Discovery of ammosesters by mining the *Streptomyces uncialis* DCA2648 genome revealing new insight into ammosamide biosynthesis

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Abstract: The ammosamides (AMMs) are a family of biologically active natural products featuring an unusual chlorinated pyrrolo[4,3,2-de]quinoline core (Fig. 1). The first discovered members, AMMs A (1) and B (2), were isolated from a marine-derived strain *Streptomyces* sp. CNR-698 (Fig. 1A) (Hughes, MacMillan, Gaudêncio, Fenical, et al., 2009; Hughes, MacMillan, Gaudêncio, Jensen, et al., 2009). Subsequent efforts in the total syntheses of 1 and 2 and generation of additional analogs for structure-activity relationship (SAR) studies enabled the discovery of AMM C (3), the third member of the AMM family from *Streptomyces* sp. CNR-698 and the likely biosynthetic precursor to 1 and 2 upon nucleophilic oxidation or sulfuration at C-2, respectively (Fig. 1A) (Hughes & Fenical, 2010). Since then, an oxidatively ring-opened analog AMM D (4) and two amidine analogs AMMs E (5) and F (6) have been isolated from another marine-derived strain *Streptomyces variabilis* SAN-020, together with 1 and 2 (Fig. 1A) (Pan et al., 2012, 2013). Supplementing the culture medium of *S. variabilis* SAN-020 with a variety of aryl and alkylamines further afforded the production of AMMs G-P (7–16) (Fig. 1B) (Pan et al., 2013). Subsequently, 3 was established as the only bona fide natural product of the AMM biosynthetic machinery, and all other AMMs isolated to date were

**Graphical abstract**

Discovery of ammosesters (AMEs) by mining the *Streptomyces uncialis* DCA2648 genome reveals a unified pathway for AME and ammosamide biosyntheses diverging from a common intermediate with pathway-specific tailoring enzymes.

**Introduction**

The ammosamides (AMMs) are a family of biologically active natural products featuring an unusual chlorinated pyrrolo[4,3,2-de]quinoline core (Fig. 1). The first discovered members, AMMs A (1) and B (2), were isolated from a marine-derived strain *Streptomyces* sp. CNR-698 (Fig. 1A) (Hughes, MacMillan, Gaudêncio, Fenical, et al., 2009; Hughes, MacMillan, Gaudêncio, Jensen, et al., 2009). Subsequent efforts in the total syntheses of 1 and 2 and generation of additional analogs for structure-activity relationship (SAR) studies enabled the discovery of AMM C (3), the third member of the AMM family from *Streptomyces* sp. CNR-698 and the likely biosynthetic precursor to 1 and 2 upon nucleophilic oxidation or sulfuration at C-2, respectively (Fig. 1A) (Hughes & Fenical, 2010). Since then, an oxidatively ring-opened analog AMM D (4) and two amidine analogs AMMs E (5) and F (6) have been isolated from another marine-derived strain *Streptomyces variabilis* SAN-020, together with 1 and 2 (Fig. 1A) (Pan et al., 2012, 2013). Supplementing the culture medium of *S. variabilis* SAN-020 with a variety of aryl and alkylamines further afforded the production of AMMs G-P (7–16) (Fig. 1B) (Pan et al., 2013). Subsequently, 3 was established as the only bona fide natural product of the AMM biosynthetic machinery, and all other AMMs isolated to date were
Fig. 1. Structures of known ammosamides (AMMs). (A) Naturally isolated AMMs: 1–3 were produced by Streptomyces sp. CNR-698 wild-type strain and 4–6 were produced by S. variabilis SAN-020 wild-type strain. (B) Amidine analogs of AMMs: 7–16 were produced by precursor-directed biosynthesis in S. variabilis SAN-020 wild-type strain. (C) Engineered AMMs, and ammosamaic acid and congener: 17–20 were produced by Streptomyces sp. CNR-698 mutant strains.

