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Mutational Biosynthesis of Ansamitocin Antibiotics: A Diversity-Oriented Approach to Exploit Biosynthetic Flexibility

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New ansamitocin derivatives were prepared by feeding aminobenzoic acid derivatives to cultures of *Actinosynnema pretiosum* HGF073, a mutant strain blocked in the biosynthesis of the required 3-amino-5-hydroxybenzoic acid (AHBA) starter unit. Use of several aminobenzoic acids as precursors led to a spectrum of products, reflecting the sequence of post-PKS tailoring steps involved in the generation of ansamitocins and adding novel aspects to the published suggestion model of post-PKS tailoring logic and flexibility. The studies provide insights into the substrate flexibility of the enzymes required for ansamitocin biosynthesis in *A. pretiosum*, whereas preliminary biological testing of the derivatives isolated and fully characterized by NMR spectroscopy allowed structure–activity relationship assignments to be made for a variety of intermediates occurring during the post-PKS tailoring sequence in ansamitocin biosynthesis.

Introduction

Natural products still represent a very important source of lead structures for antiinfectives, immunosuppressives, and anticancer agents. However, their structural complexity often complicates the generation of derivatives for structure–activity relationship (SAR) studies.[1] Mutational biosynthesis, or mutasynthesis, utilizes genetically engineered organisms for the generation of complex secondary metabolites by precursor-directed biosynthesis (PDB) with modified biosynthetic intermediates.[2] This concept was first suggested by Birch in 1963 as an alternative to classical PDB,[3a] whereas the term "mutasynthesis" was coined by Rinehart and co-workers in the late 1970s.[3b] Mutasynthesis has recently found application in the preparation of several small natural product libraries.[4] In most of these experiments the formation of new derivatives was only detected by high-resolution mass spectrometry (HRMS). When sufficient amounts of new products could be generated for isolation and characterization, the fermentation commonly did not yield more than two products for biological testing.

We have recently disclosed mutasynthetic studies on the ansamitocin antibiotics **3a–c** (Scheme 1).[5] These compounds are often termed maytansinoids, after their structural congener maytansine (**3d**) and are known for their remarkably high potencies as antitumor agents.[6] Ansamitocins belong to the group of ansamycin macrolactam antibiotics and exhibit cytotoxic activity, evident in the growth inhibition of different leukemia cell lines and human solid tumors at very low concentrations (10^{-3} to 10^{-7} μg mL^{-1}).[7] Their antimitotic mode of action is based on interaction with β-tubulin, preventing polymerization of tubulin and thereby promoting depolymerization of microtubules. Notably, maytansinoids are currently attracting great clinical interest as "warheads" in tumor-targeted immunoconjugates.[8]

The generation of new ansamitocin analogues for structure–activity relationship (SAR) studies has so far been restricted to selected semisynthetic or biological transformations. Although total synthesis approaches towards maytansinoids have been described, none has been able to provide essential information on the role of pharmacophores in these compounds.[6c] However, SAR studies were able to assign key importance for bioactivity to the presence of the ester side chain and the cyclic carbinolamide in its hemiaminal form.[9] Maytansine (**3d**) analogues bearing a variety of different C-3 acyloxy substituents were prepared by semisynthesis and revealed the nature of the side chain to be of only modulating influence on bioactivity,[6a,9a] making the ester moiety the preferred point of attachment for conjugates.[10]

In view of the compact structural quality of ansamitocins, the aromatic moiety might serve as another flexible platform for conjugate synthesis. The basic structural features of the aromatic moiety are established early in the biosynthesis, with some modifications occurring at later stages. In summary, the biosynthesis of the ansamitocins **3a–c** in *Actinosynnema pretiosum* utilizes biosynthetic machinery consisting of a modular type I polyketide synthase (PKS), followed by a cyclizing ansamycin amide synthase and additional post-PKS tailoring enzymes (Scheme 1). The generation of the ansamitocins **3a–c** starts from 3-amino-5-hydroxybenzoic acid (AHBA, **1**), a building block supplied by the aminoshikimate biosynthetic pathway.[6a–b,11] The starter unit **1** is activated and loaded onto the PKS by an adenylating loading module reminiscent of a nonribosomal peptide synthetase (NRPS). After processing, it is presumed that the final linear product of the PKS is cyclized and released by an independent ansamycin amide synthase. The

