Phytochemistry xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Verticillosides A–M: Polyoxygenated pregnane glycosides from *Asclepias verticillata* L.

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ARTICLE INFO

Article history:
Received 3 November 2011
Received in revised form 11 January 2012
Available online xxxx

Keywords:
Asclepias verticillata
Pregnane glycosides
Verticillosides

ABSTRACT

As part of our ongoing effort to explore the chemical diversity of plants of the United States Midwest region, the isolation and identification of 13 pregnane glycosides named verticillosides A–M from *Asclepias verticillata* L. are reported. The structures of these compounds were elucidated by various spectroscopic techniques, including 1D and 2D NMR, IR, UV, and HRMS. The cytotoxicity of the isolates was evaluated against paired breast cell lines Hs578T (cancer) and Hs578Bst (normal), however, no significant growth inhibition was observed.

1. Introduction

Pregnane glycosides are C_{21} steroidal compounds conjugated with sugars that commonly occur in the family Asclepiadaceae. They comprise a structurally diverse group that combines an aglycone skeleton and a variable number and type of sugar units, typically attached at the 3-position (Panda et al., 2006). Anticancer and immunomodulating activities have been reported for this class of compounds (Cao et al., 2006; Chen et al., 2010; Kim et al., 2005; Kuroda et al., 2010; Li et al., 2006; Peng et al., 2008; Ye et al., 2005).

Members of the *Asclepias* genus, commonly known as "milkweeds", are widely distributed across the United States. They are especially abundant in the Midwest, with over 130 plant species and subspecies reported, many of them endemic to this geographical region. Although *Asclepias* is a rich source of cardiac and pregnane glycosides, it has been poorly investigated chemically and biologically. For instance, *Asclepias verticillata* has not been investigated phytochemically despite its traditional use as medicine and food. The leaves and young shoots were boiled with meat and eaten by the Hopi (Fewkes, 1896). The most common medicinal use was the consumption by the Lakota and Hopi of a tea made from the whole plant to increase the production of mother's milk (Fewkes, 1896; Rodgers, 1980). This may have been a Doctrine of Signatures type use as the milky sap might indicate its value for this purpose (Kindscher, 1992). The Choctaw used the root as a diaphoretic to promote sweating, and the root tea as both a stimulant, and in a stronger concentration, as an external and internal snakebite remedy (Campbell et al., 1951).

As part of the ongoing effort to explore the biodiversity of the American Great Plains, reported herein is the isolation and identification of 13 new pregnane glycosides named verticillosides A–M (Fig. 1) from *A. verticillata* L. This report represents the first phytochemical investigation of this species.

2. Results and discussion

Dried aerial parts of *A. verticillata* were extracted and subjected to a series of purification steps, including a variety of chromatographic techniques, to afford 13 new pregnane glycosides: verticillosides A (**1**; 38 mg), B (**2**; 42 mg), C (**3**; 10 mg), D (**4**; 36 mg), E (**5**; 27 mg), F (**6**; 37 mg), G (**7**; 11 mg), H (**8**; 19 mg), I (**9**; 10 mg), J (**10**; 13 mg), K (**11**; 12 mg), L (**12**; 9 mg), and M (**13**; 14 mg).

Verticilloside A (**1**) was obtained as a white, amorphous powder. The HRMS displayed a $[M+Na]^+$ ion at *m/z* 1055.5276 consistent with a molecular formula of $C_{50}H_{80}NaO_{22}$ (calcd 1055.5039). The ^1H NMR spectrum showed four singlet signals (δ 2.49, 2.08, 1.94, 1.35) and an olefinic proton (δ 4.99) that indicated the presence of an acetylated pregn-5-en-20-one skeleton (Table 2). The proposed carbon skeleton was supported by the HMBC correlations (Fig. 2) between the proton signal δ 1.94 (Me-18) and the carbon resonances δ 58.3 (C-13), 73.9 (C-12), 89.8 (C-14), and 92.8 (C-17); and proton signal δ 1.35 (Me-19) and carbon resonances of δ 39.2 (C-1), 139.6 (C-5), 44.8 (C-9), and 38.0 (C-10). Also, the HMBC correlations between the methyl signal of the acetyl group (δ 2.49) and C-12 (δ 73.9) clarified the attachment position of the methyl

