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Highly Active Ansamitocin Derivatives: Mutasynthesis Using an AHBA-Blocked Mutant

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Despite the fact that natural products represent a very important source of drugs in several therapeutic fields such as anti-infective agents and cancer therapy, the development of natural product based drugs is often hampered by their structural complexity. This fact precludes facile total synthetic access to analogues or the development of natural product libraries. Therefore, semisynthetic and biotechnological approaches are commonly pursued in pharmaceutical research and development.[1] A very interesting strategy combines chemical semisynthesis with biosynthesis using genetically engineered microorganisms, a technique termed mutational biosynthesis or mutasynthesis (Figure 1).[2,3] Recently, mutasynthesis has experienced a renaissance as the number of fully sequenced biosynthetic gene clusters of pharmaceutically potent natural products has substantially increased, setting the stage for easier creation of specific blocked mutants and therefore efficient access to modified drug candidates.[4])

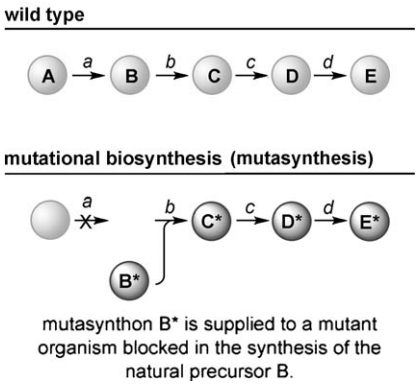
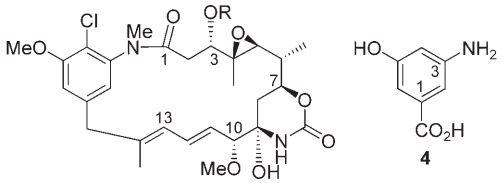


Figure 1. The concept of mutational biosynthesis: A = starting substrate, B–D = biosynthetic intermediates, E = natural product, B*–D* = modified biosynthetic intermediates, E* = modified natural product; a–d = enzymes.

Maytansine (**1**; Figure 2), first isolated from the Ethiopian plant *Maytenus serrata*,[5] and the related ansamitocins P-1, P-2, P-3 (**2**), and P-4 (**3**)[6,7] are highly potent antitumor compounds of microbial origin (*Actinosynnema pretiosum*). They consist of a 19-membered macrolactam ring and differ in the side chain at C3. These ansamycin antibiotics inhibit the growth of various leukemia cell lines as well as human solid tumors at very low concentrations (10^{-3}–10^{-7} μg mL^{-1}). Clinical development of these antitumor agents was stopped in phase II[8] owing to gastrointestinal side effects and neurotoxicity.[9] Total synthetic approaches to analogues of maytansinoids have been pursued but have not provided any new insight into their structure–activity relationships.[10] Nevertheless, there is continued interest in maytansinoids as highly toxic agents for use in antibody conjugates, and they have performed well as immunoconjugates in phase I studies.[11]



1: R = –COCH(Me)N(Me)COMe (maytnsine)
2: R = –COCHMe$_2$ (ansamitocin P-3)
3: R = –COCH$_2$CHMe$_2$ (ansamitocin P-4)

Figure 2. Structures of maytansine (**1**) and ansamitocins P-3 and P-4 (respectively **2** and **3**).

The present high interest in the maytansinoid family fuels the new search for analogues not previously obtained by either synthesis or semisynthesis. The producing microorganism creates ansamitocins through biosynthetic machinery typical for bioactive polyketides, in which the polyketide chain is assembled on a modular polyketide synthase (PKS) and further modifications are made by additional decorating enzymes. The biosynthesis of **2** is primed by a starter unit that originates from a different biosynthetic pathway, which is an ideal situation to access new analogues by a mutasynthetic approach.[2b] In the present case, 3-amino-5-hydroxybenzoic acid (AHBA, **4**) is the starter unit that originates from a shikimate-type biosynthetic pathway (Scheme 1),[12] so that blocked mutants can be generated without affecting the modules of the polyketide biosynthetic gene cluster (PKS 1). Therefore, we initiated a