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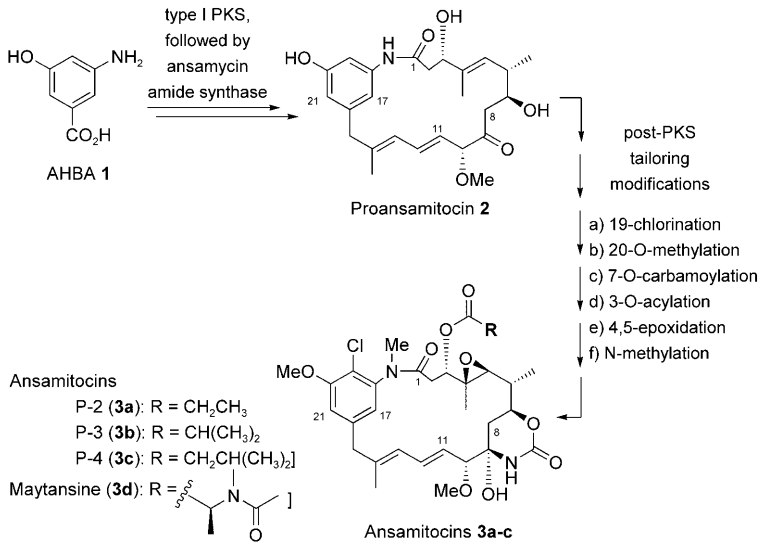


Scheme 1. Principal biosynthesis of the ansamitocins **3a–c** in *Actinosynnema pretiosum*: Proansamitocin (**2**) is generated from the 3-amino-5-hydroxybenzoic acid (**1**) starter unit and undergoes subsequent processing to afford the final products **3a–c**.

resulting proansamitocin (**2**) is then further modified by a set of tailoring enzymes that follow a particular preferred sequence as determined by gene inactivation analysis.[12] Post-PKS processing starts with the introduction of chlorine into proansamitocin, with the phenolic group subsequently being subjected to O-methylation. The introduction of the carbinol amide moiety follows and precedes the attachment of the ester side chain. Because of the flexibility of the corresponding acyl transferase in acyl building block usage this step results in structural diversification [with generation of, for example, the ansamitocins P-2 to P-4 (**3a–c**)]. Epoxidation and N-methylation complete the biosynthesis.

To generate novel structural features at the aromatic moiety, our mutasynthetic approach relies on a mutant strain of the producing actinomycete *Actinosynnema pretiosum* that is unable to generate the required AHBA (**1**) biosynthetic starter unit. The AHBA(−) phenotype of our workhorse strain *A. pretiosum* HGF073 is the product of deletion of a chromosomal region harboring a gene cluster incorporating a set of genes required for AHBA biosynthesis.[13] Feeding of AHBA (**1**, 1.25 mmol L^{-1} of culture volume) to *A. pretiosum* HGF073 restores the production of the ansamitocins **3a–c** to amounts comparable with those observed in the wild-type strain (ca. 65 mg L^{-1}, relative to 45 mg L^{-1} for the WT strain),[5d,14] whereas supplementation with AHBA analogues can result in the formation of novel ansamitocins.

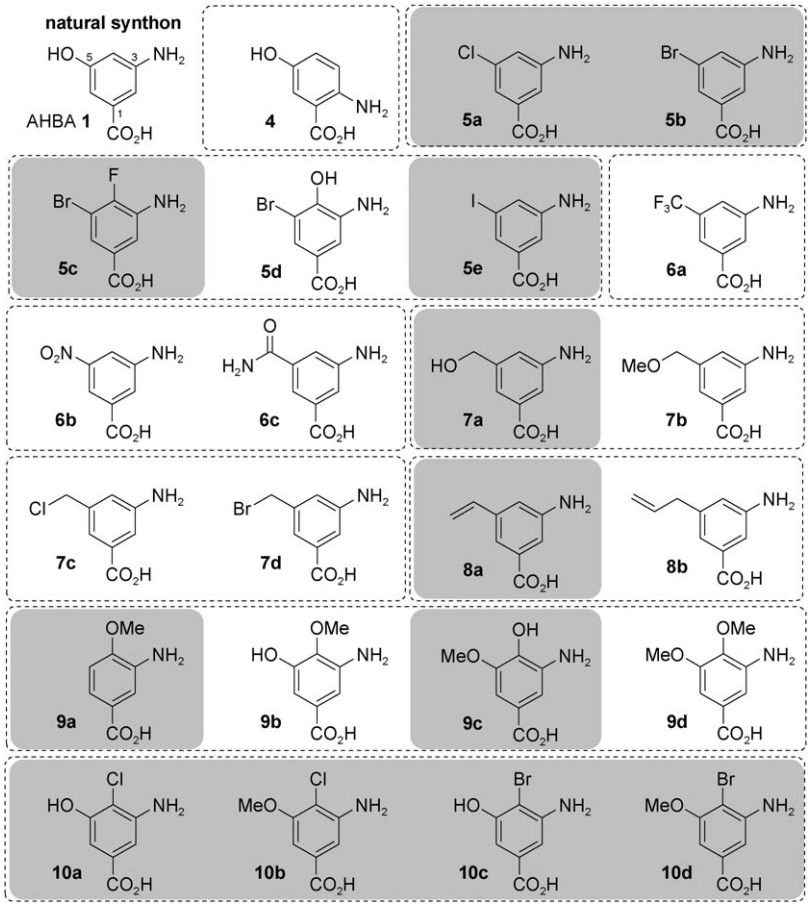
In this report we disclose the extension of our work on the mutasynthetic generation of new ansamitocin analogues.[5b–c] Particularly interesting for SAR studies on a given class of natural products are mutasynthons that are inefficiently processed to the final product by post-PKS tailoring enzymes. Although the structural diversification introduced by these transformations often demands lengthy workup procedures, a variety of structurally related mutaproducts can be generated in a single experiment. Such collections can reveal the stage of biosynthetic processing at which inactive precursors acquire biological activity. Additionally, a basic understanding of the substrate flexibility of the enzymes involved can be deduced from the relative levels of the intermediates' occurrence.