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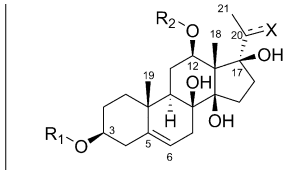
0031-9422/$ - see front matter © 2012 Published by Elsevier Ltd.
doi:10.1016/j.phytochem.2012.02.019



	X	R_1	R_2
1	O	R_A	Ac
1a	O	H	Ac
2	O	R_B	Ac
3	O	R_C	Ac
4	O	R_D	Ac
5	α-H,β-OH	R_A	H
5a	α-H,β-OH	H	H
6	α-H,β-OH	R_B	H
7	O	R_A	H
8	O	R_B	H
9	O	R_C	H
10	α-H,β-OH	R_C	H
11	α-H,β-OH	R_A	Bz
11a	α-H,β-OH	H	Bz
12	α-H,β-OH	R_B	Bz
13	α-H,β-OH	R_D	Bz

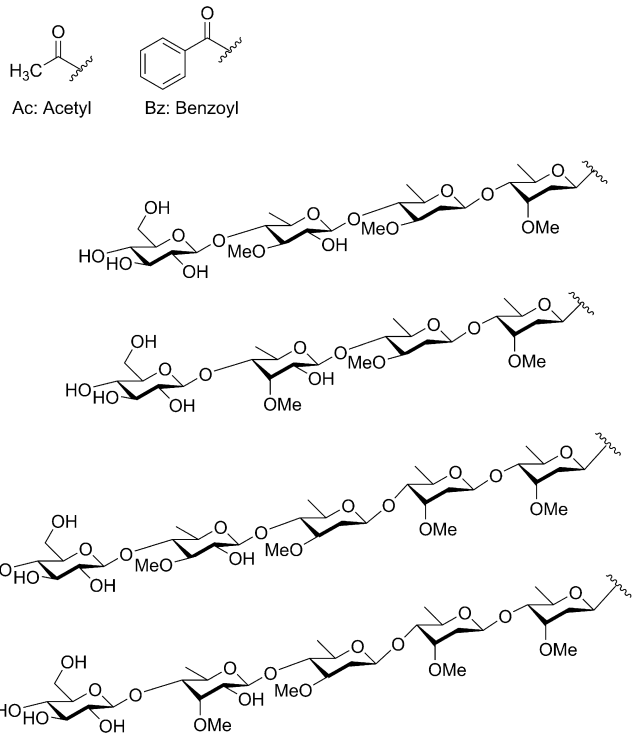


Fig. 1. Structure of compounds **1**–**13**.

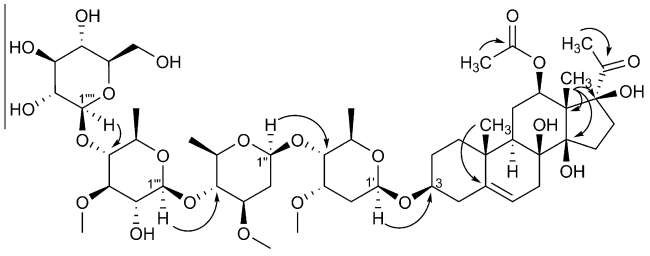


Fig. 2. Selected HMBC correlations observed in compound **1**.