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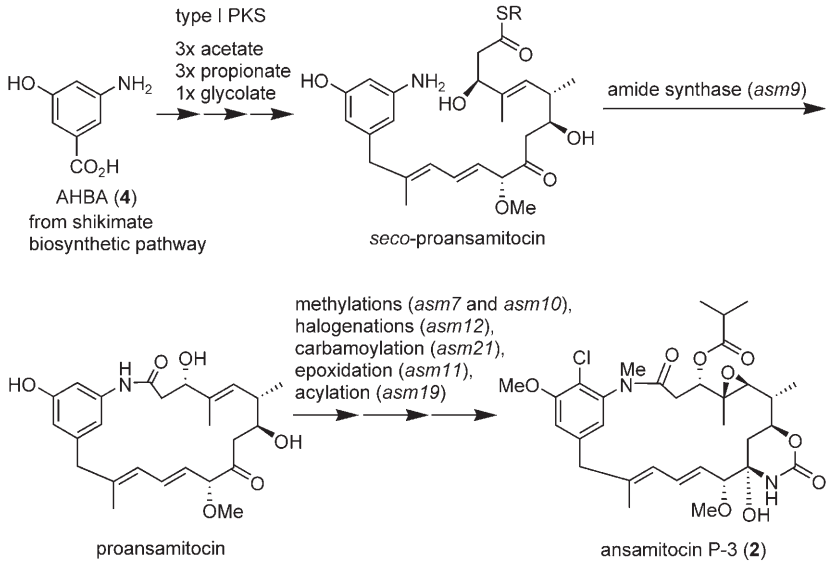


Scheme 1. The principal biosynthetic pathway of ansamitocin P-3 (**2**). Codes in parenthesis refer to corresponding genes.

research program[13] dedicated to synthetically exploiting the AHBA-blocked mutant of the ansamitocin P-3 producer, *Actinosynnema pretiosum* (HGF073; Scheme 1).[12a] Herein we describe the generation of highly active ansamitocin analogues by the combination of chemical synthesis and biosynthesis.

Experimental Section

We chose to feed a series of 3-aminobenzoic acid derivatives that varied in the functionality at the 5- position (compounds **6**–**12**) or contained an additional oxy group at C2 (compound **13**) (Figure 3). Additionally, we prepared and fed the 4-functionalized 3-aminobenzoic acid derivatives **14**–**23**, which lack the hydroxy group at C5. Related to these examples are benzoic acids **24** and **25**, which are functionalized at C6 instead of C4. *N*-Acetylcysteamine (SNAC) ester **5**, an AHBA-coenzyme A mimic, was also prepared. Mutant HGF073, which is blocked in the biosynthesis of AHBA,[12a] supplemented with aminobenzoic acid **4** and the corresponding SNAC ester **5**, produced amounts of AP-3 similar to those of the wild-type fermentation (60–70 mg L^{-1}).[13a] No AP-3 was detected in the extracts of the fermentation of HGF073 without supplementation. Clearly, AHBA (**4**) is first transformed into the aryl adenylate, which then is attacked by the thiol group of 4′-phosphopantetheine to form the enzyme-bound thioester.[14]

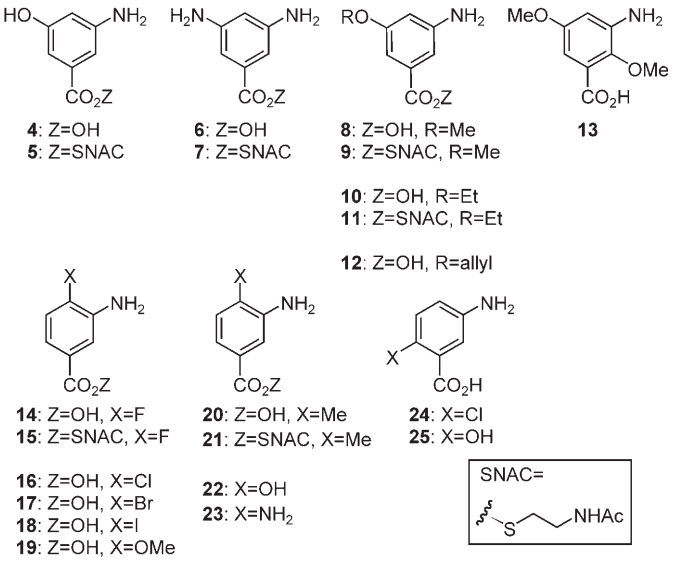
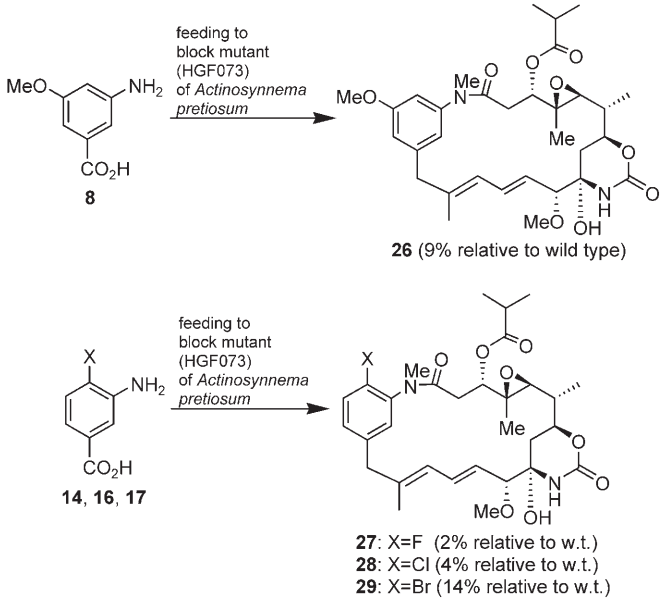


