

## REVIEW

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# Biosynthesis and bioactivities of microbial genotoxin colibactins

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Covering: up to 2021

Colibactin(s), a group of secondary metabolites produced by the *pks* island (*clb* cluster) of *Escherichia coli*, shows genotoxicity relevant to colorectal cancer and thus significantly affects human health. Over the last 15 years, substantial efforts have been exerted to reveal the molecular structure of colibactin, but progress is slow owing to its instability, low titer, and elusive and complex biosynthesis logic. Fortunately, benefiting from the discovery of the prodrug mechanism, over 40 precursors of colibactin have been reported. Some key biosynthesis genes located on the *pks* island have also been characterised. Using an integrated bioinformatics, metabolomics, and chemical synthesis approach, researchers have recently characterised the structure and possible biosynthesis processes of colibactin, thereby providing new insights into the unique biosynthesis logic and the underlying mechanism of the biological activity of colibactin. Early developments in the study of colibactin have been summarised in several previous reviews covering various study periods, whereas the two most recent reviews have focused primarily on the chemical synthesis of colibactin. The present review aims to provide an update on the biosynthesis and bioactivities of colibactin.

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1. Introduction
2. Discovery of precolibactins and colibactins
  - 2.1. Prodrug mechanism in colibactin biosynthesis
  - 2.2. Identification of various precolibactins
    - 2.2.1. Brief overview of precolibactin discovery and nomenclature suggestion
    - 2.2.2. Discovery of precolibactins deciphers the colibactin(s) biosynthesis logic
  - 2.3. Identification of various colibactins
    - 2.3.1. Discovery of colibactin-645
    - 2.3.2. Discovery of colibactin-770
    - 2.3.3. Discovery of colibactin-788
  - 2.4. Late-stage colibactin biosynthesis
    - 2.4.1. ClbL and ClbQ participate in precolibactin-1491 (24) biosynthesis
    - 2.4.2. Cyclisation
  - 2.5. Degradation
3. Unique mechanisms in colibactin biosynthesis
  - 3.1. Unusual PKS extender unit AM-ACP and deteriorated AT domain in PKS
  - 3.2. ClbH uses SAM to form a spiro-cyclopropane ring
  - 3.3. ClbQ, a type-II TE, is responsible for the offloading procedure
  - 3.4. ClbL, an amidase, is responsible for amide-bond formation
  - 3.5. Self-resistance mechanism and MATE transporter ClbM
4. Biological activities of *clb* metabolites
  - 4.1. DNA-damage activity of colibactins and the underlying mechanisms
  - 4.2. Other bioactivities of *clb* metabolites
    - 4.2.1. Cytotoxicity
    - 4.2.2. Antibacterial activity
    - 4.2.3. Inflammation-related activities
    - 4.2.4. Analgesic activity
5. Relationship between colibactin and CRC formation
  - 5.1. Colibactin plays a carcinogenic role in CRC
  - 5.2. Colibactin modulates the tumour microenvironment to promote cancer growth
6. Discussion and perspectives
  - 6.1. Proposed functions of ClbQ and ClbL in the late-stage biosynthesis
  - 6.2. Is *clb* a flexible or rigorous NRPS/PKS assembly line?

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- 6.3. Is the genotoxic colibactin a single compound or a mixture?
- 6.4. Ecological role of *clb* in *E. coli*
- 6.5. Is ROS a possible trigger for the biosynthesis of colibactin?

- 6.6. Significance and therapeutic potential
7. Author contributions
8. Conflicts of interest
9. Acknowledgements
10. References



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Yuan Qian's group in 2021. His current research field focus on the biosynthesis of novel bioactive natural products from deep-sea fungi and Streptomyces.

## 1. Introduction

The human gut microbiome comprises trillions of bacterial and fungal cells and virus particles, and thereby affects various physiological processes.<sup>1</sup> An imbalance between the host and the microbiota could lead to human diseases.<sup>2</sup> For instance, many studies have shown that gut microbial dysbiosis induced by pathogenic bacteria, such as *Bacteroides fragilis* and *Escherichia coli*,<sup>3,4</sup> causes colorectal cancer (CRC).<sup>5,6</sup> Considering that the interactions between the gut microbiome and its host are critically regulated by microbe-related metabolites, the characterisation of these mysterious molecules is a key step to



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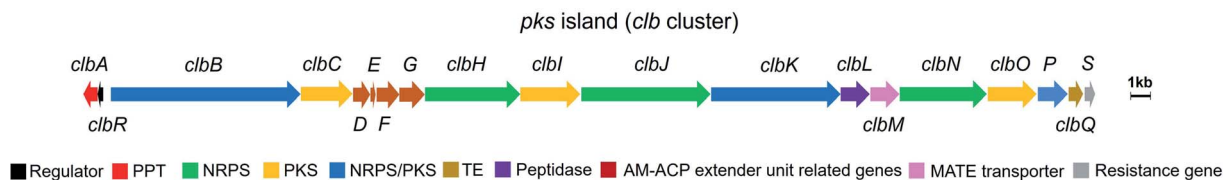


Fig. 1 Organisation of the *pks* island with annotations (modified figure based on ref. 7, 19 and 20). PPT: phosphopantetheinyl transferase, NRPS: nonribosomal peptide synthetase, PKS: polyketide synthase, NRPS/PKS: hybrid nonribosomal peptide–polyketide synthase, TE: thioesterase, AM-ACP extender unit related genes include *clbD* and *clbF* (which encode two dehydrogenases), *clbE* (which encodes a free-standing acyl carrier protein), and *clbG* (which encodes a free-standing acyl transferase).

understanding the molecular mechanisms underlying host–microbiome interactions.

Colibactins represent a group of cryptic microbial metabolites.<sup>7</sup> In 2006, Nougayrède and co-workers<sup>7</sup> observed for the first time that *E. coli* strains harbouring the *pks* island (a 54 kb genomic island also known as the *clb* gene cluster) (Fig. 1) cause DNA double-strand breaks (DSBs), leading to cell-cycle arrest in the G2/M phase, megalocytosis, and even cell death. This gene cluster containing 19 genes (from *clbA* to *clbS*) encodes 3 non-ribosomal peptide synthetases (NRPSs), 3 polyketide synthases (PKSs), 2 NRPS/PKS hybrid synthases, and 11 accessory and tailoring enzymes with respective functions for synthesising and modifying the genotoxin(s) (which are called colibactin(s)). Significantly, systematic mutagenesis studies of 18 genes (*clbR* was not tested in this research) revealed that these genes, except for *clbM* and *clbS*, are essential for the genotoxicity of colibactin(s).<sup>7</sup> To identify the missing link between certain gut microbes and DNA DSBs and to reveal the possible mechanisms underlying colibactin-induced DNA damage, researchers have attempted to elucidate the molecular identity of genotoxic colibactin. They have made significant progress regarding the relationship between the *pks* island and the symptoms caused by DNA DSBs, and have further demonstrated that colibactin induces DSBs, leading to genomic instability, senescence, and apoptosis.<sup>7–10</sup> However, the structural information of colibactin was seldom obtained from *pks*+ wild-type or heterologous strains until the discovery of the prodrug activation mechanism of colibactin,<sup>11–14</sup> which paved the way for the analysis of the biosynthesis logic through the mutagenesis processes. Finally, several colibactins, especially genotoxic colibactin-645 (**18**) and colibactin-770 (**19**), have been characterised using integrated bioinformatics, metabolomics, and chemical synthesis.<sup>15–18</sup>

The study of colibactin serves well as a significant example for natural product research. The instability of colibactin seriously impedes the traditional isolation-based strategy to access its bioactive products. Accordingly, methods such as bioinformatics and metabolomics are required to clarify this issue. Additionally, the biosynthesis logic of *clb* offers new insights into the mechanism of NRPS/PKS assembly lines. Most importantly, colibactin is critically involved in human health.<sup>21,22</sup> Studies have reported that *pks*+ *E. coli* strains are commonly found in the human colon and can promote tumour formation in mouse models of CRC.<sup>3,8,22–24</sup> Moreover, the colibactin-induced mutational signature is notably enriched in CRC

patients.<sup>25,26</sup> Thus, exposure to *pks*+ *E. coli* represents a great health risk.

Several previous reviews have highlighted the structures, biosynthesis, and genotoxicity of colibactin during their respective research periods,<sup>20,27,28</sup> whereas two recent reviews have focused on the structure determination of colibactin through DNA adductomics coupled with chemical synthesis.<sup>29,30</sup> In the present review, we focus on the biosynthesis logic of *clb*, summarise all currently known biosynthesis mechanisms, and discuss the possible mechanisms and undisclosed process(es) in late-stage biosynthesis. We also provide a summary of the biological activities of *pks*+ *E. coli*, especially the mechanism of colibactin-770 (**19**) and colibactin-645 (**18**)-induced DNA DSBs, as well as the potential relationship between colibactin(s) and CRC. Furthermore, this review discusses several questions: (1) What are the possible functions of ClbL and ClbQ in colibactin(s) biosynthesis? (2) Is the genotoxic colibactin(s) a single compound or a mixture? (3) Does the *clb* gene cluster specifically produce colibactin or simultaneously release other shunt metabolites to perform diverse functions? (4) What is the ecological function of *clb* for *E. coli* in natural settings? Moreover, the regulatory mechanism for activating colibactin(s) and their therapeutic potential are also discussed.

## 2. Discovery of precolibactins and colibactins

### 2.1. Prodrug mechanism in colibactin biosynthesis

The prodrug mechanism has already been described in detail by Balskus and co-workers<sup>28</sup> in a previous review. Herein, this mechanism is briefly described to ensure that readers can easily grasp the following content.

Prodrug activation is a general procedure in the biosynthesis of ribosomally synthesised and post-translationally modified peptides to afford mature peptides, but it is rare in non-ribosomal peptide biosynthesis. Several exceptional cases do exist,<sup>31</sup> such as those of amicoumacin,<sup>32</sup> xenocoumacin,<sup>33</sup> and zwittermicin,<sup>34</sup> which use a membrane-located D-asparagine-specific peptidase (AmiB in amicoumacin biosynthesis, XcnG in xenocoumacin biosynthesis, and ZmaM in zwittermicin biosynthesis) to convert inactive precursors into bioactive compounds. In colibactin biosynthesis, ClbP is hypothesised to exhibit homology to XcnG and ZmaM in the phylogenetic analysis of the peptidase domain and to act as the colibactin-



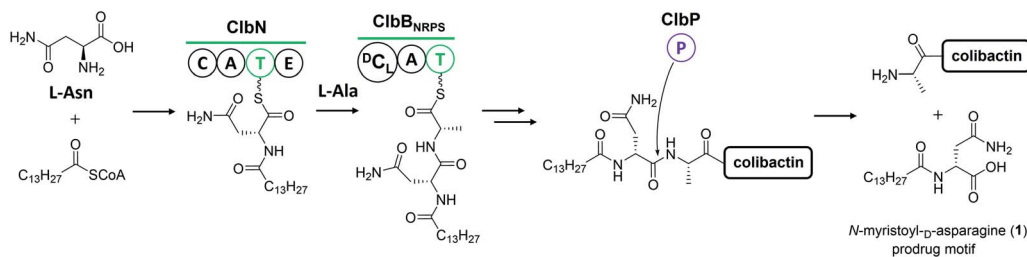


Fig. 2 Illustration of the prodrug activation mechanism (modified figure based on ref. 13 and 28). ClbN and ClbB<sub>NRPS</sub> utilise L-Asn, L-Ala, and myristoyl-CoA to install the prodrug motif at the beginning of colibactin biosynthesis. At the final step, ClbP cleaves the prodrug motif to generate colibactin.

maturing enzyme based on analyses of crystal structure, mutagenesis experiments, and structure–function relationship.<sup>11,12</sup> Later, inspired by the biosynthesis of xenocoumacin and zwittermicin, Balskus and coworkers<sup>13</sup> proposed that the prodrug motif *N*-myristoyl-D-asparagine (**1**) could be synthesised at the initial NRPS module with C–A–T–E domains and transferred to another NRPS module, which should recognise the upstream D-amino acid to construct an amide bond as the eventual peptidase substrate. An NRPS ClbN with C–A–T–E domains and an NRPS-PKS hybrid ClbB with a <sup>D</sup>C<sub>L</sub> domain meet this requirement (Fig. 2). Subsequent *in vitro* experiments supported their hypothesis. ClbN specifically selects L-asparagine, performs the *N*-acylation of myristoyl-CoA to form *N*-acyl-asparagine, and subsequently epimerases in the E domain to form *N*-myristoyl-D-asparagine. The NRPS module of ClbB accepts *N*-myristoyl-D-asparagine and adds L-alanine (L-valine) to generate *N*-myristoyl-D-Asn-L-Ala. The peptidase ClbP could specifically cleave *N*-acyl-D-asparagine substrates to generate the prodrug motif. Notably, ClbP completely loses activity when cleaving the *N*-acyl-L-asparagine substrates, suggesting that the D-asparagine is the major component responsible for the specificity in the prodrug motif. Based on these discoveries, they proposed that the prodrug mechanism of colibactin biosynthesis is as follows: two NRPS modules (ClbN and ClbB) are critical for installing the prodrug motif on colibactin at the early stage, and the peptidase ClbP then cleaves *N*-myristoyl-D-asparagine–colibactin by releasing the prodrug motif at the final stage.<sup>13</sup> Müller and co-workers also verified this mechanism with *in vivo* evidence by characterising the prodrug motif **1** in wild-type *E. coli* Nissle 1917 and analysing the metabolites of *clb*-containing heterologous *E. coli* and target-gene-deficient mutants for each gene.<sup>14</sup>