ester functionality. Finally, ^{1}H,^{1}H DQFCOSY, HSQC and HMBC spectra allowed for the full assignment of the ^{1}H and ^{13}C signals (Tables 1 and 2), and the aglycone moiety was deduced to be metaplexigenin (**1a**). The NMR spectroscopic data were in good agreement with previously reported values for metaplexigenin (Warashina and Noro, 2000; Ye et al., 2004). The initially proposed stereochemistry was based on dipolar interactions observed in the ROESY spectrum (Fig. 3) and further confirmed by X-ray crystallography of the aglycone obtained by acid hydrolysis of the glycoside (Fig. 4). In addition, four anomeric protons were observed in the ^{1}H NMR spectrum (δ 5.29, 5.16, 4.90 and 4.70) suggesting that the same number of sugars were attached at the 3-position. Furthermore, the presence of three methyl doublets in the aliphatic region of the ^{1}H NMR spectrum (δ 1.78, 1.68 and 1.44) and three methoxy groups (δ 3.96, 3.56 and 3.49), indicated the presence of 6-deoxy-3-methoxy sugars, commonly found in the *Asclepias* glycosides. Using ^{1}H,^{1}H DQFCOSY, ^{1}H, ^{1}H-TOCSY, and HSQC-TOCSY spectra, the proton spin systems and the carbon resonances of each sugar were fully assigned (Tables 1 and 2). The sugar units were then identified as cymarose, oleandrose, thevetose, and glucose by NMR spectroscopic data analysis and comparison with previously reported values. The connectivity of the sugars was established by the following key HMBC correlations: cymarose anomeric proton H-1′ (δ 5.29) and C-3 (δ 77.9); oleandrose anomeric proton H-1″ (δ 4.70) and C-4′ (δ 83.6); thevetose anomeric proton H-1‴ (δ 4.90) and C-4″ (δ 83.6), and glucose anomeric proton H-1⁗ (δ 5.16) and C-4‴ (δ 83.8) (Fig. 2). The β-linkages of the four sugars were established by the large coupling constants (J = 7.8–9.7) observed for the anomeric protons. Finally, after acid hydrolysis of compound **1**, the optical rotation of the purified monomeric sugars was measured in aqueous solution after 24 h equilibration period and compared with previously reported values showing that the sugars present in this compound had D-configuration. Therefore, the structure of **1** was determined to be metaplexigenin 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-thevetopyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranose. To the best of our knowledge, compound **1** represents a previously unreported metaplexigenin glycoside that we named verticilloside A.

Verticilloside B (**2**), an amorphous white powder, showed a HRMS [M+Na]$^+$ ion at m/z 1052.5271, suggesting the same molecular formula of $C_{50}H_{80}NaO_{22}$ (calcd 1055.5039) as for compound **1**. First, the aglycone present in **2** was determined to be metaplexigenin and it was identified as described before. Second, the ^{1}H NMR spectroscopic also showed the presence of four anomeric protons (δ 5.27, 5.26, 5.00 and 4.67); however, only the chemical shift of H-1⁗ changed significantly when compared with the anomeric protons of **1**. After analysis of the spin systems of each sugar aided by analyses of ^{1}H,^{1}H DQFCOSY, and ^{1}H, ^{1}H-TOCSY and HSQC-TOCSY spectra, the ^{1}H and ^{13}C signals were completely assigned and the four sugars were elucidated as cymarose, oleandrose, 6-deoxy-3-*O*-methyl allopyranose, and glucose. The HMBC correlations unambiguously established the connectivity of the sugars: anomeric proton of cymarose H-1′ (δ 5.26) with C-3 (δ 78.1); anomeric

Please cite this article in press as: Araya, J.J., et al. Verticillosides A–M: Polyoxygenated pregnane glycosides from *Asclepias verticillata* L. Phytochemistry (2012), doi:10.1016/j.phytochem.2012.02.019

Table 1
^{13}C NMR (125 MHz, C_5D_5N) spectroscopic data for compounds **1–13**.[a,b]

