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Nat Chem. Author manuscript; available in PMC 2020 March 23.

Published in final edited form as:

Nat Chem. 2019 October ; 11(10): 890–898. doi:10.1038/s41557-019-0338-2.

Synthesis and reactivity of precolibactin 886

Alan R. Healy[a,b], **Kevin M. Wernke**[a], **Chung Sub Kim**[a,b], **Nicholas R. Lees**[a], **Jason M. Crawford**[*,a,b,c], **Seth B. Herzon**[*,a,d]

[a]Department of Chemistry, Yale University, New Haven, Connecticut 06520, United States.

[b]Chemical Biology Institute, Yale University, West Haven, Connecticut 06516, United States.

[c]Department of Microbial Pathogenesis, Yale School of Medicine, New Haven, Connecticut 06536, United States.

[d]Department of Pharmacology, Yale School of Medicine, New Haven, Connecticut 06520, United States

Abstract

The *clb* gene cluster encodes the biosynthesis of metabolites known as precolibactins and colibactins. The *clb* pathway is found in gut commensal *E. coli*, and *clb* metabolites are thought to initiate colorectal cancer via DNA cross-linking. Here we report confirmation of the structural assignment of the complex *clb* product precolibactin 886 via a biomimetic synthetic pathway. We show that a α-ketoimine linear precursor undergoes spontaneous cyclization to precolibactin 886 upon HPLC purification. Studies of this α-ketoimine and the related α-dicarbonyl revealed that these compounds are unexpectedly susceptible to nucleophilic cleavage under mildly basic conditions. This cleavage pathway forms other known *clb* metabolites or biosynthetic intermediates and explains the difficulties in isolating fully mature biosynthetic products. This cleavage also accounts for a recently identified colibactin–adenine adduct. The colibactin peptidase ClbP deacylates synthetic precolibactin 886 to form a non-genotoxic pyridone, suggesting precolibactin 886 lies off-path of the major biosynthetic route.

Graphical Abstract

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* seth.herzon@yale.edu; jason.crawford@yale.edu.

Author contributions. A.R.H., K.M.W., and N.R.L carried out the synthetic experiments. C.S.K conducted purification of precolibactin 886 (**1**) and ClbP deacylation studies. S.B.H. and J.M.C. oversaw the project. S.B.H. wrote the manuscript. All authors discussed the results and commented on the manuscript.

Competing interest. The authors declare no competing interests.

Data availability statement. All relevant data are available from the authors, and/or are included with the manuscript and Supplementary Information.

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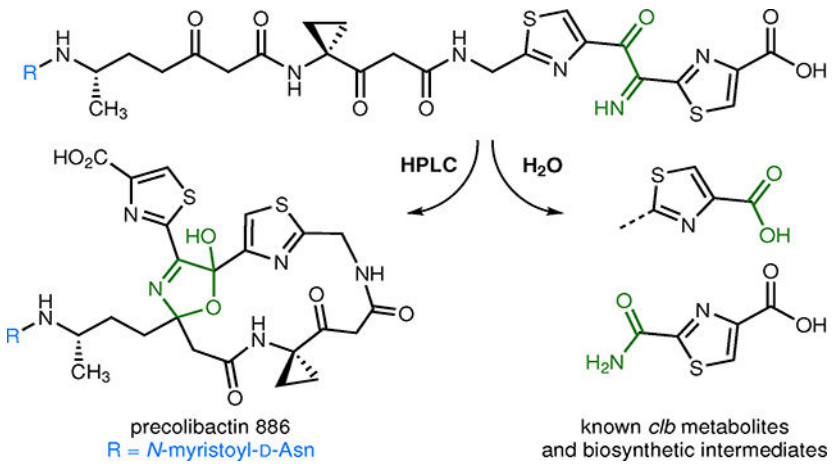

In a seminal study, Oswald and co-workers discovered that strains of commensal *E. coli* containing a biosynthetic gene cluster – referred to as *clb* or *pks* – induce DNA damage in eukaryotic cells.[1] Subsequent, the presence of *clb*$^{+}$ *E. coli* was shown to be epidemiologically correlated with colorectal cancer (CRC) in humans,[2,3] and literature now suggests these bacteria are tumorigenic.[4–9] Therefore, elucidating the structures and mechanism of action of *clb* metabolites may help to understand if and how these metabolites contribute to the formation of CRC.

Precolibactin 886 (**1**, Fig. 1a)[10] is one of the most complex isolated metabolites derived from the *clb* pathway; it accounts for all but three (*clbL*, *clbO*, and *clbQ*) biosynthetic enzymes in the gene cluster.[10,11] The C37 and C38 atoms of precolibactin 886 (**1**) derive from an α-aminomalonate building block which originates biosynthetically from serine.[10,12,13] Bacteria deficient in the genes responsible for the biosynthesis and transfer of this polyketide extender unit (*clbDEFG* and an adenylation (A) domain of *clbH-A*$_1$) are not genotoxic, indicating that the α-aminomalonate building block is required for cytotoxicity.[1,12,13]

Precolibactin 886 (**1**) was isolated by Qian, Moore, and co-workers from the fermentation broth of a *clbP/clbQ* double mutant.[10] Recently, a derivative of precolibactin 886 (**1**) bearing a terminal oxazole was disclosed.[14] Deactivation of the pathway-dedicated serine protease ClbP[15,16] has been widely used to facilitate the isolation of *clb* metabolites. This modification leads to the persistence of a terminal *N*-myristoyl-D-Asn residue (blue in **1**)[17–19] and accumulation of the more stable precolibactins. Deactivation of the type-II thioesterase ClbQ,[20] which is required for genotoxicity,[1] increased the production of precolibactin 886 (**1**) 22-fold by reducing the release of advanced intermediates from the biosynthetic assembly line.[10] Despite the use of this strategically-optimized double mutant, only 2.8 mg of precolibactin 886 (**1**) were obtained from a 1000-L fermentation.[10] Qian and Moore proposed that the 15-atom macrocycle and 5-hydroxy-3-oxazoline ring (green in **1**) found in precolibactin 886 (**1**) arises from the enzymatic oxidation and cyclization of a linear α-ketoamine precursor. This proposal is supported by literature demonstrating the addition of α-ketoimines to ketones.[21]

