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New Amide N-glycosides of Ansamitocins Identified from *Actinosynnema pretiosum*

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By using preparative TLC as the critical isolation procedure, two compounds, including one new amide N-glycosides of ansamitocin (**2**), were isolated from *Actinosynnema pretiosum*. The compounds were elucidated as *N*-demethyl-*N*-β-D-glucopyranosyl ansamitocin P-2, named ansamitocinoside P-2 (**1**), and *N*-demethyl-*N*-β-D-glucopyranosyl ansamitocin P-1, named ansamitocinoside P-1 (**2**) on the basis of their spectral data. The ^{1}H-NMR and ^{13}C-NMR assignments were made for **1** and **2** while the ^{13}C-NMR assignment for **1** was revised. Bioassay results showed that **1** had antineoplastic activity.

Key words: *Actinosynnema pretiosum*, Ansamitocin, Ansamitocinoside, Antineoplastic activity

INTRODUCTION

Maytansinoids are a family of 19-membered macrocyclic lactams having extraordinary cytotoxic and antineoplastic activities (Kupchan *et al.*, 1972, 1977), and are products of a bacterium (*Actinosynnema pretiosum*) (Higashide, *et al.*, 1977), mosses (Sakai, *et al.*, 1988; Suwanborirux *et al.*, 1990) and three closely related plant families, Celastraceae, Rhamnaceae and Euphorbiaceae. They are structurally related to ansamycin antibiotics of microbial origin. Recently, Floss and coworkers have reported the cloning, sequencing and characterization of the maytansinoid, ansamitocin, biosynthetic gene cluster (*asm*) from a cosmid library of *A. pretiosum* ssp. *auranticum* ATCC 31565 (Yu *et al.*, 2002), and the product of the gene *asm25* was deduced to be glycosyltransferase from the sequence comparison which was not evident by the structures of the ansamitocins.

To explore the potential of *A. pretiosum* to generate glycosides, this strain was cultivated on solid state ISP2 media as based on our previous work. One novel ansamitocin N-glycoside, *N*-demethyl-*N*-β-D-glucopyranosyl ansamitocin P-2, named ansamitocinoside P-2 was isolated and characterized (Lu *et al.*, 2004). In the present study, another new *N*-demethyl-*N*-β-D-glucopyranosyl ansamitocin has been isolated.

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MATERIALS AND METHODS

General procedures

Optical rotations were measured with a JASCO DIP-370 digital polarimeter using a MeOH solution. Mass spectra were measured on a VG Auto Spec-3000 spectrometer and Thermo Finnigan LCQ Advantage. The NMR spectra were measured on a Bruker DRX-500 NMR spectrometer with TMS as the internal standard. The reversed-phase (RP) C_{18} silica gel used for column chromatography was obtained from Merck, the Sephadex LH-20 from Amersham Biosciences and the Silica gel from Qingdao Marine Chemical Factory.

Materials

The strain *A. pretiosum* ssp. *aurantium* ATCC31565 was obtained from Dr. T.-W. Yu and H. G. Floss of the University of Washington (Seattle, Washington State, U.S.A.), and was conserved in 20% glycerol at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences (Kunming, Yunnan Province, P. R. China). *A. pretiosum* was used to inoculate a slope of YMG media in

a test tube at 28°C for 5 days to afford a working seed culture. The YMG media had the following composition (g/L): glucose 4.0, malt extract 10.0, and yeast extract 4.0; pH 7.2. Solid fermentation was performed using YMG media (3 L) at 28°C for 7 days.

Extractions and isolation

The culture was extracted five times with EtOAc-MeOH-AcOH (80: 15: 5, v/v) exhaustively to give an extract (21 g). The extract was subjected to MPLC over RP C_{18} silica gel (130 g) eluted with 30%, 50%, 70% and 100% MeOH (2 L each) and yielding four fractions. The 50% MeOH fraction (864 mg) was subjected to column chromatography over Sephadex LH-20 (130 g) eluted with MeOH, and then further subjected to MPLC over RP C_{18} silica gel (130 g) eluted with 30%, 35%, 40%, and 80% acetone (0.6 L each) to obtain fractions numbered 1 to 6. Fraction number 4 (240 mg) was subjected to MPLC over RP C_{18} silica gel (130 g) eluted with 30% (0.5 L), 38% (2 L), and 80% acetone (0.6 L) yielding five fractions. Fraction (56 mg) was subjected to thin-layer chromatography by using separating plates (Qingdao, P. R. China, GF254) with the particular developing agent as the solvent. The particular developing agent was blended with ethyl acetate-methanol (20: 1). Densitometry analyses of the chromatograms were carried out with a ternary wave-length TLC scanner ZF-I set at 254 nm.