## 2.2. Identification of various precolibactins

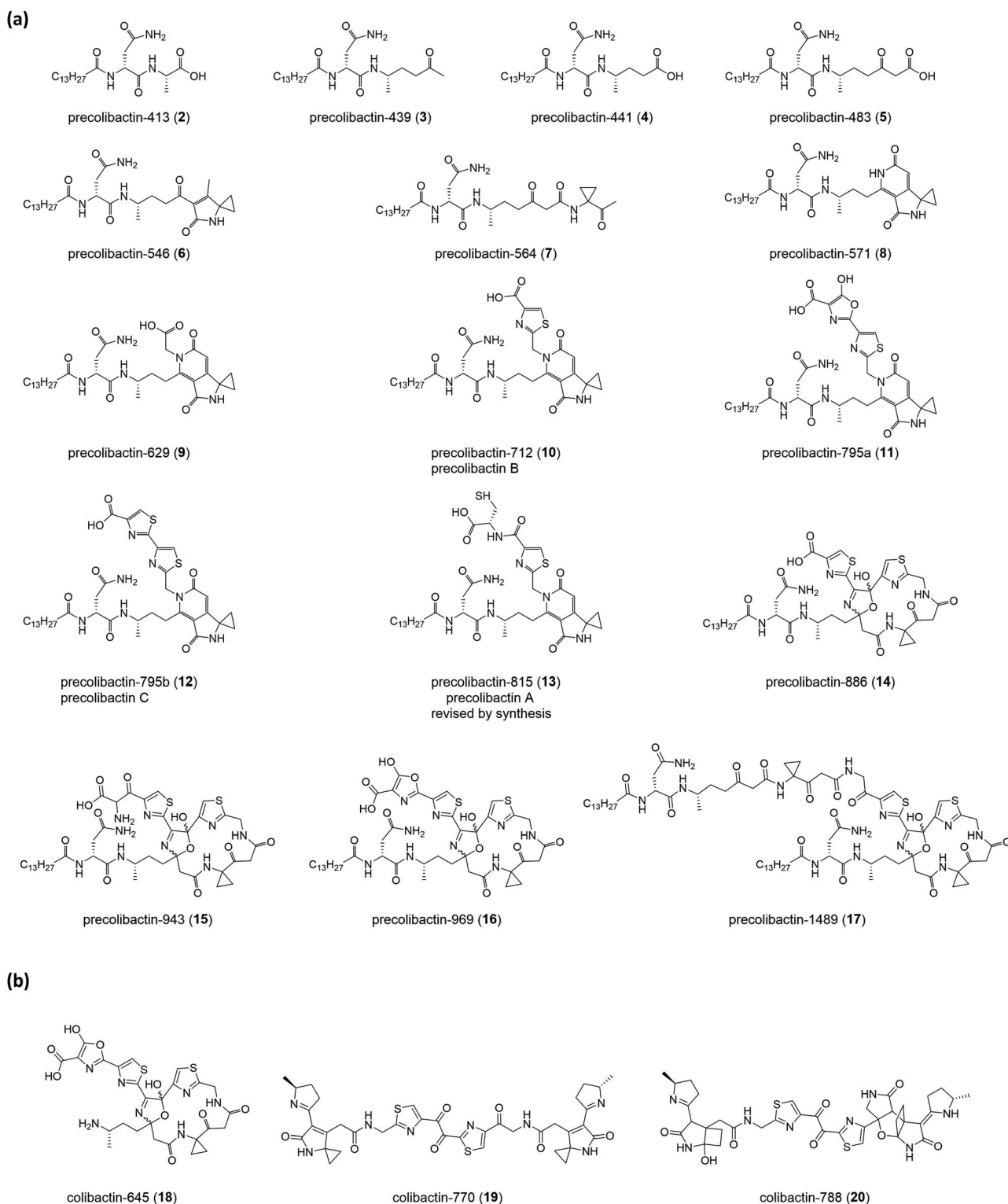
**2.2.1. Brief overview of precolibactin discovery and nomenclature suggestion.** In earlier studies, it was impossible to isolate colibactin directly from *pks*<sup>+</sup> wild-type strains because mature colibactin may be highly unstable and produced in trace amounts. Therefore, an alternative strategy of mutating the *clbP* gene was developed to obtain the premature but much more stable precursor-colibactin (denoted as precolibactin),<sup>19</sup> followed by the mutagenesis of *E. coli* with the knockdown of other genes to accumulate precolibactins. Although the production of

precolibactins is improved by mutagenesis, isolating and structurally elucidating most precolibactins through NMR experiments remain difficult. Therefore, comparative metabolomics combined with tandem MS (MS/MS) fragment analysis and isotropic labelling experiments is used to identify nearly inseparable precolibactins, which are ultimately confirmed or revised through chemical synthesis. To date, more than 40 precolibactins have been identified from genetically modified *E. coli*. These precolibactins provide substantial insight into the structure and biosynthesis of colibactin.

The nomenclature of precolibactins is relatively disorganised because authors do not provide scientific names for individual compounds. Instead, they label the relevant compounds with numbers. This practice is conventional in biosynthesis studies that illustrate the intermediates of the final product but is confusing for the identification of precolibactins. In our previous study, we used a precolibactin nomenclature of the form ‘precolibactin-molecular weight’ to name precolibactin-886 (**14**)<sup>35</sup> and precolibactin-969 (**16**),<sup>18</sup> which is much easier to follow (Fig. 3) and avoids confusion.

**2.2.2. Discovery of precolibactins deciphers the colibactin(s) biosynthesis logic.** The first precolibactin, precolibactin-439 (**3**), was isolated and characterised from the  $\Delta clbP$  mutant by Crawford and co-workers in 2014.<sup>19</sup> They utilised comparative metabolomics of the *pks*<sup>+</sup> strain and  $\Delta clb$  and  $\Delta clbP$  mutants to identify the dominant metabolites in the  $\Delta clbP$  mutant. Additionally, precolibactin-413 (**2**), precolibactin-441 (**4**), and precolibactin-483 (**5**) were deduced based on MS/MS fragment analysis. These findings serve as *in vivo* evidence of the initial steps of colibactin biosynthesis, which uses the ClbN and ClbB<sub>NRPS</sub> modules to install the prodrug motif and successively catalyses the malonate extension in the ClbB<sub>PKS</sub> module (Fig. 4). In 2015, three research groups independently isolated precolibactin-546 (**6**) bearing a spiro-cyclopropane ring from  $\Delta clbP$  mutants.<sup>36–38</sup> Feeding experiments and metabolite analyses of systematic gene knockout mutants identified ClbC, ClbH, and ClbI as essential genes for the biosynthesis of **6** and suggested that ClbH may act as a 1-amino-cyclopropanecarboxylic acid (ACC) synthase. However, later studies have shown that ClbH directly activates the unusual nonproteinogenic amino acid, *S*-adenosylmethionine (SAM), as the building block to yield a cyclopropane ring.<sup>36,38,39</sup> Moreover, the spiro-cyclopropane ring is shown to be a key moiety for the

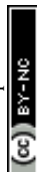




**Fig. 3** (a) Representative structures of precolibactins (structures that help in deciphering the biosynthesis logic that have been presented). (b) Structure of colibactin-645 (18),<sup>18</sup> colibactin-770 (19),<sup>15,16</sup> and colibactin-788 (20).<sup>17</sup>

genotoxicity of colibactin through irreversible covalent binding to DNA.<sup>36</sup> With the identification of **6**, based on biosynthesis-guided isotopic labelling and MS/MS analysis, Crawford and co-workers proposed the structure of precolibactin A (**13**), which

was soon revised by the same group.<sup>40</sup> In the same year, our group isolated three new cyclopropane-containing compounds, namely, precolibactin-564 (**7**), precolibactin-571 (**8**), and precolibactin-712 (**10**), through a series of gene-knockout



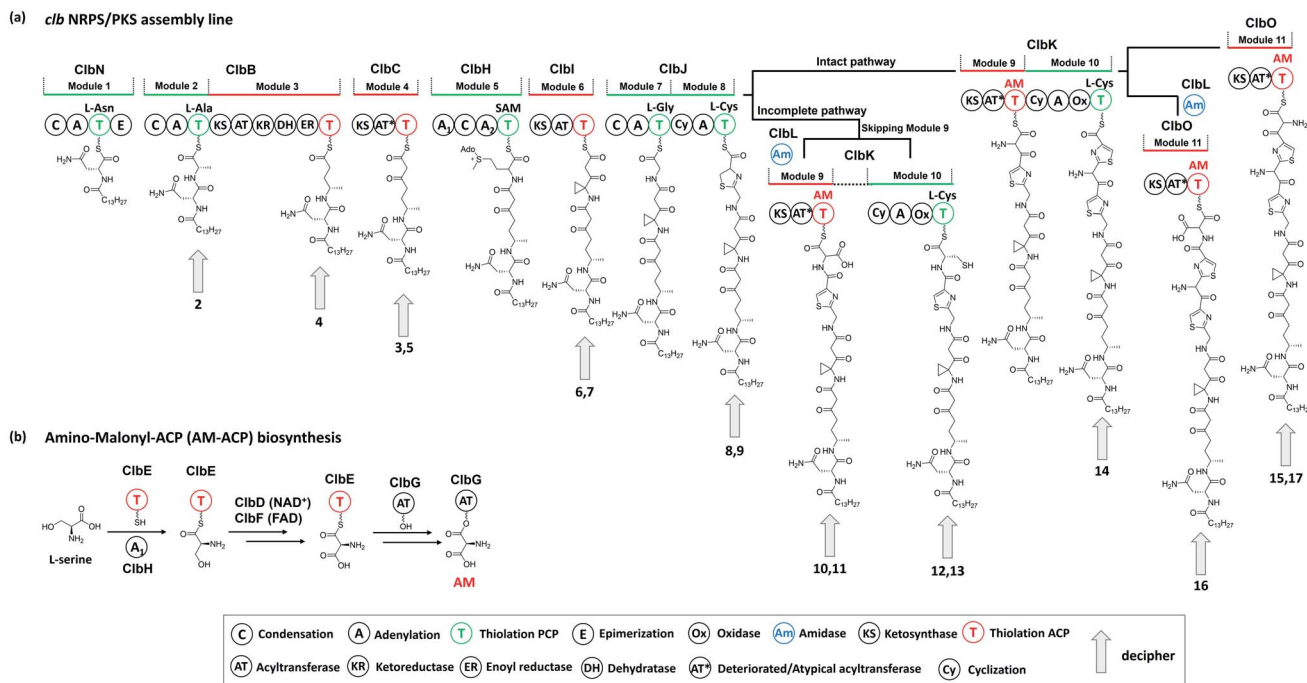
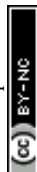


Fig. 4 (a) Proposed biosynthesis of colibactin based on the discoveries of precolibactins (late-stage biosynthesis involving ClbL, ClbP, and ClbQ is not shown in this figure). (b) Biosynthesis of the unusual PKS extender unit AM-ACP (ClbH-A1 activates L-serine, which is then transferred to the holo-ClbE and finally forms AM-ACP-ClbE through the dehydrogenases ClbD and ClbF; ultimately, ClbG transfers AM to ClbPKS and ClbO for subsequent biosynthesis).

experiments and large-scale fermentation of mutants.<sup>41</sup> Clearly, precolibactin-712 (**10**) possesses a pyridone moiety and a thiazole ring, suggesting that ClbJ and ClbK, featuring the heterocyclisation and the oxidase domain,<sup>42,43</sup> are the modules after ClbI catalysis. Moreover, based on MS/MS fragment analysis and isotropic-labelling experiments, we proposed bithiazole-containing precolibactin-795b (**12**), which is also known as precolibactin C. Further systematic mutagenesis experiments suggest that all the predicted NRPS and PKS megasynthases except for ClbO participate in the biosynthesis of **10** and **12**. In 2016, Balskus and co-workers isolated and verified the proposed precolibactin-795b (**12**) from a  $\Delta clbP/\Delta clbG$  double mutant<sup>44</sup> and showed that ClbG, as a free standing AT, could transfer the unusual PKS extender unit amino-malonyl (AM) (Fig. 4b), which is synthesised by ClbH<sub>A1</sub>, ClbD, ClbE, and ClbF,<sup>45</sup> to PKS modules in the colibactin assembly line. Later, our group characterised precolibactin-886 (**14**), a macrocyclic precolibactin containing the highly sought aminomalonate extender unit, along with precolibactin-629 (**9**) from a  $\Delta clbP/\Delta clbQ$  double mutant.<sup>35</sup> This discovery, together with further systematic mutagenesis experiments, provided *in vivo* evidence for the generation and incorporation of AM in colibactin biosynthesis. Furthermore, with the *in vivo* and *in vitro* evidence obtained in our study,<sup>35</sup> we then proposed that ClbQ, a type-II thioesterase (TE), may mediate the offloading procedure, especially in early-stage biosynthesis. In 2019, our group discovered precolibactin-969 (**16**) from a  $\Delta clbP/\Delta clbQ/\Delta clbS$  triple mutant.<sup>18</sup> The results of systematic mutagenesis experiments revealed

that the inactivation of any component of the colibactin NRPS/PKS assembly line, excluding ClbQ, abolishes the production of **16**. In contrast, the inactivation of ClbS, a self-resistance enzyme, hydrolyses the cyclopropane ring to avoid self-inflicted DNA damage,<sup>46–49</sup> resulting in a fourfold increase in the titer of **16**. In the same study, we also characterised precolibactin-795a (**11**) from a  $\Delta clbP/\Delta clbQ/\Delta clbS/\Delta clbO$  quadruple mutant. Structural comparison with the previously reported precolibactin-795b (**12**) shows that **11** contains a 5-hydroxyoxazole moiety next to the thiazole group rather than the two consecutive thiazole groups in **12**. The structural differences between **11** and **12**, as well as **14**, shed light on the biosynthesis logic of ClbK. The AM unit can be incorporated into downstream products in two forms: one is through the nucleophilic attack of the amino group at the upstream peptidyl-S-T thioester of ClbJ to generate **11**, and the other is through the nucleophilic attack of the  $\alpha$ -carbon to generate **14**. Additionally, the AM unit can avoid incorporation by skipping the PKS module, and the L-cysteine in the NRPS module performs the nucleophilic attack of the amino group at the upstream peptidyl-S-T thioester of ClbJ to generate **12**. These findings, together with the detection of precolibactin-943 (**15**), further facilitate the characterisation of ClbO. Mutagenesis experiments also show that ClbL, an amidase, is essential for the production of **11** and **16**, but not for that of **15**, suggesting that the nucleophilic attack of the amino in AM is catalysed by ClbL. Otherwise, the nucleophilic attack of the  $\alpha$ -carbon in AM is performed by the intrinsic KS domain in ClbO. The discovery



of these compounds (especially **15** and **16**) fills almost all gaps in the biosynthesis process of this assembly line.

### 2.3. Identification of various colibactins

**2.3.1. Discovery of colibactin-645.** Precolibactin-943 (**15**) and precolibactin-969 (**16**)<sup>18</sup> were considered to be products of the intact colibactin NRPS-PKS assembly line and serve as candidates for the generation of genotoxic colibactin at that moment. However, accumulating a sufficient amount of precolibactin-943 (**15**) to conduct a cleavage experiment to generate the corresponding mature colibactin is difficult because of its low titer. Fortunately, a sufficient amount of precolibactin-969 (**16**) was isolated and subsequently cleaved by ClbP to generate colibactin-645 (**18**). Additionally, **18** has been detected by a comparative metabolite analysis of the native *pks*<sup>+</sup> *E. coli* strain (CFT073), confirming that this small molecule is native of the *pks* pathogenicity island rather than a non-natural biosynthesis pathway in a heterologous host. Further mode-of-action study revealed that **18** directly induces DNA DSBs through copper-mediated oxidative cleavage<sup>18</sup> (details in Section 4). These results suggest that **18** is likely a genotoxic component of *pks*<sup>+</sup> *E. coli*.

