First Ring-Expanded Maytansin Lactone Accessed by a New Mutasynthetic Variant

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A multiblocked mutant strain (ΔAHBA and Δ*asm12*, *asm21*) of *Actinosynnema pretiosum*, the producer of the highly toxic maytansinoid ansamitocin, has been used for the mutasynthetic production of new proansamitocin derivatives. The use of mutant strains that are blocked in the biosynthesis of an early building block as well as in the expression of two tailoring enzymes broadens the scope of chemo-biosynthetic access to new maytansinoids. Remarkably, a ring-expanded macrolactone derived from ansamitocin was created for the first time.

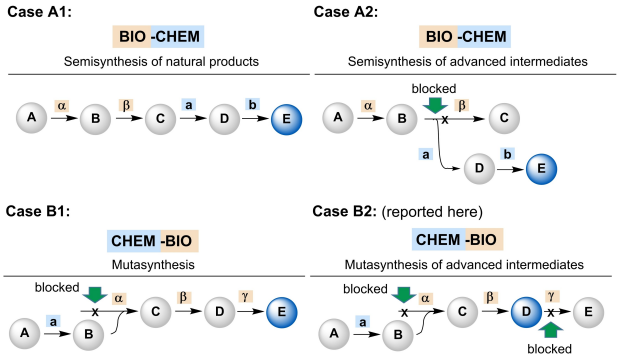

Figure 1. Selected concepts of chemo-biosynthetic strategies (blue: chemical synthesis, orange: biosynthesis, dark blue: final product, green: position of block; semisynthesis = BIO-CHEM and three concepts of mutasynthesis = CHEM-BIO; α, β, γ = enzymes; a, b = chemical agents/catalysts).

A promising strategy for creating natural product libraries is to combine biotechnologically oriented synthetic biology[1] with synthetic chemistry.[2] The former allows complex molecular architectures to be created through multistep biosynthesis while the latter provides large synthetic flexibility for introducing unnatural but pharmacologically important functions and functional groups.

Sensibly, chemo-biosynthetic strategies can be categorized by dissecting a synthesis into chemo- and biosynthetic elements (Figure 1). Semisynthesis is an example for a BIO-CHEM strategy (case A1) in which first a natural product is formed naturally followed by chemical derivatization.[2b] If the biosynthesis is blocked at a late stage the organism can provide advanced biosynthetic intermediates derived from important secondary metabolites (Figure 1, case A2).[2c]

An example for the reversed CHEM-BIO scenario (case B) is mutational biosynthesis (short "mutasynthesis") in which for example a mutant strain of a producer organism is blocked in the formation of an early biosynthetic pathway intermediate.[3] Administration of so called mutasynthons that are commonly prepared by chemical synthesis – these are structural analogs of biosynthetic intermediates – to the mutant strain then results in new metabolites sometimes referred to as mutaproducts (Figure 1, case B1). So far, no reports have appeared that utilize mutant producer strains that are blocked both at an early as well as at a late stage of the biosynthesis (Figure 1, case B2).

Following the concept B2, we disclose the creation and mutasynthetic exploitation of a mutant strain of *Actinosynnema pretiosum*, the producing strain of the highly cytotoxic polyketide ansamitocins P-3 (**3**), a maytansinoid.[4] Only in 2013 it was approved by the FDA as an antibody drug conjugate against HER2-positive breast cancer.[5] The strain is genetically blocked in the formation of 3-amino-5-hydroxybenzoic acid (AHBA, **1**) as well as in two tailoring steps, namely the chlorination (Asm12) and the amidination (Asm21) leading to almost complete loss of post-PKS enzyme activity (Scheme 1).[6]

As a consequence, new proansamitocin derivatives can be obtained that can principally be further modified semisynthetically thereby further expanding the opportunities to generate new nature-derived macrolactams.