“artifacts,” derived from 3 via a nonenzymatic nucleophilic addition of the varying nucleophiles at C-2 followed by air oxidation, during fermentation and isolation of the natural products (Reimer & Hughes, 2017). While 1 and 2 were initially shown to be highly cytotoxic against the HCT-116 colon cell line, targeting the motor protein myosin (Hughes, MacMillan, Gaudêncio, Fenical, et al., 2009; Hughes, MacMillan, Gaudêncio, Jensen, et al., 2009), 2 and several of its synthetic analog have been shown to be potent inhibitors against quinone reductase 2, a cytosolic protein that has been implicated as a target for cancer chemoprevention (Reddy et al., 2012; Reimer & Hughes, 2017). Discovery of novel AMM congeners could therefore enrich the structural diversity around this privileged scaffold and expand the SAR of AMMs for potential drug discovery.

The ammosamide (amm) biosynthetic gene cluster (BGC) has been cloned from Streptomyces sp. CNR-698 (Fig. 2A) and expressed in Streptomyces coelicolor M512, leading to the production of 1–3 and providing a genetically amenable platform to interrogate AMM biosynthesis in vivo (Jordan & Moore, 2016). The deceptively simple AMM scaffold is of a surprisingly complex biosynthetic origin, as the amm BGC encodes a precursor peptide (Amm6) and LanB-like proteins (such as Amm8, 9, 11, and 18), only known to be associated with ribosomally synthesized and posttranslationally modified peptides (RiPPs) at the time (Arnison et al., 2013). Deletion of the genes encoding either Amm6 or the LanB-like proteins yielded mutants that completely lost AMM production, confirming their necessity for AMM production but failing to reveal any insight into their roles in AMM biosynthesis (Jordan & Moore, 2016). In contrast, deletion of the genes encoding selected tailoring enzymes, such as a F420-dependent oxidase (Amm4) and an N-methyltransferase (Amm23), afforded mutants that accumulated ammosamaic acid (17) and N-desmethyl AMM C (19), as well as their corresponding air-oxidized (at C-2) congeners (18 and 20), shedding light on the late steps of AMM biosynthesis (Jordan & Moore, 2016). Most recently, it has been discovered that the AMMs belong to an emerging family of amino acid-derived natural products whose biosynthesis features a ribosomally synthesized scaffold peptide that undergoes a nonribosomal peptide extension, catalyzed by the LanB-like proteins, followed by tailoring modifications of the extended amino acid and an eventual proteolytic
removal of the scaffold peptide to afford the final natural products (Ting et al., 2019). This was demonstrated for AMMs by in vitro extension of an Amm6 (also known as AmmA) variant with L-Trp, catalyzed by Amm9 (also known as AmmB2) (Ting et al., 2019). Pending experimental confirmation, tailoring modifications of the extended L-Trp followed by proteolytic removal of the Amm6 scaffold peptide would yield a biosynthetic intermediate featuring the pyrroloquinoline core with a free carboxylic acid, in agreement with the isolation of 17 and 18 (Fig. 1C) (Jordan & Moore, 2016). Comparison of the BGCs encoding AMMs and novel congeners therefore promises to reveal new insights into the use of a scaffold peptide in the biosynthesis of amino acid-derived natural products and provide new opportunities to mimic Nature’s combinatorial biosynthetic strategies to generate natural product structural diversity.

*Streptomyces uncialis* DCA2648, a lichen-associated actinomycete, is known to produce uncialamycin (UCM) (Davies et al., 2005), a member of the enediyne family of extremely potent antitumor antibiotics (Adhikari et al., 2020), and the cladoniamides (CLAs) (Williams et al., 2008), bis-indole alkaloids with modest cytotoxicity against selected human cancer cell lines. The cla BGC was cloned previously by screening a cosmid library of *S. uncialis* (Ryan 2011). We cloned the ucm BGC by sequencing and mining the *S. uncialis* genome for genes encoding the characteristic enediyne polyketide synthase cassette (Yan et al., 2016). Genome mining has revolutionized microbial natural product discovery in the genomics era (Kalkreuter et al., 2020; Ziemert et al., 2016). We have applied multiple strategies in our current efforts to leverage the large strain collection at The Scripps Research Institute for natural product and drug discovery by genome mining (Steele et al., 2019), as exemplified by the discovery of new enediynes (Yan et al., 2016; Yan et al., 2017) and the leinamycin family of natural products (Pan et al., 2017). The *S. uncialis* genome harbors minimally 30 BGCs (Yan et al., 2016), providing an outstanding opportunity to mine for novel natural products. Here we report the discovery of the ammosesters (AMES), a sub-family of new AMM congeners that feature a C-4a methyl ester (Fig. 2), differing from the C-4a amide functional group characteristic to the AMMs (Fig. 1), from *S. uncialis* SB18002. Comparative analysis of the amm and ame BGCs supports the use of a scaffold peptide in the biosynthesis of the common pyrroloquinoline carboxylic acid intermediate 17, from which AME and AMM biosynthesis diverges by evolving a pathway-specific O-methyltransferase (Ame24) for AMEs and amide synthetase (Amm20) for AMMs, respectively (Fig. 3). The AMEs exhibit modest cytotoxicity against selected human cancer cell lines, further expanding the SAR for the pyrroloquinoline family of natural products.