**2.3.2. Discovery of colibactin-770.** In 2019, two groups separately established the structure of colibactin-770 (**19**, known as colibactin).<sup>15,16</sup> Evidence indicates that the putative colibactin causes DNA DSBs, probably by directly alkylating and crosslinking DNA.<sup>36,50,51</sup> Crawford, Herzon, and co-workers used MS/MS to analyse the relatively stable colibactin–DNA adducts<sup>52</sup> by treating *pks*<sup>+</sup> *E. coli* with exogenous pUC19 plasmid DNA from *pks*<sup>+</sup> wild-type and heterotrophic mutants fed with isotopic-labelling amino acids, glucose, and ammonium chloride. Using this strategy, they directly obtained the structural information of colibactin without any isolation or purification procedures and then used chemical synthesis to test the information from DNA adductomics and biosynthetic logic. In the MS/MS analysis, they observed an 18.5 *m/z* shift (*z* = 2) and a 4 *m/z* shift (*z* = 2) as a result of feeding with D-[U-<sup>13</sup>C]-glucose and [<sup>15</sup>N]-ammonium chloride, respectively, suggesting that the

DNA adduct possesses 37 carbon and 8 nitrogen atoms (Fig. 5). A similar procedure with L-[U-<sup>13</sup>C]-Met, L-[U-<sup>13</sup>C]-Cys, L-[U-<sup>13</sup>C]-Gly, and L-[U-<sup>13</sup>C]-Ser showed that two cyclopropanes, two thiazoles, one glycine, and two aminomalonates are involved in the DNA adduct. Based on these results, they determined the structure of **19** and verified it through chemical synthesis. Using a similar strategy, they identified precolibactin-1489 (**17**), the macrocyclic product of a predicted colibactin precursor, precolibactin-1491 (**24**) (Fig. 5). The mode-of-action study showed that **19** induces DNA DSBs by alkylating and cross-linking DNA (the relevant mechanism is shown in Section 4).

Balskus and coworkers<sup>16</sup> established the structure of colibactin based on the characterisation of ClbL. In their analysis of the remaining uncharacterised components in colibactin biosynthesis, they focused on the function of ClbL, as bioinformatics analysis indicates that ClbL is an amidase containing a Ser-*cis*-Ser-Lys catalytic triad to hydrolyse the amide bond, and that the mutation of any active residues leads to the loss of genotoxicity in *pks*<sup>+</sup> strains. Thus, comparison of the metabolites of the ClbL-expressing strain and of the  $\Delta clbP/\Delta clbL$  mutant provided the unhydrolysed precursor from the  $\Delta clbP/\Delta clbL$  mutant. However, they did not obtain any unique metabolite from the  $\Delta clbP/\Delta clbL$  mutant, but obtained precolibactin-728 (**21**) containing an indole-derivative moiety in the ClbL-expressing strain (Fig. 6a). They proposed that ClbL catalyses amide-bond formation, not the putative hydrolysis. In *in vitro* experiments, ClbL recognises mimics of the ClbC- and ClbJ-bound intermediates and appears to prefer the use of a mimic of the ClbI-bound thioester as an electrophile (Fig. 6b). For the nucleophile,  $\alpha$ -aminoketone is crucial for ClbL recognition, and the AM-incorporated intermediates generate  $\alpha$ -aminoketone through decarboxylation. Accordingly, they tested the reaction between the mimic of the ClbI-bound thioester and a mimic of the  $\alpha$ -aminoketone derived from ClbO. The successful formation of an amide bond in the experiment verified that ClbL is a possible enzyme responsible for the final step of the formation of intact precolibactin. An additional

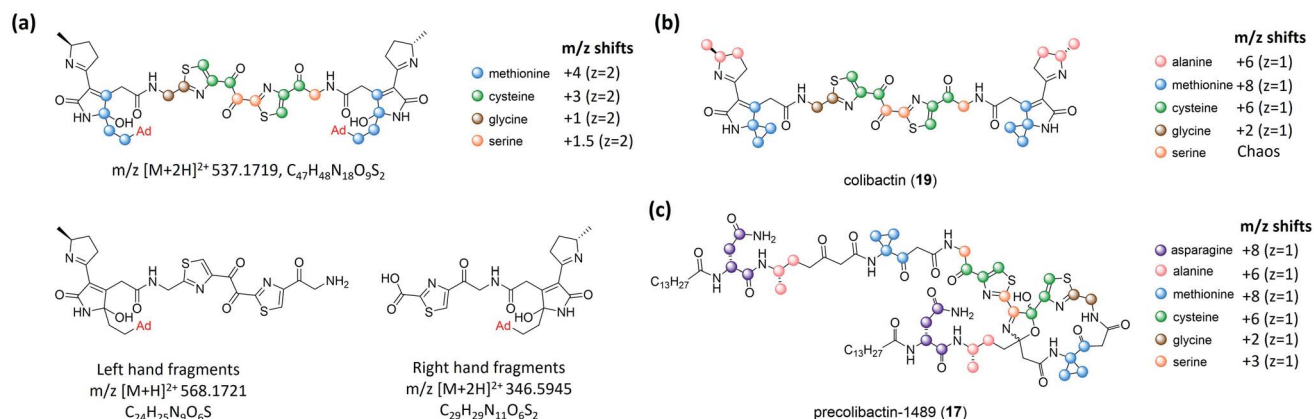


Fig. 5 (a) Structure of the colibactin–bisadenine adduct with different isotope-labelling patterns and degenerated adenine adducts (left- and right-hand fragments). (b) Isotopic-labelling pattern of colibactin-770. (c) Isotopic-labelling pattern of precolibactin-1489 (**17**) (modified figure based on ref. 15).



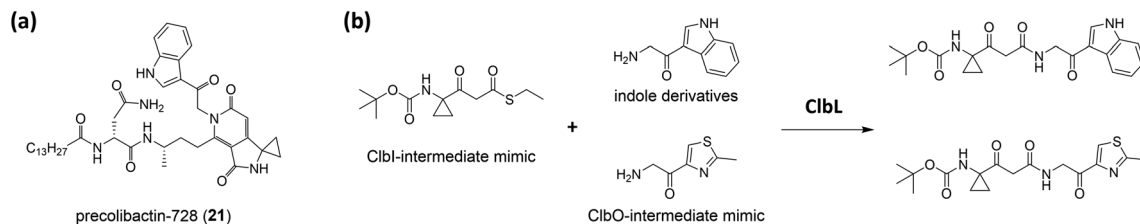


Fig. 6 (a) Structure of precolibactin-728 (21). (b) Structure of the ClbI-bound thioester mimic, indole derivative, ClbO-bound thioester mimic, and their ClbL catalysis products.

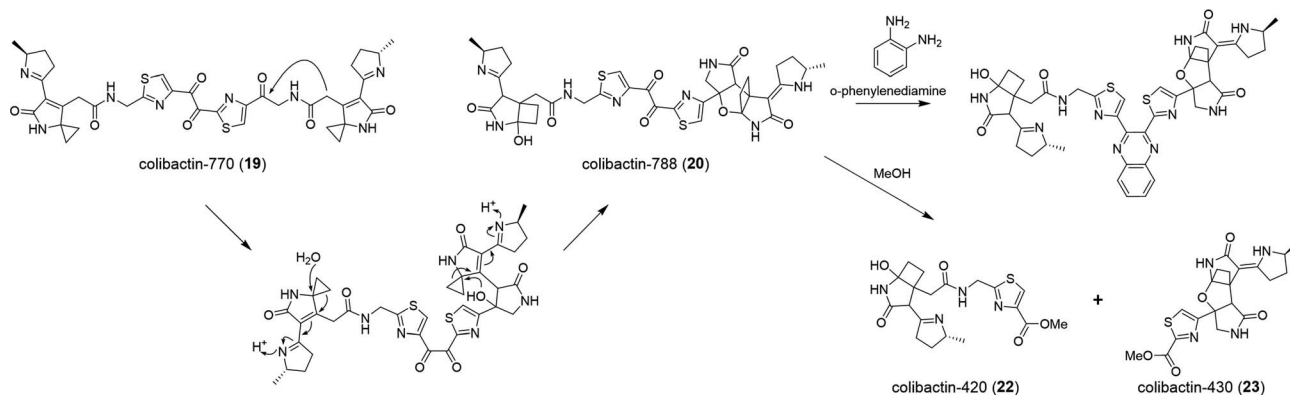


Fig. 7 Biosynthesis of colibactin-788 (20) and its degenerated compounds colibactin-420 (22) and colibactin-430 (23) in MeOH.

labelling experiment using DNA adductomics<sup>53</sup> enabled them to propose the structure of 19.

**2.3.3. Discovery of colibactin-788.** The first colibactin derivative, colibactin-788 (20)<sup>17</sup> (Fig. 7), was isolated from *pks+* wild-type by Japanese scientists this year. Amongst several *E. coli* strains isolated from human CRC tissue<sup>54</sup> they identified the strain *E. coli*-50, which is capable of producing 26-fold more 19 than *E. coli* Nissle 1917, through fluorescent probe-based high-throughput screening. Based on its molecular weight of 788, they proposed that this compound is a hydroxylated derivative of colibactin-770 (19), but larger-scale fermentation of *E. coli*-50 and subsequent purification failed to yield the pure target compound. Instead, they obtained two degenerated compounds, colibactin-420 (22) and colibactin-430 (23). These results suggest spontaneous hydrolysis in 20, consistent with the 1,2-diketone cleavage reaction proposed by Herzon and co-workers.<sup>55</sup> To stabilise the structure to make it amenable to isolation, they added *o*-phenylenediamine to the culture medium to capture the 1,2-diketone moiety. Finally, they obtained colibactin-788 (20), providing isolation-based evidence of the structure and biosynthesis of colibactin.

## 2.4. Late-stage colibactin biosynthesis

**2.4.1. ClbL and ClbQ participate in precolibactin-1491 (24) biosynthesis.** With the structure of colibactin-770 (19) established, the precursor(s) of 19 could be deduced. Precolibactin-1491 (24) (Fig. 8) requires a linkage between the final product from ClbO and an intermediate from the assembly line after module 6 (ClbI). Based on a study of ClbL,<sup>16</sup> Balskus and co-

workers proposed that after the ClbQ-bound intermediate is offloaded (no mention is made of whether ClbQ is involved), further spontaneous decarboxylation forms 25. ClbL recognises the  $\alpha$ -aminoketone of 25 and activates the amino to attack the carbonyl of the ClbL-bound intermediate to yield 24. In contrast, Crawford, Herzon, and co-workers proposed that ClbL acts as a hydrolase<sup>15,30</sup> based on their observation that ClbL cleaves the amide bonds of asparagine to form aspartic acid by releasing ammonia *in vitro*. Accordingly, they proposed that the ClbJ<sub>NRPS1</sub>-bound intermediate offloads the assembly line through ClbQ. ClbL then cleaves the amide bond to release glycine, and during this procedure, the  $\alpha$ -amino of the ClbO-bound intermediate attacks the carbonyl to form 24. These two proposed biosynthesis pathways of 24 require similar precursors, but ClbL plays a completely different role in the processes.

**2.4.2. Cyclisation.** After its synthesis, the intact precolibactin-1491 (24) is further cleaved and cyclised to generate colibactin-770 (19). Unlike the thiazole and cyclopropane rings that are catalysed on the NRPS/PKS assembly line, the 3,4-dihydro-2H-pyrrole and 2H-pyrrol-2-one rings in colibactin-770 (19) originate from the spontaneous intramolecular amino nucleophilic addition and Knoevenagel condensation, respectively<sup>15,50</sup> (Fig. 9a). The timing of the 2H-pyrrol-2-one ring formation remains obscure. The 2H-pyrrol-2-one ring could be rapidly formed after it is offloaded from the assembly line according to an *in vitro* study;<sup>39</sup> it is also supposed to be stable after assembly line offloading, supported by evidence of the detection of the linear (pre)-colibactin products



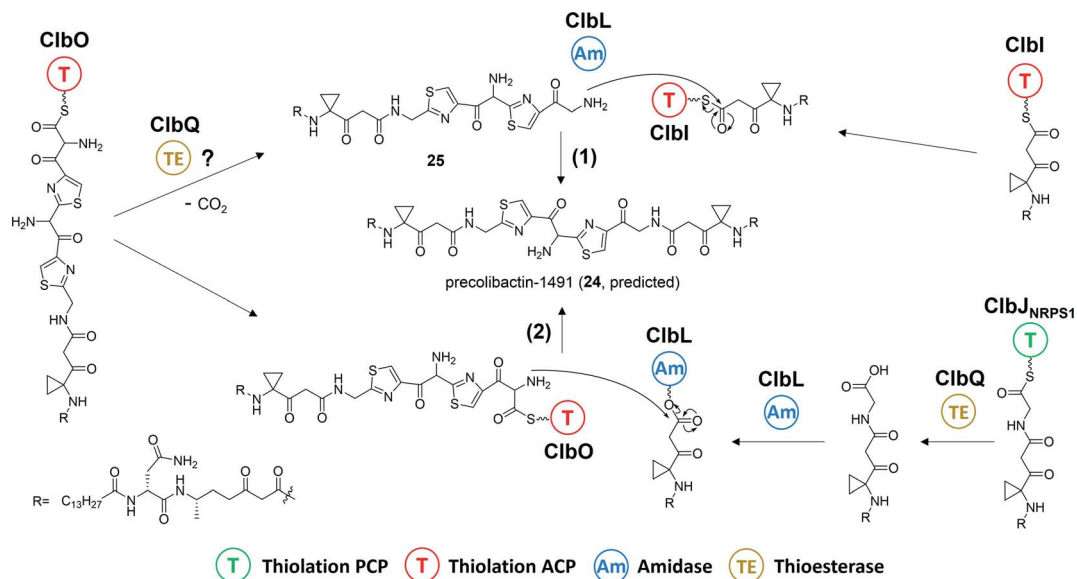


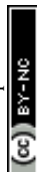
Fig. 8 Proposed biosynthesis of predicted precolibactin-1491 (24): pathway (1) from the intermediates ClbO and ClbI was proposed by Balskus and co-workers, and pathway (2) from intermediates ClbO and ClbJ<sub>NRPS1</sub> was proposed by Crawford, Herzon, and co-workers.

or the macrocyclic precolibactin without a 2*H*-pyrrol-2-one ring in several studies.<sup>15,18,35,41</sup> Additionally, once the 2*H*-pyrrol-2-one ring forms, precolibactin possesses the warhead moiety, which damages DNA and is rapidly hydrolysed by ClbS.<sup>46–49</sup> Thus, 2*H*-pyrrol-2-one tends to form in the periplasm to avoid self-damage. Nevertheless, the possibility of cyclisation in the cytoplasm and subsequent rapid exportation to the periplasm is not necessarily excluded. Regarding the timing of the 3,4-dihydro-2*H*-pyrrole ring formation, the 3,4-dihydro-2*H*-pyrrole forms only after the cleavage of the prodrug motif by ClbP in the periplasm.<sup>11–13</sup> Notably, the imine moiety in the 3,4-dihydro-2*H*-pyrrole is important for the genotoxicity of colibactin. The loss of the imine does not abolish the activity, but the existence of the imine enhances the DSB level.<sup>50</sup> Therefore, the prodrug motif blocks the 3,4-dihydro-2*H*-pyrrole formation in precolibactin, which may also be a self-resistance strategy in complement with the direct hydrolysis enzyme ClbS. Interestingly, if the prodrug motif cannot be cleaved, it forms a pyridone ring.<sup>50</sup> Taking the proposed biosynthesis of precolibactin-712 (10) as an example, in the  $\Delta clbP$  mutant, the predicted precursor generates 10 through an intramolecular amino nucleophilic addition to form a pyridone ring (Fig. 9b). These pyridone-containing precolibactins are viewed as artefacts of the  $\Delta clbP$  mutant on account of their lack of genotoxicity and inability to be hydrolysed by ClbS.<sup>48</sup> However, from another perspective, forming a pyridone ring may also be a complement strategy of *pks+* *E. coli* self-resistance. In addition to the aforementioned ring forms, 2,5-dihydro-5-hydroxyoxazole is another type of ring found in (pre)-colibactins. For the cyclisation of precolibactin-886 (14), we propose that the amino at C-37 of intermediate 34 attacks the carbonyl at C-23, and the resultant oxyanion further attacks the carbonyl at C-36 to form the 5-oxazolidinol ring. With subsequent oxidation (catalysed by the ClbK oxidation domain or spontaneously occurring), a double

bond is introduced in the 5-oxazolidinol ring and it becomes the 2,5-dihydro-5-hydroxyoxazole ring in 14. Another possibility is the oxidation of the amino at C-37 of intermediate 34 to first generate an imine (intermediate 35), which then performs similar reactions to the amino in 34, finally affording 14. The first proposal may be more plausible, because the amino in 35 is a better nucleophilic group than the imine in 35, which should be more ready to attack the ketone group.