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In wild-type strains possessing functional ClbP, *N*-deacylation initiates an alternative reaction pathway that ultimately forms unsaturated imines resembling **4** (Fig. 1b).[11,22] Synthetic derivatives of **4** alkylated linearized plasmid DNA by nucleotide addition to the cyclopropane and activated DNA damage response pathways in human cells.[22,23] A dimer of **4** formed stable DNA cross-links, and a derivative possessing a *gem*-dimethyl group in place of the cyclopropane did not damage DNA, providing robust support for DNA alkylation by cyclopropane opening.[16] Further supporting this model, the adenine adduct **5** was subsequently identified in *clb*$^+$ *E. coli* cultures treated with exogenous DNA.[24] This product was later established as an N3-substituted adenine adduct, which could also be detected in human cells and in colonic epithelial cells of a mouse model when treated with *clb*$^+$ *E. coli*.[25] These studies suggest a basis for *clb*$^+$ *E. coli* genotoxicity and explain earlier determinations that functional ClbP is required for cytopathic effects.[1,15–19]

Here we report a biomimetic synthesis of precolibactin 886 (**1**) and the surprising discovery that the C36–C37 bond in **2** is unstable toward nucleophilic cleavage. This degradation generates other known *clb* metabolites and biosynthetic precursors, and explains the difficulties in isolating advanced *clb* metabolites. We show that ClbP deacylation of precolibactin 886 (**1**) forms a non-genotoxic pyridone, suggesting it lies off of the major colibactin biosynthetic pathway.

Results

Total synthesis of the linear precursor 2

Our retrosynthetic analysis is shown in Fig. 1c. As the α-ketoimine motif was found to be unstable, it was introduced in the final step. We envisioned accessing **2** by double oxidation of the amino alcohol **9**. The latter was readily deconstructed to the β-ketothioesters **10** and **11**[26] and the azido alcohol **12**.

The synthesis of the azido alcohol **12** is shown in Fig. 2. The thiazole **13** is accessible in one step and 74% yield from commercial reagents.[26] Semi-reduction of the ester (di-*iso*-butylaluminum hydride, DIBAL-H) provided the aldehyde **14** (88%). Alternatively, removal of the carbamate (hydrochloric acid) followed by condensation with benzophenone imine generated the imine **15** (85%, two steps). This approach exploits the pseudo-symmetry within the thiazoles and greatly expedited material throughput. Silver-catalyzed coupling[27,28] of **14** and **15**, followed by hydrolysis, then provided the amino alcohol **16** (2:1 mixture of diastereomers, stereochemistry not assigned). Wong diazo transfer[29,30] to **16** using imidazole sulfonyl azide[31] in methanol yielded the azido alcohol **17** with concomitant transesterification of the ester (56% over three steps). Treatment of the diazo transfer product **17** with hydrochloric acid then formed the primary ammonium ion **12**. Coupling with the β-ketothioester **11** (silver trifluoroacetate, triethylamine) generated the β-ketoamide **18** (85%, two steps).[26] Saponification of the ester (lithium hydroxide, 87%) followed by removal of the *tert*-butyl carbamate (hydrochloric acid) produced the carboxylic acid **19**. A second silver-mediated fragment coupling between **19** and the β-ketothioester **10** provided the linear intermediate **20** (78%, two steps). Staudinger reduction of **20** (trimethylphosphine) proceeded smoothly to generate the target amino alcohol **9** (>99%).

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Oxidation and cyclization studies

We then probed the oxidation of **9** to the α-ketoimine **2** (Fig. 3a). However, the reaction was difficult to monitor by thin-layered chromatography (TLC) or liquid chromatography/mass spectrometry (LC/MS), and all attempts to isolate the product derived from two-fold oxidation of **9** were unsuccessful. We surmised that the α-ketoimine **2**, if formed, was hydrating or hydrolyzing on attempted isolation.[32,33] To circumvent this, we studied the oxidation of **9** by NMR spectroscopy. The addition of 4.0 equiv of 2-iodoxybenzoic acid (IBX) to a solution of **9** in methyl sulfoxide-d_6 at 23 °C resulted in consumption of the amino alcohol **9** within 30 min. A sharp singlet that we attributed to the *N*-H resonance of the α-ketoimine emerged at δ 12.02 ppm. However, this resonance under-integrated relative to the C20 methyl group, suggesting transformation of the starting material to other unidentified products.

Nonetheless, we attempted to promote macrocyclization of the α-ketoimine **2** under thermal, basic, or acidic conditions. However, in each instance we were unable to detect (by ^{1}H NMR spectroscopy) any signals that could be attributed to precolibactin 886 (**1**). Under most conditions, we observed hydrolysis to the α-diketone **22**, as evidenced by a gradual disappearance of the *N*–H resonance and the appearance of a new signal at 185.8 ppm in the ^{13}C NMR spectrum (assigned as the C37 carbonyl). The hydrolysis was faster under acidic conditions, as expected. Surprisingly, under basic conditions precolibactin B (**7**) was formed by an unforeseen hydrolytic cleavage of the C36–C37 bond, followed by cyclodehydration.[26] Attempts to promote the cyclization thermally led to decomposition.

In light of these difficulties, we hypothesized that in the isolation studies[10] cyclization to precolibactin 886 (**1**) occurred during HPLC (high pressure liquid chromatography) analysis and purification. Such reactivity would be undetectable because all reported fermentations of *clb*$^+$ *E. coli* have been analyzed (and purified) by HPLC, to our knowledge. To test this, we directly injected solutions of the α-ketoimine **2** onto a semipreparative HPLC system. Under these conditions cyclization did occur, and from 10 mg of **2** we obtained ~0.3 mg of precolibactin 886 (**1**, ~3%). Spectroscopic data for synthetic precolibactin 886 (**1**) were in full agreement with natural material[10] (See Supplementary Table 1 and Supplementary Fig. 2). The C36 stereocenter was formed as a ~1:1.9 mixture of diastereomers in the ring closure (stereochemistry not assigned). The dr of natural precolibactin 886 (**1**), which also formed non-stereoselectively, was not reported.[10] Importantly, H32 are magnetically-equivalent in the linear precursor **2** and appear as an apparent doublet (δ 4.52, J = 6.0 Hz). Formation of the macrocycle breaks this degeneracy leading to a doublets of doublets resonating at δ 4.27 (J = 17.2, 5.0 Hz) and δ 4.79 (J = 17.2, 7.1 Hz). We did not observe cyclization of the amino alcohol **9** or the monooxidation product **21**, suggesting the imine is formed prior to macrocyclization in the bacterial extracts. Finally, precolibactin 886 (**1**) could also be accessed in comparable yield from the azido alcohol **20**. Oxidation of **20** with 2-iodoxybenzoic acid (IBX) formed the α-ketoimine **2** directly, presumably via loss of dinitrogen from an α-azidoketone intermediate.[30] The generation of precolibactin 886 (**1**) from the azido alcohol **20** provides further support for the intermediacy of the α-ketoimine **2**.