**Materials and Methods**

**Bacterial Strains and Culture Conditions**

Bacterial strains, plasmids, and oligonucleotides used in this study are listed in Supplementary Tables S1 and S2. The cosmid
Fig. 3. A unified proposed pathway for ammosester and ammosamide biosynthesis diverging from a common intermediate with pathway-specific tailoring enzymes. (A) The use of a scaffold peptide as an emerging paradigm for the biosynthesis of the pyrroloquinoline family of natural products. (B) AME and (C) AMM biosynthesis diverges from the common intermediate by evolving the pathway-specific Ame24 O-methyltransferase (color-coded blue) and Amm20 amide synthetase (color-code red), respectively. The structures in brackets represent proposed intermediates; bold arrows represent the proposed major routes and dashed arrows depict nonenzymatic paths or enzyme substrate promiscuity. PEARL, peptide-amino acid tRNA ligase (Ting et al., 2019); SP, scaffold peptide; [O], oxidation; [S], sulfuration; [NH2R], aryl and alkylamines.

Experimental Procedures

Bioinformatics analyses and comparative analyses of nucleotide or amino acid sequences were conducted using BLAST. Fermentation was carried out in New Brunswick Scientific Innova 44 incubator shakers. The 1H and 13C, HSQC, and HMBC spectra were collected with a Bruker Avance III Ultrashield 700 at 700 MHz for 1H and 175 MHz for 13C nuclei. HPLC was carried out on an Agilent 1260 Infinity LC equipped with an Agilent SB-C18 column (250 mm × 4.6 mm, 5 μm) at a flow rate of 1 ml/min. The column was equilibrated with 10% acetonitrile with 0.1% formic acid, and developed with a 20-min analytical program consisting of a 15-min linear gradient from 10% to 70% acetonitrile with 0.1% formic acid followed by a 5-min linear gradient from 70% to 100% acetonitrile with 0.1% formic acid. LC-MS was performed on an Agilent 1260 Infinity LC coupled to a 6230 TOF (HR-ESI) equipped with an Agilent Poroshell 120 EC-C18 column (50 mm × 4.6 mm, 2.7 μm) using a linear gradient of CH3CN in H2O with 0.1% formic acid (0–15 min, 10–70% CH3CN; 15–20 min, 70–100% CH3CN) at a flow rate of 0.4 ml/min. Column chromatography was conducted on Sephadex LH-20. Semipreparative HPLC was carried out on a Varian liquid chromatography system with a YMC-pack ODS-A (250 mm × 10 mm, 5 μm) column. All common biochemicals and...
Table 1. NMR Spectroscopic Data ($^1$H at 700 MHz and $^{13}$C at 175 MHz, DMSO-$d_6$) for AMEs (21–23) and the Ammosamaic Acid Congener 24