Two compounds **1** and **2** were achieved from pre-TLC and they were subjected to column chromatography over Sephadex LH-20 (50 g) eluted with acetone for eliminating any impurities from the last purification. The final weight of each compound was 5 mg.

***N*-Demethyl-*N*-β-D-glucopyranosyl ansamitocin P-2 (1)** Straw yellow solid, $[\alpha]_D^{15}$ -42 (*c* 7.9, MeOH). ESI-MS *m/z*: 791 $[M + Na]^+$. IR (KBr): 3431, 1692 (s), 1082 cm^{-1}. UV (MeOH) λ_{max} (log ε) 203.0 (4.57), 230.4 (4.35), 253.4 (4.31), 282.2 (3.71).

***N*-Demethyl-*N*-β-D-glucopyranosyl ansamitocin P-1 (2)** Straw yellow solid, $[\alpha]_D^{19}$ -28 (*c* 6.5, MeOH). ESI-MS *m/z*: 777 $[M + Na]^+$. IR (KBr): 3432, 1690 (s), 1641, 1082, 1054, 1039 cm^{-1}. UV (MeOH) λ_{max} (log ε) 203.0 (4.57), 230.4 (4.33), 253.4 (4.25), 282.2 (3.72).

Biological activity

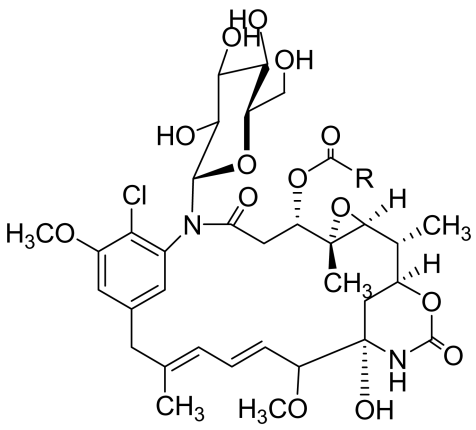
The antitumor assays were performed by the National Center for Drug Screening (Shanghai, P. R. China).

RESULTS AND DISCUSSION

Identification of compounds

For compound **1**, the HR-FAB-MS determined the molecular formula to be $C_{36}H_{48}N_2O_{14}Cl$ (*m/z* 767.2811 $[M - H]^-$, calcd: 767.2794). The UV (MeOH) spectra showed absorption at λ_{max} (log ε) 203.0 (4.57), 230.4 (4.35), 253.4 (4.31), 282.2 (3.71). The ^{13}C-NMR and DEPT spectra of **1** showed 36 carbon signals including 6 methyls, 5 methylenes, 15 methines and 10 quaternary carbons (Table I). The ansamitocin moiety was readily recognized by inspecting the NMR data (^{1}H, ^{13}C, DEPT, HMQC and HMBC) (Table I) that was obtained and by comparison with data cited in the literature (Sakai *et al.*, 1988; Suwanborirux *et al.*, 1990; Spiteller *et al.*, 2003). The proton signal attributed to $\underline{CH_3}$N-18 at δ 3.18~3.22 ppm in the ^{1}H-NMR spectra we obtained, however, was missed (Kupchan *et al.*, 1977; Asai *et al.*, 1979). Instead an additional six-carbon unit was observed which was determined to be the β-D-glucopyranosyl group as based on the NMR assignments. The HMBC experiment showed the ^{1}H-^{13}C long-range correlations between the anomeric proton at δ 5.71 (H-1") and the carbons at δ 172.9 (C-1) and δ 137.6 (C-18), indicating the glycosylation at the amide nitrogen of C-18. Therefore, compound **1** was determined to be *N*-demethyl-*N*-â-D-glucopyranosyl ansamitocin P-2, and named ansamitocinoside P-2 (Figure). The NMR data of this compound were assigned by Lu *et al.* (2004), and in the present study, the ^{1}H- and ^{13}C-NMR assignments for compound **1** were carried out on the basis of the DEPT, HMQC and HMBC experiments. Our results were consistent, by reference, with data reported (Lu *et al.*, 2004) except for carbon signals at C-5", C-19, C-16 and NH$\underline{C}$O-9 which were mistake in the ^{13}C-NMR spectra. Those carbon signals at C-5", C-19, C-16 and NH$\underline{C}$O-9 were designated (Table I).