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After confirming the formation of precolibactin 886 (**1**) during HPLC purification, we attempted to form precolibactin 886 (**1**) deliberately by evaluating the cyclization of the α-ketoimine **2** in aqueous methanol or aqueous acetonitrile. While we were able to observe precolibactin 886 (**1**) by HPLC/MS analysis, we did not observe an increase in isolated yield after incubating for 24 h before purification. As a result, we conclude that a small percentage of the fleetingly stable α-ketoimine **2** cyclizes to form macrocylic precolibactin 886 (**1**) during LC/MS analysis/HPLC purification and that this isolate is an artifact of these analytical methods.

On the chemical instability of the α-ketoimine 2

The low yield of precolibactin 886 (**1**) and the unexpected generation of precolibactin B (**7**) from **9** motivated us to study the reactivity of the model α-ketoimine **25** and its hydrolysis product **26** in detail (Fig. 3b and Table 1). The amino alcohol **23** (see Supplementary Fig. 1 for synthesis) was efficiently oxidized to the α-ketoimine **25** by treatment with excess Swern reagent. ^{1}H NMR analysis indicated that the sample was >95% pure (nominally 93% yield). The imine *N*–H resonance appeared at δ 12.07 ppm in methyl sulfoxide-d_6, in good agreement with the fully elongated α-ketoimine **2** (δ 12.02 ppm). As expected, the imine readily hydrolyzed. For example, the α-diketone **26** began to form if the α-ketoimine **25** was briefly exposed to aqueous conditions on work-up. Alternatively, the addition of silica gel to solutions of the α-ketoimine **25** formed the α-diketone **26** (38% from **23**). The α-diketone **26** was independently synthesized by Wong diazo transfer[29,30] to **23** using imidazole sulfonyl azide[31] (96%), followed by oxidation with the Dess–Martin periodinane[34] (DMP, 2.5 equiv, 54%). The C37 carbon atom of **26** was observed at δ 184.6 in methyl sulfoxide-d_6, in good agreement with the fully elongated α-diketone **22** (δ 185.8).

Studies of the oxidation of the *N*-(*tert*-butoxycarbonyl)-1,2-amino alcohol **27** provided unexpected results (Fig. 3c). Oxidation with an excess of DMP, under Swern conditions, or using sulfur trioxide–pyridine complex formed the product of two-fold oxidation, the hemiaminal **29**. Attempted oxidation of **27** using 0.9 equiv of DMP, followed by immediate purification by flash-column chromatography, generated the expected α-amino ketone **28**, but this spontaneously oxidized to **29** after prolonged exposure to air. These data suggest intermediates such as **28** undergo facile oxidation and support a pathway for the spontaneous generation of the α-ketoimine in the precolibactin 886 precursor **2** from the α-aminoketone substrate expected to be formed based on biosynthetic logic.

We then studied the reactivity of the α-ketomine **25** and the α-diketone **26** in detail (Table 1). Unexpectedly, **25** and **26** readily underwent cleavage of the C36–C37 bond [precolibactin 886 (**1**) numbering]. For example, dissolution of **25** in methanol followed by the addition of sodium bicarbonate resulted in formation of the ester **30a** (42%) and the carboxamide **30d**. Bond cleavage even occurred in the absence of base, albeit more slowly: the half-life of the α-ketoimine **25** in neutral, distilled methanol-d_4 was ~12 h at 24 °C (^{1}H NMR analysis). Alternatively, the α-ketoimine **25** was transformed to the carboxylic acid **30b** by treatment with saturated aqueous sodium bicarbonate solution in tetrahydrofuran (51%), and the amide **30c** was formed by treatment with excess pyrrolidine (36%). In the case of entries 2 and 3 the products derived from the right-hand thiazole were not readily identified. The α-diketone

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26 underwent parallel transformations forming the methyl esters **30a** and **30e**, the carboxylic acids **30b** and **30f**, and the amide **30c**. The remainder of the mass balance in these reactions is accounted for by the formation of unidentified decomposition products.

In light of this, we re-examined the products resulting from oxidation of the amino alcohol **9** by LC/MS and tandem MS. As expected, we detected hydrolysis of the α-ketoimine **2** to its corresponding α-diketone **22** (Fig. 4a, Supplementary Fig. 4). We also detected hydrolytic cleavage of the C36–C37 bond to generate **32**, and, as reported in an earlier study,[26] double ring closure of **32** to provide precolibactin B (**7**). The addition of L-cysteine resulted in generation of **33**, the linear precursor to precolibactin A (**6**, Fig. 1b). The structures of **31–33** and precolibactin B (**7**) were confirmed by tandem MS and LC/MS co-injection with the bacterial extracts derived from a *clb*$^{+}$ *ΔclbP E. coli* culture (Supplementary Figs. 3, 5–7). We also detected competitive hydrolysis of the C23–C24 bond to generate the acylasparagine derivative **31**, which has previously been detected in *clb* cultures.[19] The formation of these products from the α-ketoimine **2** under mild conditions suggests that this intermediate is decomposing during HPLC purification and provides an explanation for the low overall yield of precolibactin 886 (**1**).

To establish precolibactin 886 (**1**) as a potential substrate of the promiscuous colibactin peptidase ClbP, we added **1** to cultures of *E. coli* expressing functional ClbP (pPEB018)[19] and a control strain lacking ClbP (pBAD18) (Fig. 4b). The expected deacylation product **34** was formed in the ClbP expressing strain but it was undetectable in the ClbP-deficient strain. The concentration of **34** increased in a time-dependent fashion. However, we determined that the macrocycle **34** was unstable under the cultivation conditions. As it was produced, **34** underwent conversion to the pyridone **35**. The structure of **35** formed in this experiment was confirmed by LC/MS co-injection and tandem MS with a synthetic standard (Supplementary Fig. 8). The pyridone **35** derives from C36–C37 bond cleavage and double cyclodehydration.