## 2.5. Degradation

Herzon, Crawford, and co-workers<sup>55</sup> found that the C–C bond in the 1,2-diketone moiety in 19 or the  $\alpha$ -imineketone moiety in 31 is readily broken by nucleophilic attack in the environment. Based on this discovery, they proposed that some precolibactins, such as precolibactin-712 (10) and precolibactin-815 (13), may be partly derived from the degradation of downstream products rather than solely from the *clb* assembly line offloading. The degradation dose provides another perspective on the biosynthesis of (pre)-colibactins. Taking 10 as an example, based on the knockout data in our early study,<sup>35</sup> the production of 10 was found to be independent of ClbDEFG, indicating that 10 is likely to be derived from the ClbJ-bound intermediate (Fig. 10a). Thus, theoretically, the disruption of the subsequent ClbK and ClbO may increase or at least not decrease the production of 10. However, domain-targeted metabolomics<sup>56</sup> showed that the titer of 10 in the ClbL and ClbP mutants is higher than that in mutants without ClbK domain function. This finding suggests that the degradation of downstream products, such as the offloaded product of ClbO, indeed affords 10. Furthermore, the titer difference is more pronounced for 13 (that of the ClbL mutant is significantly higher than that in the mutants without ClbK domain function and the ClbQ knockout mutant), which suggests that the degradation pathway is dominant in the biosynthesis of 13



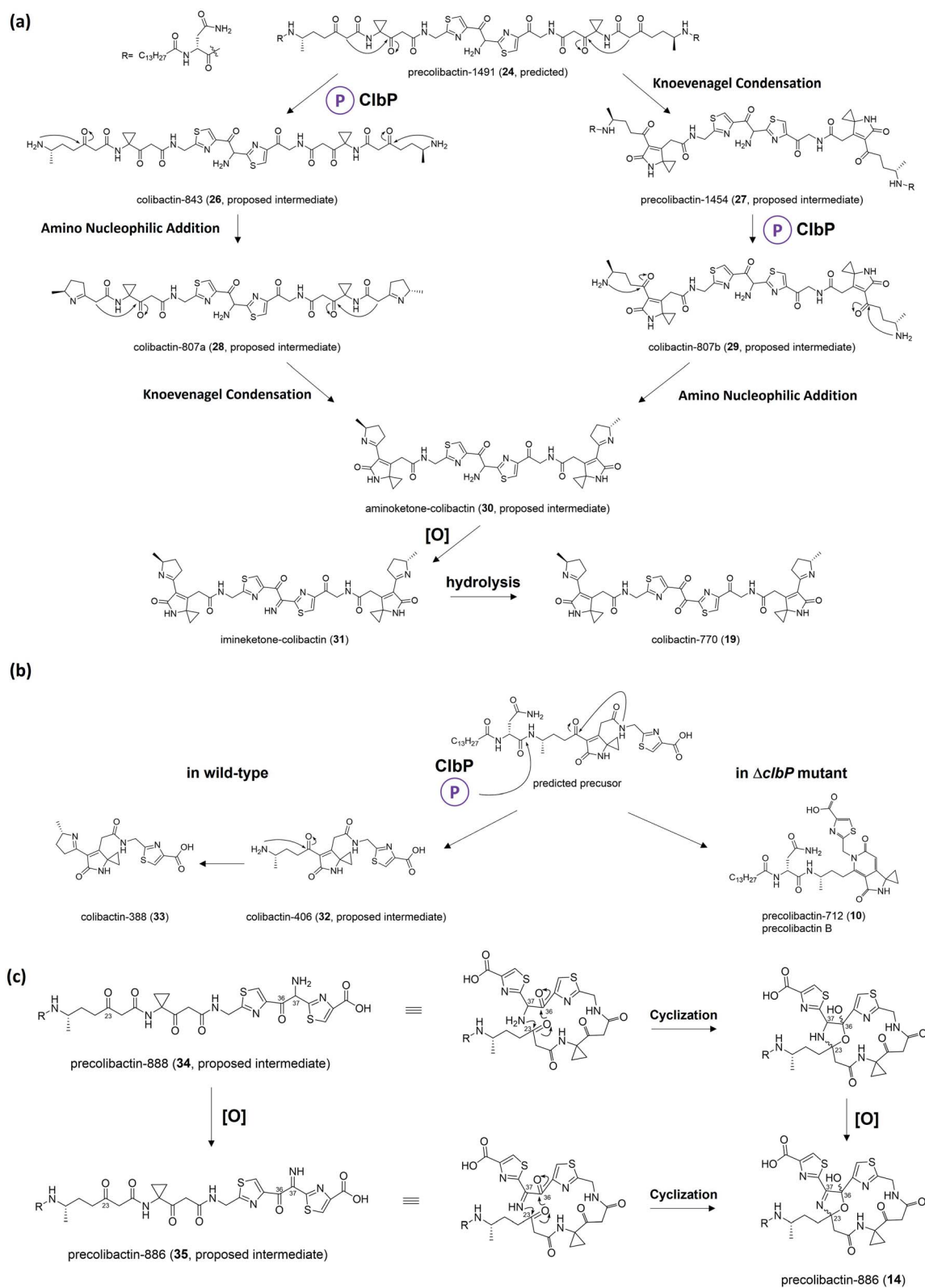


Fig. 9 (a) Proposed biosynthesis from precolibactin-1491 (**24**) to colibactin-770 (**19**). (b) Proposed divergent biosynthesis with or without ClbP. (c) Generation mechanism of macrocyclic precolibactin.



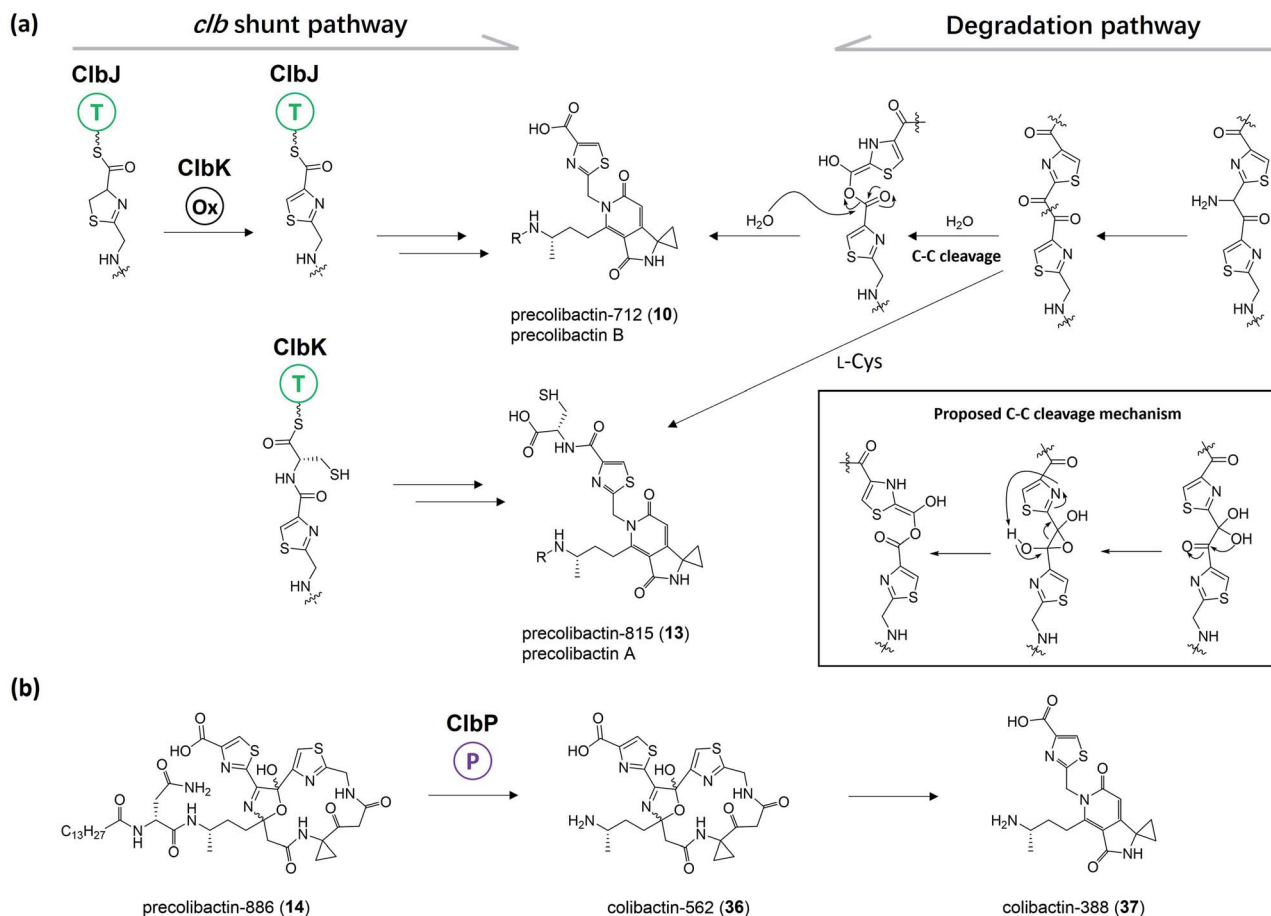


Fig. 10 (a) Previously proposed biosynthesis pathway of **10** and **13** (from left to middle) and proposed degradation pathway of **10** and **13** (from right to middle); the C–C cleavage mechanism was proposed by Herzon and Crawford (modified illustration in black box based on ref. 55). (b) Observation of the conversion of **14** to **37** in Herzon and Crawford's research (modified figure based on ref. 55).

rather than assembly line offloading. The degradation may have also occurred in macrocyclic (pre)-colibactins. Herzon and Crawford's research<sup>55</sup> indicated that colibactin-562 (**36**), the ClbP-cleaved product of precolibactin-886 (**14**), can be spontaneously converted into colibactin-388 (**37**) through degradation (Fig. 10b). However, in our study,<sup>18</sup> we could not detect any degraded compounds such as **37** from **36**. This contradictory observation is difficult to explain, and may involve some imperceptible factors that could affect the stability of the macrocyclic precolibactins in these two independent studies. Therefore, more in-depth studies on degradation are required in the future to obtain a conclusive result.

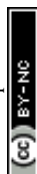
### 3. Unique mechanisms in colibactin biosynthesis

Unlike most modular PKS/NRPS assembly lines, colibactin biosynthesis involves many unique and rare biosynthesis mechanisms, such as the utilisation of a noncanonical PKS extender unit (AM-ACP), the existence of three functionless (deteriorated) *cis*-AT domains in the PKS module (ClbC, ClbK<sub>PKS</sub>, and ClbO), and the formation of a spiro-cyclopropane ring

directly using SAM as the building block. Additionally, the freestanding type-II TE ClbQ is proposed to be responsible for the offloading of colibactin, but several independent studies have shown the release of intermediates without ClbQ. The amidase ClbL has been suggested to be responsible for amide-bond formation in the final step to generate precolibactin-1491 (**21**). However, ClbL is also involved in the biosynthesis of **11** and **16**, thereby increasing the complexity of the biosynthesis logic in ClbK and ClbO. Therefore, it is necessary to specifically discuss these unique biosynthesis mechanisms of this intriguing NRPS/PKS assembly line.

#### 3.1. Unusual PKS extender unit AM-ACP and deteriorated AT domain in PKS

The PKS module in colibactin biosynthesis contains *cis*-AT and *trans*-AT domains, which are exclusive in most module PKSs.<sup>57,58</sup> In 2014, Crawford and co-workers<sup>19</sup> revised the annotation of the *pks* island and found an inactive AT domain in ClbC, ClbK<sub>PKS</sub>, and ClbO, which lacks the canonical GxSxG motif (the catalytically active site serine is responsible for covalently tethering the extender unit).<sup>59</sup> They proposed that these inactive AT-containing PKSs are inactivated AT ancestral relics of *cis*-AT but



have evolved to use a freestanding *trans*-AT domain.<sup>19,59</sup> Given that ClbC lacks the active AT domain but remains responsible for the malonate extension, they suggested that ClbC may load malonyl-CoA by the help of other *trans*-AT PKS systems or by interacting with fatty acid biosynthesis.<sup>36</sup> This hypothesis was later proven by Balskus and co-workers, who found that ClbC can load malonyl-CoA in the presence of ClbB<sub>PKS</sub>, ClbI, or the *trans*-AT domain FabD from fatty acid synthesis *in vitro*.<sup>44</sup> The other two deteriorated AT PKSs, ClbK<sub>PKS</sub> and ClbO, were found to accept the unusual PKS building block AM. AM is rarely found in common PKS products. To the best of our knowledge, only zwittermixin,<sup>34,60</sup> guadinomine<sup>61</sup> and lumiquinone A<sup>62</sup> involve AM as an extender unit in their biosynthesis. In 2015, Piel and co-workers discovered that ClbD and ClbF are homologous to ZmaG and ZmaI in zwittermixin biosynthesis and produce the AM-ACP extender unit.<sup>45</sup> The biosynthesis of the AM unit occurs through the following steps: ClbH-A1 activates L-serine, which is then transferred to the holo-ClbE and finally forms AM-ACP-ClbE through the dehydrogenases ClbD and ClbF (Fig. 4b). Later, Balskus and co-workers characterised the ClbG responsible for transferring AM-ACP to multiple PKS modules. Moreover, their *in vitro* experiment showed that not only these deteriorated AT domains containing PKS (like ClbK<sub>PKS</sub> and ClbO), but also the *cis*-AT PKS ClbI, recognised the AM-ACP.<sup>44</sup>