Over the past years[7] we mostly employed ΔAHBA mutant strains (resembling case B1). AHBA **1**[8] is formed from UDP-glucose through a unique variation of the shikimate acid pathway (Scheme 1).[9] Processing through seven PKS modules furnishes *seco*-proansamitocin that undergoes macrolactamization by an amide synthase that is not part of the PKS to yield proansamitocin (**2**).[10,11] Finally, six tailoring steps provide ansamitocin P-3 (**3**).[10] Commonly, the protocols can be conducted with unmodified benzoic acids, because activation to the corresponding SCoA esters occurs before the in vivo uploading onto the PKS.[12]

The new mutant strain (AH1) was constructed according to the procedure of Yu et al.[10] The plasmid pHGF9029 was delivered into *A. pretiosum* Δ*asm12/21*[6] by conjugation with *Escherichia coli*. Positive clones where detected by feeding experiments with and

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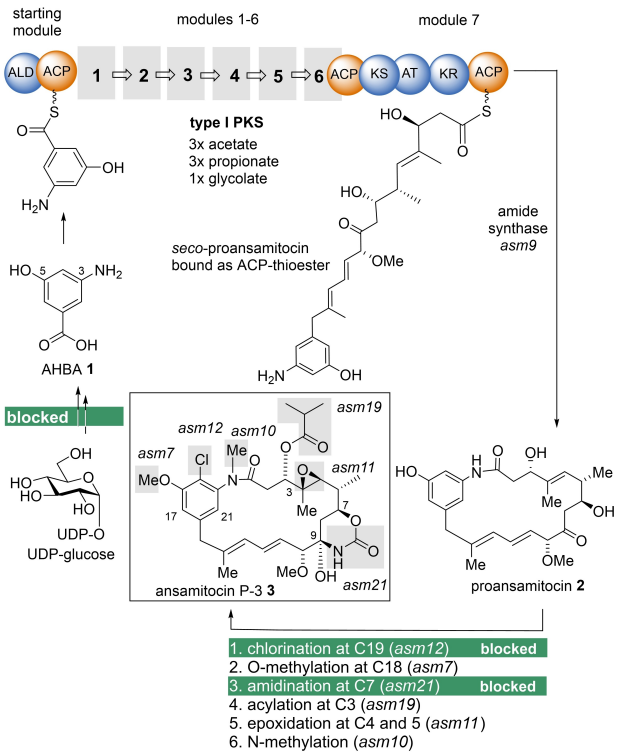

Scheme 1. Overview of ansamitocin **3** biosynthesis via proansamitocin (**2**), genes of tailoring enzymes and genes blocked in new strain AH1 of *Actinosynnema pretiosum;* (green: position of blocks in this work).[6,10]

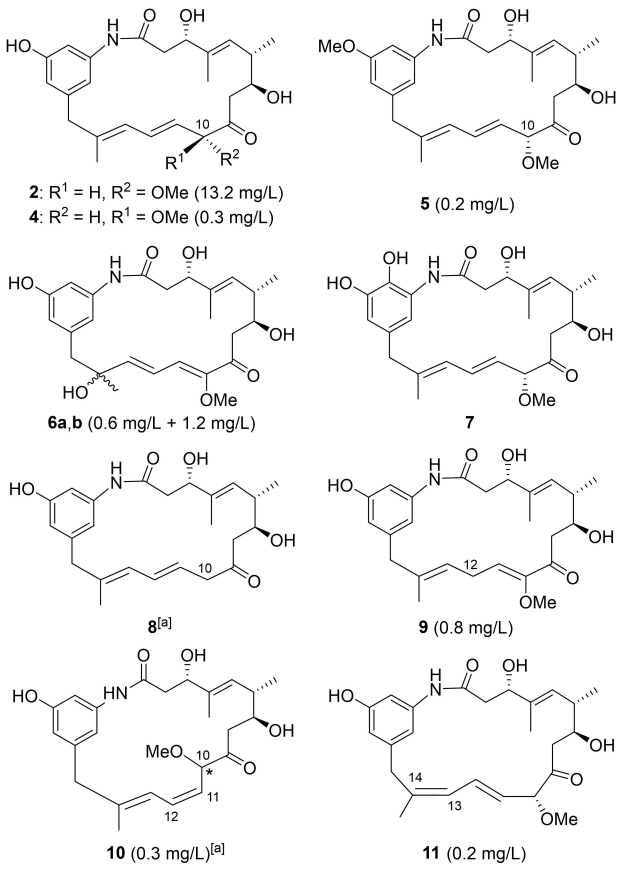

Figure 2. Proansamitocin (**2**) and (oxygenated) derivatives **4**–**11** produced by *A. pretiosum* (strain AH1) after supplementation of AHBA **1**. [a] Stereochemistry at C10 not assigned.

