Natural Products

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The Interplay between Mutasynthesis and Semisynthesis: Generation and Evaluation of an Ansamitocin Library**

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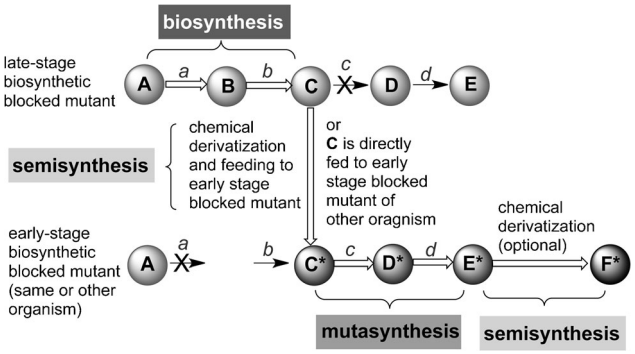
The broad use of natural products in the development of new drugs is currently hampered by the fact that they are too complex and too expensive in pre-commercial development, disregarding their potential that results from being receptor-optimized during evolution.[1,2] From a synthetic point of view, the extraordinary structural complexity of many natural products limits the options for accessing compound libraries through semisynthesis and total synthesis. Mutasynthesis and related concepts, which are based on interference with the biosynthesis of natural products, have emerged as an additional tool in natural product synthesis for generating structural diversity (Scheme 1).[3] Mutasynthesis[4] requires the generation of mutants of an organism wherein the formation of an intermediate for the biosynthetic pathway is blocked. Administration of mutasynthons to the blocked mutant then results in new metabolites.[5] Only in a few cases has this technique been combined with chemical synthesis, specifically semisynthesis,[6] even though such a combination should allow the best of both worlds: a) long linear biosyntheses towards structurally complex molecules and advanced intermediates and b) the flexibility of chemical synthesis to introduce any functional group, even at "unnatural" positions. Structural diversity and structure–activity relationship (SAR) studies should thus become more feasible for natural products.



Scheme 1. The concept of combined mutasynthesis and semisynthesis towards natural product derivatives. **A**: starting substrate, **B**–**D**: biosynthetic intermediates, **E**: natural product, **C***: chemically modified intermediate, **E***: modified natural product; **F***: chemically modified natural product derivative from **E***; *a*–*d*: enzymes (different for different organisms).

To explore the possibilities of combining chemical synthesis with mutasynthesis (Scheme 1) we chose the ansamycin antibiotics ansamitocin[7] and geldanamycin[8] as models. In fact, we have used the microbial producers of both cytotoxic natural products before to conduct mutasynthetic studies. Besides two (early stage) blocked mutants that we established as powerful "synthetic" tools[9] a (late stage) blocked mutant of *Actinosynnema pretiosum*, which produces ansamitocin, has now been added to our synthetic portfolio (Scheme 1).

It was our goal to achieve the synthesis of new ansamitocin derivatives by switching between semi- and mutasynthesis such that the product of one method serves as the starting material for the second method. This approach requires that all new derivatives are generated in sufficient amounts (mg scale). Besides demonstrating the principal synthetic opportunities of such a combined synthetic strategy we designed our syntheses such that the pharmacophoric role of the ester side chain could be addressed in conjunction with the cyclic carbinolamide in the ansamitocins.

Geldanamycin (**7**; Scheme 2), ansamitocins P2–4 (**3**–**5**), and the plant-derived maytansine (**6**) show structural similarities but exert different biological activities. Geldanamycin (**7**) is a highly potent antitumor drug[11] that binds to the N-terminal ATP-binding domain of the heat shock protein 90 (Hsp90) and inhibits its ATP-dependent chaperone activities,[12] thus preventing restructuring of mutated cancer-