### 3.2. ClbH uses SAM to form a spiro-cyclopropane ring

The cyclopropane ring is a very intriguing structure that is extensively present in terpenoids yet rarely present in NRPS/PSK products. Along with the identification of **6**, Crawford and co-workers added L-[U-<sup>13</sup>C]-methionine and [2,2,3,3-<sup>2</sup>D]-labelled ACC to distinguish the formation of cyclopropane through the direct or indirect incorporation of ACC.<sup>36</sup> They observed a mass shift of 4 Dalton when adding the former, but no shift when adding the latter, suggesting that the ACC unit is involved in the formation of cyclopropane in an indirect way. Similar results

were reported by Müller and his co-workers for feeding experiments with L-[U-<sup>13</sup>C, <sup>15</sup>N]-methionine and L-[methyl-<sup>2</sup>D<sub>3</sub>]-methionine.<sup>38</sup> L-methionine is involved as a precursor in cyclopropane formation, possibly through an intramolecular S<sub>N</sub>2-type attack of a carbanion in ethylene biosynthesis, which requires ACC synthase to transform SAM into ACC.<sup>63</sup> Thus, the deficiency of ACC synthases in *E. coli* as indicated by bioinformatics analysis inspired them to propose that ClbH acts as an ACC synthase. Later, the ClbH-A1 domain was found to activate L-serine and load it onto ClbE to participate in further AM-ACP synthesis; the remaining domain C-A2-T seems unable to function as an ACC synthase. Thus, Balskus and co-workers re-analysed the data and proposed that the ClbH-A2 domain activates an unusual non-proteinogenic amino acid, SAM, as the building block to form cyclopropane.<sup>39</sup> Further biochemical experiments have been conducted to verify this hypothesis by testing the SAM activation and loading activity of ClbH, but no ACC-ClbH thioester has been detected in experiments. Thus, ClbH cannot work alone to generate cyclopropane. Results of *in vivo* experiments further indicate that ClbI may participate in malonate extension and cyclopropane formation. Interestingly, bioinformatics analysis of the KS domain in ClbI shows that the conserved active-site cysteine, which is responsible for transiently tethering the upstream intermediate and waiting for decarboxylation-driven Claisen condensation,<sup>42,64</sup> was replaced by serine. A similar mutation has been found in the starter KS<sup>S</sup> domains, such as PksF<sup>65</sup> and TaK,<sup>66</sup> which perform the decarboxylation of the ACP-tethering extender unit. Thus, the KS domain in ClbI may not participate in malonate extension. Furthermore, mutating serine (S178) to alanine abolishes the downstream products. Based on these observations, they proposed two possible functions of the KS domain in ClbI that participated in cyclopropane ring formation: (1) the serine (S178) of ClbI potentially serves as a general base for promoting the cyclisation of the ClbH-tethered intermediate, and (2) the serine (S178) may perform a similar function to cysteine in the canonical KS domain to tether an

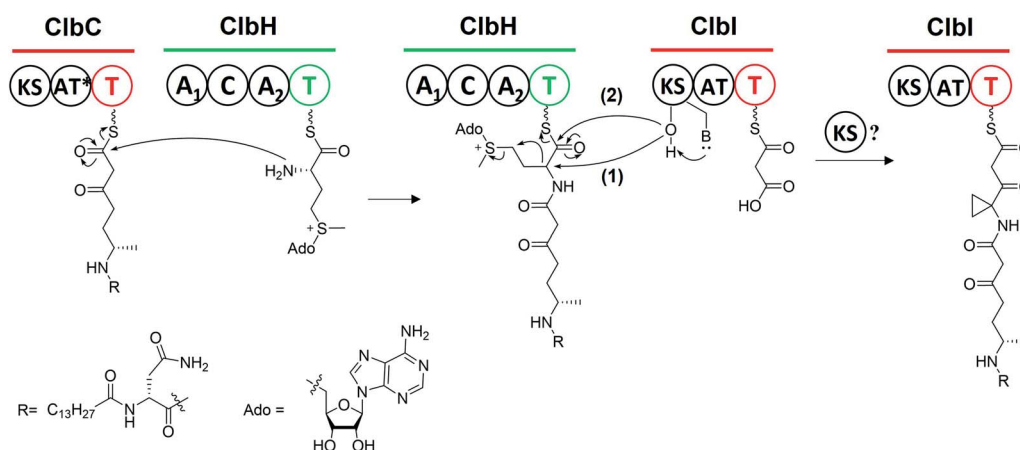


Fig. 11 Biosynthesis machinery of the cyclopropane ring (ClbH utilises a non-proteinogenic amino acid SAM as the building block, with subsequent nucleophilic attack of the upstream acyl-S-T-ClbC to afford ClbH-tethered intermediates; later, the serine (S178) of the KS domain in ClbI (1) potentially acts as a general base to promote the transformation from SAM to cyclopropane or (2) to tether an intermediate onto ClbI during cyclopropane formation; the intermediate is then catalysed by itself or other KS domains to finish another round of malonate extension).



intermediate onto ClbI during cyclopropane formation. The question of whether the malonate extension can be performed solely by ClbI or involves other KS domains in the assembly line, such as ClbB and ClbC, requires further study (Fig. 11).

### 3.3. ClbQ, a type-II TE, is responsible for the offloading procedure

There are two types of thioesterase (TE) in NRPS/PKS. Type-I TE is located at the terminus of the final module of PKS and NRPS megasynthase to mediate the offloading of the final product, whereas type-II TE is free-standing and is in charge of diverse functions.<sup>67,68</sup> Type-II TE is commonly used as a corrective cleaner through hydrolysis to remove incorrect acyl or peptidyl groups from misprimed PKS and/or NRPS assembly lines.<sup>69–71</sup> The NRPS/PKS assembly line of colibactin does not contain intrinsic type-I TE. Type-II TE ClbQ is assumed to be in charge of the offloading of colibactin. In an early work by our group, a  $\Delta clbP/\Delta clbQ$  mutant showed dramatically decreased amounts of upstream intermediates and a 22-fold increase in the amount of precolibactin-886 (**14**).<sup>35</sup> Our *in vitro* experiments have also shown that ClbQ readily hydrolyses the *N*-acetylcysteamine thioester (SNAC) derivatives of upstream precolibactins (Fig. 12). Similar conclusions have been drawn by the Brunauer laboratory.<sup>72</sup> They also found that ClbQ shows low specificity but a strong preference for upstream intermediates. Additionally, they found that ClbQ offloads the AM unit from AM-ACP-ClbE. Accordingly, they provided another explanation for the increase in the production of **14** in the  $\Delta clbQ$  mutant, *i.e.*, it may be due to not only the release of upstream products caused by the presence of ClbQ, but also the stable existence of the AM unit ensured by

the absence of ClbQ, thereby increasing the yield of **14**. Furthermore, many enzyme-bound thioester intermediates in the assembly line were observed to be released in the absence of ClbQ in the domain-targeted metabolomics analysis conducted by Crawford and co-workers.<sup>56</sup> Interestingly, their analysis shows that **14** is independent of ClbQ, but the titer has significant differences (a slightly decreased yield in the ClbQ inactivation mutant) compared with our data. This finding may be attributed to the difference in the site-mutagenesis methods used by Crawford and co-workers and the gene knockout in our study. Nevertheless, these findings suggest that ClbQ releases early-stage intermediates of *clb* and that some of the downstream intermediates may be released independently. Notably, the domain-targeted metabolomics analysis of precolibactin-1489 (**17**) shows that **17** is ClbQ dependent.<sup>15</sup> Additionally, we detected precolibactin-943 (**15**), the offloaded product of intermediate-ClbO, in the  $\Delta clbP/\Delta clbQ$  mutant, suggesting that the offloading of intermediate-ClbO may be ClbQ independent.<sup>18</sup> These observations indicate that ClbQ may participate in the final-stage biosynthesis of colibactin-770 (**19**) through an uncharacterised process(es). The function(s) of ClbQ deserves more in-depth study.

### 3.4. ClbL, an amidase, is responsible for amide-bond formation

ClbL, an amidase containing a conserved Ser179-Ser155-Lys80 catalytic triad,<sup>73,74</sup> is involved in the biosynthesis of **11** and **16** and is responsible for the final step of precolibactin-1491 (**24**) formation. Amidases commonly function as hydrolases, consistent with the observation that ClbL cleaves the amide

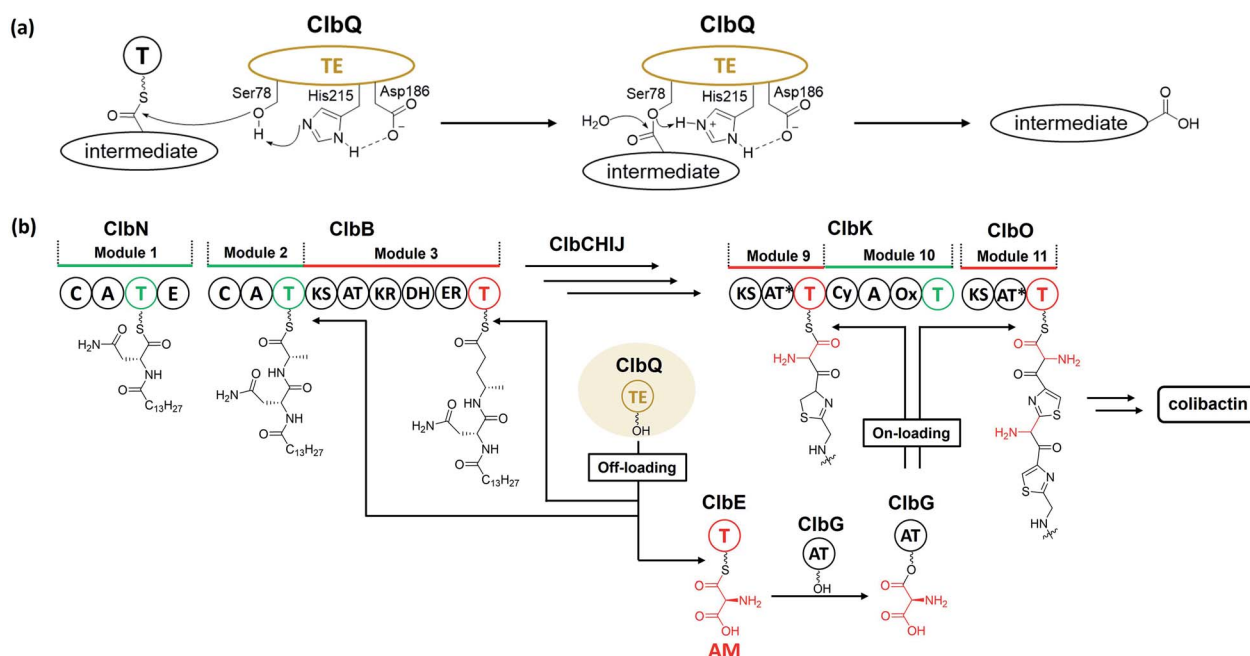


Fig. 12 (a) Offloading mechanism of ClbQ (the nucleophilic oxygen atom of Ser78 is activated by His215 and Asp186, and then attacks the thioester bond of the thiolation domain to yield intermediate-O-Ser78; later, other nucleophiles such as H<sub>2</sub>O attack intermediate-O-Ser78 to finally afford the hydrolysed intermediate). (b) Verified functions of ClbQ (ClbQ offloads the early-stage intermediates; it also offloads the AM unit, a key building block, to interrupt the biosynthesis of late-stage intermediates).



bond of asparagine *in vitro*.<sup>30</sup> It also exhibits amide-/acid-/acyl-transferase activity,<sup>75</sup> which is assumed to be due to the fact that the nucleophilicity of different nucleophiles towards the intermediate-*O*-Ser is stronger than that of oxygen in H<sub>2</sub>O. However, the catalytic machinery of ClbL remains obscure. The proposed role of ClbL in the biosynthesis of **24** is illustrated in Section 2.4.1. Regarding the role of ClbL in the biosynthesis of **11** and **16**, a systematic mutagenesis experiment<sup>18</sup> revealed that ClbL catalyses the amide-bond formation between the amino of the AM unit and the carbonyl of intermediate-ClbJ<sub>NRPS2</sub> and intermediate-ClbK (Fig. 13). Knocking out ClbK and ClbO also abolishes the production of **11** and **16**, respectively, suggesting that the AM unit originates from AM-ACP on the PKS module instead of the free-standing AM-ACP-ClbG. Moreover, considering that **11** and **16** do not undergo malonate extension in ClbK and ClbO, based on PKS chemistry logic, it is unclear whether the biosynthesis of **11** and **16** requires the KS domain in ClbK and ClbO, respectively. Nevertheless, on the basis of these discoveries, we hypothesise that the ClbL-mediated amide-bond formation of **11** and **16** may be a shunt reaction during the amino-malonate extension. Taking the biosynthesis of **11** as example, when the KS domain transiently binds the upstream intermediate of ClbJ<sub>NRPS2</sub>, decarboxylation-driven Claisen condensation (C–C bond formation) and ClbL-driven amide bond formation occur competitively. The amino-malonate

extension product is subjected to the action of downstream enzymes, ClbK<sub>NRPS</sub> module and ClbO, to afford **15** and **16**. The amide extension product cyclises under mediation by ClbL with two proposed mechanisms: (1) ClbL again acts as a general base to remove the proton of NH and promote cyclisation in the ClbK<sub>PKS</sub> domain; and (2) ClbL acts as a TE to offload the intermediate, Lys80 deprives the proton of intramolecular NH with subsequent electron transfer, and oxygen attacks the acyl-*O*-Ser179 to yield **11**. These hypotheses require further testing through site-mutagenesis and *in vitro* experiments in the future.