For compound **2**, the HR-ESI-MS determined the molecular formula to be $C_{35}H_{47}N_2O_{14}Cl$ (*m/z* 777.2621, [M +



1 R = CH_2CH_3
2 R = CH_3

Fig 1. The structures of compounds **1** and **2**

Table I. NMR Data of compounds in CD_3OD

Position	Compound **1**			Compound **2**	
	^{13}C	^{1}H	HMBC	^{13}C	^{1}H
1	172.9	/	/	172.9	/
2	34.7	2.57 m 2.18 dd (4.5,13.7)	C-1, C-3	34.7	2.47 m 2.18 m
3	77.8	4.74 dd (2.7,11.9)	C-2, C-4, C-5, C-4a, C-1'	79.3	4.74 dd(3.5,12.0)
4	62.0	/	/	62.0	/
5	68.0	2.8 m	C-3, C-4, C-6, C-6a	68.0	2.7 m
6	39.2	1.58 m	C-4, C-5, C-7, C-8, C-6a	39.1	1.58 m
7	75.9	4.20 dd (3.1,10.5)	/	75.9	4.20 m
8	37.4	1.58 m	C-6, C-7, C-9	37.6	1.58 m
9	81.9	/	/	82.0	/
10	89.8	3.58 m	C-8, C-9, C-12, MeO-10	89.8	3.63 m
11	129.3	5.53 dd (9.0,15.3)	C-13	129.5	5.57 dd (9,15.5)
12	134.1	6.6 dd (11.0,15.3)	C-10, C-13, C-14	134.0	6.63 dd(11,15.0)
13	125.5	6.25 d (10.8)	C-11, C-12, C-15, C-14a	125.6	6.27 d (11.5)
14	141.5	/	/	141.5	/
15	47.5	3.58 m 3.30 m	C-13, C-14, C-17, C-21, C-14a, C-16	47.5	3.63 m 3.30 m
16	141.4	/	/	141.4	/
17	126.3	7.19 s	C-15, C-19, C-18, C-20, C-21	126.2	7.22 d (2.0)
18	137.6	/	/	137.6	/
19	123.4	/	/	123.5	/
20	157.1	/	/	157.2	/
21	115.1	7.14 s	C-16,C-15,C-19,C-17,C-18,C-20	115.2	7.16 d (2.0)
NHCO-9	155.3	/	/	155.3	/
MeO-10	56.9	3.34 s	C-10	56.9	3.36 s
MeO-20	57.1	3.96 s	C-20	57.1	3.97 s
4a	12.1	0.78 s	C-3, C-4, C-5	12.1	0.70 s
6a	14.7	1.21 d (6.37)	C-5, C-6, C-7	14.6	1.22 d (6.37)
14a	15.8	1.74 s	C-13, C-14, C-15	15.8	1.75 s (3H)
1''	84.7	5.71 d (9.3)	C-1, C-18, C-2'', C- 3''	84.7	5.72 d(9.5)
2''	71.8	3.04 m	C-1'', C-3''	71.8	3.04 m
3''	79.3	3.46 t (8.9)	C-2'', C-4''	80.2	3.46 m
4''	71.8	3.04 m	C-3'', C-5'', C-6''	71.9	3.04 m
5''	80.2	3.30 m	/	78.2	3.30 m
6''	63.3	3.80 dd (2.2,11.7) 3.58 m	C-4'', C-5''	63.3	3.64 m 3.64 m
1'	175.3	/	/	173.0	/
2'	27.7	2.80 m 2.57 m	C-1', C-3'	21.6	1.25 s
3'	8.5	1.11 t (7.2)	C-1'		

Na]$^+$, calcd: 777.2613). The DEPT spectra of **2** showed 35 carbon signals, including 6 methyls, 4 methylenes, 15 methines and 10 quaternary carbons (Table I). The glucose moiety was recognized by inspection of the NMR data (^{1}H- and ^{13}C-NMR) (Table I) and compared with compound **1**. The UV, IR and NMR data of **2** were very similar to those of compound **1** except that the ester group of C-3 at compound **1** was the propionyl group

instead of the acetyl group in compound **2** (Table I). Thus, comparison of the NMR data of compound **2** with that of compound **1** and the literature citations (Sakai *et al.*, 1988; Suwanborirux *et al.*, 1990; Spiteller *et al.*, 2003), indicated compound **2** to be *N*-β-D-glucopyranosyl Ansamitocin P-1, named ansamitocinoside P-1 (Figure).

Biological activity

The inhibitory effects of ansamitocinoside P-2 on the growth of various tumor cell lines were performed by the National Center for Drug Screening. Growth of all cell lines evaluated were inhibited and inhibited at different dose levels: the IC_{50} against P388 was 0.07 μM and the IC_{50} against A-549 was 6.47 μM.

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