| Position | AME A (21) | AME B (22) | AME C (23) | Ammosamaic acid congener (24) |
|----------|------------|------------|------------|-------------------------------|
|          | $\delta_C$, type $^a$ | $\delta_H$ (J in Hz) | $\delta_C$, type $^a$ | $\delta_H$ (J in Hz) | $\delta_C$, type $^a$ | $\delta_H$ (J in Hz) |
| 1a       | 38.4, CH$_3$ | 4.34, s | 29.1, CH$_3$ | 3.61, s | 29.1, CH$_3$ | 3.60, s |
| 2        | 124.3, C   | 7.95, s | 164.1, C   | 8.00, s | 165.2, C | 8.10, s |
| 2a       | 121.5, C   |          | 130.7, C   |          | 131.9, C |          |
| 3        | 121.0, CH  | 8.48, s | 119.0, CH  | 8.38, s | 119.3, CH | 8.36, s |
| 4        | 138.6, C   |          | 142.1, C   |          | 119.1, C |          |
| 4a       | 166.5, C   |          | 165.8, C   |          | 165.9, C |          |
| 5a       | 142.0, C   |          | 133.6, C   |          | 132.8, C |          |
| 5b       | 119.5, C   |          | 119.6, C   |          | 120.4, C |          |
| 6        | 142.9, C   |          | 132.4, C   |          | 132.7, C |          |
| 7        | 106.4, C   |          | 105.9, C   |          | 105.1, C |          |
| 8        | 154.3, C   |          | 140.9, C   |          | 140.5, C |          |
| 8a       | 119.1, C   |          | 107.0, C   |          | 105.2, C |          |
| 1N-H     | 52.7, CH$_3$ | 3.90, s | 53.0, CH$_3$ | 3.96, s | 53.0, CH$_3$ | 3.95, s |
| 4a-CH$_3$ | 52.7, CH$_3$ | 3.90, s | 53.0, CH$_3$ | 3.96, s | 53.0, CH$_3$ | 3.95, s |
| 6-NH$_2$ | 8.31, s    | 6.18, s  | 6.08, s    | 6.20, s  | 6.20, s  |
| 8-NH$_2$ | 6.34, s    | 6.35, s  | 6.37, s    | 6.50, s  | 6.50, s  |

$^a$Carbon chemical shifts were based on HSQC and HMBC data.

$^b$No carbon signal was detected, which was consistent with reported $^{13}$C NMR spectrum for 18 (Jordan & Moore, 2016).

Culture medium components were purchased from commercial sources and used as it.

### Extraction and Isolation

After the fermentation, 1% Amberlite XAD-16 and 1% Dianion HP-20 resins were added to the fermentation broth and incubated overnight at 28°C. The cell pellets and resins were collected by centrifugation, dried on air, and then extracted three times with methanol. The extract was concentrated and subjected to HPLC or LC–MS for analysis. For large-scale fermentation, 2-l baffled flasks (1 mg), respectively. The EC$_{50}$ was determined by computerized curve fitting using GraphPad Prism (Table 2 and Supplementary Fig. S6).

### Construction of the $\Delta$ame24 Mutant Strain S. uncialis SB18003

Inactivation of the $\Delta$ame24 gene was performed by gene replacement following literature procedures (Supplementary Fig. S7) (Gust et al., 2003; Kieser et al., 2000). Briefly, pBS18005, a cosmid containing a partial $\Delta$ame gene cluster, was transformed into E. coli BW25113/pJL790. The $\Delta$ame24 gene was replaced with the apramycin resistance cassette from pJL773 using λ-red-mediated PCR-targeting strategy to afford pBS18006. Then pBS18006 was introduced into S. uncialis SB18002 by intergeneric conjugation. Exconjugants with the desired double crossover recombination event were selected based on resistance to apramycin and sensitivity to kanamycin, yielding the $\Delta$ame24 mutant strain SB18003.

The genotype of SB18003 was confirmed by Southern blot analysis using DIG High Prime DNA Labeling and Detection Starter Kit I (Roche) (Supplementary Fig. S7). DNA probe was PCR-amplified using oligonucleotides SA-ame24-F/SA-ame24-R (Supplementary Table S2) and S. uncialis SB18002 genomic DNA as template.