### 3.5. Self-resistance mechanism and MATE transporter ClbM

*pks+* *E. coli* uses a combination strategy to avoid self-toxicity. At the onset of NRPS/PKS assembly, ClbN and ClbB install the prodrug motif on colibactin,<sup>13,14</sup> thereby preventing the formation of the bioactive imine moiety. *pks+* *E. coli* also produces ClbS,<sup>46–49</sup> a cyclopropane hydrolase that opens the genotoxic cyclopropane in these offloaded intermediates. Furthermore, the multidrug and toxic compound extrusion (MATE) inner-membrane transporter ClbM pumps intermediates into the periplasm.<sup>76,77</sup> Notably, in Jobin and co-workers' research,<sup>78</sup> crystallographic analysis of ClbM indicated that the binding pocket spans nearly 40 Å. Based on this discovery, they hypothesised that ClbM specifically transports 700–900 Da precolibactins, which is obviously inconsistent with the size of

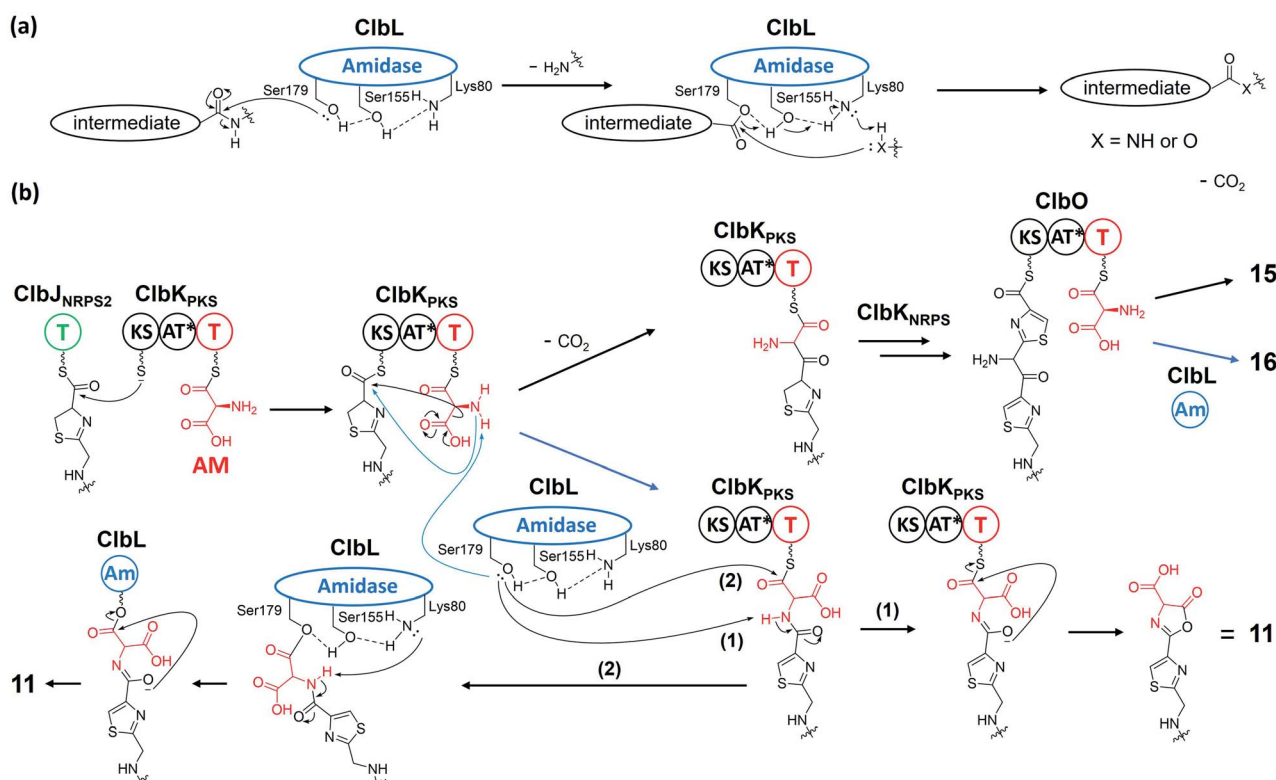
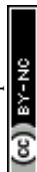


Fig. 13 (a) Proposed mechanisms of ClbL acting as a hydrolase or amide-/acid-/acyl-transferase (the nucleophilic oxygen atom of Ser179 is activated by Ser155 and Lys80 before attacking the carbonyl group to yield intermediate-*O*-Ser179; later, nucleophiles such as H<sub>2</sub>O, NH<sub>2</sub>-, and OH- attack the carbonyl of intermediate-*O*-Ser179 to yield the respective products). (b) Proposed mechanisms of ClbL for the amide-bond formation of precolibactin-795a (**11**) and precolibactin-969 (**16**) (the nucleophilicity of the amino group of AM is increased by ClbL; then, during the subsequent decarboxylation-derived malonate extension, the activated amino competitively attacks the transiently bound acyl-S-KS).



the colibactin precursor precolibactin-1491 (24). To be clear, knocking out *clbM* does not lead to a loss of genotoxicity, indicating that precolibactin-1491 (24) can also be transported to the periplasm by other means. However, given that ClbM is the only natural transporter on *clb*, the fact that the size of its binding pocket is incapable of satisfying the final product raises two questions: (1) Is the hypothesis of specifically transporting 700–900 Da precolibactins rigorous? (2) If the hypothesis is correct, should ClbM work for the ‘shunt products’ or the ‘real *clb* natural product’ rather than the ‘final product (like 24)’ of the *clb* assembly line?

## 4. Biological activities of *clb* metabolites

### 4.1. DNA-damage activity of colibactins and the underlying mechanisms

Colibactin-770 (19) and colibactin-645 (18) are two mature colibactins with different skeletons that induce DNA DSBs. Intriguingly, they inflict DNA damage through different mechanisms. DNA DSBs are induced by 19 through DNA alkylation and crosslinking and by 18 through copper-mediated oxidative cleavage.

The DNA damage-inducing mechanism of linear colibactin 19 was elucidated based on its structure, *i.e.*, 19 contains two ‘warheads’ with the azaspiro[2.4]bicyclic-ring substructure. The  $\alpha,\beta$ -unsaturated imines of this substructure render the cyclopropane rings electrophilic, thereby facilitating the formation of colibactin–DNA adducts. Thus, the generation of DNA interstrand crosslinks (ICLs) is initiated by twofold cyclopropane ring opening. At present, one of the two warheads is well-known to react at the N3 of adenine, whereas the second alkylation site remains an open question. Notably, precolibactin-546 (6) with a single warhead also causes ICLs *in vitro* in the presence of dithiothreitol or  $\beta$ -mercaptoethanol, which suggests the involvement of cyclopropane ring opening and Michael addition.<sup>36</sup> However, the DNA crosslinking activity of 6 is relatively weak, and a reducing agent is necessary, indicating that it cannot be the mode of ICL formation observed in *pks+* *E. coli*-infected cells.

Colibactin can induce intracellular DNA ICLs and DSBs, whereas extracellular DNA exposed to *pks+* *E. coli* exhibits DNA ICLs instead of DSBs,<sup>36</sup> suggesting that the DSBs are derived from the repair pathway of DNA ICLs (Fig. 14). HeLa cells infected with *pks+* *E. coli* respond to the replication stress induced by ICLs through the Ataxia Telangiectasia Mutated-and Rad3-related (ATR) signalling pathway, which increases the phosphorylation of ATR, Chk1, and RPA.<sup>51</sup> Subsequently, the Fanconi anaemia (FA) repair pathway is recruited to repair the damage.<sup>51</sup> The FANCD2 is mono-ubiquitinated and colocalises with  $\gamma$ H2AX, permitting incisions of ICLs followed by DSBs. Finally, the homologous recombination (HR) pathway is activated. The HR pathway is characterised by increased phosphorylation of ATM, H2AX, and Chk2, together with the formation of p53-binding protein 1 (53BP1) foci.<sup>7,51</sup>

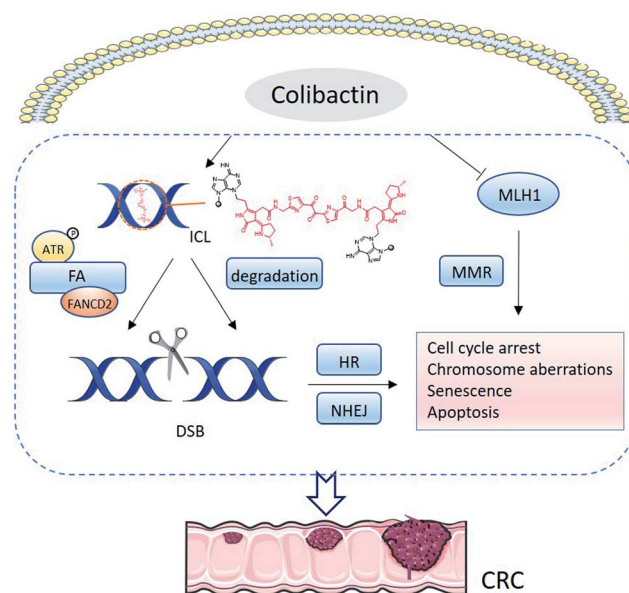
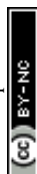


Fig. 14 Mechanism of conventional colibactin-induced DNA damage. Dual electrophilic cyclopropane warheads are adducted with adenine residues by ring opening, forming ICLs. In mammalian cells, ICL-induced replication stress activates ATR signalling and recruits the FA pathway to repair the damage. In the repair process, the co-localisation of FANCD2 with  $\gamma$ H2AX permits incisions of the ICLs, resulting in DSBs, which are repaired by the HR pathway. Additionally, colibactin-derived ICLs can be spontaneously degraded through depurination and elimination in 3'-phosphate, generating DSBs. This phenomenon likely activates the NHEJ repair pathway. Colibactin can also inhibit MLH1, disturbing MMR. DNA damage arises from DNA DSBs, and the inhibition of MMR leads to cell-cycle arrest and chromosome aberration. When cell repair fails, the cell undergoes senescence or apoptosis. The accumulation of DNA damage results in CRC.

In addition to the FA repair pathway, DNA DSBs also arise from ICL degradation. Colibactin-derived ICLs are reportedly unstable in depurination and 3'-phosphate elimination, and repeating the degeneration at the remaining intact alkylated nucleotide leads to a DSB.<sup>79</sup> HR and nonhomologous-end joining (NHEJ) are the two main DNA DSB repair pathways. Cells deficient in Ku80 are sensitive to *pks+* *E. coli*, suggesting the involvement of NHEJ in DNA repair.<sup>8</sup> FA is linked only to the HR pathway, so DSBs generated from the spontaneous depurination are proposed to activate the NHEJ repair pathway. Furthermore, *pks+* *E. coli* can induce ROS and inhibit the mismatch repair (MMR) protein mutL homologue 1 (MLH1), further leading to DNA damage (Fig. 14).<sup>80</sup>

Colibactin-645 (18) with a macrocyclic skeleton exerts direct DNA-DSB activity through copper-mediated oxidative cleavage (Fig. 15) rather than through the formation of ICLs.<sup>18</sup> In the presence of Cu(II) *in vitro*, 18 causes significant DNA DSBs. HeLa cells show the formation and co-localisation of foci derived from  $\gamma$ H2AX and 53BP1 when treated with 18 or *pks+* wild-type *E. coli*. The addition of a Cu-sequestering agent significantly decreases the levels of DNA damage. Copper-mediated oxidative DNA cleavage is generally proposed to involve copper-complex-induced ROS or reactive metal-oxo species (RMOS). Subsequently, the ROS or RMOS attack the DNA and initiate DNA



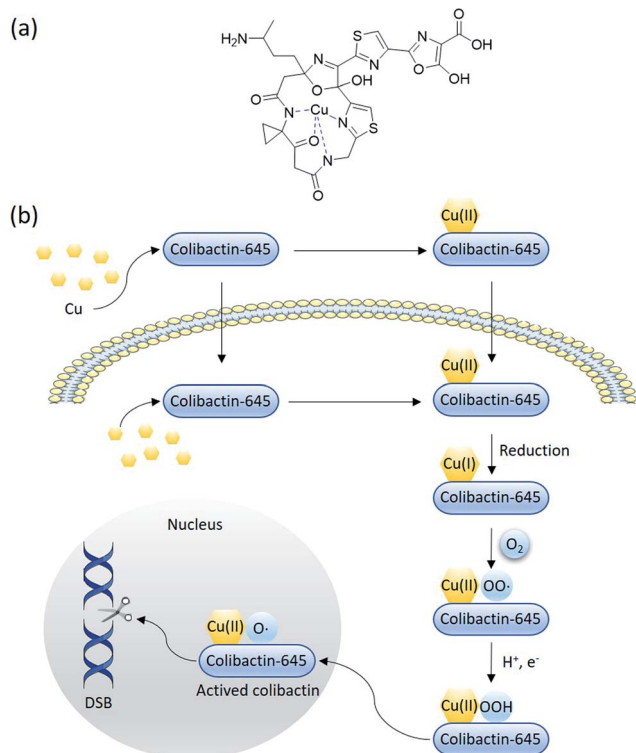


Fig. 15 Proposed mechanism of macrocyclic colibactin-induced oxidative DNA cleavage (modified figure based on ref. 18). (a) The proposed structure of the colibactin-Cu(II) complex. (b) Colibactin-645 binds to copper in the intestinal lumen to form the colibactin-Cu(II) complex, which is quickly transported into epithelial cells. Colibactin-645 may also quickly enter the cell after being secreted whilst forming the colibactin-Cu(II) complex with intracellular copper. Colibactin-645 reduces this complex to colibactin-Cu(I), which then coordinates with  $O_2$  to generate colibactin-Cu(II)- $OO^\bullet$ . In the presence of  $H^+$  and  $e^-$ , colibactin-Cu(II)- $OO^\bullet$  is converted to colibactin-Cu(II)-OOH, which can in turn be converted into  $HO^\bullet$  and colibactin-Cu(II)- $O^\bullet$ . The latter is the active species responsible for DNA carbon-hydrogen bond activation.

cleavage. A suitable reductant or photo-irradiation is initially required to reduce Cu(II) to Cu(I), which then reacts with  $O_2$  or  $H_2O_2$  to generate ROS. In this case, the macrocyclic scaffold of colibactin can serve as a reductant and a binding site. The colibactin-Cu(II) complex, the structure of which is proposed in Fig. 15a, is reduced by the ligand to form colibactin-Cu(I). The addition of mannitol, dimethylsulfoxide, or superoxide dismutase (SOD) (the former two are hydroxyl radical scavengers and the latter one catalyses the conversion of the superoxide radical into  $O_2$  and  $H_2O_2$ ) does not influence the activity of precolibactin-969.<sup>18</sup> Conversely, the  $H_2O_2$  blockers potassium iodide and catalase significantly inhibit the cleavage reaction, suggesting that  $H_2O_2$  is involved in mediating DNA cleavage *in vitro*.<sup>29</sup> This is consistent with the previous observation that the fluorescence of  $H_2$ -DCFDA (which acts as a sensor of hydroxyl and peroxy radicals, and of hydrogen peroxide production) increases significantly in cells treated with colibactin-producing *E. coli*.<sup>9</sup> The colibactin-Cu(I) complex is proposed to coordinate with  $O_2$  to generate colibactin-Cu(II)-OOH, which can be

converted into  $HO^\bullet$  and colibactin-Cu(II)- $O^\bullet$  (Fig. 15b). Colibactin-Cu(II)- $O^\bullet$  is the active species responsible for DNA carbon-hydrogen bond activation.<sup>81</sup> This mechanism is akin to the proposed mechanism for the generation of activated bleomycin *in vivo*,<sup>82</sup> with differences in the metal usage and the intrinsic metal-reduction activity of the compounds.

Based on structure-activity relationship analyses, we hypothesise that the loss of the N-terminal fatty acyl-asparagine residue helps mature colibactins to enter epithelial cells; the 'active colibactin' intercalates into DNA through the thiazole/5-hydroxyoxazole tail. However, further investigation should be conducted to verify this hypothesis and to confirm the mechanism of copper-mediated oxidative DNA cleavage.

The *pks* island produces multiple active metabolites with various modes of action. They may work synergistically to achieve DNA-damage activity. Thus, the elucidation of the mechanisms by which various colibactins induce DNA damage will provide insight into the development of specific gene-therapy strategies for CRC.