Results and Discussions

The 24 different aminobenzoic acids **5a–10d** (Scheme 2) were purchased or synthesized[15] and individually added to cultures of *Actinosynnema pretiosum* HGF073 (preferably 1.25 mmol L^{-1} of culture volume). The fermentation was carried out with inclusion of L-valine (3 g L^{-1}) in the basic fermentation broth. The amino acid serves as a productivity-enhancing additive and promotes incorporation of isobutyric acid as the dominant ester side chain (P-3; see **3b**).[14,16]

After completion of the cultivation, samples of the fermentation broths including biomass were mixed with ethanol and directly screened for novel products by UPLC-MS (ultra performance liquid chromatography-coupled ESI-MS). In the absence of detection of novel products, the whole remaining fermentation broth, including the mycelial biomass, was extracted with ethyl acetate. The crude organic extract was filtered over silica gel and the concentrate was subjected to UPLC-MS analysis. For mutasynthons resulting in production levels of novel ansamitocins judged to be too low for scale-up and production in shake-flask cultures, high-resolution mass spectrometry and MS/MS data are provided (Schemes 3 and 4, below). In cases of higher-yielding mutasyntheses (> 0.2 mg L^{-1}), fermentation was repeated on a larger scale and the fermentation broth with the mycelial biomass was extracted with ethyl acetate and subjected to several chromatographic purification steps (silica gel chromatography, size exclusion chromatography, and reversed phase-HPLC).

As a minimum, conversion of analogous starter units into novel (pro)ansamitocin derivatives demands acceptance by the loading module, followed by PKS processing and cyclisation by the ansamycin amide synthase to afford a first free proansamitocin derivative. Half of the aminobenzoic acids tested in this study were indeed converted into novel ansamitocins (Schemes 2–4). Although several 4- or 5-substituted and 4,5-disubstituted 3-aminobenzoic acids were accepted, supplementation of the 2-aminobenzoic acid **4**, a derivative of anthranilic acid, did not yield novel ansamitocins. In accordance with our previous observations,[5b–c] this underlines the criteria for building blocks suitable for ansamitocin mutasynthesis: a 3-aminobenzoic acid substitution pattern with available modification sites at the 4- and 5-positions. Nevertheless, not all types of substituents at these positions are accepted. This might in some cases be due to steric or electronic effects of individual substituents disturbing biosynthetic processing, but it is worthy of note that detoxification of a mutasynthon by other pathways of the bacterial metabolism will give the same negative result as rejection.[17]



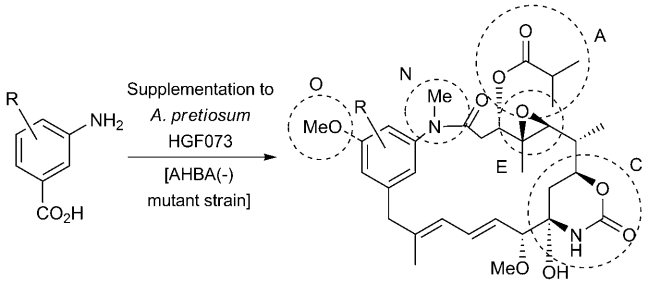
Scheme 2. Starter unit analogues (mutasynthons) added to cultures of *A. pretiosum* HGF073 for mutasynthetic generation of novel ansamitocin derivatives (successful conversions marked in gray); **1**, **5a**, **6a**, **6b**, **7a–d**, **8b**, **9b–d**, and **10a–c** were prepared as their corresponding hydrochloride salts.

To minimize such side reactions, mutasynthons were usually fed to production cultures by slow continuous addition over 3–4 days through autoclavable, syringe-pump-driven feeding capillaries. This procedure has been shown to increase yields up to 1.5-fold,[18] and we have not yet encountered a mutasynthon that was not also accepted when added to cultures in larger portions.