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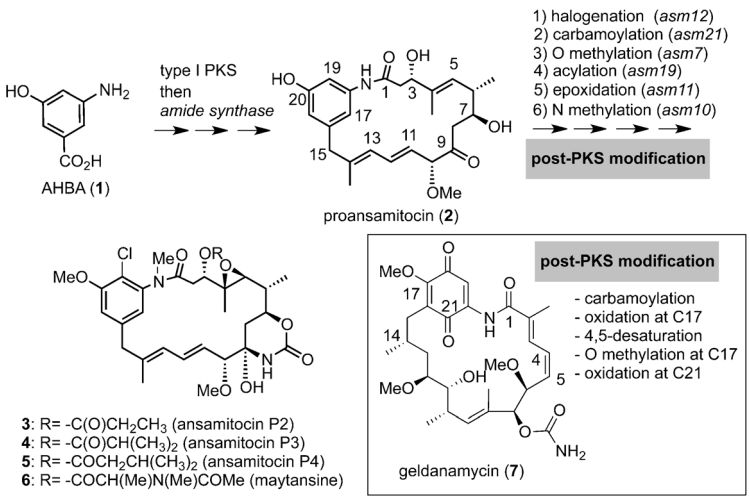


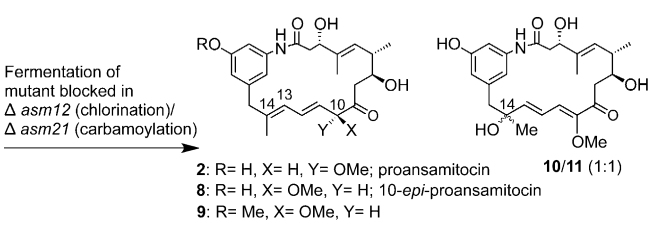
Scheme 2. Biosyntheses of ansamitocins P2 (**3**), P3 (**4**), P4 (**5**), and maytansine (**6**) via proansamitocin (**2**) (postketide transformations from **2** to **3**–**5** are listed along with gene annotations according to the predominant route of metabolic flux) and geldanamycin (**7**) and the post-PKS modifications.[7c,9,10]

relevant proteins such as p53.[13] The ansamitocins inhibit the growth of different leukaemia cell lines as well as human solid tumors at very low concentrations (10^{-3} to 10^{-7} μg mL^{-1}). They prevent polymerization of tubulin, thus causing G2/M cell cycle arrest, and subsequently lead to apoptosis.[14,15] Their activity was evaluated in human clinical trials, but although potent in vitro, they displayed a poor therapeutic window in vivo.[16,17] Nevertheless, they recently performed very well as antibody conjugates in phase II studies.[18,19] So far, the importance for bioactivity has been assigned to the presence of the ester side chain at C3 and the cyclic carbinolamide in its half-aminal form (C7–C9).

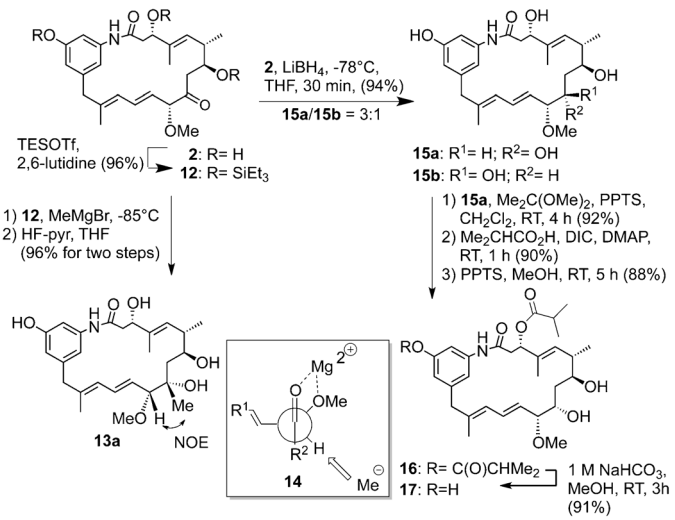
The structural similarities of ansamitocins and geldanamycin are due to related polyketide (PK) biosyntheses in which aminohydroxybenzoic acid (AHBA; **1**) is used as a starter unit (Scheme 2).[20] After being extended in seven consecutive PK synthase modules, proansamitocin (**2**) and progeldanamycin, respectively, are cyclized and released from the PKS by an amide synthase.[21] Both biosyntheses are completed by two different sets of post-PKS tailoring enzymes to furnish the ansamitocins **3**–**5** and geldanamycin (**7**).[9,22]

