A Novel Amide N-Glycoside of Ansamitocins from *Actinosynnema pretiosum*†

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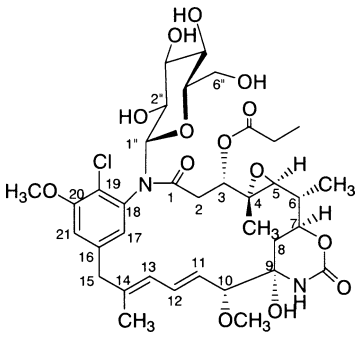
Maytansinoids are a family of 19-membered macrocyclic lactams having extraordinary cytotoxic and antineoplastic activities,[1,2] and are products of a bacterium (*Actinosynnema pretiosum*),[3] mosses[4,5] and three closely related plant families, Celastraceae, Rhamnaceae and Euphorbiaceae.[1,2] They are structurally related to ansamycin antibiotics of microbial origin. Recently, FLOSS and coworkers have reported the cloning, sequencing and characterization of the maytansinoids, ansamitocins, biosynthetic gene cluster (*asm*) from a cosmid library of *Actinosynnema pretiosum* ssp. *auranticum* ATCC 31565.[6]

By cultivating *A. pretiosum* on YMG agar media (yeast extract 4 g/liter, malt extract 10 g/liter, glucose 4 g/liter), we have isolated a novel ansamitocin amide N-glycoside, *N*-demethyl-*N*-β-D-glucopyranosyl ansamitocin P-2, named ansamitocinoside P-2 (compound **1**) (Figure 1), through column chromatography over reversed-phase C_{18} silica gel and Sephadex LH-20, respectively. The glycosides of other ansamitocins such as P-0, P-1, P-3 and P-4 were detected by LC-ESI-MS (data not shown). The structure elucidation of ansamitocinoside P-2 was unambiguously carried out on the basis of HRFABMS (high resolution fast atom bombardment mass spectrometry), and 1D and 2D NMR data.

Fig. 1. The structure of ansamitocinoside P-2 (**1**).



Compound **1** was determined to have the molecular formula $C_{36}H_{48}ClN_2O_{14}$ by negative HRFABMS (*m/z* 767.2811, cacld.: 767.2794). The ^{13}C NMR spectra including DEPT experiments showed signals for 36 carbons including 7 methyls, 5 methylenes, 15 methines and 9 quaternary carbons. The ansamitocin moiety was readily recognized by inspecting the NMR data (proton, carbon, DEPT, ^1H-^1H COSY, HMQC and HMBC, Table 1) and comparing with literature data.[4,5,7,8] The coupling constants $J_{H\text{-}2,\text{-}3}$, $J_{H\text{-}5,\text{-}6}$, and $J_{H\text{-}10,\text{-}11}$ were consistent with those assigned in ansamitocin P-3,[7] indicating that the aglycone moiety of **1** had the same stereochemistry as ansamitocins. However, the proton signal attributed to CH_3N-18 at 3.15~3.18 ppm was missing, instead, an additional six-carbon unit was observed which was revealed to be hexosyl group. The sugar moiety was determined to be β-D-glucopyranosyl based on the unambiguous NMR assignments, particularly the ^{13}C NMR assignments indicated the D-configuration and the $J_{H\text{-}1'',\text{-}2''}$ 9.4 Hz indicated the β-form of the anomeric proton. The anomeric proton at δ 5.74 had ^1H-^{13}C long-range correlations with the carbons at δ 172.9 (C-1) and 137.3 (C-18), indicating the glycosylation at the amide nitrogen of C-18. Therefore, compound **1** was determined to be *N*-demethyl-*N*-β-D-glucopyranosyl ansamitocin P-2, a novel amide N-glycoside, named ansamitocinoside P-2.

Experimental

Spectral Analysis

Optical rotations were measured with a JASCO DIP-370 digital polarimeter in MeOH solution. Mass spectra were measured on a VG Auto Spec-3000 spectrometer. NMR spectra were Bruker AM-400 or DRX-500 NMR spectrometers with TMS as internal standard.

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† This paper is dedicated to my mentor professor Dr. HEINZ G. FLOSS in University of Washington at the occasion of his 70th birthday.

Table 1. The ^{1}H and ^{13}C NMR assignments and ^{1}H-^{13}C long-range correlations (HMBC) for ansamitocinoside P-2[a].