Atom	1	2	3	4	5	6	7	8	9	10	11	12	13
1	39.2 *t*	39.4 *t*	39.3 *t*	39.3 *t*	39.4 *t*	39.4 *t*	39.3 *t*	39.3 *t*	39.3 *t*	39.4 *t*	39.2 *t*	39.2 *t*	39.2 *t*
2	30.2 *t*	30.4 *t*	30.2 *t*	30.2 *t*	30.3 *t*	30.3 *t*	30.3 *t*	30.3 *t*	30.3 *t*	30.3 *t*	30.3 *t*	30.3 *t*	30.3 *t*
3	77.9 *d*	78.1 *d*	78.0 *d*	78.0 *d*	78.1*d*	78.1*d*	78.1*d*	78.1*d*	78.1 *d*	78.1 *d*	78.0 *d*	78.0 *d*	78.1 *d*
4	39.5 *t*	39.7 *t*	39.6 *t*	39.6 *t*	39.7 *t*	39.7 *t*	39.7 *t*	39.7 *t*	39.7 *t*	39.7 *t*	39.6 *t*	39.6 *t*	39.6 *t*
5	139.6 *s*	139.8 *s*	139.6 *s*	139.6 *s*	139.5 *s*	139.5 *s*	139.5 *s*	139.5 *s*	139.7 *s*	139.5 *s*	139.5 *s*	139.5 *s*	139.5 *s*
6	119.6 *d*	119.7 *d*	119.5 *d*	119.5 *d*	120.2 *d*	120.2 *d*	119.9 *d*	120.2 *d*	119.9 *d*	120.1 *d*	120.0 *d*	120.0 *d*	120.0 *d*
7	35.0 *t*	35.2 *t*	35.0 *t*	35.0 *t*	35.7 *t*	35.7 *t*	35.4 *t*	35.4 *t*	35.4 *t*	35.6 *t*	35.4 *t*	35.4 *t*	35.4 *t*
8	74.6 *s*	74.1 *s*	74.6 *s*	74.6 *s*	74.4 *s*	74.4 *s*	74.6 *s*	74.6 *s*	74.1 *s*	74.1 *s*	74.6 *s*	74.6 *s*	74.6 *s*
9	44.83 *d*	45.0 *d*	44.8 *d*	44.8 *d*	44.9 *d*	44.9 *d*	45.3 *d*	45.3 *d*	44.9 *d*	44.9 *d*	44.5 *d*	44.4 *d*	44.5 *d*
10	38.0 *s*	38.2 *s*	37.7 *s*	37.7 *s*	37.7 *s*	37.7 *s*	37.7 *s*	37.7 *s*	37.7 *s*	37.7 *s*	37.7 *s*	37.6 *s*	37.6 *s*
11	25.2 *t*	25.4 *t*	25.2 *t*	25.2 *t*	29.5 *t*	29.5 *t*	29.8 *t*	29.8 *t*	29.8 *t*	29.5 *t*	26.0 *t*	26.0 *t*	26.0 *t*
12	73.9 *d*	74.8 *d*	73.9 *d*	73.9 *d*	71.1 *d*	71.1 *d*	69.2 *d*	69.3 *d*	69.2 *d*	71.1 *d*	75.7 *d*	75.7 *d*	75.7 *d*
13	58.3 *s*	58.4 *s*	58.3 *s*	58.2 *s*	59.2 *s*	59.2 *s*	60.7 *s*	60.7 *s*	60.7 *s*	58.4 *s*	57.8 *s*	57.8 *s*	57.8 *s*
14	89.8 *s*	90.0 *s*	89.8 *s*	89.8 *s*	89.2 *s*	89.2 *s*	89.7 *s*	89.7 *s*	89.7 *s*	89.2 *s*	89.0 *s*	89.0 *s*	89.0 *s*
15	34.1 *t*	34.3 *t*	34.1 *t*	34.1 *t*	34.9 *t*	34.9 *t*	34.6 *t*	34.6 *t*	34.6 *t*	34.9 *t*	34.6 *t*	34.6 *t*	34.6 *t*
16	33.2 *t*	33.4 *t*	33.2 *t*	33.2 *t*	34.6 *t*	34.6 *t*	33.1 *t*	33.1 *t*	33.2 *t*	34.6 *t*	33.2 *t*	33.2 *t*	33.2 *t*
17	92.8 *s*	92.0 *s*	92.8 *s*	92.8 *s*	89.4 *s*	89.3 *s*	92.9 *s*	92.9 *s*	92.9 *s*	89.4 *s*	89.2 *s*	89.2 *s*	89.2 *s*
18	10.8 *q*	11.0 *q*	10.8 *q*	10.8 *q*	11.8 *q*	11.8 *q*	9.8 *q*	9.8 *q*	9.8 *q*	11.8 *q*	12.2 *q*	12.1 *q*	12.1 *q*