We have utilized mutant strains (*Actinosynnema pretiosum* HGF073 for ansamitocins and *Streptomyces hygroscopicus var. geldanus* K390-61-1 for geldanamycin) that are blocked in the formation of the starter unit **1** (early stage blocked mutant; Scheme 1).[6–8] The new blocked mutant is unable to carry out postketide modifications [blocked in *asm12* (chlorination) and *asm21* (carbamoylation)] and thus should generate proansamitocin (**2**; late-stage blocked mutant).[9] We found that this blocked mutant (Δ*asm12*/Δ*asm21*) of *A. pretiosum* produces **2** in good yield (up to 106 mg L^{-1}; Scheme 3). Additionally, small amounts of 10-*epi*-proansamitocin (**8**; 3.5 mg L^{-1})[23] and the O-methyl proansamitocin **9** (2.3 mg L^{-1}) were isolated.[24] It is likely that **8** may arise from α epimerization of the keto group at C9 during work-up. Interestingly, we also isolated two diastereomeric by-products, **10** and **11** (7.6 mg L^{-1}; 1:1 ratio), which contain an additional hydroxy group at C14 and the diene moiety is now in conjugation with the keto group. The two diastereomers were separated by HPLC methods, but thus far the absolute configuration at C14 has not been elucidated. These two diastereomers may result from an intermediate oxirane at C13–C14, an intermediate resulting from a nonspecifically operating epoxidase present in *A. pretiosum*. The epoxide opens after activation of the proton α to the keto group at C9. Oxidative formation of a benzyl alcohol (at C15) or a hydroxymethyl group at C14, which have been reported for ansamitocins before,[25] can be excluded here.[26]

We next developed routes to derivatize **2** by utilizing the keto moiety at C9 as a reactive functional group (Scheme 4). First, **2** was transformed into the per-O-silylated derivative **12** before addition of a methyl group to the ketone could be conducted. Methyl lithium led to a mixture of diastereomeric alcohols **13a**,**b** (**13a**/**13b** = 5:1) after removal of the silyl group. When methylmagnesium bromide was employed at



Scheme 3. The preparation of proansamitocin (**2**), 10-*epi*-proansamitocin (**8**), the O-methyl proansamitocin **9**, and oxidation by-products **10** and **11**. Biosynthesis using a late-stage mutant.



Scheme 4. Semisynthetic modifications of **2**. A combined biosynthesis/semisynthesis approach using a late-stage mutant is shown. DIC = diisopropylcarbodiimide, DMAP = 4-dimethylamino pyridine, PPTS = pyridinium *para*-toluene hydrosulfonate, pyr = pyridine, TES = triethylsilyl, Tf = trifluoromethanesulfonyl, THF = tetrahydrofuran.

−85 °C, only the diastereomer **13a** (d.r. > 80:1) was formed in excellent yield. The stereo-preference can be rationalized by assuming the transition-state **14** wherein there is chelation control through the neighboring methoxy group. Likewise, the keto group can be reduced with $LiBH_4$ to yield the diastereomeric triols **15a**,**b** (d.r. = 3:1).[27] After separation, the 1,3-diol moiety in the major diastereomer **15a** was protected as an acetonide and the remaining hydroxy groups were esterified with isobutyric acid to yield the diester **16** after acetonide cleavage. Selective hydrolysis of the phenoxy ester furnished the corresponding monoester **17**.[28] This sequence paves the way to specifically acylating O3, which is of general importance for generating cytotoxic ansamitocin derivatives (see below).