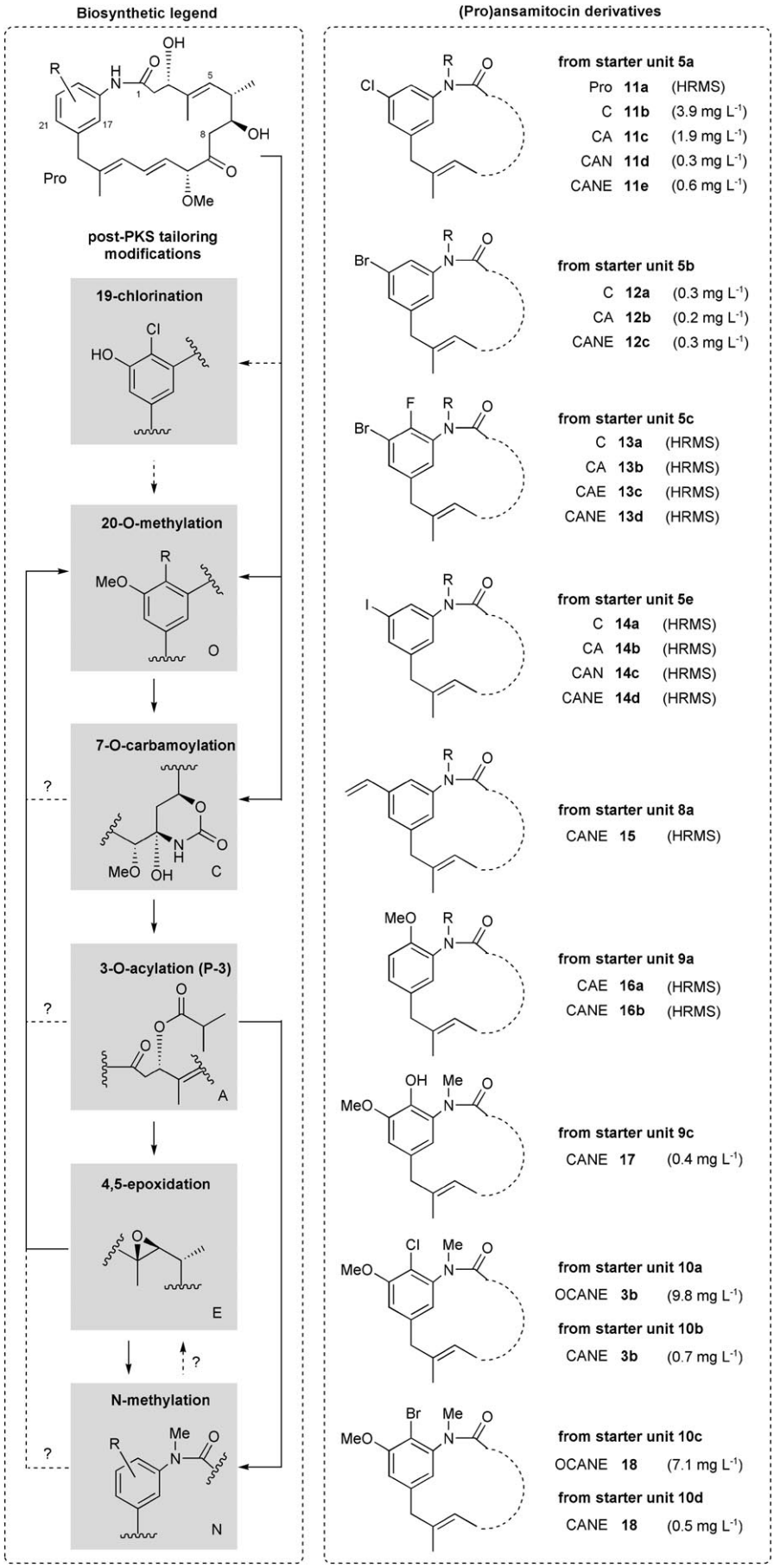
In general the biosynthetic machinery coped well with the formal replacement of the hydroxy group in AHBA (**1**) by different halogen substituents (compounds **5a–c** and **5e**), although increasing steric demands of the halogen atom series led to lower yields (Cl > Br > I, Scheme 3). The derivatives **14a–d**, each bearing an iodine moiety, could only be detected and characterized by mass spectrometric methods. Replacement of the hydrogen at the 4-position of 3-amino-5-bromobenzoic acid (**5b**) by an isosteric fluorine substituent (to give **5c**) was also tolerated. In contrast, a hydroxy group at the same position (compound **5d**) was not accepted, although novel ansamitocins could be generated from a starter unit bearing the 4-hydroxy group with an adjacent 5-methoxy substituent rather than bromine (compound **9c**). Supplementation with 3-amino-5-trifluoromethylbenzoic acid (**6a**) did not yield novel ansamitocins, likely due to the strongly electron-withdrawing character of the substituent in contrast to the halogen atoms in **5a–c** and **5e**. The same reasoning applies to the negative results for the mutasynthons **6b** (nitro group) and **6c** (benzamide group), with the increasing steric demands also likely to play an additional role in these cases.

