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Cyclization of Synthetic *seco*-Proansamitocins to Ansamitocin Macrolactams by *Actinosynnema pretiosum* as Biocatalyst

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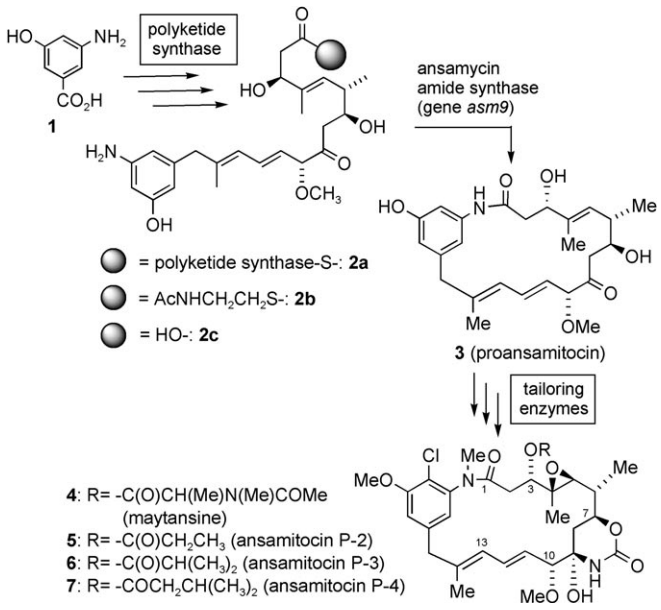
The plant-derived maytansinoids **4**[1] and their microbial counterparts, the ansamitocins P-2 to P-4 **5**–**7** (Scheme 1)[2] exert strong in vitro and in vivo antitumor activity by blocking the assembly of tubulin into functional microtubules.[3] Ansamitocin biosynthesis involves construction of the carbon framework on a type I modular polyketide synthase (PKS)[4] from 3-amino-5-hydroxybenzoic acid **1** (AHBA)[5] via chain extension by one "glycolate", three propionate, and three acetate units. The last PKS module holds the *seco*-proansamitocin **2a**, which is released and cyclized, presumably by an ansamycin amide synthase (gene *asm9*),[6] to yield the 19-membered macrocyclic lactam, proansamitocin **3** (Scheme 1).[7] The latter enzyme is particularly interesting because the corresponding chemical macrolactamizations are known to be challenging, due, in part, to the lack of nucleophilicity of the aniline moiety.[7–9] The amide synthases are not part of the PKS,[10] but are separate enzymes with homology to arylamine acyl transferases.[4,11,12]

We have studied the substrate specificity of the ansamitocin biosynthetic machinery[13,14] using a mutant strain (HGF073) of *Actinosynnema pretiosum* blocked in the biosynthesis of the PKS starter unit AHBA **1**.[7] Through mutasynthetic approaches, we could show that the PKS, the amide synthase, and the tailoring enzymes accept several substrates that are modified in the aromatic moiety of the biosynthetically advancing ansamitocins. As the amide synthase is thought to use PKS-bound *seco*-proansamitocin **2a** as substrate, it is unclear whether simple thioester analogues or free carboxylic acids can also act as substrates in mutabiosyntheses. Acceptance of very advanced biosynthetic intermediates and analogues would be highly desirable because it would allow one to bypass the "conservative" PKS with modified substrates that the PKS machinery has difficulty processing. In addition, this approach would also shed light on mechanistic aspects of the amide synthase and on its substrate specificity. So far, the difficulty in accessing potential substrates of the amide synthase has been a major hurdle in this research. Although it is a time-consuming task, total synthesis is a reliable strategy including access to analogues.



Scheme 1. Biosynthesis of ansamitocins **5**–**7** via *seco*-proansamitocin **2a** and proansamitocin **3**, and comparison with maytansine **4**.

We previously reported the total synthesis of *N*-acetylcysteamine thioester (SNAC ester) **2b**.[13] Herein we describe the synthesis of *seco*-acid **2c** and of the SNAC ester of the new *seco*-proansamitocin derivative **16**, as well as the use of all three advanced substrates in conversion experiments with *A. pretiosum* mutant HGF073.

SNAC ester **2b** (2.3 mg, 4.1 μmol) was added to a culture (50 mL) of *A. pretiosum* strain HGF073 (Scheme 2).[15] The culture was harvested after seven days, and extracts were analyzed by UPLC–MS (ultra-high-performance LC-coupled ESI-MS). The spectra of the biosynthetic samples were compared with those of authentic AP-3 (**6**) and found to be identical, with a parent ion at *m/z* 657 $[M+Na]^+$ and collision-induced fragmentation giving daughter ion spectra with a base peak at *m/z* 569 (from *m/z* 657) due to loss of the ester function at C3. In earlier studies, we could unequivocally exclude background synthesis of AP-3 with blocked mutant HGF073.[16] The yield of AP-3 formed from SNAC ester **2b** was 0.2% based on quantitation by UV absorption at $\lambda = 248$ nm after preparative HPLC purification.[17] That equals 2% of the incorporation observed with AHBA supplementation (~80 mg L^{-1}).