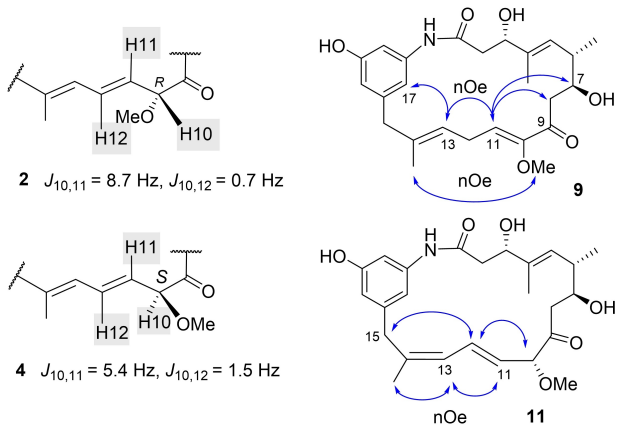

Figure 3. Coupling constants of 10-H in (10*R*)-**2** and (10*S*)-proansamitocin **4**, 2D NOESY correlations in 1,3-diene **8** and selected 1D nOe correlations in 1,4-diene **11**.

without AHBA **1**. Fermentation conditions were adapted to those previously described for the ΔAHBA mutant strain (HGF073) of *A. pretiosum*.[7a] The monitoring of proansamitocin titers during fermentation revealed that highest concentrations are observed on day ten post inoculation (p.i.). For ansamitocin production[13] the addition of the amino acid L-valine was beneficial. Unexpectedly, also in the present case increased proansamitocin production was observed upon supplementation of L-valine. In fact, L-valine is a biosynthetic precursor for the ester side chain at C3 and this transfer does not occur in strain AH1. We also found that pulse feeding of 2.5 g/L fructose on days 3, 4 and 5 p.i. led to significantly increased proansamitocin (**2**) titers (Figure S2 in the Supporting Information) which was also reported for ansamitocin P-3 (**3**) titers produced by the wild-type strain.[14]

Upon feeding of AHBA **1** to strain AH1 proansamitocin (**2**) and several derivatives **2**, **4**–**11** were isolated after extensive HPLC. As expected, proansamitocin (**2**) was the main product formed (Figure 2). Macrolactams **4**–**6** were disclosed before when using a *Δasm12/21* mutant strain of *A. pretiosum*.[15] 19-Hydroxy proansamitocin (**7**), 10-desmethoxy proansamitocin (**8**) and macrolactams **9**–**11** that mainly differ in the stereochemistry and location around the diene moiety (C10–C14) are new members of the proansamitocin family. The structure elucidation relied on NMR spectroscopic analyses that included the determination of nuclear Overhauser effects (nOe; Figure 3).

Having established the ability of the new multiblocked mutant strain AH1 to accept and process AHBA **1**, we next administered different aromatic acids **12**–**31** to a growing culture of strain AH1 (scale 3 x 50 mL). The formation and presence of new proansamitocin derivatives was determined by UPLC-HRMS/MS analyses after extraction with ethyl acetate.

Halogenated 3-aminobenzoic acids **12**–**16** were processed by strain AH1 to the corresponding macrolactams (Figure 4). Mass