Figure 3. AHBA (**4**), SNAC compound **5**, and mutasynthons **6**–**25** fed to strain HGF073.

Each of the benzoic acids and SNAC derivatives **6**–**25** were then fed to growing cultures of *Actinosynnema pretiosum* mutant HGF073 according to the cultivation parameters A (Supporting Information). Each time parallel fermentations were carried out for comparison with the wild-type strain, the mutant HGF073 supplemented with the respective AHBA analogues and the 'natural' AHBA, and without supplementation to test the viability of strain HGF073. The cultures were harvested after seven days and extracted with ethyl acetate. The extracts were subjected to high-resolution electrospray ionization mass spectrometry (HR ESI-MS) for primary screening. The crude extracts collected from mutasynthons **8**, **9**, and **14**–**17** clearly revealed *m/z* peaks for the suggested ansamitocin analogues. Interestingly, ethyl- and allyl derivatives **10** and **12**, which are closely related to methyl derivative **8**, were not processed to any macrocycles. A 5-amino group as in **6** or **7** is not tolerated, and mutasynthon **13** bearing an additional oxy functionality at position 2 also failed to be transformed into any products or advanced intermediates. The same observation applies to the 4-iodo-, 4-methoxy-, 4-methyl-, 4-hydroxy-, and 4-aminobenzoic acids **18**–**23**, as well as 6-substituted aminobenzoic acids **24** and **25**. On the basis of these analytical results, fermentations with supplemented benzoic acid derivatives **8** and **14**, **16**, and **17** were repeated with cultures of *Actinosynnema pretiosum* mutant HGF073 (cultivation parameters B, Supporting Information) on a larger scale to obtain sufficient amounts of new AP-3 derivatives (Scheme 2).

Indeed, mutasynthon **8** was efficiently processed to 19-deschloroansamitocin P-3 (**26**).[15] The corresponding D_3-methoxy derivative was converted into D_3-**26**, proving that **8** is completely incorporated intact. Adding 3-amino-4-halobenzoic acids **14**, **16**, and **17** to growing cultures of mutant HGF073 (cultivation parameters B, Supporting Information) afforded the corresponding demethoxy-AP-3 analogues **27**–**29**, which were



Scheme 2. Successful mutasyntheses with strain HGF073.

isolated as pure compounds by applying HPLC purification procedures. These primary mutasynthetic experiments point out that modifications at the 2- and 6-positions of the aminobenzoic acid are not tolerated by the biosynthetic apparatus.

In principle, mutasynthesis not only provides new natural product analogues for testing their biological properties but can serve to introduce new functional groups for further chemical derivatization (semisynthesis) as shown in Figure 4.