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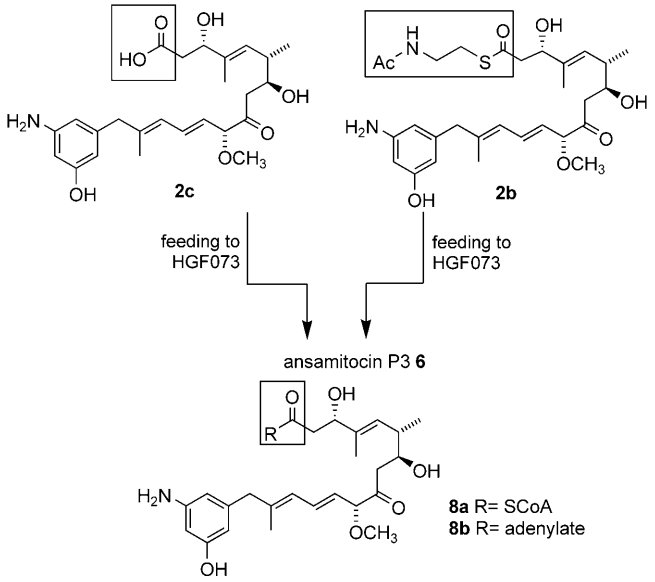


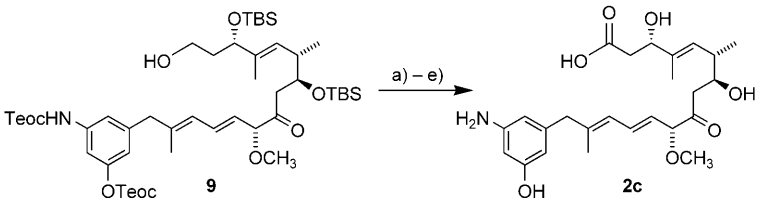
Scheme 2. Feeding experiments with *seco*-proansamitocin **2c** and SNAC ester **2b** to yield ansamitocin P3 **6** and proposed activated intermediates of *seco*-acids **8a** and **8b.**

The SNAC ester **2b** may have been accepted as substrate by the amide synthase directly; alternatively, **2b** could have been initially loaded onto the last PKS module by transesterification before macrolactamization occurs. Surprisingly, *seco*-proansamitocin **2c** (0.4 mg, 0.9 μmol), prepared from a published advanced synthetic precursor **9**[13] via an established sequence (Scheme 3),[18] also gave AP-3 (**6**) in 1.5% yield relative to AHBA supplementation (0.13% absolute yield), when incubated with *A. pretiosum* strain HGF073 (50 mL). Macrolactamization of **2c** can only occur after activation of the carboxylate to the CoA ester **8a**, for example, or the adenylate **8b**. The amide synthase then promotes macrolactamization either directly or following transfer onto the final PKS module.

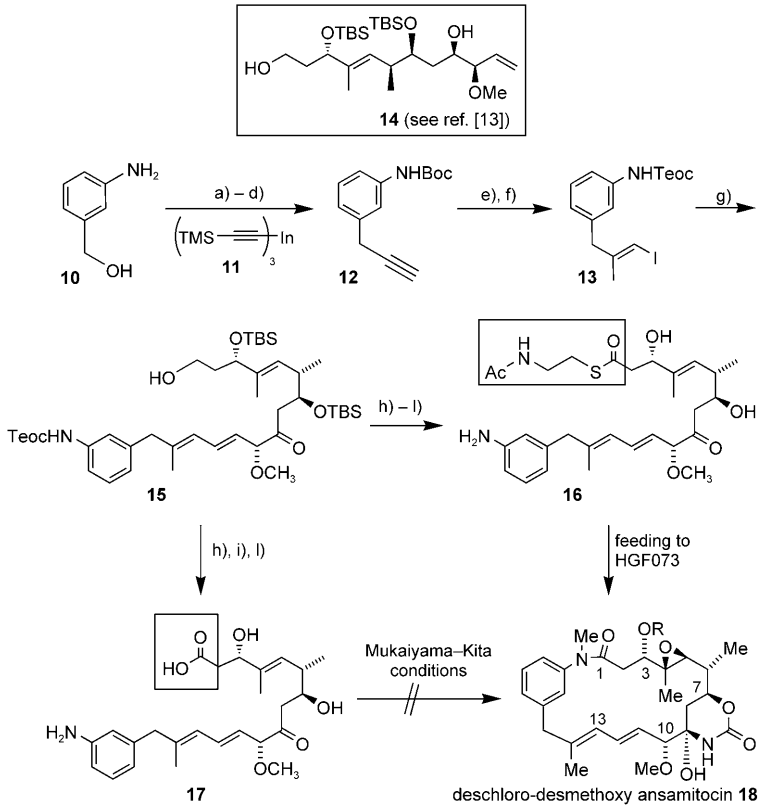
As a second complex substrate, we prepared the SNAC ester of 20-deoxy-*seco*-proansamitocin **16**, which was chosen because we had observed that supplementing a culture of HGF073 with aminobenzoic acid **19** (260 mg, 1.5 mmol) unexpectedly gave the corresponding ansamitocin derivative **18** in very low yield, as determined by UPLC–MS (see Scheme 5 below).[19] The total synthesis approach relates to the successful preparation of SNAC ester **2b** (Scheme 4).[13] Thus, starting from benzyl alcohol **10**, intermediate propargyl aniline **12** was prepared via the *N*-Boc-protected benzyl bromide, which was subjected to a palladium-catalyzed cross-coupling with alkynylindium derivative **11** (Scheme 4).[20] After desilylation, carboalumination,[21] during which the Boc group was also removed, followed by Teoc protection yielded vinyl iodide **13** in good yield and with good stereocontrol.

