Published in final edited form as:
Chembiochem. 2010 December 10; 11(18): 2517–2520. doi:10.1002/cbic.201000422.

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

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Keywords

Ansamitocin; Amide synthase; SNAC-esters; Mutabiosynthesis; Total synthesis

The plant-derived maytansinoids **4**[1] and their microbial counterparts, the ansamitocins P-2 to P-4 **5-7** (Figure 1) [2] exert strong *in vitro* and *in vivo* antitumor activity, blocking the assembly of tubulin into functional microtubules.[3] The biosynthesis of the ansamitocins involves the 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 in part due 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] In 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 supposed to utilize PKS-bound *seco*-proansamitocin **2a** as substrate it is unclear whether simple thioester analogues or free caboxylic acids can also act as substrates in mutasbiosyntheses. Acceptance of very advanced biosynthetic intermediates and analogues would be highly desirable because it would allow to bypass the "conservative" PKS with modified substrates that the PKS machinery has difficulty to process. In addition, this approach would also shed light on mechanistic aspects of the amide synthase and on its substrate specificity. So far, the difficulty to access potential substrates of the amide

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Supporting Information Available: Synthesis and spectra of new compounds, protocols of feeding experiments and their analysis. Supporting information for this article is available on the WWW under http://www.chembiochem org or from the author.

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.

We have previously reported the total synthesis of *N*-acetylcysteamine-thioester (SNAC-ester) **2b**.[13] In the present work we describe the synthesis of *seco*-acid **2c** and of the SNAC-ester of the new *seco*-proansamitocin derivative **16**, as well as the utilization 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 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 unequivocally could exclude background synthesis of AP-3 with blocked mutant HGF073.[17] The yield of AP-3 formed from SNAC ester **2b** was 0.2 % based on quantitation by UV absorption at λ=248 nm after preparative HPLC purification.[16] That equals 2% of the incorporation seen with AHBA-supplementation (ca. 80 mg/L).

The SNAC ester **2b** may have been accepted as substrate by the amide synthase directly or alternatively, **2b** was first loaded onto the last PKS module by transesterification before macro-lactamization 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 compared to AHBA-supplementation (0.13 % absolute yield), when incubated with strain HGF073 of *A. pretiosum* (50 mL). Macrolactamization of **2c** can only occur after activation of the carboxylate, e.g. to the CoA-ester **8a** 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 yielded the corresponding ansamitocin derivative **18** in a very low yield as determined by UPLC-MS (Scheme 5).[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 alkinylindium derivative **11** (Scheme 3).[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 to merge vinyl iodide **13** and complex alkene **14**[13] under Jeffery conditions[22] furnishing 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 which for example included 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 reduced 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. Again, UPLC-MS served to identify the metabolite revealing the parent ion at m/z 571 $(M+H)^+$ and collision-induced fragmentation giving daughter ion spectra with a base peak at m/z 483 (from m/z 571). In order to confirm the structure of **18**, we conducted another mutational biosynthesis with *Actinosynnema 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). Pd-catalyzed debromination finalized the alternative synthesis of 19-deschloro-20-demethoxy AP-3 **18**. The product was identical in all respects with the fermentation product collected from feeding SNAC ester **16**. The semisynthetic material also served for quantifying the feeding experiment with SNAC-ester **16**. This was achieved with an authentic standard utilizing diagnostic UV absorption at λ = 248 nm.[16] The degree of incorporation for all three seco-acid derivatives is rather low and in magnitude is comparable with incorporation yields of tri- or tetraketide PKS intermediates.[17] 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 last 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. Obviously, only when cyclization has occurred undesired degradations are 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.

The new AP-3 derivative **18**[26] was tested for its inhibitory effects on the proliferation of different cancer cell lines in comparison to AP-3 **6**. It showed very strong activity in the pg/ml 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**.

These results demonstrate for the first time that open chain *seco* acid precursors can in principal be cyclized by *A. pretiosum* when a mutant blocked in AHBA-biosynthesis is utilized. 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.

Acknowledgments

We thank the US National Institutes of Health (grant CA 76461), the Deutsche Forschungs= gemeinschaft (grant Ki 397 / 13-1) and the Fonds der Chemischen Industrie for financial support. We are grateful to T. Knobloch for helpful discussions. We thank M. Quitschalle and Dr. T. Frenzel for expert synthetic support.