Despite the different DNA-damage-inducing modes of the two colibactins, various colibactin-producing *E. coli* strains can initiate DNA damage. Short-term exposure to *pks+* *E. coli* leads to the formation of anaphase bridges and chromosomal abnormalities in dividing cells,<sup>9</sup> whereas persistent exposure promotes the accumulation of mutations.<sup>8</sup> To maintain genome integrity, cells respond to DNA damage by activating the DNA-repair pathway. Nevertheless, improper DNA repair triggers the senescence or apoptosis of the target cells. Colibactin induces DNA DSBs in lymphocytes, followed by cell-cycle arrest and cell death by apoptosis.<sup>10</sup> Infection with *pks+* *E. coli* leads to chronic DSBs, prolonged cell-cycle arrest, and cellular senescence.<sup>9</sup> *pks+* *E. coli* upregulates miR-20a-5p expression through the c-Myc transcription factor in infected cells. The binding of miR-20a-5p to SUMO Specific Peptidase 1 (SEN1) mRNA 3'-UTR triggers the transcriptional silencing of SEN1, leading to the accumulation of SUMO-conjugated p53, thereby resulting in cellular senescence.<sup>83</sup> Moreover, the senescence is transmissible and promotes tumour-cell growth through the secretion of hepatocyte growth factor (HGF) *in vitro* and *in vivo*.<sup>9,83</sup> In short, *pks+* *E. coli* exposure can also promote the progress of CRC through senescence.

#### 4.2. Other bioactivities of *clb* metabolites

In addition to the DNA-damage genotoxicity inflicted by colibactin-producing *E. coli*, *pks+* *E. coli* also has cytotoxic, antibacterial, inflammation-promoting, anti-inflammation, and analgesia bioactivities. These diverse bioactivities are associated with the different moieties of colibactin, precolibactins, and other bactins produced by various *pks+* *E. coli*.

**4.2.1. Cytotoxicity.** Although mature colibactins are responsible for the DNA-damage activity, some precolibactins also show moderate cytotoxicity. Compared with precolibactin-441 (4), precolibactin-546 (6), and precolibactin-795b (12), precolibactin-886 (14) is more than five times more toxic toward HCT-116 cells and HeLa cells (IC<sub>50</sub> values of 22.3  $\mu$ M and 34.0  $\mu$ M, respectively).<sup>35</sup> The fatty acid chain in 14 appears to affect



only its physicochemical properties, likely making it more hydrophobic and inaccessible to eukaryotic cells; consequently, it can still exert cytotoxicity at higher concentrations.

**4.2.2. Antibacterial activity.** *pks+* *E. coli* shows antibiotic activity against pathogenic *Staphylococcus aureus* species including multiresistant isolates such as methicillin- and antistaphylococcal-antibiotic-resistant *S. aureus* strains.<sup>84</sup> The small molecule *N*-myristoyl-D-Asn (**1**) released by ClbP from precolibactin inhibits the growth of *Bacillus subtilis*.<sup>19</sup> The weak antibacterial activities of precolibactin-441 (**4**) and precolibactin-546 (**6**) against *B. subtilis* and *E. coli* (EC<sub>50</sub> 30.3–71.6 µg mL<sup>-1</sup>) have also been reported.<sup>38</sup> This antibacterial activity may also offer *pks+* *E. coli* a niche advantage and facilitate the colonisation of *pks+* *E. coli* in the gut, resulting in a significantly higher level of colonisation of the *pks+* *E. coli* strain 11G5 in the mouse gut than in mice colonised with *pks-* *E. coli* K-12.<sup>3</sup> The total clearance of the *E. coli* strain K-12 in the gut after 50 days of treatment indicates the important role of colibactin in colonisation ability and the bacterium-killing potency of colibactin and colibactin moieties.<sup>3</sup>

**4.2.3. Inflammation-related activities.** Different *pks+* *E. coli* strains exhibit inflammation-promoting and anti-inflammation bioactivities, possibly due to the diverse colibactin moieties produced by various colibactin biosynthesis gene clusters. No significant differences in inflammatory score are observed between mice infected with colibactin-producing *E. coli* and non-colibactin-producing *E. coli*,<sup>23,85,86</sup> although colibactin-producing *E. coli* generates a proinflammatory microenvironment.<sup>87</sup> The *pks+* *E. coli* strain CFT073 activates the expression of pro-IL-1β mRNA and the cleavage of caspase-1, and leads to the release of IL-1β from human neutrophils. This activity is mediated by the NLRP3 inflammasome and serine proteases in an NF-κB- and cathepsin B-dependent manner. The inhibition of NLRP3 and caspase-1 also increases neutrophil ROS production, phagocytosis, and the ability of neutrophils to suppress *pks+* *E. coli* growth.<sup>88</sup> Conversely, proinflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, and IFN-γ) trigger increased CFT073 growth,<sup>89</sup> thereby facilitating the colonisation of *pks+* *E. coli* in intestinal inflammation.

The *pks* island is also related to anti-inflammatory activity. Compared with wild-type Nissle 1917, Δ*clbA* Nissle (an isogenic mutant of Nissle 1917) does not show DNA-damage activity in eukaryotic cells, whereas its anti-intestinal inflammation effect is abrogated.<sup>75</sup> The DNA-damage and anti-inflammatory activities are restored when the wild-type *clbA* gene is reintroduced to the chromosome of Nissle Δ*clbA* (Nissle Δ*clbA* + *clbA*). The abolition of the ACP activator ClbA possibly results in the abrogation of the colibactin, precolibactin, and colibactin moieties, which may contribute to the anti-inflammation activity. However, anti-inflammation activity of these colibactin moieties cannot be observed in the pathogenic *pks+* *E. coli* strains CFT073 and M1/5. We cannot exclude the possibility of ClbA involvement in the syntheses of other anti-inflammatory products because *clbA* participates in siderophore biosynthesis. Therefore, the functional *pks* island appears to be a double-edged sword in terms of inflammation in various *pks+* *E. coli* strains with diverse genomes.

**4.2.4. Analgesic activity.** The *pks* island also synthesises lipopeptides with analgesic activity. For instance, C12AsnGA-BAOH, a γ-aminobutyric (GABA) acid dependent on *clbA*, *clbB*, and *clbN*, can cross the epithelial barrier and inhibit the calcium flux induced by nociceptor activation in sensory neurons through the GABA<sub>B</sub> receptor, thereby inhibiting visceral hypersensitivity in mice.<sup>90</sup>

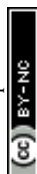
## 5. Relationship between colibactin and CRC formation

### 5.1. Colibactin plays a carcinogenic role in CRC

Previous studies have shown a high prevalence of *pks+* *E. coli* in CRC.<sup>21,22</sup> Around 55–67% of CRC patients carry *pks+* *E. coli*, whereas less than 20% of healthy people carry *pks+* *E. coli*.<sup>21,22</sup> In animal experiments, *pks+* *E. coli* enhances tumourigenesis in FAP and CRC models and promotes invasive carcinoma in azoxymethane (AOM)-treated interleukin-10-deficient mice (IL10<sup>-/-</sup>).<sup>14</sup> The deletion of the *pks* island decreases tumour multiplicity and invasion.<sup>22</sup> Multiple intestinal neoplasia (Min) mice infected with *pks+* *E. coli* 11G5, a strain isolated from a human CRC biopsy, display a remarkably increased number of visible colonic polyps after seven weeks of injection.<sup>3</sup> Data obtained from an AOM/dextran sodium sulphate (DSS) colon-cancer mouse model,<sup>83</sup> xenograft model,<sup>83,91</sup> and other models<sup>92</sup> also support the carcinogenic properties of colibactin. However, these models are essentially tumour models, so the development of cancer may be attributed to genetic susceptibility. Whether colibactin initiates or promotes carcinogenesis remains unclear.

Recent evidence shows that colibactin plays a causative role in CRC. The first and most important clue is that the mutational fingerprint of colibactin is detected in CRC patients.<sup>25,26</sup> Whole-genome sequencing or whole-exome sequencing analyses reveal the mutant signature of colibactin. Mammalian cells exposed to *pks+* *E. coli* show increased single-base substitutions (SBSs) and the induction of a characteristic small indel signature (ID-*pks*).<sup>25</sup> SBSs show a preference for T > N substitutions within AT-rich DNA regions, which is defined as a *pks*-specific SBS signature (SBS-*pks*), whereas ID-*pks* is characterised by single T deletions at T homopolymers.<sup>25</sup> The substitutions in specific AT-rich motifs are consistent with the sites at which colibactin adducts with adenine by alkylation. Moreover, the same mutational signature is detected in human cancer genomes.<sup>25,26</sup> Out of 5876 CRC samples, SBS-*pks* and ID-*pks* are enriched in around 6.5% and 7.1% of patients, respectively, whereas 4.65% of samples are high in SBS-*pks* and ID-*pks*.<sup>25</sup> Furthermore, *APC* is the most frequently mutated gene in CRC,<sup>93</sup> in which 5.3% of cancer-driven mutations match the motifs induced by colibactin.<sup>25</sup> In short, the high level of colibactin-induced mutational fingerprints in CRC patients suggests that colibactin plays a direct role in CRC.

Studies using models without genetic susceptibility and carcinogens provide another important evidence. Short-term *pks+* *E. coli* infection inflicts DNA damage to healthy primary colon epithelial cells, resulting in genomic instability.<sup>24</sup> Short-term exposure is closer to the dynamic process of crypt regeneration



in the colon. Organoids from primary murine colon epithelial cells exposed to *pks+* *E. coli* for 3 h exhibit DNA damage and Wnt independence. Wnt-independent organoids exhibit higher organoid-forming capacity and proliferation, which is highly similar to the character of CRC cells.<sup>24</sup> Furthermore, infection with *pks+* *E. coli* triggers the formation of invasive colonic tumours in a DSS-induced chronic inflammation mouse model.<sup>23</sup>

Overall, the high abundance of *pks+* *E. coli* in CRC patients and the mutant fingerprint suggest the carcinogenic role of colibactin in CRC development. However, the colibactin mutational signature is also found in healthy human colon crypts,<sup>25,26</sup> suggesting that colibactin may initiate cancer only under specific conditions or certain mutant accumulations. The underlying molecular mechanism of carcinogenicity remains obscure.

## 5.2. Colibactin modulates the tumour microenvironment to promote cancer growth

The tumour microenvironment is the environment surrounding the tumour and can affect cancer development. Colibactin directly interacts with DNA, induces mutations, and promotes a pro-carcinogenic environment. Colibactin contributes to the colonisation capacity of *E. coli*<sup>94</sup> and alters the microbial diversity in the gut.<sup>95</sup> Microbial dysbiosis exerts a cancer-promoting effect.<sup>96</sup> After 35 days of colonisation by *pks+* *E. coli*, the gut microbiota substantially changes at the taxonomical and functional levels.<sup>89</sup> This phenomenon may be due to the antibiotic activity of *pks+* *E. coli*, which helps genotoxic bacteria to create their own niches.<sup>97</sup> Once niches are created, the genotoxic activity of the bacteria helps the bacteria to expand their niches by targeting unrelated microbial taxa in the gut. *pks+* *E. coli* can also impair the permeability of the host intestinal mucosa, leading to increased ROS and in turn altering the composition and function of the gut microbiota.<sup>95</sup>

Furthermore, *pks+* *E. coli* shows immune-modulation activity. In CRC patients, a decrease in CD3+ T-cells is correlated with the colonisation of colibactin-producing *E. coli*.<sup>87</sup> In an APC<sup>Min/+</sup> mouse model, *pks+* *E. coli* 11G5 significantly increases gut inflammation and reduces tumour-infiltrating T lymphocytes (CD3+ and CD8+ T cells) as well as antitumour T cells in mesenteric lymph nodes.<sup>87</sup> CD45+ T cells and neutrophil percentage decrease in tumour areas treated with *E. coli* 11G5. Moreover, *pks+* *E. coli* infection shows resistance to PD-1 immunotherapy in an MC38 CRC model.<sup>87</sup> *pks+* *E. coli* also impairs intestinal permeability in newborn rats and thus promotes the *trans*-epithelial passage of luminal antigens and hyperimmune response, thereby contributing to the development of immune-dysregulation diseases.<sup>98</sup> These results indicate that colibactin-producing *E. coli* induces a procarcinogenic immune microenvironment and facilitates the development and progress of CRC.<sup>87</sup>

Additionally, *pks+* *E. coli* alters the physiology of epithelial cells by inducing the apoptosis of epithelial cells, which is compensated by the abnormal proliferation of epithelial cells.<sup>98,99</sup> Colibactin-induced senescence is accompanied by the secretion of HGF, which promotes the proliferation of mutant cells,<sup>83</sup> which in turn facilitates the development of colon cancer cells.

## 6. Discussion and perspectives

### 6.1. Proposed functions of ClbQ and ClbL in the late-stage biosynthesis

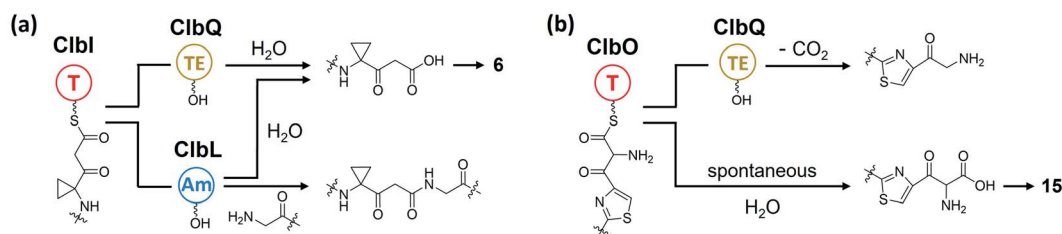
Research of the mechanisms of ClbL and ClbQ in the final-step biosynthesis of precolibactin-1491 (24) is inadequate. Nevertheless, according to the research of Balskus and co-workers,<sup>16</sup> ClbL likely participates in the offloading of intermediate-ClbI. During this offloading, the amino group of  $\alpha$ -aminoketone in 25 possesses stronger nucleophilicity than the oxygen in H<sub>2</sub>O, thereby forming an amide bond. Additionally, in the study of Crawford, Herzon, and co-workers,<sup>15,30</sup> ClbL plays a role similar to that of amide transferase. In the process of hydrolysing the amide bond, the amino group of  $\alpha$ -aminoketone attacks intermediate-*O*-Ser-ClbL to form an amide bond. However, neither of these two proposed mechanisms is backed by sufficient evidence to prove the real function of ClbL. In our re-analysis of all the information to date, we made an interesting discovery about ClbL. In Müller and co-workers' research of precolibactin-546 (6),<sup>38</sup> the knockout of *clbL* significantly decreased the production of 6, indicating that it may be produced by ClbL through the hydrolysis of the larger precolibactin. In contrast, knocking out the downstream *clbJ* increases the production of 6. These observations imply that 6 may originate not from the hydrolysis of the larger precolibactin, but instead from the ClbL-dependent offloading. A similar observation was reported by Balskus.<sup>16</sup> Conversely, in Crawford's research,<sup>56</sup> the S179A site-mutation S179A of ClbL did not affect the yield of 6. These contradictory observations may be due to the different methods used in their research (the Müller and Balskus laboratories used gene knockout, and the Crawford laboratory used site mutagenesis). Nevertheless, the two independent studies discussed above provide new insights into the function of ClbL, suggesting that it behaves like a TE to offload intermediate-ClbI (Fig. 16a).