From earlier studies in our laboratories using the fully synthetic proansamitocin we knew that **2** could be transformed into ansamitocin P3 (**4**) when added to a culture of the (−)-AHBA-blocked mutant of *A. pretiosum* (HGF073).[29] Having established protecting group strategies and semisynthetic modifications for **2** we extended our mutasynthetic studies to the biosynthetically advanced derivatives **8**, **13a**, and **15a**,**b**. After adding the derivatives to either of the two (−)-AHBA-blocked mutants of *A. pretiosum* HGF073 or to *hygroscopicus* var. *geldanus* (K390-61-1), which produces the geldanamycin, new carbamoylated metabolites **18**–**22** were isolated, sometimes in excellent yields (Figure 1).[30]

Feeding of **8** to K390-61-1 led to the carbamoylated product **18** as well as to a mixture of **2** and the starting **8**. The HRMS/MS also revealed traces of the oxidation products **10**, **11**, and the carbamoylated proansamitocin **30** (see below).

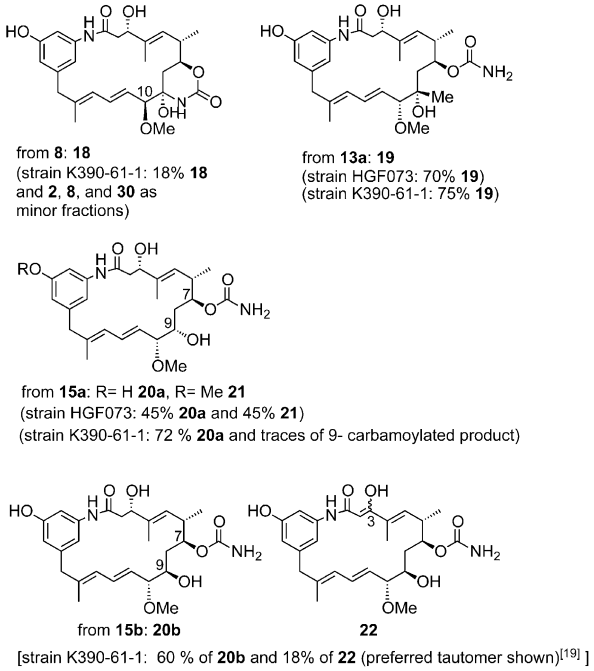


Figure 1. Preparation of the ansamitocin derivatives **18**–**22** after mutasynthetic derivatization of the modified proansamitocins **8**, **13a**, and **15a,b**. The compounds were prepared using a combined biosynthesis/mutasynthesis approach using a late-stage-blocked mutant followed by an early stage blocked mutant.

Clearly, these results demonstrate that epimerization at C10 takes place under the fermentation conditions on proansamitocin and not during the PKS assembly, and that the natural *R* configuration at C10 is thermodynamically favored (see below). The methyl-branched proansamitocin **13a** turned out to be an excellent substrate for the carbamoyl transferase, thus resulting in the mutaproduct **19**. The *anti*-1,3-diol **15a**, when fed to both (−)-AHBA-blocked mutants, yielded the 7-O-amidinated proansamitocin derivative **20a**. Strain HGF073 also gave a small amount of the O-methylated product **21**, which again is indicative of some degree of flexibility in the timing of the postketide transformations. The corresponding 9-epimeric diol **15b** also gave the corresponding 7-O-amidinated biotransformed product **20b**, but this time along with the 3-oxo derivative **22**. Indeed, we occasionally noted oxidations at various positions of the carbon backbone of proansamitocin (see **27** and **31**). It is interesting to note, that the carbamoyl transferase present in *S. hygroscopicus* is able to utilize proansamitocin derivatives as substrates and it addresses the same position in the macrocycle as does the carbamoyl transferase from *A. pretiosum*.[31]

Unfortunately, all these mutasynthetic transformations did not provide derivatives esterified at C3. As this post-PKS transformation is considered essential for full cytotoxic activity we pursued two synthetic routes. The first strategy relied on the semisynthetically esterified proansamitocin **17**. This C9 epimer was chosen because, as in **4**, the hydroxy group at C9 has an α orientation. Feeding *A. pretiosum* (HGF073) with **17** yielded a set of new metabolites, **20a**, **21**, and **23**–**29**, which were isolated in an overall excellent yield (Figure 2). Carbamoylation is expected to be the first step, however, the regioisomers **25** and **29** which are carbamoylated at C9 were also formed. In addition N glucosylation also took place as demonstrated for metabolites **28** and **29**. This postketide transformation has been described before by us[7c] and others[32] in related work; it is commonly accompanied by loss of strong cytotoxic activity.