### Gene Expression and Protein Overproduction and Purification

The $\Delta$ame24 gene was amplified by PCR from the cosmid pBS18005 using the primers GE-ame24-F/GE-ame24-R (Supplementary Table S2) and Q5 High-Fidelity DNA Polymerase (NEB) following the protocol provided by the manufacturer. The PCR product was purified, treated with T4 polymerase (NEB), and cloned into pBS3080 using the primers SA-ame24-F/SA-ame24-R (Supplementary Table S2) and Q5 High-Fidelity DNA Polymerase (NEB) following the protocol provided by the manufacturer. The PCR product was purified, treated with T4 polymerase (NEB), and cloned into pBS3080 according to ligation-independent procedures to give pBS18007 (Lohman et al., 2013). pBS18007 was transformed into E. coli BL21 (DE3), and cells were grown in LB at 37°C until an OD$_{600}$ of 0.6 was reached. The cells were cooled to 4°C, added 0.1 mM of isopropyl β-D-1-thiogalactopyranoside to induce gene expression, and further grown at 18°C for 16 hr. The cells were harvested at 4000 x g for 15 min at 4°C, and the pellet was resuspended in lysis buffer.
(100-mM Tris, pH 8.0, containing 300-mM NaCl, 15-mM imidazole, and 10% glycerol). After sonication, the cell debris was removed by centrifugation at 15,000 x g for 15 min at 4°C. The lysate was loaded onto a HisTrap 5-ml column equilibrated with washing buffer (50-mM Tris, pH 8.0, containing 100-mM NaCl and 15-mM imidazole). The column was washed with washing buffer and the His6-tagged protein was eluted using elution buffer (50-mM Tris, pH 8.0, containing 100-mM NaCl and 300-mM imidazole). Following elution, the protein was diluted three times using 50-mM Tris, pH 8.0 buffer. The protein was loaded onto a HiTrap Q HP 5 ml equilibrated with washing buffer (50-mM Tris, pH 8.0, containing 1-M NaCl). Finally, the protein was loaded onto a Superdex S200 16/600 gel filtration column using 50-mM Tris, pH 8.0, buffer containing 100-mM NaCl, affording 9.7 mg of Ame24 to homogeneity from 0.8 l of E. coli recombinant culture, and stored at −80°C (Supplementary Fig. S8A).

In vitro Assay of Ame24 as a C-4a O-Methyltransferase

Enzymatic reactions were performed in 50-mM Tris buffer, pH 7.5, containing 200 μM of Ame24, 500-μM SAM, and 200 μM 24, in a total volume of 50 μl. After incubation at 30°C for different times, 50 μl of methanol were added to quench the reactions. The reaction mixture was then centrifuged and 10 μl of the supernatant were injected and analyzed by analytical HPLC (Supplementary Fig. S8C).

Results and Discussion

Identification of a Putative BGC Encoding Ammosamide Family of Natural Products Through Genome Mining of S. uncialis DCA2648

In silico analysis of the S. uncialis genome by antiSMASH (Blin et al., 2019) led to the identification of the aemc BGC that has a nearly identical genetic organization to the amm BGC from Streptomyces sp. CNR-698 (Fig. 2A). Comparative analysis of the gene products within the two BGCs revealed high amino acid sequence identity (52–76%), indicative of homologous biosynthetic machinery (Supplementary Table S3). While the ame and amm BGCs consist of 22 and 24 open reading frames (ORFs), respectively, 21 of them are considered between the two BGCs. Notably, the homolog of amm20, which is predicted to encode an amide synthetase, is missing in the ame BGC, together with amm21, encoding a transporter, and amm5, encoding a hypothetical protein, while the aemc BGC harbors an extra gene, aemc24 encoding an O-methyltransferase, the homolog of which is absent in the amm BGC (Fig. 2A). The similarity and difference between the two BGCs suggest that S. uncialis may produce novel congeners of the AMM family of natural products.

Production, Isolation, and Structural Elucidation of Ammosesters from S. uncialis SB18002

To determine whether S. uncialis produces AMM congeners, we initially carried out fermentation of the wild-type S. uncialis DCA2648 on a small scale (50 ml). However, detection of new metabolites was obscured by the CLAs, which were the predominate natural products under the optimized fermentation condition. To facilitate detection of the trace metabolites overshadowed by CLAs, we deleted the cla BGC in the wild-type strain to afford the Δcla mutant strain of S. uncialis SB18002. S. uncialis SB18002 was fermented and the fermentation broth was extracted and subjected to HPLC and LC-MS analysis, resulting in identification of several metabolites with a UV absorbance of 520–540 nm and MS isotope signatures of chlorine-containing natural products, characteristic of the AMMs (Fig. 2B and Supplementary Figs. S1–S4). These results suggested the production of new chlorinated pyrroloquinoline analogs by S. uncialis, and we then performed a large-scale fermentation of S. uncialis SB18002 (1 l) and isolated four major products from the fermentation broth for structural characterization. They were identified as AME A (21), B (22), and C (23), named after the characteristic C-4a methyl ester functional group unprecedented in the AMM scaffold known to date, as well as a new ammosamaic acid congener (24) (Fig. 2C), by extensive spectroscopic analysis and direct comparison of their NMR data (Table 1 and Supplementary Figs. S1–S4) with those reported for AMMs (Hughes & Fenical, 2010; Hughes, MacMillan, Gaudêncio, Jensen, et al., 2009; Reddy et al., 2010; Williams et al., 2008).