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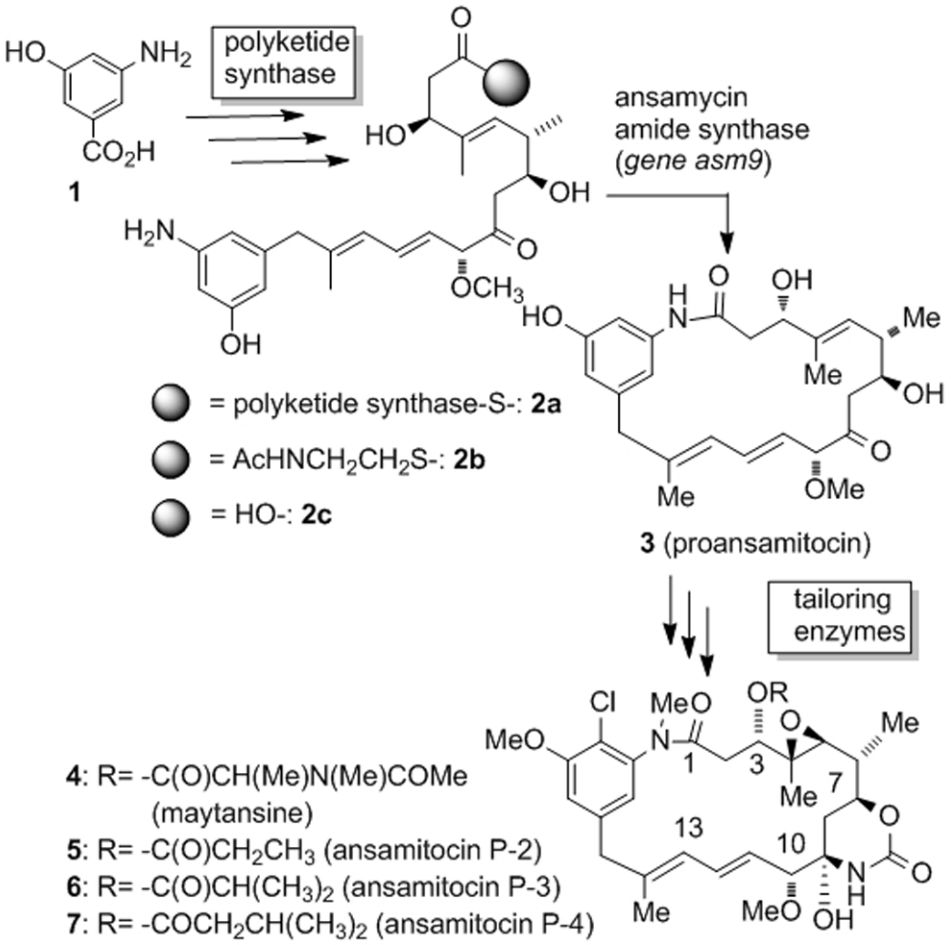
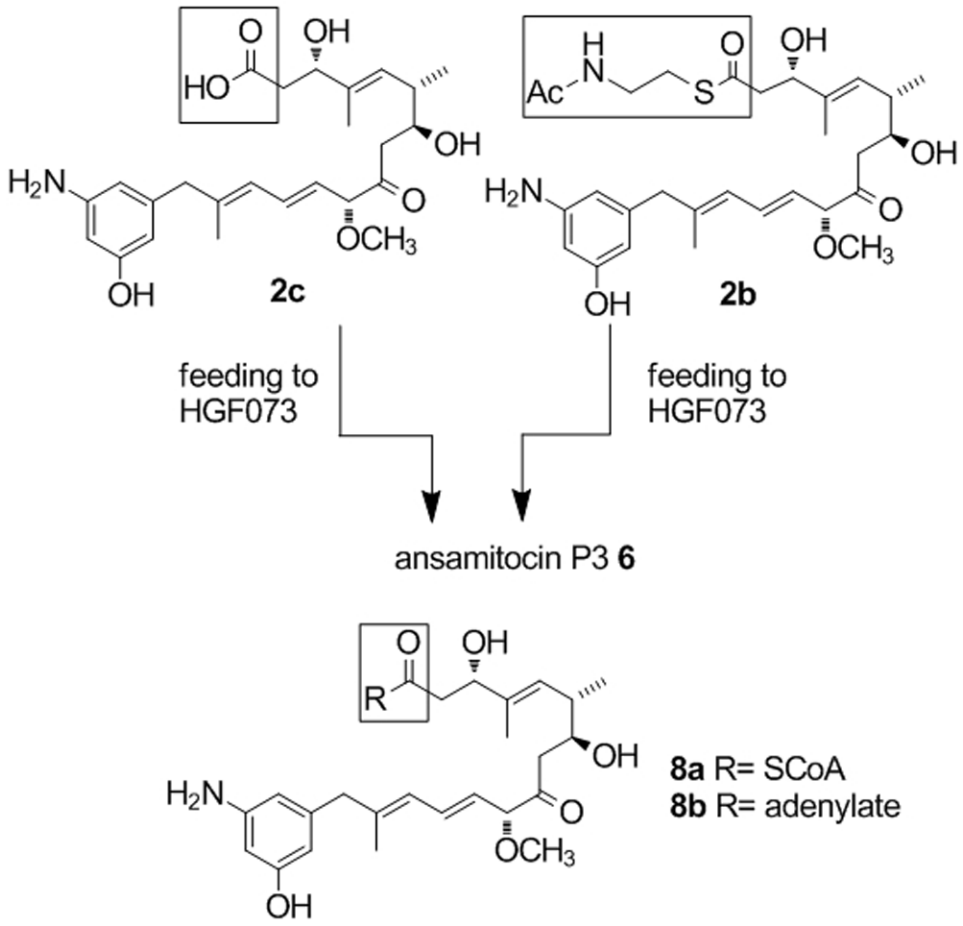
25. Indeed, one may argue that macrocyclization is not only a tool of Nature to create macrocycles that are able to adopt different defined conformations to suit the receptor's requirements but also allows to chemically stabilize open chain polyketide metabolites.