Again, also O-methylation (metabolites **21**, **24** and **27**) occurred occasionally. Surprisingly, the phenol **27** that is hydroxylated at C21, was formed; formation of **27** is unprecedented in ansamitocin metabolism.[23] Interestingly, these biotransformations are sometimes accompanied by 3-O-deacylation as seen in metabolites **20a**, **26**, and **27**. Notably, when the fermentation of the proansamitocin derivative **17** was repeated with the (−)-AHBA-blocked mutant of *S. hygroscopicus* only the two regioisomeric carbamoylated proansamitocins **23** and **25** could be detected and isolated. From a biological perspective the ansamitocin derivative **23** is highly interesting as it contains the expected key pharmacophoric groups, the ester side chain at C3 and the carbamoyl group at C7, and it is reduced at C9 with the correct α orientation of the hydroxy group.

The second strategy is based on a sequence reversal. First, the carbamoyl group at C7 was introduced with subsequent semisynthetic introduction of the ester side chain at C3 (Scheme 5). Strain HGF073 is not well suited for the carbamoylation of **2** because this organism would utilize the complete set of postketide enzymes.[29] However, by feeding proansamitocin **2** to the (−)-AHBA-blocked mutant of

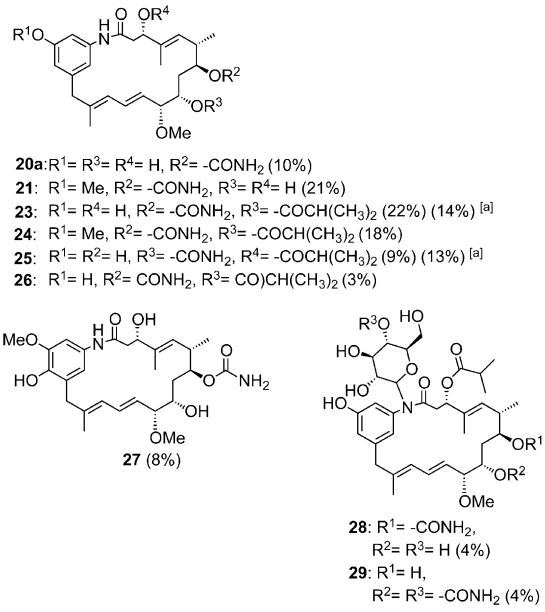
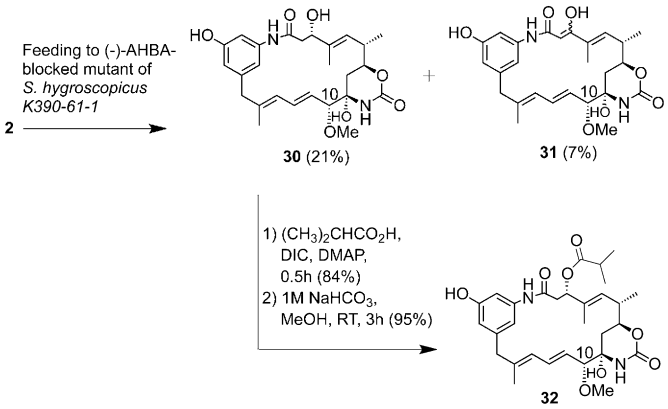