Out of the four 3-aminobenzoic acids **7a–d**, bearing different benzyl substituents at the 5-position, only 3-amino-5-hydroxymethylbenzoic acid (**7a**) was accepted by the biosynthetic machinery. The analogous O-methylated derivative **7b** likely failed to undergo processing to novel ansamitocin derivatives for steric reasons, whereas the same outcome for the mutasynthons **7c–d**, bearing halogenated benzyl moieties, might also be attributed to their chemical instability.

With regard to the 5-hydroxymethyl-substituted mutasynthon **7a**, 3-amino-5-vinyl-benzoic acid (**8a**) also appeared to be a comparable building block with a high chance of acceptance. Indeed **8a** afforded the novel derivative **15**, but in yields too low for isolation and full characterization. The vinyl moiety is certainly less flexible than the hydroxymethyl substituent of mutasynthon **7a**, whereas the hydroxy function in the latter building block might also favorably interact with the biosynthetic machinery through hydrogen bonding.



Scheme 3. Novel ansamitocins generated from AHBA analogues by fermentation with *A. pretiosum* HGF073. The pattern and sequence of post-PKS tailoring steps as established by this study and by Spiteller et al.[12] is given. Fermentation yields refer to isolated ansamitocin derivatives fully characterized by NMR spectroscopy, whereas HRMS indicates that compounds were detected by high-resolution mass spectrometry with supporting MS/MS characterization (see the Supporting Information). Pro: proansamitocin derivative, O: O-methylation, C: carbamoylation, A: acylation, N: N-methylation, E: epoxidation.



Scheme 3 (continued).

Although the 5-allyl substituent in the 3-aminobenzoic acid **8b** has features of free rotation comparable to those in **7a**, the bulky terminal alkene group probably impeded its processing by the biosynthetic assembly line.

The series of starter units **9a**–**d**, with alternating methoxy and hydroxy substitution patterns, was tested for mutasynthetic processing. Successful isolation and characterization of an ansamitocin derivative resulting from feeding of 3-amino-5-methoxybenzoic acid has been reported previously,[5c] and indeed a similar starter unit bearing an additional hydroxy function at position 4 (compound **9c**) also yielded the novel mutaproduct **17**. The mutasynthons **9a**, **9b**, and **9d**, with the bulky, electron-donating methoxy substituent situated at position 4 in 3-aminobenzoic acid, performed less well. Novel ansamitocin derivatives could be generated in trace amounts from the monosubstituted mutasynthon **9a**. The aminobenzoic acids **9b** and **9d**, with additional hydroxy or methoxy moieties at position 5, were not processed to the corresponding ansamitocin derivatives. In contrast, all 3-aminobenzoic acid mutasynthons bearing methoxy or hydroxy groups at position 5 and halogen substituents (Cl, Br) at position 4 (compounds **10a**–**d**) were processed to ansamitocins.

Whereas both **10a** and **10b** unsurprisingly yielded authentic ansamitocin P-3 (**3b**), the novel 19-bromo-ansamitocin P-3 (**18**) could be generated from the mutasynthons **10c** and **10d**. In general, mutasynthons containing a 5-methoxy group (compounds **10b** and **10d**) performed about ten times less well than those containing the smaller hydroxy group (compounds **10a** and **10c**). These differences indicate that in the latter cases the bulky 20-O-methoxy substituent was apparently installed after PKS processing by the corresponding methyltransferase involved in the natural set of tailoring modifications (Scheme 3). Because no products lacking 20-O-methylation could be detected upon feeding with **10c**, it should be noted that the sterically demanding bromo substituent apparently represents no substantial obstacle for the methylating enzyme.

When AHBA (**1**) is fed to *A. pretiosum* HGF073, fully processed ansamitocin P-3 (**3b**) is obtained as the dominant product, accompanied by ansamitocins P-2 (**3a**) and P-4 (**3b**) in low amounts. Interestingly, for several mutasynthons a whole series of biosynthetic intermediates differing in the pattern of known post-PKS tailoring modifications could be detected (Schemes 3 and 4). The occurrence of these intermediates indicates inefficient processing of modified (pro)ansamitocin derivatives by subsequent enzymes. The published suggestion for the sequence of post-PKS tailoring occurring during ansamitocin biosynthesis in *A. pretiosum* was established by gene inactivation mutant analysis[12] and provides a good idea of the substrate flexibility of the enzymes involved. These studies led to the conclusion that certain modifications have to take place before the intermediate is accepted for processing by the next enzyme (Scheme 3). In these linear one-way pathways in the post-PKS modification sequence, it can be postulated that an observed accumulation of a precursor is the direct result of limited substrate flexibility of the enzyme that would be expected to process it to the next intermediate. Nevertheless, the observations gleaned from these multicomponent systems do not yield the same information as in vitro data established by isolated enzyme kinetic measurements. Slow processing further downstream in the post-PKS tailoring sequence might cause a backlog of intermediates, resulting in an accumulation of a precursor at a point further upstream where a modifying enzyme is susceptible to product inhibition. With these considerations in mind, the observed patterns of post-PKS tailoring for the different mutasynthons are discussed below.