26. Antiproliferative activities of **21** are described in reference [14].

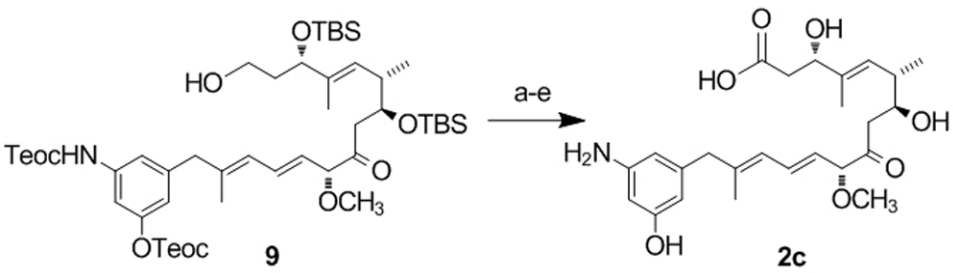


Scheme 1.
Biosynthesis of ansamitocins **5 - 7** via *seco*-proansamitocin **2a** and proansamitocin **3** and comparison to maytansine **4**.



Scheme 2.

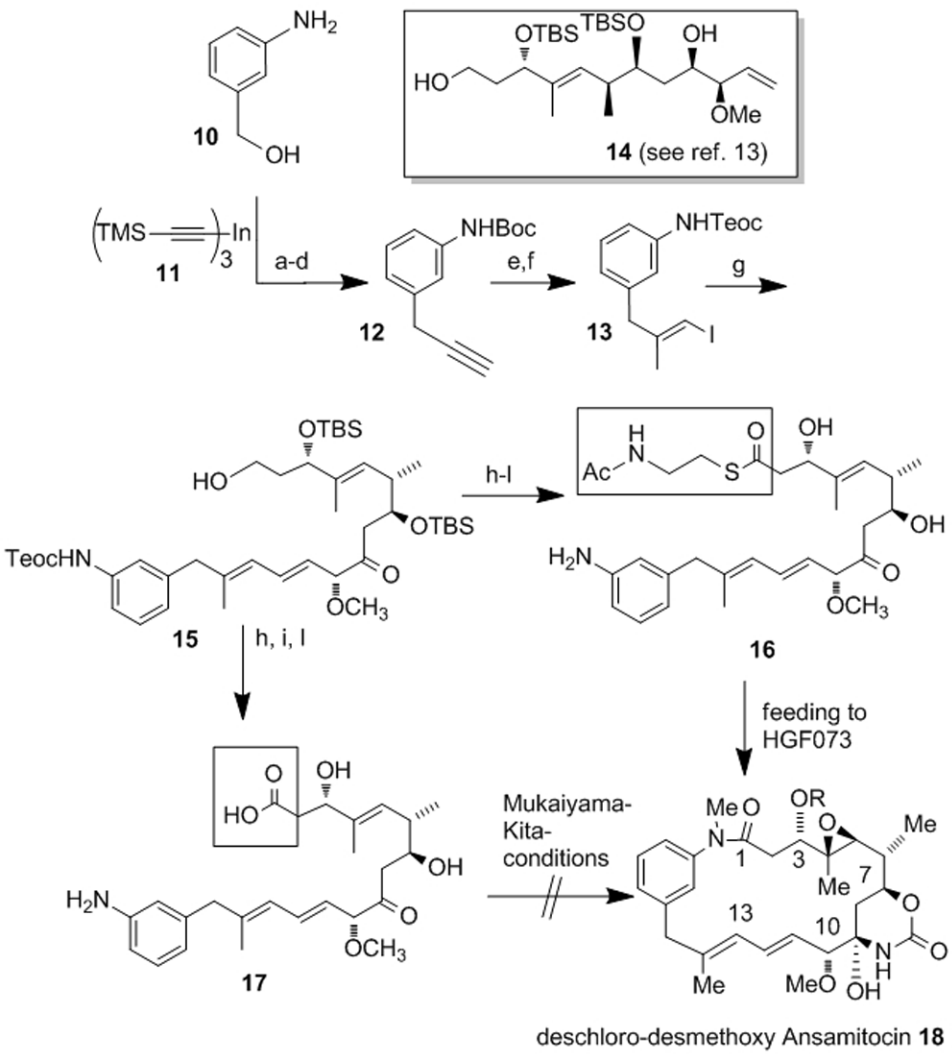
Feeding experiments with *seco*-proansamitocin **2c** and SNAC-ester **2b** yielding ansamitocin P3 **6**.