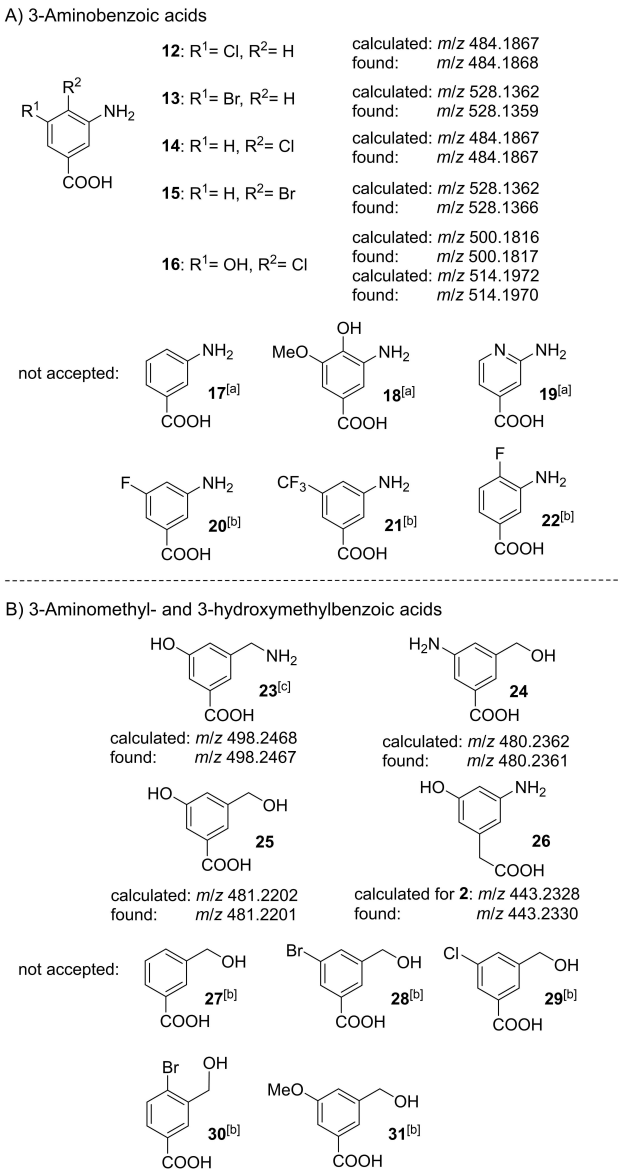

Figure 4. Aromatic acids **12**–**31** tested as mutasynthons for strain AH1. First MS data ($m+Na^+$) of expected proansamtocin derivatives collected from crude products are given. [a] No PKS-processed products detected; [b] detoxification products (benzamides) or starting benzoic acids detected; [c] processing occurred but only *seco*-acids detected. Test fermentations were carried out in three parallel experiments accompanied by two positive controls (with AHBA addition) and one negative control (without addition of mutasynthon).

spectrometric analyses of the crude extracts obtained after feeding of 3-amino-(het)arenecarboxylic acids **17**–**22** revealed no acceptance and processing to the corresponding proansamtocin derivatives.

A second series of benzoic acids that are not anilines but rather 3-aminomethyl- (**23**) and 3-hydroxymethylbenzoic acids (**24**, **25** and **27**–**31**) as well as phenylacetic acid derivative **26**. HRMS analysis of crude extracts obtained from feeding experiments conducted with **24** and **25** revealed the presence of macrocyclic products while the corresponding *seco*-acid was the result from mutasynthetic processing of **23**.

Surprisingly, feeding experiments with phenylacetic acid **26** revealed formation of proansamitocin (**2**). One possible explanation could be the oxidation of **26** to the corresponding 2-oxo derivative which is followed by oxidative decarboxylation (like pyruvate→acetyl-CoA) to AHBA **1** being activated as CoA thioester. The other carboxylic acids **27**–**31** did not act as processible mutasynthons in this study.

With these results in hand we carried out large-scale fermentations (up to 3.6 L) choosing the most promising benzoic acids **12**, **14**–**16** and **25**. The selection was based on semi-quantitative analysis of signal intensities in the UPLC chromatograms (for details see the Supporting Information). Consequently, we did not include aminobenzoic acids **13** as well as aminobenzyl **23** and hydroxymethylbenzoic acids **24** for large-scale fermentations despite the fact that test fermentations revealed PKS processing.

As a result we isolated new proansamitocin derivatives **32**–**38** after purification by column chromatography (SiO_2), size exclusion chromatography (LH-20) and preparative HPLC (C_{18} and CN columns). Most remarkably, supplementation of 3-hydroxy-5-(hydroxymethyl)benzoic acid (**25**) to a growing culture of strain AH1 yielded three products that resulted from complete PKS-processing. Besides the *seco*-acid **38**, the two epimeric, ring-expanded macrolactones **37 a** and **37 b**, were collected (Figure 5). Support for their formation was gained when comparing the chemical shift (δ) of the benzylic methylene protons of benzyl alcohols **25** and **38** with the epimeric macrolactones **37 a**,**b** [ArCH_2O-: **25** ([D_6]DMSO) $\delta = 4.46$ (s, 2H) ppm; **37 a** and **37 b** ([D_6] benzene) $\delta = 5.05$ (d, $J = 13.0$ Hz, 1H) and 4.97/4.92 (d, $J = 13.0$ Hz,