Discussion

The prevailing model for colibactin biosynthesis and genotoxicity involves off-loading of fully linear products (see **3**, Fig. 1b) from the biosynthetic assembly line. In wild-type strains these intermediates are deacylated in the periplasm by ClbP and transform, via spontaneous cyclization reactions, to genotoxic unsaturated imines (see **4**, Fig. 1b). In mutant strains lacking functional ClbP, artificial cyclization reactions prevail resulting in the generation of non-genotoxic pyridone-containing compounds (*e.g.*, **6**–**8**, Fig. 1b).[7,22] The studies reported herein allow us to expand this model to accommodate the structure of precolibactin 886 (**1**, Fig. 5a). Our data suggest a biosynthetic pathway for **1** involving off-loading of the linear α-amino ketone **36** from the assembly line. It has previously been suggested that **36** is oxidized by the enzyme ClbK;[10] however, our studies of the amino alcohol **27** (Fig. 3c) raise the possibility that oxidation occurs spontaneously. Irrespective of how it is formed, the resulting α-ketoimine **2** then partitions among several reaction pathways. We speculate that in wild-type or mutant strains hydrolysis of the α-ketoimine to the α-diketone **22** is rapid and predominates. In mutant strains lacking functional ClbP, the hydrolysis product **22** degrades by cleavage of the C23–C24 bond and/or cleavage of the C36–C37 bond. The latter pathway generates metabolites such as precolibactin A (**6**)[26,35] and precolibactin B (**7**)[36] that

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have previously been detected in *ΔclbP* cultures. In wild-type strains, deacylation of **22** followed by cyclocondensation generates unsaturated imines **4** structurally analogous to those formed from simpler *clb* metabolites. With the difficulties we encountered in inducing cyclization of the linear α-ketoimine **2** to precolibactin 886 (**1**), and the very low yield of the latter from prior isolation studies,[10] we suggest that macrocyclization is a minor pathway. This is also consistent with our observation that ClbP cleavage of **1** leads to the non-genotoxic pyridone breakdown product **35** (Fig. 4b) as the major detectable product in cell culture.

The facile C36–C37 bond cleavage we have observed has several important implications for colibactins and precolibactins First the adenine adduct **5** (Fig. 1b) was characterized in the digestion mixtures of DNA that had been cross-linked by *clb*$^+$ *E. coli*.[24,25] While it is conceivable that **5** derives from potentially more abundant monoalkylation products, the facile cleavage of the α-dicarbonyl residue we have documented raises the possibility that **5** is generated by degradation of a cross-linked product. While genetic studies indicate that every gene in the *clb* cluster is required for cytopathic effects,[1] implicating the fully elaborated colibactin as the causative genotoxic agent, cleavage of the C36–C37 bond provides an explanation for the difficulties encountered in identifying this molecule (or the corresponding precolibactin). The remaining biosynthetically unaccounted *clb* enzymes ClbL and ClbO, which are required for genotoxicity, append substituents to the terminal thiazole of advanced *clb* metabolites. C36–C37 bond cleavage would effectively remove these substituents from advanced precolibactins, rendering isolation of the corresponding precolibactin or colibactin products impossible. A possible mechanism for the hydrolytic cleavage of the C36–C37 bond in the α-ketoimine **2** is shown in Fig. 5b. Formation of the hemiaminal **37**, followed by rearrangement would generate the enolester **39**. Hydrolysis would generate the carboxylic acid **32** and liberate the enol **40**. Oxidation of the enol **40** would generate the observed carboxamide **30d**. This mechanism is reminiscent of thiazolium ion reactivity,[37] but employs a thiazole rather than a thiazolium ion as the nascent leaving group. Related pathways for the cleavage of 1,2-dicarbonyl compounds by thiazolium ions have been proposed.[38]

Conclusion

In sum, we have reported the first total synthesis of precolibactin 886 (**1**), one of the most advanced *clb* products. Our studies confirm the structural assignment of this unusual metabolite, and suggest it may be an artifact deriving from HPLC analysis and purification of the bacterial extracts. Significantly, our data have revealed the unexpected electrophilic reactivity of the α-ketoimine of precolibactin 886 (**1**); the mild cleavage of the C36–C37 bond we have observed provides an explanation for the difficulties in isolating advanced *clb* metabolites and accounts for the structures of recently isolated colibactin–adenine adducts. Finally, our synthetic studies suggest the unusual α-ketoimine may arise by spontaneous oxidation of an unstable α-amino ketone. This work defines the unexpected reactivities of advanced *clb* metabolites, provides a path for characterizing the structures of DNA crosslinked products, and explains the underlying cellular and chemical degradation pathways of (pre)colibactins.

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Supplementary Material

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Acknowledgement

We thank Professor David Spiegel for helpful discussions regarding the mechanism of hydrolytic cleavage of **2**. Financial support from the National Institutes of Health (R01GM110506 to S.B.H., 1DP2-CA186575 to J.M.C., R01CA215553 to S.B.H. and J.M.C.), the Chemistry Biology Interface Training Program (T32GM067543 to K.M.W.), the Charles H. Revson foundation (postdoctoral fellowship to A.R.H.) and Yale University is gratefully acknowledged.