Regarding the role of ClbQ, the observation of 15 in the  $\Delta clbQ$  mutant<sup>18</sup> suggests that the release of the ClbO-bound intermediate may be ClbQ independent. Moreover, if ClbL does indeed catalyse amide-bond formation upon offloading of the intermediate of ClbI, then ClbQ cannot be involved in the offloading of ClbI- and ClbO-bound intermediates. Therefore, it is difficult to propose the role of ClbQ, which may participate in the decarboxylation of intermediate-ClbO (Fig. 16b), similarly to SgcE10<sup>100</sup> and CalE7.<sup>101</sup> It may also play a similar role such as being a 'waiting room'<sup>68</sup> to tether the intermediate-ClbO and search for ClbL.

### 6.2. Is *clb* a flexible or rigorous NRPS/PKS assembly line?

The *clb* NRPS/PKS assembly line appears to be highly flexible. The starting module, ClbN, prefers myristoyl-CoA (C14), but it can also prefer *N*-acylates and other substrates ranging from hexanoyl-CoA (C6) to lauryl-CoA (C12).<sup>13</sup> This finding is consistent with identified precolibactins with varying chain lengths and patterns in the prodrug motif, ranging from decaacyl to palmitoyl,<sup>19,36</sup> similar to xenocoumacin biosynthesis.<sup>33</sup> The biosynthesis logic in ClbK and ClbO is also very complex,





**Fig. 16** (a) Proposed biosynthesis of **6** and machinery of ClbL catalysis of amide-bond formation. ClbQ and ClbL offload intermediate-ClbI to generate **6**; in ClbL-mediated offloading, the amino group of  $\alpha$ -aminoketone is a stronger nucleophile than the oxygen in H<sub>2</sub>O, thereby forming an amide bond. (b) Proposed role of ClbQ in offloading, decarboxylation of intermediate-ClbO, and biosynthesis of **15**.

because AM can be incorporated or not into downstream products with different mechanisms.<sup>18,35</sup> The offloading mechanism and the amidase ClbL are also promiscuous.<sup>16</sup> In addition to the intrinsically flexible logic of *clb*, some components of *clb* also participate in the biosynthesis of other metabolites in *E. coli*, which further increases the diversity of *clb*-related metabolites. For instance, ClbA is involved in the biosynthesis of yersiniabactin,<sup>102</sup> and AM-ACP-ClbE cooperates with fatty acid biosynthesis enzymes to generate  $\gamma$ -lactam derivatives.<sup>103</sup> These results indicate that the *clb* NRPS/PKS assembly line tends to be flexible, similar to the thalassospiramide biosynthesis gene cluster,<sup>104,105</sup> and can synthesise various metabolites *in vivo*.

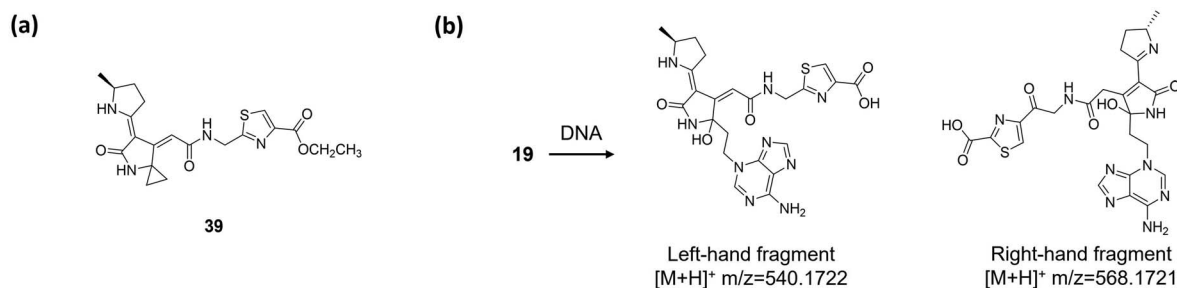
In contrast, *clb* shows extremely tight control over the synthesis of colibactin-770 (**19**)<sup>15,16</sup> as the final product. The formation of **19** requires an intact intermediate and an incomplete intermediate of the assembly line. These intermediates are heterodimerically linked through amide-bond formation. Later, the heterodimer is exported from the cytoplasm to the periplasm and cleaves the prodrug motif. Through subsequent spontaneous intramolecular nucleophilic amino addition and Knoevenagel condensation, the genotoxic colibactin finally forms. In this procedure, any mistake abolishes the production.

In summary, *clb* possesses the paradoxical character of being either flexible or rigorous. It is rigorous in synthesising the final product colibactin-770 (**19**), which requires the precise designation of the synthesis route, especially for the two-step spontaneous reaction that forms the bioactive moiety. Meanwhile, its flexibility lies in the promiscuity of multiple enzymes and unpredictable offloading mechanism. These properties enable

*clb* to specifically act on DNA DSBs *via* colibactin coupled with diverse bioactivities on other targets *via* shunt metabolites.

### 6.3. Is the genotoxic colibactin a single compound or a mixture?

At present, colibactin-770 (**19**)<sup>15,16</sup> is viewed as being the long-sought genotoxic colibactin produced by *pks+* *E. coli*, or at least its major component. However, owing to the promiscuous activities of multiple enzymes in the *clb* assembly line, we must reconsider whether there are other genotoxic shunt/final products and underlying genotoxic mechanisms that have not yet been characterised. For example, the macrocyclic colibactin-645 (**18**)<sup>18</sup> exerts direct DNA DSB activity through copper-mediated oxidative cleavage. In addition, as we mentioned in the previous section, ClbA participates in biosynthesis of yersiniabactin,<sup>102</sup> a siderophore responsible for mediating the copper density of *E. coli*.<sup>106</sup> We cannot reach a conclusion as to whether yersiniabactin plays a role in the DNA DSBs caused by colibactin-645 (**18**) *in vivo*, but it does provide a new perspective on the role of *clb* metabolites in causing DNA DSBs. Furthermore, the research of Balskus and co-workers<sup>53</sup> inspired us to make a more inclusive reconsideration. They observed that compound **39** (Fig. 17a), an ethyl-ester-modified colibactin possessing only one cyclopropane ring, induces DNA DSBs in HeLa cells at 20  $\mu$ M. The mechanism of this induction is proposed to go through DNA alkylation. This observation suggests that shunt metabolites of *clb* containing a cyclopropane ring may also be a component of colibactin in inducing DSBs. In addition, we note that in their DNA adductomics analysis, only the *m/z* 540.1772 adduct,



**Fig. 17** (a) Structure of compound **39**. (b) DNA adductomics of colibactin (**19**) (spontaneous breakage of the C-C bond of the 1,2-diketone moiety generates the left-hand and right-hand fragments).



which is the left-hand fragment of colibactin-770 (19), is detected. Later, in their subsequent research,<sup>16</sup> they detected the right-hand fragment of 19, the *m/z* 568.1721 adduct, by exposing *pks+* *E. coli* to plasmid DNA. Despite the influences of the different DNA adductomic methods in their two studies, these observations still raise the question of whether the *clb* metabolites, specifically colibactins, which are produced by the same/different strains, are the same or not in different studies. For instance, we noted that in several studies, different strains of *pks+* *E. coli* show different genotoxicities and cartridges of colibactin(s).<sup>17,24,107</sup> When comparing the *E. coli* M1/5 strain with *E. coli* Nissle 1917 in terms of the induction of DNA DSBs and ICLs, *E. coli* Nissle 1917 causes a lower-level induction of DSBs and ICLs and fails to induce Wnt-independent organoids in healthy primary colon epithelial cells.<sup>24</sup> Apparently, while it is known that the expression level of the *clb* cluster differs in different strains, the question of whether colibactin acts as a single compound or a mixture remains to be discussed.

In addition, considering the fact that the *clb* cluster produces various active products, we propose distinguishing between *pks+* *E. coli* and colibactin. Most studies of colibactin utilise *pks+* *E. coli* to assess the activities of colibactin *in vivo* and use the  $\Delta pks$  or  $\Delta clbA/clbQ/clbP$  mutants as a negative control. The deletion of the *clb* cluster or the functional *clb* gene abolishes colibactin production and affects the production of other metabolites, such as lipopeptides and yersiniabactin. Thus, the observed differences in activities between *pks+* *E. coli* and *E. coli* mutants *in vivo* may not be attributed solely to colibactin. To be more rigorous, the biological effects should be linked to the *clb* metabolites instead of colibactin.

#### 6.4. Ecological role of *clb* in *E. coli*

In evolutionary terms, the commensal *E. coli* featuring *clb* gene clusters may aim to adapt to the ecological niche instead of killing the host. Colibactin encoded by *clb* confers *E. coli* with stronger colonisation capacity, and its antibacterial activity offers *E. coli* a competitive edge over other gut bacteria. Meanwhile, *pks+* *E. coli* induces inflammation by releasing proinflammatory cytokines,<sup>87</sup> which in turn promotes the growth of *E. coli*.<sup>89</sup> The immunomodulation activity facilitates the escape of *E. coli* from immunity. *clb* is also involved in the synthesis of siderophores,<sup>102</sup> which import iron and copper to *E. coli* as nutrients.<sup>106,108</sup> Therefore, *E. coli* can thrive under iron- or copper-limited conditions. The self-resistant gene *clbS* inactivates colibactin and protects the bacterial DNA from nucleolytic degradation.<sup>47</sup> At present, in our opinion, the ecological function of *clb* in *E. coli* remains a mystery because of the limited data. Therefore, future studies should further explore the real natural product of the *clb* pathway. More physiological studies of ClbM, the *clb* natural transporter, may provide some answers to these questions, considering the fact that the mutation of ClbM failed to abolish the genotoxic colibactin-induced DSBs. Moreover, all the alleged final *clb* natural products are highly complicated. It is difficult to imagine that the *E. coli* hosts consume large amounts of energy to synthesise these complex

molecules without using them for specific physiological functions.

#### 6.5. Is ROS a possible trigger for the biosynthesis of colibactin?

To date, knowledge regarding the regulation of the *clb* cluster is limited. Nevertheless, several studies demonstrated that bacterial growth state and composition of the growth medium affect *clb* expression.<sup>109</sup> Heat shock protein HtpG also regulates *clb* via a non-transcriptional pathway.<sup>110</sup> As the sole transcriptional regulator in the *clb* cluster, ClbR, a LuxR family regulator, has been demonstrated to be the main transcriptional activator for the biosynthesis of colibactin.<sup>111</sup> However, unlike the classical LuxR family proteins, ClbR lacks the N-terminal receiver (REC) domain (responsible for recognizing signal molecules), suggesting that ClbR is an autonomous effector domain regulator. Thus, the triggers for *clbR* expression and the relative regulation mechanism of *clb* remain uncharacterized. Several indirect studies on this topic warrant brief discussion, although the information is limited.

It is a consensus that inflammation is associated with cancer.<sup>112,113</sup> In inflammatory disorders, ROS production is a typical event in most cases.<sup>114</sup> Thus, the frequent observation of a higher amount ROS in enteritis and CRC patients compared with healthy populations<sup>114</sup> indicates the potential role of ROS in the development of CRC caused by *pks+* *E. coli*. These results lead us to consider the possibility of ROS as a trigger for colibactin biosynthesis. The ROS-initiated oxidative-stress response regulator OxyR,<sup>115</sup> which belongs to LysR transcriptional factor family, has also been reported as a positive regulator in *E. coli*.<sup>116</sup> The activation of biosynthesis-related genes also regulates the secondary metabolites of *Streptomyces avermitilis*<sup>117</sup> and *Streptomyces coelicolor*.<sup>118</sup> According to the known mechanism of OxyR regulation,<sup>119</sup> ROS initiates the conformational change of OxyR from the reduced -SH state to the -S-S state; the oxidised OxyR-S-S binds to the upstream DNA sequences adjacent to the target gene,<sup>120</sup> which further recruits RNA polymerase to promoter<sup>121</sup> and subsequently initiates the expression. The sequences in the noncoding region similar to the conserved motifs<sup>120</sup> binding to OxyR have also been found in the upstream of *clbR*. Thus, further validation of the possibility that OxyR acts as a regulator of *clb* is imperative in future studies.

#### 6.6. Significance and therapeutic potential

Colibactin-producing *E. coli* exhibits diverse biological activities, including genotoxicity intermediated by colibactin, antibacterial activities to facilitate colonisation, decreasing the number of immune cells to achieve immune escape, analgesic activity for the survival of *pks+* *E. coli*, alteration of intestinal-tract physiology via inflammation-inducing activity to generate more ROS, and causing CRC in humans. Recent studies have made substantial progress in illustrating the structure and carcinogenicity of colibactin and the distinct mutational signatures that could act as potential diagnostic markers of CRC. Given that short-term infection with colibactin induces DNA damage in healthy colon epithelial cells without the



mutational signature,<sup>24</sup> the colibactin–DNA adduct (instead of the signature) could also serve as a biomarker for early diagnosis.<sup>122</sup> To retard the development and progress of CRC, diverse strategies should be developed to abolish or impede colibactin biosynthesis. The following approaches could be considered: (i) the knockout of key genes responsible for colibactin biosynthesis, such as *clbP*, *clbA*, and *clbR*; (ii) the addition of a Cu(II) chelator such as EDTA to deprive colibactin of Cu(II), thereby at least partially reducing its action; (iii) the introduction of the colibactin-resistant gene *clbS* to the host to transform colibactin into other adducts, thereby reducing the genotoxicity of colibactin; (iv) the introduction of a dCas9 element that targets the key genes for colibactin biosynthesis to abolish the expression of these genes, thereby eliminating colibactin production; and (v) the development of an inhibitor of the regulators of *clbR*, HtpG heat-shock protein, and other uncharacterised regulators to impede the production of colibactin. Furthermore, from another perspective, the utilisation of *pks+* *E. coli* such as Nissle 1917 and the *clb* gene cluster to induce the apoptosis of tumour cells should also be considered. For instance, the attenuated *Salmonella enterica* strain, which can selectively target tumour tissues, could be an ideal host for the expression of the *clb* gene cluster to produce colibactin in cancer, induce tumour-cell apoptosis and death, and ultimately impede CRC development and progress. Additionally, colibactin-645 (**18**) causes DNA damage in various eukaryotic cells at the nanomolar level<sup>18</sup> and the cytotoxicity of precolibactin-886 (**14**) indicates **18**'s potential cytotoxicity. Thus, this compound could provide a new scaffold template for anticancer drug development.

## 7. Author contributions

P. Y. Qian conceptualised the project in response to an invitation from editors of NPR. P. Y. Qian and Z. R. Li drafted and submitted the synopsis. J. W. Tang, X. Liu and W. Ye drafted the manuscript with input from Z. R. Li. (J. W. Tang drafted the chemistry and biosynthesis related part; X. Liu drafted the bioactivity related part). All authors contributed to the revision of the manuscript.

## 8. Conflicts of interest

All authors in this manuscript declare no conflict of interest.

## 9. Acknowledgements

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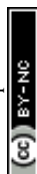
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