Scheme 3.

Synthesis of *seco*-proansamitocin **2c**.[a]

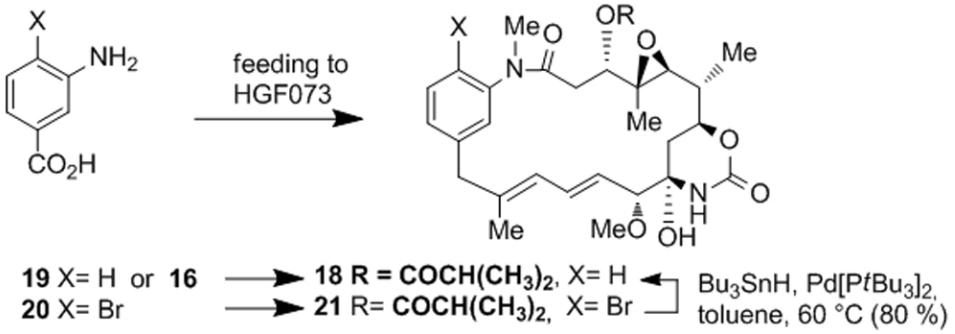
[a] Reagents and conditions: (a) **9**[13] DMSO, $(COCl)_2$, -60 °C, 1 h then Et_3N, -40°C (75%); (b) $NaClO_2$, NaH_2PO_4 H_2O, *t*-BuOH, 2-methyl-2-butene, 0 °C to rt, 30 min (86%); (c) HF-pyr, THF, rt, 6h, (48 %); (d) $ZnCl_2$, $MeNO_2$, ultrasound, rt, 1 h, (47%) (TBS= *tert.*-butyldimethylsilyl, Teoc= trimethylsilylethoxycarbonyl).



Scheme 4.

Total synthesis of SNAC ester **16** and seco acid **17**[a] and feeding experiments with **16** towards deschloro-desmethoxy AP3 **18**.

[a] Reagents and conditions: (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*3H_2O, THF, -20 °C, 1 h (88%); (e) i. $AlMe_3$, Cp_2ZrCl_2, $(CH_2Cl)_2$, rt, 1 h, then addition of **11**[13] at 0°C rt, 72 h, ii. I_2, THF, -30 °C to 0 °C, 1.5 h (53%); (f) TeocCl, $NaHCO_3$, CH_2Cl_2, rt, 10 min (91%); (g) **14**, $Pd(OAc)_2$, $CsCO_3$, 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 to rt, 30 min (85%); (j) $AcHN(CH_2)_2SH$, 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 %; towards **17**: 11%). (TMS= trimethylsilyl Boc= *tert*-butyloxycarbonyl; dppf= bis(diphenylphosphino) ferrocene, TBAF= tetra-*n*-butylammonium fluoride; DIC= diisopropyl carbodiimide, DMAP= 4-dimethylamino pyridine



Scheme 5.
Preparation of deschloro-desmethoxy AP-3 **18**.

Table 1

Antiproliferative activity, IC_{50} [ng/mL], of **18** compared to **6** with different cancer cell lines.

	Cell line					
	KB-3-1 Cervix carc.	**U-937 Lymp-homa**	**PC-3 Prostate carc.**	**SK-OV-3 Ovarian carc.**	**A-431 Epiderm carc.**	**A-549 Lung carc.**
18	0.022	0.015	0.020	0.040	0.080	0.095
6[a]	0.11	0.0035	0.035	0.030	0.050	0.095

[a]The IC_{50} with primary human fibroblasts from foreskin was 3.0 ng/ml.