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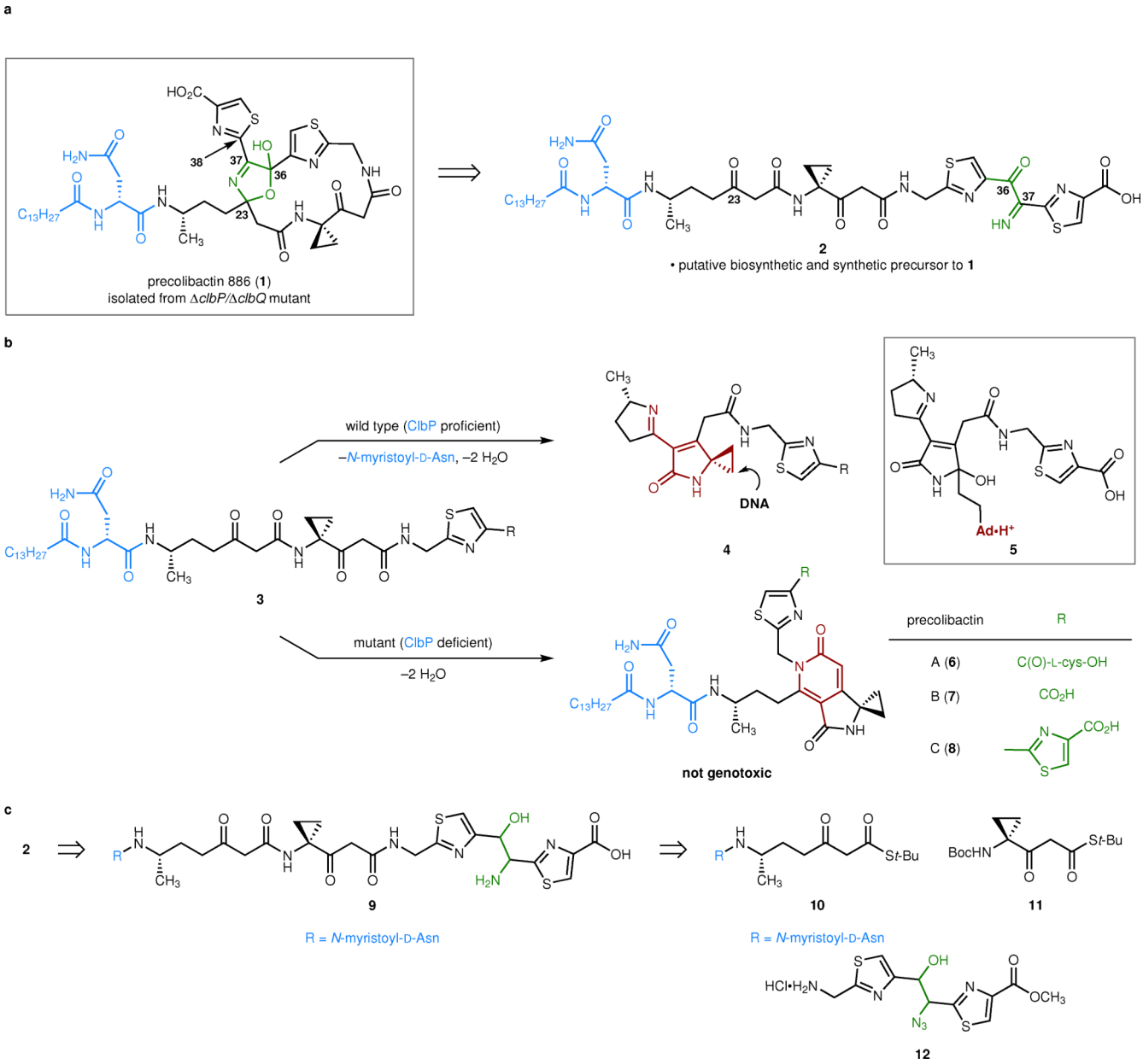

Fig. 1.

Overview of (pre)colibactin reactivity, mechanism of genotoxicity, and the synthetic strategy pursued herein. **a**, Structure of precolibactin 886 (**1**) and the putative biosynthetic precursor **2**. The α-ketoimine **2** served as a synthetic precursor to precolibactin 886 (**1**). **b**, Genetic studies established that linear intermediates resembling **3** are off-loaded from the *clb* biosynthetic assembly line. In wild-type strains, these products undergo ClbP deacylation and transformation to the genotoxic imines **4**. In mutant strains lacking functional ClbP, they transform to non-genotoxic pyridones, such as precolibactins A–C (**6**–**8**). The adenine adduct **5** was derived from DNA that had been exposed to *clb*$^{+}$ *E. coli* cultures. **c**, Retrosynthetic analysis of the α-ketoimine **2**.

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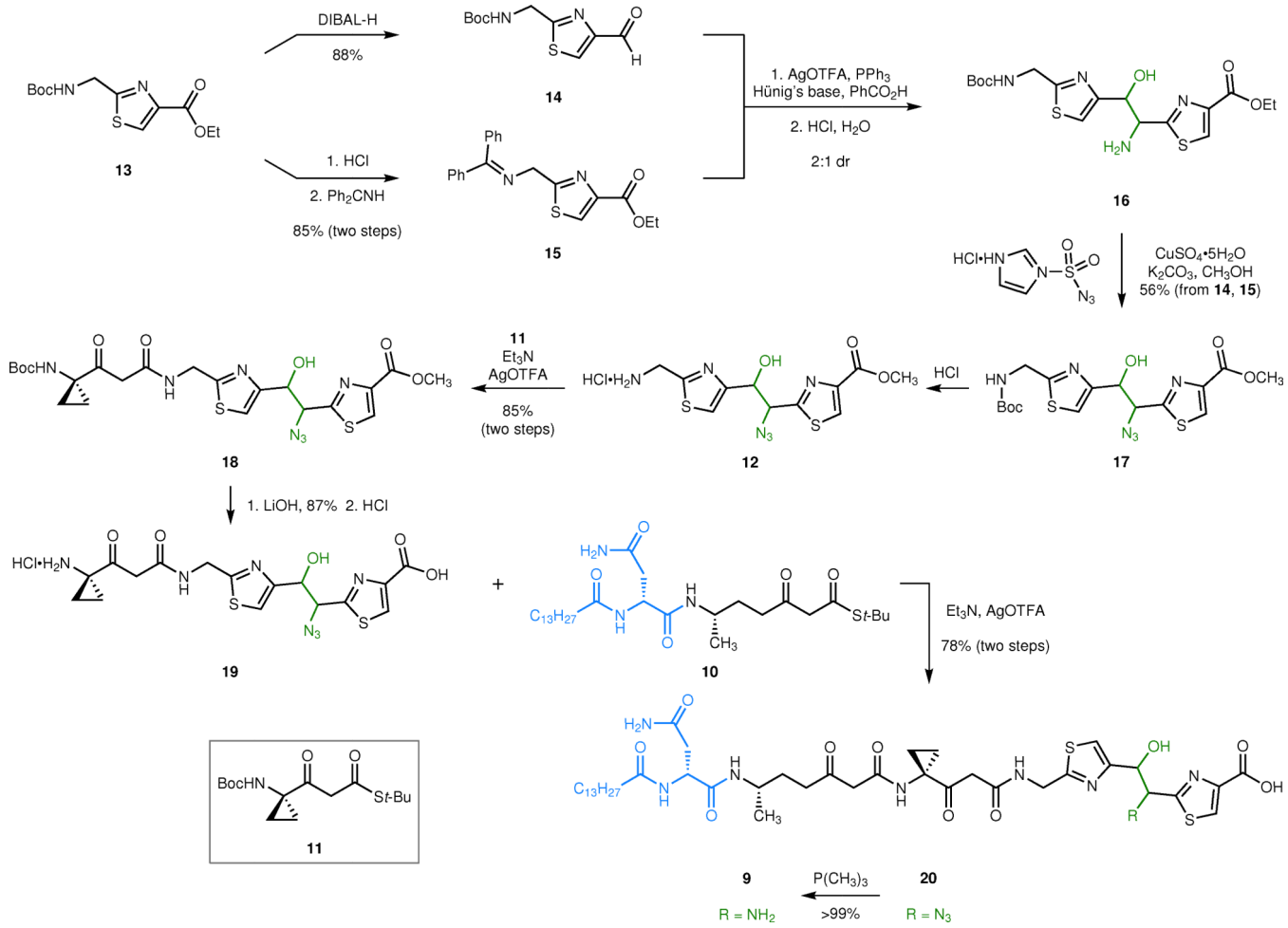