No	^{13}C	^{1}H[b]	HMBC
1	172.9s	/	/
2	34.7t	2.50 (m), 2.20 (m)	C-1, C-3, C-4
3	77.8d	4.76 (dd, 2.9, 10.0)	C-1, C-2, C-4, C-4a, C-5
4	62.0s	/	/
5	68.0d	2.72 (d, 9.3)	C-3, C-4, C-6a
6	39.2d	1.18 (m)	C-6a
7	75.9d	4.20 (dt, 2.8, 11.1)	COO-7
8	37.4t	1.53 (m)	C-7, C-9, C-10
9	81.8s	/	/
10	89.7d	3.55 (m)	C-9
11	129.3d	5.53 (dd, 8.1, 15.2)	C-9, C-13
12	134.0d	6.63 (dd, 11.0, 15.8)	C-10, C-13, C-14
13	125.5d	6.26 (d, 10.8)	C-11, C-12, C-14, C-15, C-15a
14	141.5s	/	/
15	47.5t	3.61 (m), 3.32 (m)	C-1, C-13, C-14, C-14a, C-21
16	123.4s	/	/
17	126.3d	7.21 (s)	C-15, C-16, C-19, C-21
18	137.3s	/	/
19	155.3s	/	/
20	157.1s	/	/
21	115.2d	7.17 (s)	C-15, C-17, C-20
MeO-10	56.9q	3.36 (s, 3H)	C-10
NHCO-9	159.3s	/	/
MeO-20	57.1q	3.98 (s, 3H)	C-20
1'	175.2s	/	/
2'	27.7t	2.77 (m), 2.56 (m)	C-3'
3'	8.5q	1.08 (t, 7.4, 3H)	C-1', C-2'
4a	12.1q	0.8 (s, 3H)	C-3, C-4, C-5
6a	14.7q	1.21 (d, 6.3, 3H)	C-5, C-6, C-7
14a	15.8q	1.75 (s, 3H)	C-13, C-14, C-15
1''	84.4d	5.74 (d, 9.4)	C-1, C-18, C-2''
2''	71.7d	3.13 (t, 9.3)	C-1'', C-5''
3''	78.6d	3.48 (m)	C-2''
4''	73.0d	4.25 (t, 9.4)	C-3'', C-5''
5''	77.0d	3.57 (m)	C-1'', C-3''
6''	62.9t	3.64 (m), 3.45 (m)	C-4''

[a] ^{1}H, ^{13}C NMR and HMBC spectra were obtained at 500MHz, 125MHz and 500MHz, respectively, and recorded in $CDCl_3$ at room temperature.
[b] Coupling constants are presented in Hertz. Unless otherwise indicated, all proton signals integrate to 1H.

LC-ESI-MS Analysis

Samples were subjected to gradient elution on a reverse phase HPLC system (HPLC Waters 2695, C_{18} reversed-phase column 3.5 μ, 3.0×50 mm), Mobile phase solution A is methanol; solution B is distilled water containing 1% formic acid. A multistep gradient was set up for maytansine isolation, with an initial injection volume of 10 μl and a flow rate 0.2 ml·min^{-1}. ESI-MS analysis was carried out with electrospray ionization mass spectrometer (ESI-MS) on Thermo Finnigan LCQ Advantage instrument. The retention time and molecular mass were monitored under the following conditions: 63% A (0~20 minutes) and A (20~25 minutes) and 63% A (25~30 minutes). The high gas temperature (275°C) and gas flow (50 psi) were applied to the LC-MS parameters. Scan ranges of 200~1000 amu were used for positive ion collection.

Chromatography Materials

Silica gel (200~300 mesh) for column chromatography and precoated TLC plates (Si gel G) were purchased from

the Qingdao Marine Chemical Factory, Qingdao, P. R. China. Reversed-phase C_{18} silica gel for column chromatography and C_{18}-RP-TLC plates were obtained from Merck. Sephadex LH-20 for column chromatography was purchased from Amersham Biosciences.

Culture Conditions and Extraction

A. pretiosum ssp. *aurantium* ATCC31565, stored in glycerol, was used to inoculate on slope of YMG media in a test tube at 28°C for 5 days to afford seed cultures. The YMG media had the following composition (g/liter): glucose 4.0, malt extract 10.0, yeast extract 4.0, pH 7.2. Solid state fermentation was performed with YMG media (3.5 liters) at 28°C for 7 days, and the seed culture was inoculated with inoculating loop. The cultured agar was chopped, diced and extracted with EtOAc - MeOH - AcOH (80 : 15 : 5, 3.5 liters) at room temperature for over night. The organic solution was collected through filtration, and the remaining agar residue was extracted several times more as described above until the filtrate colourless. The combined filtrates were concentrated under vacuum to remove organic solvents. The aqueous solution was extracted five times with chloroform. The removal of solvents under vacuum afforded $CHCl_3$ extract (11.77 g) and H_2O portion (15 g), respectively.

Isolation of Ansamitocinoside P-2

The H_2O portion (15 g) was subjected to MPLC over reversed-phase C_{18} Si gel (130 g) eluted with 30%, 50%, 70% and 100% MeOH (1 liter each) to produce 4 fractions. After the removal of solvents under vacuum, all fractions were subjected to antifungal assay against *Penicillium avellanceum* UC-4376, which indicated that the 50% and 70% MeOH fractions were active. The 50% MeOH fraction (500 mg) was subjected to column chromatography over Sephadex LH-20 (130 g) and eluted with methanol to produce an antifungal fraction (98 mg). The 70% MeOH fraction (200 mg) was subjected to column chromatography over Si gel (10 g) and eluted with EtOAc - MeOH (8 : 1) to afford another antifungal fraction. TLC analysis indicated that those two active fractions had similar compositions, therefore, were combined and subjected column chromatography over Si gel (20 g) and eluted with EtOAc (100 ml), EtOAc - MeOH (14 : 1, 300 ml, 12 : 1, 200 ml) and MeOH (100 ml), and 10 ml was collected for each fraction. The antifungal assay indicated that fractions 1～18, 29～46 and 61～69 had modest activity, fractions 19～21, 22～28 and 47～76 had strong activity, and that methanol wash-off was inactive. The fractions 22～28 (28 mg) was further subjected to column chromatography over reversed-phase C_{18} Si gel (28 g) and eluted with 50% methanol to afford the relatively pure ansamitocinoside P-2 (5 mg).

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