+$ *E. coli* Δ*clbP* (top), synthetic **7** (middle), and co-injection (bottom).

Discussion and Conclusion.

The colibactins are a fascinating family of natural products that are produced by certain strains of commensal and extraintestinal *E. coli*, and the pathway has been implicated in the progression of colorectal cancer.[7,8] Despite over a decade of intensive research, their complete structures and mechanism of action

have remained unresolved. As highlighted in Figure 1, many of these compounds have been isolated in astoundingly low yields (μg/L) by painstaking fermentation experiments. By bringing the power of modern bioinformatics, enzymology, and mass spectrometry to bear on this problem, the structures of additional precolibactins, which are recalcitrant to isolation, have been predicted.

At the time we began our work, **3**–**6** represented the most complex precolibactin structures in the literature. Precolibactin B (**3**) and **4** were fully characterized by isolation,[10] while **5a**[10a] and precolibactin C (**6**)[11] were predicted. While this manuscript was in preparation, Balskus and co-workers[12] reported the isolation of precolibactin C (**6**; 0.5 mg from a 48 L fermentation) from a mutant strain. Biosynthetic studies now suggest that additional precolibactins incorporating an aminomalonyl unit exist,[12,16] but no evidence for their structures has been presented, to our knowledge.

We have developed a high-yielding and modular synthetic route to the most advanced known precolibactin structures. The left hand fragment **10**, which is common to all of the precolibactins, is prepared in six steps and 63% overall yield from pent-4-en-al (Scheme 1). We have executed the synthesis of four distinct heterocyclic side chain fragments, in 4–7 steps and 37–41% overall yield (Schemes 2 and 5). Finally, these intermediates are elaborated to advanced precolibactins in three steps and ~50% overall yield (Schemes 3, 4, and 5). We have confirmed the structures of the isolated precolibactins B (**3**) and C (**6**) by chemical synthesis, and revised the structure of precolibactin A, from **5a** or **5b** to **7**. This structural revision also supports an unexpected biosynthetic route to colibactin bithiazole formation, in which biosynthesis of the first thiazole ring may precede heterocyclization and oxidation of the C-terminal L-cysteine moiety. This is in contrast to bioinformatic proposals for bleomycin bithiazole biosynthesis.[24] Our synthetic studies also provide insights into the reactivities of these structures, and the facile cyclization of the linear precursors **29a–c** to pyridones suggests this element as a common substructure. We envision that the synthetic strategy we have presented will be amenable to the synthesis of precolibactins incorporating an aminomalonyl substituent or other modifications, as their structures are proposed.

The synthetic route outlined herein provides a means to procure sufficient quantities of material to study the cellular responses to isolated colibactins and elucidate their mechanism of action for the first time. As noted in the introduction, mammalian cells were shown to accumulate DNA DSBs when co-cultured with *pks*$^+$

E. coli cells,[3a] but no essential follow-up studies employing single metabolites derived from the *clb* pathway have been reported, to our knowledge. We expect that our modular synthetic strategy will finally open the door to examining these types of questions regarding colibactin's mode of action with molecular-level resolution.[25]

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Supporting Information Available. Supplementary Figures S1–S6, Supplementary Tables S1–S5, Supplementary Scheme S1, general experimental procedures, and detailed experimental procedures and characterization data for all new compounds. This data is available free of charge at www.pubs.acs.org.

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25. Our data suggest that prior proposals of the colibactin mechanism of action may require revision. See the Supporting Information for a discussion.

TOC Graphic.