Fig. 2.

Synthesis of the azide **20** and the amino alcohol **9**. Our synthetic approach exploits the pseudo-symmetry within the linked thiazole residues to access this fragment from the single synthetic intermediate **13**. The linear precursors **9** and **20** are assembled by sequential *N*-acylation reactions, using the β-ketothioesters **10** and **11** as acylating agents.

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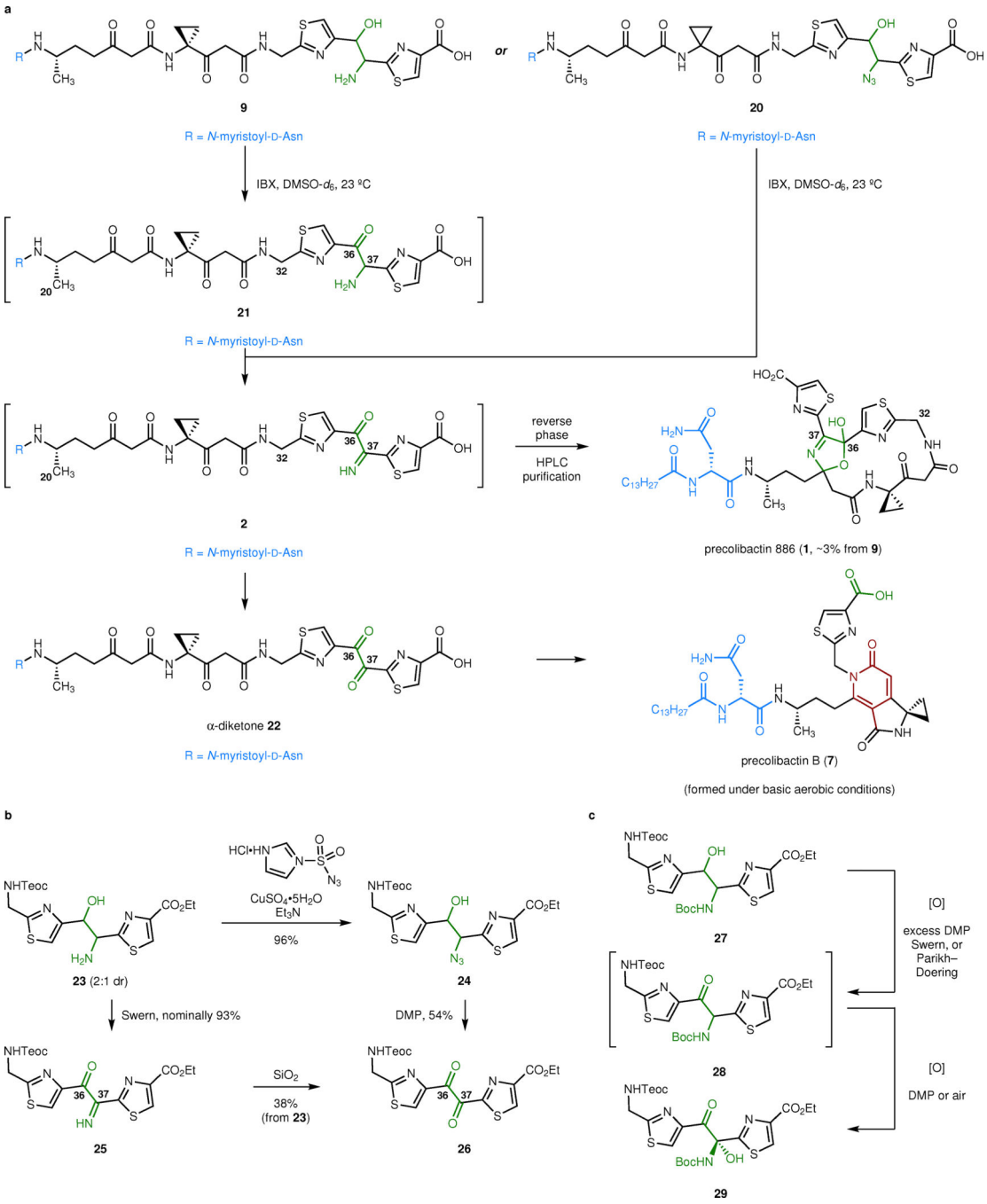

Fig. 3.

Synthesis of precolibactin 886 (**1**) and reactivity of the α-ketoimine and α-dicarbonyl residues. **a**, Two-fold oxidation of the 1,2-aminoalcohol **9** generates the α-ketoimine **2**. This intermediate is unstable toward hydrolysis to the α-diketone **22**. We were unable to effect the cyclization of **2** under a broad range of acidic, basic, or thermal conditions. However, we found that the α-ketoimine **2** converted to precolibactin 886 (**1**) upon HPLC purification. Oxidation of the azidoalcohol **20** with excess IBX also formed the α-ketoimine **2**, presumably by loss of dinitrogen from an α-ketoazide intermediate (not shown). **b**,

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Synthesis of the α-ketoimine **25** and the α-dicarbonyl **26**. The α-ketoimine **25** partially hydrolyzed to the α-dicarbonyl **26** upon exposure to water during work-up. Alternatively, this intermediate could be deliberately transformed to **26** by exposure to silica gel. **c**, Oxidation of the *N*-(*tert*-butoxycarbonyl)-1,2-amino alcohol **27**. Oxidation with excess DMP generated the *N*-(*tert*-butoxycarbonyl) aminal **29**. Alternatively, oxidation with 0.9 equiv of DMP formed the α-aminoketone **28**. The α-aminoketone **28** transformed spontaneously to the hemiaminal **29** on standing.