Except for 20-O-methylation of the ansamitocin analogues derived from the mutasynthons **10a** and **10c** (vide supra), none of the known tailoring modifications targeting the aromatic moiety occurred for the other mutaproducts described in this study. In accordance with our previous studies,[5b,c] chlorination of position 19 apparently requires a free phenol at position 20 [see proansamitocin (**2**)], which can subsequently undergo O-methylation to afford the 20-methoxy group.

The post-PKS tailoring step following decoration of the aryl subunit targets the eastern hemisphere of the (pro)ansamitocin framework at a point located as far away as possible from the potentially modifiable aromatic moiety. It can be expected that when a proansamitocin derivative is formed, carbamoylation of the hydroxy group at C-7 will occur regardless of the substituents located in the aromatic western hemisphere. Indeed, carbamoylation took place en route to fully modified ansamitocin derivatives, whereas in cases in which mutasyntheses yielded a set of late-stage analogues, those bearing a carbinol amide among other modifications clearly dominated the spectrum of products.

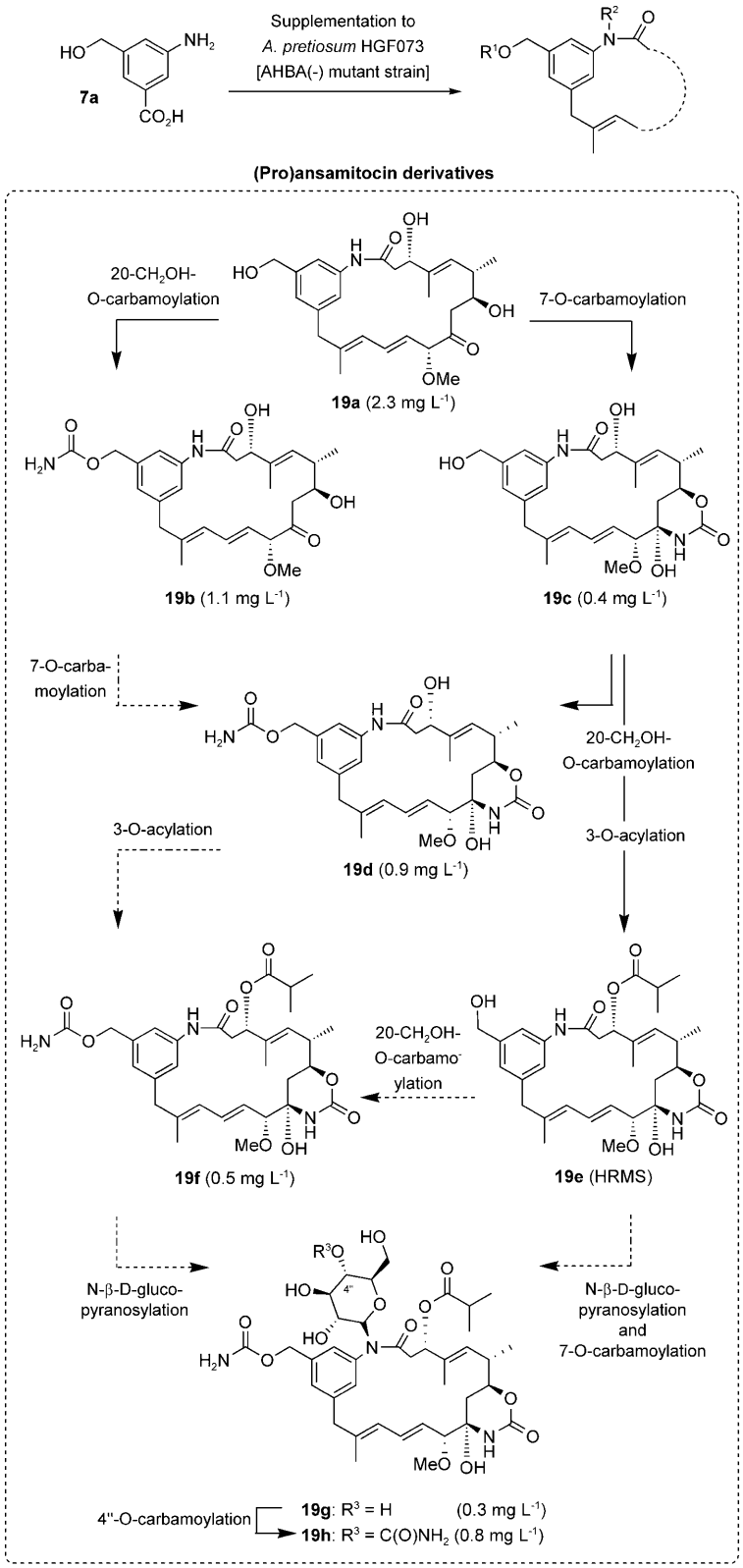
In the cases of the 20-chloro-ansamitocins **11a**–**e**, the carbamoylated derivative **11b** represented the main product of fermentation, whereas its proansamitocin-type precursor **11a** could only be detected in trace amounts. NMR analysis of compound **11b** yielded the first reliable reference for signal shifts for derivatives at this stage of modification. As described by Moss et al., a natural 7-O-carbamoylated proansamitocin derivative additionally bearing the bulky 19-chloro-20-O-methyl substitution pattern resulted in conformational instability of the macrolactam ring, evident in severe line broadening and reduction of signal intensity in NMR spectra.[19] These difficulties were also encountered in the case of the carbamoylated 20-bromoproansamitocin **12a**, whereas its 20-chloro congener **11b** gave a clear-cut set of NMR signals.