AME B (22) was isolated as purple powder. It was readily identified as a methyl ester variant of AMM B (2) (Fig. 2C) through direct comparison of the NMR and HR-ESI-MS data (Table 1 and Supplementary Fig. S2) with those reported for AMMs. The molecular formula was established as C12H9ClN4O3 by HR-ESI-MS ([M + H]+ ion at m/z 293.0444, 293.0448 (Supplementary Fig. S3), giving the molecular formula as C12H9ClN4O3. The diagnostic 1H and 13C NMR resonances showed high similarity between 22 and 23, with the only differences at N-1 and its nearest carbon chemical shifts at C-2 and C-8a (Table 1 and Supplementary Fig. S3). Together with the loss of the N-CHO signal, 23 was assigned as the N-demethyl congener of 22 (Fig. 2C), the structure of which was further supported by extensive 2D NMR analysis, as exemplified, in particular, by the clear HMBC correlations from 1-NH to C-2a and C-5b (Supplementary Figs. S3 and S5).

The new ammosamaic acid congener (24) was isolated as purple powder. The molecular formula of 24 was established as C12H9ClN4O3 by HR-ESI-MS ([M + H]+ ion at m/z 293.0444,

### Table 2. Cytotoxicity of AMEs (21–23) and the Ammosamaic Acid Congener 24 Against Selected Human Cancer Cell Lines

| Cell lines            | Cancer type | EC50 (μM)       | Doxorubicin (μM) |
|-----------------------|-------------|----------------|-----------------|
| MDA-MB-231            | Breast      | 8.5 ± 2.3      | 0.2 ± 0.8       |
| SK-MEL-5              | Melanoma    | 9.3 ± 0.9      | 0.5 ± 0.3       |
| SF-295                | CNS         | 126 ± 20       | 4.8 ± 1.1       |
| NCI-H226              | NSC lung    | 80 ± 4         | 3.0 ± 0.3       |
| OVCAR-3               | Ovarian     | 8.4 ± 1.1      | 1.4 ± 0.1       |

The results suggested the production of new chlorinated pyrroloquinoline analogs by S. uncialis, and we then performed a large-scale fermentation of S. uncialis SB18002 (1 l) and isolated four major products from the fermentation broth for structural characterization. They were identified as AME A (21), B (22), and C (23), named after the characteristic C-4a methyl ester functional group unprecedented in the AMM scaffold known to date, as well as a new ammosamaic acid congener (24) (Fig. 2C), by extensive spectroscopic analysis and direct comparison of their NMR data (Table 1 and Supplementary Figs. S1–S4) with those reported for AMMs (Hughes & Fenical, 2010; Hughes, MacMillan, Gaudêncio, Jensen, et al., 2009; Reddy et al., 2010; Williams et al., 2008).

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Supplementary Fig. S4E). With the exception of 14 mass unit less and an extra N-CH₃ signal (δ_H 3.60, 3H, s, δ_C 29.1), 24 displayed very similar NMR data to those of the C-2 oxidized ammosaic acid (18) (Jordan & Moore, 2016). Detailed comparison of overall NMR data revealed that the major differences between 24 and 18 resided mainly at N-1 and its adjacent carbons C-2 and C-8a, supporting N-methylation at N-1 for 24 (Table 1). Taken together, 24 was assigned as N-methyl and C-2 oxidized ammosaic acid (Fig. 2C), the overall structure of which is fully supported by 2D NMR analysis, including the key HMBC correlations from N-1 (δ_H 3.60) to C-2 (δ_C 164.4) and C-8a (δ_C 107.1) (Supplementary Figs. S4 and S5).