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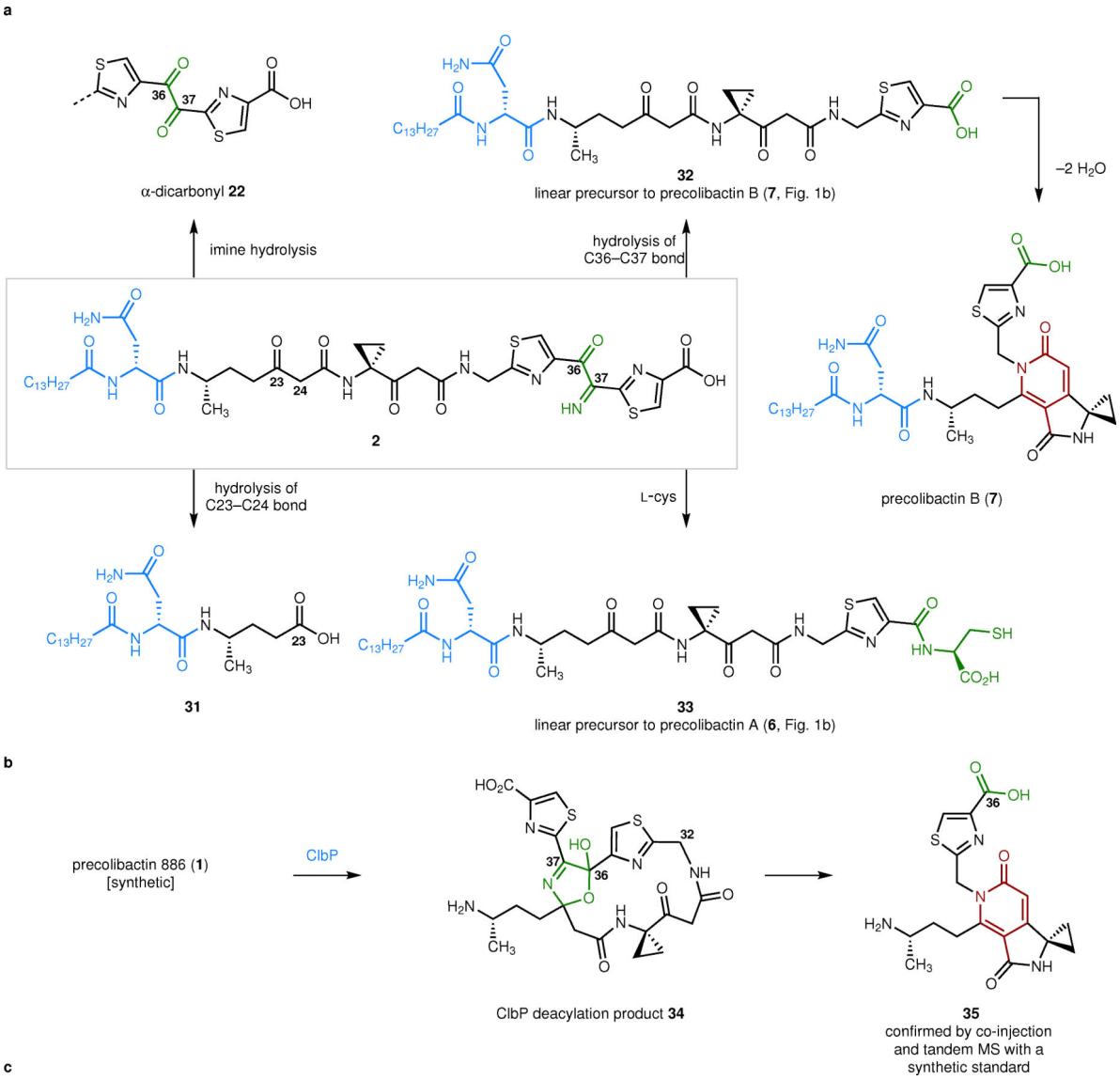

Fig. 4.

The α-ketoimine **2** undergoes transformation to known *clb* metabolites or biosynthetic intermediates under mild conditions, while ClbP deacylation of precolibactin 886 (**1**) forms the non-genotoxic pyridone **35**. **a**, Cleavage reactions of the α-ketoimine **2**. Hydrolysis of **2** generates the α-dicarbonyl **22**. Alternatively, hydrolytic cleavage of the C23–C24 or C36–C37 bonds in **2** forms the carboxylic acids **31** and **32**, respectively. The cleavage product **32** was found to convert to precolibactin B (**7**). The addition of L-cysteine to **2** results in cleavage of the C36–C37 bond and generation of the *N*-acyl cysteine derivative **33**. **b**, ClbP deacylation of synthetic precolibactin 886 (**1**) generates the amine **34**. The amine **34** was found to convert to the pyridone **35**, suggesting that precolibactin 886 (**1**) lies off of the major biosynthetic pathway **c**, Time course analysis of the conversion of synthetic precolibactin 886 (**1**) to the deacylation product **34** and the pyridone **35** when exposed to *E. coli* expressing or lacking functional ClbP. The conversion of precolibactin 886 (**1**) was only detected in the presence of a ClbP-expressing strain ($n = 1$).