In contrast with all of the other successful mutasyntheses that we have undertaken so far with *A. pretiosum* HGF073, only fermentation with the benzyl alcohol **7a** yielded the corresponding proansamitocin derivative **19a** as the major product (Scheme 4). Apparent accumulation of **19a** indicates its inefficient processing by the subsequently acting 7-O-carbamoyltransferase. Surprisingly, during workup and purification of the fermentation product it became apparent that molecular formulas (HRMS) consistent with the occurrence of different single or double carbamoylations could be assigned to several components. After an extensive purification sequence, the pure compounds were characterized by NMR spectroscopy in $[D_4]$MeOH. Whereas the derivatives **19a** and **19c** each showed a NMR resonance of the 20-benzylic protons at 4.55 ppm, reminiscent of the precursor **7a**, for the other derivatives isolated these signals appeared at 5.00 ppm. Furthermore, for each of these last compounds, a HMBC contact of the 20-benzylic protons with a quaternary carbon at 159.7 ppm was assigned. These data can be unambiguously interpreted in terms of additional carbamoylation at the benzylic alcohol. Apart from the proansamitocin derivative **19a**, analogues bearing the additional carbamoyl group clearly dominate the spectrum of compounds isolated. Apparently, the novel tailoring step is carried out quite efficiently, although it remains unclear whether the modifying enzyme is the same one as is responsible for 7-O-carbamoylation. Additionally, the low yields obtained for compound **19c** suggest its facile conversion to more advanced derivatives, with relatively high yields obtained for the 20-O-carbamoylated derivatives **19b** and **19d**, raising the question of whether these actually represent dead-ends of the post-PKS tailoring sequence. To provide definite answers to these questions, the derivatives will have to be subjected to future in vitro tests with the isolated tailoring enzymes.

As described by Spiteller et al.[12] 7-O-carbamoylation is the prerequisite for subsequent 3-O-acylation. For all derivatives bearing the carbinol amide substituent described in this study, concomitant occurrence of derivatives bearing 3-O-isobutyryl ester side chains (P-3) was also observed (Schemes 3 and 4).

In contrast with the post-PKS tailoring sequence published previously, our results indicate that N-methylation of the macrolactam amide might indeed precede 4,5-epoxidation. An N-methylated derivative lacking epoxidation (compound **11d**) could be isolated and fully characterized, and the same substitution pattern could also be detected in the product spectrum resulting from another mutasynthesis (compound **14c**).

As mentioned above, the previously published tailoring sequence was based on the fermentation of specific knock-out mutants.[12] Because the gene responsible for 4,5-epoxidation is located upstream of the one controlling N-methylation,[11] polar effects might have played a role in eliminating both tailoring steps upon in-gene deletion inactivation of the 4,5-ep-



Scheme 4. The 20-hydroxymethyl-ansamitocin derivatives **19a**–**h** generated by mutasynthesis and organized according to the proposed sequence of post-PKS tailoring steps required for their biosynthesis (dotted arrows indicate pathways of ambiguous occurrence, importance and position in the sequence).

oxidase gene. In summary, epoxidation can be the final step but, in accordance with the results of Spiteller et al.,[12] N-methylation does not need to occur prior to epoxidation, as is demonstrated in the detection of the derivatives **13c** and **16a**. With one exception all mutasyntheses described in this study yielded fully processed mutaproducts with all of the four principal non-aryl tailoring modifications.

Neither products of N-methylation of the macrolactam amide, nor products of epoxidation of the 4,5-alkene could be found in the fermentation of 3-amino-5-hydroxymethyl-benzoic acid (**7a**). This might indicate that the presence of the carbamoyl group on the 20-benzylic alcohol prevents further action of the normal tailoring enzymes. However, two relatively polar products of high molecular mass (compounds **19g** and **19h**) could be isolated and characterized by NMR spectroscopy. The two metabolites were assigned as N-β-D-glucopyranosylated at the macrolactam amide. This type of modification has recently been described for ansamitocin derivatives isolated from *A. pretiosum* during solid agar fermentation[20] as well as from a related isolate strain producing N-glucosylated ansamitocins (ansacarbamitocins).[21] The NMR signals corresponding to the sugar moiety of the derivatives **19g** and **19h** are in good agreement with those reported in the literature.[20a,21] Because the N-glucosyltransferase from *A. pretiosum* was recently isolated and shown to utilize UDP-glucose as glucosyl donor,[22] it can be postulated that the carbamate present on the sugar moiety of the derivative **19h** results from carbamoylation of **19g**.