In the following, the Heck reaction allowed the merging of vinyl iodide **13** and complex alkene **14**[13] under Jeffery conditions[22] to furnish the coupling product **15** in 68% yield. Simultaneous oxidation of both hydroxy groups and further oxidation of the aldehyde moiety to the carboxylic acid was followed by SNAC ester formation. Finally, the silyl ethers were removed by treatment with the HF·pyr complex, and deprotection of the Teoc group was achieved with $ZnCl_2$ under ultrasound conditions to yield the desired SNAC ester **16**. Standard functional group manipulations led to *seco*-acid derivative **17** using alcohol **15** as branching point. Interestingly, we were unable to chemically cyclize *seco*-proansamitocin **17** under various conditions, including those of the Mukaiyama (*N*-methyl-2-chloropyridinium iodide, Et_3N)[23] and Kita (ethoxyacetylene, cat. $[RuCl_2(p\text{-cymene})]_2$)[24] protocols. The difficulty of smoothly achieving macrolactamization in ansamycin synthesis has been encountered before, for which the decreased nucleophilicity of the arylamino group is likely responsible.[8]

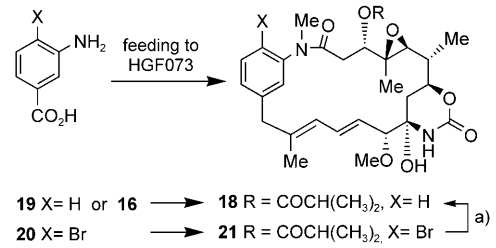
In contrast, when SNAC ester **16** (1.2 mg, 2.2 μmol) was incubated with HGF073, the new deschloro-desmethoxy-AP-3 **18** (14 μg, 1.1%) was formed (Scheme 5). Again, UPLC–MS served to identify the metabolite, revealing the parent ion at *m/z* 571 $[M+H]^+$ and collision-induced fragmentation that gave daughter ion spectra with a base peak at *m/z* 483 (from *m/z* 571). To confirm the structure of **18**, we conducted another mutational biosynthesis with *A. pretiosum* HGF073 using bromoaminobenzoic acid **20** as mutasynthon, which yielded 19-bromo-20-demethoxy-AP-3 **21**[15] in good yield (up to 15 mg L^{-1}). Palladium-catalyzed debromination finalized the alternative synthesis of 19-deschloro-20-demethoxy-AP-3 **18**. The product was identical in all respects to the fermentation product collected from feeding SNAC ester **16**. The semisynthetic material also served to quantify the feeding experiment with SNAC ester **16**. This was achieved with an authentic standard using diagnostic UV absorption at $\lambda = 248$ nm.[17] The degree of incorporation for all three *seco*-acid derivatives is rather low, and is similar in magnitude to incorporation yields for tri- or tetraketide PKS intermediates.[16] This does not necessarily mean that their further processing is inefficient. In fact, we encountered severe stability problems with all three open-chain substrates **2b**, **2c**, and **16** during the final steps of total synthesis. These included dehydration (at C2–C3), retro-aldol reaction (at C3) and double bond isomerization (C10 to C14), just to name those we could clearly identify. Clearly, only when cyclization has occurred, are undesired degradations suppressed.[25] Therefore, it cannot be excluded that substantial amounts of the free *seco*-acid derivatives decomposed during the seven days in the fermentation broth.