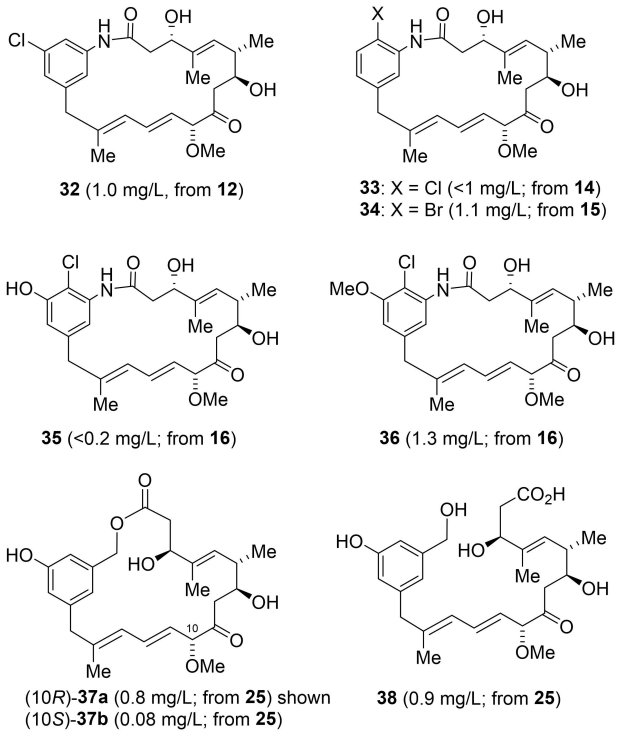

Figure 5. Fermentations using mutasynthons **12**, **14**–**16** and **25** as well as isolation of proansamitocin derivatives **32**–**36**, macrolactones **37 a** and **37 b** and *seco*-acid **38**.

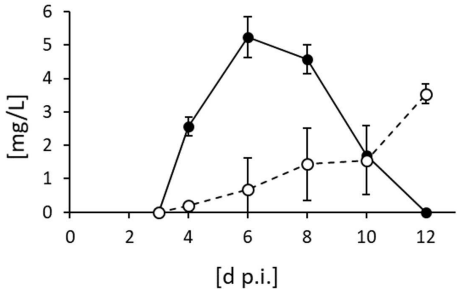

Figure 6. Monitoring the formation of macrolactone **37** (•) and *seco*-acid **38** (○) by AH1 over a period of 12 days (feeding of 1.65 mmol/L on days 3, 4 and 5 p.i.); calibration was carried out with **2** as reference (see SI).

1H); **38** $\delta = 4.50$ (s, 2H) ppm]. These downfield shifts are diagnostic for *O*-acylated secondary alcohols as found in macrolactones **37a**,**b**. It has to be noted that proansamitocins are prone to epimerization at C10, evident from the isolation of proansamitocins **2** and **4** that we have never noted for fully processed ansamitocin P3 (**3**).[7]

The two 10-epimers **37a**,**b** were identified by comparison of the coupling constants $J_{10,11}$ and $J_{10,12}$ with those found in epimeric proanamsitocins **2** and **4** (Figure 3 for major 10*R* isomer **37a**: $J_{10,11} = 7.0$ Hz and $J_{10,12} = 0.9$ Hz; for minor 10*S* isomer **37b**: $J_{10,11} = 5.3$ Hz and $J_{10,12} = 1.7$ Hz). It has to be stressed that this is the first example of an ansamitocin derivative ever reported that bears an expanded ring system and a lactone instead of a lactam substructure.

The monitoring of the mutasynthetic experiment that leads to macrolactones **37a**,**b** revealed that *seco*-acid **38** is not the precursor of the macrolactones or a side product of macrocyclization but rather its hydrolysis product (Figure 6). Over a period of 12 days the titers for macrolactones **37** first increased to about 5 mg/L before it a dramatically decreased until it could not be detected anymore. During that time, the formation of *seco*-acid **38** continuously increased to a value of about 4 mg/L. It must be noted that formation of the corresponding macrolactones using *A. pretiosum* (ΔAHBA, strain HGF073) was not found in previous experiments.[16]

In summary, expanding the concept of mutasynthesis by designing multiblocked mutant strains of *Actinosynnema pretiosum* opens the door to access new derivatives of biosynthetically advanced macrocyclic PKS intermediates. For the first time a ring expanded 20-membered ansamitocin macrolactone could be generated, revealing an unusual promiscuity and chemoselectivity of the amide synthase *asm9* that commonly catalyzes macrolactam formation. These results suggest that this amide synthase may become a chemical tool of interest for performing macrocylizations.[11]

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: ansamitocin · macrolactones · maytansinoids · mutasynthesis · proansamitocins

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