AME A (21) was isolated as purplish red solid. HR-ESI-MS analysis of 21 afforded an [M]+ ion at m/z 291.0632, giving the molecular formula as C₂₃H₂₁ClN₂O₇. The 1D NMR spectra of 21 were very similar to those of 22. The major differences were that the carbonyl carbon at C-2 (δ_C 164.1) in 22 was replaced by iminium carbon at C-2 (δ_H 7.35, s, δ_C 124.3) in 21 (Table 1), leading to the assignment of 21, in a structural analogy to AMM C (3), as an iminium salt (Fig. 2C). The structure of 21 was finally confirmed by 2D NMR analysis, specifically by HMBC correlations from H-2 to C-2a/C-5b/N-CH₃ and from N-CH₃ to C-2/C-8a (Supplementary Figs. S1 and S5) as has been observed previously for 3 (Reimer & Hughes, 2017).

Since it has been demonstrated that AMM B (2) is derived from AMM C (3) via nonenzymatic oxidation at C-2 (Reimer & Hughes, 2017), the AMEs (22–24) are likely also derived from the corresponding iminium ion precursors, such as 21 for 22, via the same nonenzymatic process (Fig. 3). Although 22 and 23 have been reported previously as synthetic intermediates of 2 (Reddy et al., 2010), we have isolated them for the first time, together with 21 and 24, as natural products, expanding the structural diversity for the AMM family of natural products.

Cytotoxicity of the Ammosesters Against Selected Human Cancer Cell Lines

The AMEs (21–23), together with the ammosaic acid congener 24, were evaluated for their cytotoxic activities against five selected human cancer cell lines, including melanoma (SKMEL-5), breast (MDA-MB-231), central nervous system (SF-295), nonsmall cell lung (NCI-H226), and ovarian (OVCAR-3), each of which was either resistant or sensitive to taxol (Table 2 and Supplementary Fig. S6) (Yan et al., 2016; Yan et al., 2017). Similar to 2, 21 exhibited modest cytotoxicity against all the tested cancer cell lines, with EC₅₀ values ranging from 15 to 140 μM, while 22 was active against the SKMEL-5 and OVCAR-3 cell lines, and 23 and 24 were inactive at the concentrations examined (EC₅₀ > 200 μM), suggesting both the O-methylation and N-methylation contribute to the cytotoxicity. The fact that 21 exhibited an increased cytotoxicity towards the selected human cancer cell lines in comparison to 22–24 is consistent with the previous findings for the AMMs with the pyrroloquinolinium congeners showing the highest cytotoxicity against HCT-116 (Reimer & Hughes, 2017).

In Vitro and In Vivo Characterization of Ame24 Confirming Its Function as an O-Methyltransferase

The characteristic structural difference between AMEs and AMMs is the presence of a methyl ester or amide at the C-4a of AMEs or AMMs, respectively (Figs. 1 and 2C). Comparative analysis of the ame and amm BGCs revealed ame24, unique to the ame BGC and predicted to encode an O-methyl transferase, serving as the candidate for O-methylation at C-4a in AME biosynthesis (Fig. 2A). To investigate AME biosynthesis, we first carried out in vivo studies by inactivating the ame24 gene in S. uncilis SB18002, using the PCR-targeting and λ-RED-mediated mutagenesis method (Gust et al., 2003; Kieser et al., 2000). The genotype of the resultant Δame24 mutant strain S. uncilis SB18003 was confirmed by Southern analysis (Supplementary Fig. S7). When cultured under the same conditions, Δame24 strain SB18003 was found to fail to produce 21–23 but still produced the free acid congener 24 (Supplementary Fig. S7). The latter phenotype would be consistent with Ame24 as an O-methyl transferase catalyzing O-methylation of a free acid precursor in AME biosynthesis. We next expressed ame24 in E. coli and purified the overproduced Ame24 to homogeneity to directly confirm its activity in vitro (Supplementary Fig. S8). In the presence of S-adenosyl methionine (SAM), Ame24 catalyzed time-dependent conversion of 24 into a new product, which was absent in the negative control using boiled Ame24 as a control (Fig. 2B). The identity of the new product as 22 was confirmed by HPLC–MS analysis in comparison with an authentic standard. While these findings unambiguously established Ame24 as an O-methyltransferase, catalyzing O-methylation at C-4a in AME biosynthesis, the low catalytic efficiency of Ame24 with 24 as a substrate, as exemplified by 6% and 12% conversion after 2 and 18 hr, respectively, under the assay conditions (Supplementary Fig. S8), suggested that 24 may not be the preferred substrate. The latter would be consistent with the isolation of 21 and 23 from S. uncilis SB18002 fermentation, indicative of the existence of alternative free acids as AME24 substrates, such as 26 and 17, respectively (Fig. 3B). All attempts to generate 26 by hydrolysis of 21, however, failed due to its intrinsic instability, and we also failed to detect 17 in S. uncilis SB18002 fermentation, both of which prevented us from directly determining the preferred substrate for Ame24 and thereby the timing of Ame24 catalysis in AME biosynthesis.