Ansamitocin derivatives obtained in sufficient amounts for full characterization by NMR spectroscopy were subsequently subjected to in vitro biological testing with different human cell lines derived from cancer growths or the umbilical vein. The results from these tests are given (Table 1) as values for the half-maximum inhibitory concentrations of the corresponding ansamitocin derivatives in relation to the "gold standard" ansamitocin P-3 (**3b**).

Because these mutaproducts reflect an almost complete series of post-PKS tailoring steps, the stage at which pronounced antimitotic activity arises during this sequence is directly visible. Our data clearly underline that the cytotoxicity of ansamitocins essentially depends on the presence of the ester side chain (**11b** vs. **11c**, **19a**–**d** vs. **19f**), whereas further epoxidation and N-methylation exert a less severe, modulating influence on the biological activity (**11b** vs. **11d**/**e**, **12b** vs. **12c**). As is evident from the example of the 20-chloro-ansamitocins **11**, N-methylation seems to have a positive influence on the biological activity (**11c** vs. **11d**), whereas further epoxidation appears to have a slightly adverse effect (**11d** vs. **11e**). Small changes at the aromatic moiety are tolerated without any substantial loss of cytotoxic potency [AP-3 (**3b**) vs. **11e**, **12c**, **18**], whereas larger and more hydrophilic substituents appear to have a slightly stronger nega-

Table 1. Antiproliferative activities (IC_{50}, nmol L^{-1}) of **11b–e**, **12b**, **12c**, **17**, **18**, **19a–d** and **19f–h**, relative to AP-3 (**3b**).

Compound	Cell line U-937	A-431	SK-OV-3	PC-3	MCF-7	HUVEC
AP-3 (**3b**)	0.01	0.08	0.05	0.06	n.d.	0.02
11b	149	>80	>800	>800	>800	>800
11c	0.05	1.6	0.66	0.3	0.9	0.32
11d	0.05	0.1	0.05	0.16	0.11	0.08
11e	0.18	0.35	0.21	0.53	0.41	0.21
12b	0.52	8.3	2.9	1.3	3.2	n.d.
12c	0.2	0.48	0.52	0.4	0.34	n.d.
17	0.52	0.94	1.5	3.4	1.8	1.6
18	0.01	0.06	0.06	0.15	0.02	n.d.
19a–d	>700	>70	>700	>700	>700	>700
19f	1.5	8.5	6.8	14.0	12.4	6.7
19g	348	>500	>500	>500	>500	>500
19h	659	>500	452	>500	>500	>500

Values shown are each the means of two determinations in parallel. Human cell lines: U-937 (histiocytic lymphoma), A-431 (epidermoid carcinoma), SK-OV-3 (ovary adenocarcinoma), PC-3 (prostate adenocarcinoma), MCF-7 (breast adenocarcinoma), HUVEC (umbilical vein endothelial cells); n.d. = not determined. No data are given for the ansamitocin **12a**, because ansamitocins lacking the ester side chain at C-3 do not show activity in cell proliferation tests.[6]

tive effect [**11c** versus **19f**, AP-3 (**3b**) versus **17**]. In accordance with previously described N-glucosylated ansamitocin derivatives,[20–21] the presence of the sugar moiety appears to lead to a pronounced loss of potency (**19f** versus **19g** or **19h**).

Conclusions

In conclusion, we have achieved the preparation of novel ansamitocin derivatives by exploiting the concept of mutational biosynthesis. Sixteen new metabolites were isolated in amounts sufficient for full structural characterization and for preliminary biological testing. Mutaproducts bearing at least a cyclic carbinol amide and ester side chain along with optional N-methyl or epoxide moieties showed pronounced cytotoxic potency.

Mutasynthesis represents a powerful strategy for accessing compound libraries of highly potent and complex natural products such as ansamitocins. Notably, the bromo derivatives **12** and **18** described in this study represent ideal precursors for further semisynthetic modification through exploitation of palladium-catalyzed cross-coupling reactions, an approach we have previously described for a 19-bromo-ansamitocin generated upon supplementation of HGF073 *A. pretiosum* with 3-amino-4-bromobenzoic acid.[5b] Mutasynthesis should indeed enhance its reputation as a powerful tool for the generation of small compound libraries if novel substituents introduced in this fashion can pave the way for novel semisynthetic derivatizations resulting in diverse sets of analogues suited for SAR studies.

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Keywords: ansamitocin · antitumor agents · mutasynthesis · polyketide biosynthesis · structure–activity relationships

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