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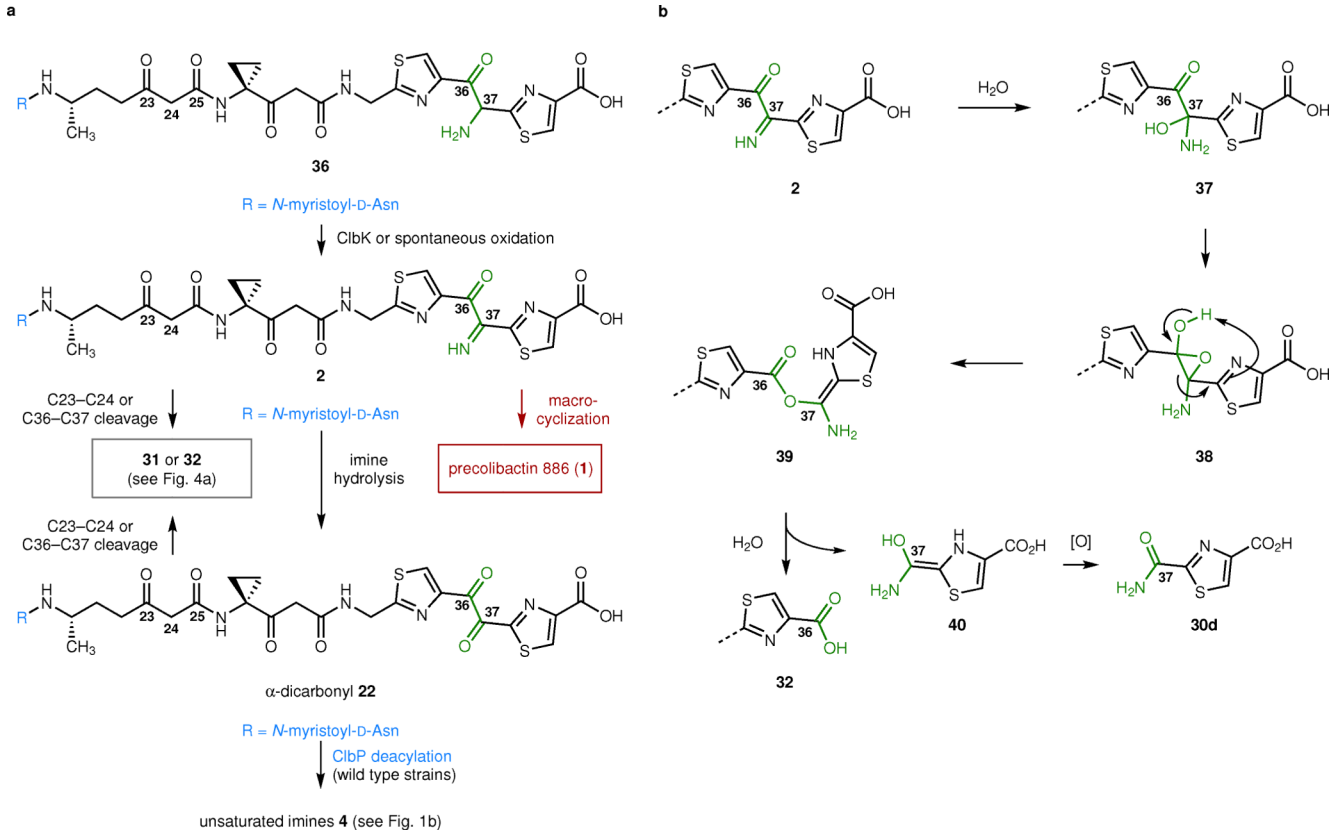

Fig. 5.

Biosynthetic and chemical reactivity model based on the studies reported herein. **a**, Our data suggest that the α-ketoamine **36** is off-loaded from the biosynthetic assembly line. This product undergoes oxidation, either spontaneously or by the enzyme ClbK. The α-ketoimine **2** that is formed then partitions among several pathways. Hydrolytic cleavage of the C23–C24 or C36–C37 bonds forms the carboxylic acids **31** or **32**, respectively (see Fig. 4a). Alternatively, macrocyclization (upon HPLC analysis or purification) generates precolibactin 886 (**1**). Hydrolysis of the imine forms the α-dicarbonyl **22**. This intermediate is also susceptible to hydrolytic cleavage, to provide the carboxylic acids **31** or **32**, respectively (see Fig. 4a). ClbP deacylation of the α-dicarbonyl in wild-type strains may generate unsaturated imines resembling **4** (Fig. 1b). **c**, Potential mechanism for hydrolytic cleavage of the α-ketoimine **2** or the α-dicarbonyl **22**. Hydration of the α-ketoimine **2** generates the hemiaminal **37**. Isomerization to the hydroxyepoxide **38**, followed by rearrangement, forms the enol ester **39**. Addition of water to the C36 carbonyl of **39**, followed by collapse of the tetrahedral intermediate, provides the carboxylic acid **32** and the aminoenol **40**. Oxidation of **40** generates the thiazole **30d**.

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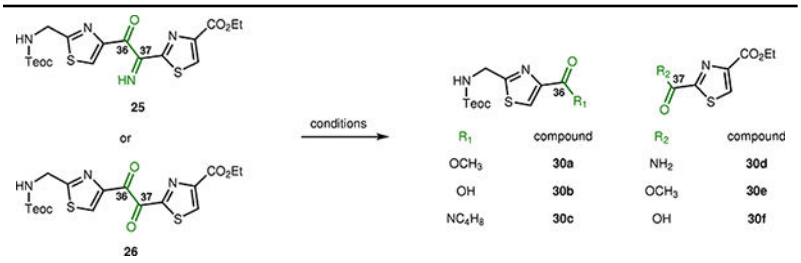
Table 1.

Nucleophilic cleavage of the α-ketoimine 25 and the α-diketone 26.



entry	substrate	nucleophile	product(s) (yield)[a]
1	**25**	methanol[b]	**30a** (42%) + **30d** (~42%)[e]
2	**25**	water[c]	**30b** (51%)
3	**25**	pyrrolidine[d]	**30c** (36%)
4	**26**	methanol[b]	**30a** (45%) + **30e** (41%)
5	**26**	water[c]	**30b** (49%) + **30f** (25%)
6	**26**	pyrrolidine[d]	**30c** (41%)

[a] Isolated yield after purification by flash-column chromatography.

[b] **25** or **26** (1 equiv), $NaHCO_3$ (9.0 equiv), methanol, 23 °C, 48 h (for **25** and **26**).

[c] **25** or **26** (1 equiv), tetrahydrofuran–saturated aqueous sodium bicarbonate solution (1:1, v/v), 23 °C, 48 h (for **25**), 72 h (for **26**).

[d] **25** or **26** (1 equiv), pyrrolidine (14 equiv), dichloromethane, 23 °C, 48 h (for **25**), 72 h (for **26**).

[e] Estimated by HPLC analysis of the product mixture.