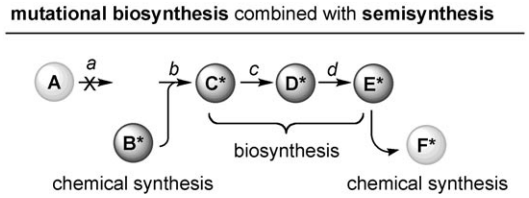
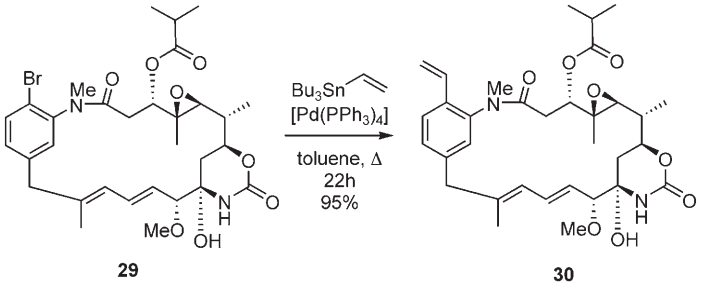


Figure 4. The concept of a combined mutasynthesis–semisynthesis approach: A = starting substrate, B*–D* = modified biosynthetic intermediates, E* and F* = modified natural products; a–d = enzymes.

To illustrate this combined approach, the brominated product **29** was used as substrate for a Pd-catalyzed Stille reaction, because Stille coupling proceeds under almost neutral conditions, unlike other Pd-catalyzed C–C coupling procedures such as the Heck–Mizoroki and Suzuki–Miyaura reactions. We were delighted to find that the coupling of tributylvinyl stannane with the brominated ansamitocin **29** proceeded in excellent yield. After chromatographic purification, 20-demethoxy-19-vinyl AP-3 (**30**) (1.3 mg, 2.2 μmol) was isolated as pure material (Scheme 3)

For the detailed evaluation of their biological profile as anticancer agents, the new derivatives were used to treat cultured human tumor cell lines; all showed strong antiproliferative activity, with IC_{50} values in the single-digit pmol mL^{-1} range or less (Table 1).



Scheme 3. Stille reaction of 19-bromo-20-demethoxy-AP-3 (**29**) with vinyl stannane.

Table 1. Antiproliferative activity of **2** and **27**–**30**.[a]

		IC_{50} [pmol mL^{-1}][16]				
Cell line	Origin	**2**	**27**	**28**	**29**	**30**
KB-3-1	cervix carcinoma	0.17	0.31	0.58	0.46	0.67
U-937	lymphoma	0.0055	0.017	0.058	0.046	0.084
PC-3	prostate carcinoma	0.055	0.059	0.20	0.15	0.17
SK-OV-3	ovarian carcinoma	0.047	0.059	0.17	0.077	0.067
A-498	kidney carcinoma	1.7	3.4	5.0	6.2	3.0
A-431	epidermoid carcinoma	0.079	0.068	0.25	0.15	0.070

[a] The activity of **26** was published previously.[15]

The 19-halogenated 20-demethoxyansamitocins **27**–**29** and the vinyl derivative **30** were tested for their inhibitory effect on cell growth of various cell lines in relative to AP-3.[16] **27**–**30** showed very strong activity against leukemia and ovarian cancer cell lines. Lower activity was found against kidney cancer cell lines. Thus, the introduction of different substituents onto the ansamitocins results in differential inhibitory action toward these cell lines. In general, the 19-fluoro-20-demethoxyansamitocin **27** and similarly the vinyl derivative **30** are the most active compounds tested, apart from the natural product **2**. The activities of **28** and **29** and 20-demethoxyansamitocin **26**[15] are generally 3- to 50-fold weaker than that of AP-3.

In conclusion, we have achieved the first mutasynthetic preparation of new 19-deschloro (compound **26**) and demethoxy derivatives of AP-3 (compounds **27**–**29**) in quantities sufficient for testing and chemical modification. Furthermore, we extended this type of "total synthesis" approach by combining mutasynthesis and semisynthesis. All new derivatives showed strong inhibitory effect on cell growth. Therefore, this mutasemisynthetic strategy has great potential for accessing compound libraries of highly potent and complex natural products like the ansamitocins.

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Keywords: ansamitocin · antitumor agents · mutasynthesis · semisynthesis · Stille reaction

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[16] Like AP-3 **2** ($IC_{50} = 0.02$ pmol mL^{-1}), demethoxyansamitocins P-3 **27**–**29** also showed strong antiproliferative activity for primary human endothelial cells (**27**: $IC_{50} = 0.04$ pmol mL^{-1}, **28**: $IC_{50} = 0.08$ pmol mL^{-1}, **29**: $IC_{50} = 0.23$ pmol mL^{-1}).

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