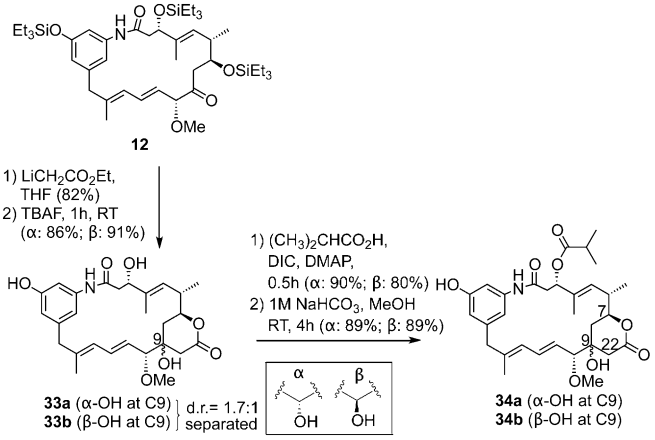
Figure 2. Mutasynthesis of the semisynthetically modified proansamitocin **17** with the (−)-AHBA-blocked mutant of *A. pretiosum* (HGF073). Yields of isolated products total 99%. [a] Yield for the fermentation experiment conducted with the (−)-AHBA-blocked mutant of *S. hygroscopicus* (K390-61-1). Prepared by a combined biosynthesis/semisynthesis/mutasynthesis approach using a late-stage-blocked mutant followed by an early stage blocked mutant.



Scheme 5. Synthesis of the ansamitocin derivative **32**. Prepared by a combined biosynthesis/mutasynthesis/semisynthesis approach using a late-stage mutant and an early stage blocked mutant.

S. hygroscopicus, the new carbamoylated derivative **30** along with the oxidized derivative **31** were formed as major products.[33,34] The established synthetic sequence (see Scheme 4) involves acylation of the phenolic group and the 3-hydroxy position with subsequent selective hydrolysis of the phenolic ester to give the ansamitocin P3 derivative **32**, which is esterified at C3.

To complement the SAR studies, we also prepared the carba-analogues of **32**, the lactones **34a** and **34b**, which open the door to studying the role of the carbamoyl group and the stereochemistry at C9 in the bioactivity of the ansamitocins (Scheme 6). These analogues were accessed by treating the silyl-protected proansamitocin **12** with lithiated ethyl acetate and subsequent TBAF-promoted desilylation, which simultaneously induces lactonization. The two diastereomeric alcohols **33a**,**b** were formed as separable diastereomers (d.r. = 1.7:1). The stereochemical assignment of **33b** was determined by NMR spectroscopy using steady-state NOE experiments ([D_6]THF), wherein contacts between the OH at C9 and H_a on C22, and 7H and H_b at C22 in the lactone ring were observed.[23] Next, introduction of the isobutyrate side chain was achieved as described above to yield the new derivatives **34a** (α-OH at C9) and **34b** (β-OH at C9).



Scheme 6. Syntheses of the carbaanalogues **34a** and **34b**. Prepared by a combined biosynthesis/semisynthesis approach using a late-stage mutant. TBAF = tetra-*n*-butylammonium fluoride

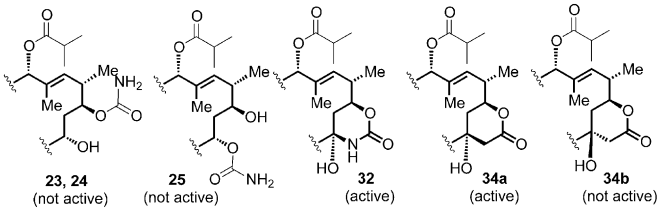
For a detailed evaluation of the biological profiles of the derivative as anticancer agents, all new derivatives were tested in cultured mouse fibroblast cells and human tumor cell lines.[34] As expected, all ansamitocines that lack both the ester side chain at C3 and the carbamoyl group (**2**, **8**, **9**, **10**, **11**, **13a**,**b**, and **15a**,**b**) do not show antiproliferative activity ($IC_{50} > 900$ nM) when exposed to mouse fibroblast cells (L-929). The second group of derivatives, **18**–**22** and **27**, either contain the carbamoyl group in the cyclic or open-chain form, but lack the isobutyrate group at C3. They too turned out to be inactive in the proliferation assay with L-929 cell lines ($IC_{50} > 900$ nM). Compounds **16** and **17** bear the isobutyrate side chain at C3 and the hydroxy group at C9 has an α orientation just as the hydroxy group of the cyclic carbinolamide in **4**). But both derivatives lack the carbamoyl group and hence do not show antiproliferative activity ($IC_{50} > 900$ nM). One may argue that in addition to the ester side chain at C3, an sp^2 center at C9, even when present in equilibrium with the open carbinolamide may also be essential for biological activity. Although supported by the fact that the derivatives **23**–**25** are inactive, despite containing both pharmacophoric key groups and the α orientation of the oxygen substituent at C9, this notion is ruled out by the result with carbaanalogue **34a**.