A Unified Pathway for AME and AMM Biosynthesis Diverging from a Common Intermediate with Pathway-Specific Tailoring Enzymes

The similarity in both structures and BGCs suggests a common pathway for AME and AMM biosynthesis (Fig. 3). Thus, inspired by the emerging paradigm for amino acid-derived natural product biosynthesis featuring a scaffold peptide, we propose that AME and AMM biosynthesis shares a unified pathway, diverging from a common intermediate, that is, the nascent product derived upon proteolytic removal of the scaffold peptide (Ting et al., 2019). Such an intermediate would feature a free carboxylic acid, in agreement with 17 (Fig. 3A), which has been isolated from the Streptomyces sp. CNR-698 Δamm4 mutant strain (Jordan & Moore, 2016). However, amm4, predicted to encode an F420-dependent oxidase, is unlikely to be involved in the formation of the C-4a amide for the AMMs as proposed previously (Jordan & Moore, 2016) on the basis of both the presence of ame5, an amm4 homolog, and the absence of an amm20 homolog in the ame BGC (Fig. 2A). Instead, we propose that amm20, predicted to encode an amide synthetase, catalyzes C-4a amidation of the common intermediate 17 to afford 19, which is finally N-methylated by Amm23 to yield 3, completing AMM biosynthesis (Fig. 3C). In a biosynthetic analog, C-4a O-methylation and N-methylation of 17 by Ame24 and Ame22, respectively, affording 21, would account for AME biosynthesis (Fig. 3B).

Finally, we carried out a time course analysis of the AME metabolite profile of S. uncilis SB18002, in an attempt to determine the timing of the Ame24-catalyzed O-methylation
and Am22-catalyzed N-methylation in AME biosynthesis (Supplementary Fig. S9). AME production could be readily observed as early as 4 days of growth, with Am22 as the dominant metabolite. After 6 days of fermentation, Am21, the precursor to the nonenzymatically C-2 oxidized product Am22, was observed as the dominant metabolite, supporting Am21 as the bona fide natural product of the AME biosynthetic machinery (Fig. 3B). As fermentation continued, Am21 started to decrease with a concomitant increase of Am22, as well as the detection of Am23 and Am24. Since Am23 and Am24 could be viewed as nonenzymatically C-2 oxidized products of intermediates Am25 and Am26, respectively, en route to Am21, the fact that Am23 and Am24 increased with a relatively constant ratio would suggest that N-methylation and O-methylation of Am21–Am24 proceed in two parallel pathways, most likely due to substrate promiscuity of both Am22 and Am24 (Fig. 3B).

In conclusion, by mining the genome of *S. uncialis*, we have identified the ame BGC that is highly homologous in both gene content and genetic organization to the amm BGC and have discovered a sub-family of new AMM congeners, named AMEs (Fig. 2). The AMEs feature a C-4a methyl ester, differing from the C-4a amide functional group characteristic to AMMs, and exhibit modest cytotoxicity against a broad spectrum of human cancer cell lines, expanding both the structure and SAR for the pyrroloquinoline family of natural products (Figs. 1 and 2C, and Table 2). Comparative analysis of the amm and ame BGCs, together with in vivo and in vitro studies of ame24, support the use of a scaffold peptide as an emerging paradigm for the biosynthesis of the pyrroloquinoline family of natural products. AME and AMM biosynthesis diverges from a common intermediate by evolving the pathway-specific Am24 O-methyltransferase and Ame20 amide synthetase, respectively (Fig. 3). Although the exact timing for each of the steps will have to be determined by additional in vivo and in vitro experiments in the future, the findings from the current study showcase once again Nature’s ingenuity to generate diverse natural products by evolving biosynthetic machineries in a combinatorial fashion.

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Supplementary Material

Supplementary material is available online at JIMB (www.academic.oup.com/jimb).

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Conflict of Interest

The authors declare no conflict of interest.

Data availability

All data generated or analysed during this study are included in this published article and its supplementary material.

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