Scheme 3. Synthesis of *seco*-proansamitocin **2c**: a) **9**,[13] DMSO, $(COCl)_2$, −60 °C, 1 h then Et_3N, −40 °C (75%); b) $NaClO_2$, NaH_2PO_4, H_2O, *t*BuOH, 2-methyl-2-butene, 0 °C→RT, 30 min (86%); c) HF·pyr, THF, RT, 6 h, (48%); d) $ZnCl_2$, $MeNO_2$, ultrasound, RT, 1 h, (47%). TBS = *tert*-butyldimethylsilyl, Teoc = trimethylsilylethoxycarbonyl.



Scheme 4. Total synthesis of SNAC ester **16** and *seco*-acid **17**, and feeding experiments with **16** toward deschloro-desmethoxy-AP-3 **18**: a) Boc_2O, Et_3N, dioxane/H_2O (2.5:1), RT, 12 h, (82%); b) PPh_3, CBr_4, CH_2Cl_2, RT, 1 h (83%); c) Pd(dppf)Cl_2, THF, 65 °C, 4 h (98%); d) TBAF·3 H_2O, THF, −20 °C, 1 h (88%); e) 1. $AlMe_3$, Cp_2ZrCl_2, $(CH_2Cl)_2$, RT, 1 h, then addition of **11**[13] at 0 °C, RT, 72 h, 2. I_2, THF, −30 °C→0 °C, 1.5 h (53%); f) TeocCl, $NaHCO_3$, CH_2Cl_2, RT, 10 min (91%); g) **14**, Pd$(OAc)_2$, Cs_2CO_3, Bu_4NBr, NEt_3, DMF, RT, 2 h (68%); h) DMSO, $(COCl)_2$, −60 °C, 1 h then Et_3N, −40 °C (90%); i) $NaClO_2$, NaH_2PO_4, H_2O, *t*BuOH, 2-methyl-2-butene, 0 °C→RT, 30 min (85%); j) AcNH$(CH_2)_2$SH, DIC, DMAP, CH_2Cl_2, RT, 2 h (56%); k) HF·pyr (70% HF)/THF, RT, 24 h (62%); l) $ZnCl_2$, $MeNO_2$, ultrasound, RT, 80 min (63%; toward **17**: 11%). TMS = trimethylsilyl, Boc = *tert*-butyloxycarbonyl; dppf = bis(diphenylphosphino)ferrocene, TBAF = tetra-*n*-butylammonium fluoride; DIC = diisopropylcarbodiimide, DMAP = 4-dimethylamino pyridine.



Scheme 5. Preparation of deschloro-desmethoxy-AP-3 **18**: a) Bu_3SnH, Pd[P*t*$Bu_3]_2$, toluene, 60 °C (80%).

These results demonstrate for the first time that open-chain *seco*-acid precursors can, in principle, be cyclized by *A. pretiosum* when a mutant blocked in AHBA biosynthesis is used. We also showed that not only activated thioesters are cyclized but also the free carboxylic acid can be accepted and is transformed into the corresponding macrolactam. Overexpression and isolation of the amide synthase should pave the way to study its mechanism in detail and will allow synthetic chemists to explore the use of the enzyme for preparative macrolactamizations using other long-chain amino acids as substrates. Such investigations are currently pursued in our laboratories.

The new AP-3 derivative **18**[26] was tested for its inhibitory effects toward the proliferation of various cancer cell lines relative to AP-3 **6** (Table 1). It showed very strong activity in the pg mL^{-1} range against all cancer cell lines tested. In the case of cervix and prostate carcinoma, **18** had an even higher activity than AP-3 **6**.

Table 1. Antiproliferative activity of **18** compared with that of **6** toward various cancer cell lines.[a]

	IC_{50} [ng mL^{-1}]					
	KB-3-1 cervical	U-937 lymphoma	PC-3 prostate	SK-OV-3 ovarian	A-431 epidermal	A-549 lung
18	0.022	0.015	0.020	0.040	0.080	0.095
6	0.11	0.0035	0.035	0.030	0.050	0.095

[a] The IC_{50} values toward primary HUVEC (from LONZA) were 0.021 and 0.031 ng mL^{-1} for **18** and **6**, respectively.

Acknowledgements

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Keywords: amide synthase · ansamitocin · mutabiosynthesis · SNAC esters · total synthesis

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[18] Details on the synthesis of **2c** from **9** are provided in the Supporting Information.
[19] Formation of **18** was determined to be 0.5% relative to supplementation with AHBA (see also ref. [17]).
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[25] Indeed, one may argue that macrocyclization is not only Nature's way of creating macrocycles that are able to adopt various defined conformations to suit a given receptor's requirements, but also allows the chemical stabilization of open-chain polyketide metabolites.
[26] Antiproliferative activities of **21** are described in ref. [14].

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