Finally, the new derivatives **32** and **34a**,**b** were tested (Table 1). Compound **32**, which resembles **4** but lacks several

Table 1: Antiproliferative activity IC_{50} [nmol L^{-1}] of **23**, **25**, **32**, and **34a**,**b** in comparison to **4**.[a]

Cell line	23	25	32	34a	34b	4
KB-3-1	>3500	>3500	0.88	34	4100	0.17
U-937	n.d.	n.d.	0.77	6.3	1200	0.01
PC-3	>7000	>7000	2.3	144	>7000	0.06
SK-OV-3	>7000	4000	2.9	27	4500	0.05
A-431	>7000	5900	2.9	40	7000	0.08
MCF-7	n.d	n.d	1.3	16	2300	n.d.
HUVEC	>7000	4400	2.2	22	3800	0.02

[a] Values shown are mean values of two determinations run in parallel. n.d. = not determined.[23,35]



postketide modifications, shows good antiproliferative activity. However, its activity is generally reduced about 5–100-fold compared to the parent natural product **4**. Remarkably, the carba-analogue **34a**, bearing the correct stereochemistry at C9 but lacking the nitrogen atom, is also a cytotoxic derivative; and upon inversion of the stereochemistry at C9, as in **34b**, all of this activity is lost. Clearly, the ester side chain at C3 and a six-membered ring between C7 and C9 that bears a carbonyl group is crucial for biological activity, provided that the configuration of the stereogenic center at C9 is *R*. Clearly, the carbamate nitrogen atom is important but not essential in a pharmacophoric model of the ansamitocins whereas the *R* configuration at C9 is crucial.

In conclusion, we disclose a flexible application of chemical synthesis/semisynthesis and biosynthesis/mutasynthesis sequences for creating a library of 25 ansamitocin derivatives. The similarity of the biosyntheses of the two ansamycin antibiotics ansamitocin (**4**) and geldanamycin (**7**), paved the way to successfully feeding advanced and chemically modified ansamitocin intermediates to an (−)-AHBA-blocked mutant of *S. hygroscopicus*, which produces geldanamycin, and commonly resulted in selective carbamoylation. These are rare examples of mutasynthetic studies with highly advanced mutasynthons. Semisynthesis broadened the range of derivatives available, including access to analogues in which the nitrogen atom of the cyclic carbinolamide is replaced by a carbon atom. Antiproliferation assays revealed that the stereochemistry at C9 is crucial whereas the carbamate nitrogen atom does not play a strong role in the biological activity of the ansamitocins. We are certain that a combination of chemical synthesis and mutasynthesis holds great promise for accessing compound libraries of highly potent and complex natural products like the ansamitocins.

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[33] Additionally, 10-*epi*-proansamitocin (**8**), the two diastereomers **10** and **11**, and the carbamoylated 10-*epi*-proansamitocin **18** were detected in trace amounts, again indicating the facile epimerization at C10.

[34] The UPLC/HRMS/MS data also revealed the presence of **8**, **10**, and **11**.

[35] Human cell lines: L-929 (mouse fibroblasts), KB-3-1 (cervix carcinoma), U-937 (histiocytic lymphoma), PC-3 (prostate adenocarcinoma), SK-OV-3 (ovary adenocarcinoma), A-431 (epidermoid carcinoma), MCF-7 (breast adenocarcinoma), HUVEC (umbilical vein endothelial cells).