19	18.5 *q*	18.7 *q*	18.5 *q*	18.5 *q*	18.6 *q*	18.7 *q*	18.7 *q*	18.7 *q*	18.8 *q*	18.7 *q*	18.5 *q*	18.5 *q*	18.5 *q*
20	210.6 *s*	210.8 *s*	210.6 *s*	210.6 *s*	73.5 *d*	73.5 *d*	209.9 *s*	209.9 *s*	209.9 *s*	73.5 *d*	71.3 *d*	71.3 *d*	71.3 *d*
21	28.0 *q*	28.2 *q*	28.0 *q*	28.0 *q*	18.2 *q*	18.2 *q*	28.2 *q*	28.2 *q*	28.2 *q*	18.2 *q*	19.8 *q*	19.8 *q*	19.8 *q*
	12-Ac	**12-Ac**	**12-Ac**	**12-Ac**							**12-Bz**	**12-Bz**	**12-Bz**
1	170.3 *s*	170.5 *s*	170.3 *s*	170.3 *s*							167.0 *s*	167.0 *s*	167.0 *s*
2	21.1 *q*	21.3 *q*	21.1 *q*	21.1 *q*							132.1 *s*	132.1 *s*	132.1 *s*
3											130.8 *d*	130.8 *d*	130.8 *d*
4											129.2 *d*	129.2 *d*	129.2 *d*
5											133.6 *d*	133.6 *d*	133.6 *d*
	D-Cym	**D-Cym**	**D-Cym**	**D-Cym**	**D-Cym**	**D-Cym**	**D-Cym**	**D-Cym**	**D-Cym**	**D-Cym**	**D-Cym**	**D-Cym**	**D-Cym**
1′	96.7 *d*	96.9 *d*	96.7 *d*	96.7 *d*	96.7 *d*	96.7 *d*	96.7 *d*	96.7 *d*	96.7 *d*	96.7 *d*	96.7 *d*	96.7 *d*	96.7 *d*
2′	37.6 *t*	37.7 *t*	37.3 *t*	37.6 *t*	37.6 *t*	37.6 *t*	37.6 *t*	37.5 *t*	37.6 *t*	37.5 *t*	37.6 *t*	37.6 *t*	37.6 *t*
3′	78.2 *d*	78.4 *d*	78.4 *d*	78.4 *d*	78.2 *d*	78.7 *d*	78.2 *d*	78.2 *d*	78.4 *d*	78.4 *d*	78.2 *d*	78.2 *d*	78.4 *d*
4′	83.6 *d*	84.0 *d*	83.7 *d*	83.7 *d*	83.6 *d*	83.8 *d*	83.9 *d*	83.8 *d*	83.7 *d*	83.7 *d*	83.5 *d*	83.8 *d*	83.8 *d*
5′	69.3 *d*	69.2 *d*	69.4 *d*	69.4 *d*	69.3 *d*	69.3 *d*	69.3 *d*	69.2 *d*	69.4 *d*	69.4 *d*	69.3 *d*	69.3 *d*	69.4 *d*
6′	19.0 *q*	19.0 *q*	18.8 *q*	18.8 *q*	19.0 *q*	19.0 *q*	19.0 *q*	19.0 *q*	18.7 *q*	18.8 *q*	19.0 *q*	19.0 *q*	18.9 *q*
3′-OMe	59.2 *q*	59.4 *q*	59.3 *q*	59.3 *q*	59.2 *q*	59.0 *q*	59.2 *q*	59.0 *q*	59.2 *q*	59.2 *q*	59.2 *q*	59.2 *q*	59.2 *q*
	D-Ole	**D-Ole**	**D-Cym**	**D-Cym**	**D-Ole**	**D-Ole**	**D-Ole**	**D-Ole**	**D-Cym**	**D-Cym**	**D-Ole**	**D-Ole**	**D-Cym**
1″	102.3 *d*	102.5 *d*	100.8 *d*	100.8 *d*	102.3 *d*	102.3 *d*	102.3 *d*	102.3 *d*	100.8 *d*	100.8 *d*	102.3 *d*	102.3 *d*	100.8 *d*
2″	37.7 *t*	37.9 *t*	37.3 *t*	37.3 *t*	37.7 *t*	38.0 *t*	38.0 *t*	38.0 *t*	37.3 *t*	37.3 *t*	38.0 *t*	38.0 *t*	37.3 *t*
3″	79.6 *d*	79.8 *d*	78.1 *d*	78.0 *d*	79.6 *d*	79.6 *d*	79.6 *d*	79.6 *d*	78.7 *d*	78.7 *d*	79.6 *d*	79.6 *d*	78.0 *d*
4″	83.6 *d*	83.7 *d*	83.5 *d*	83.5 *d*	83.6 *d*	83.3 *d*	83.6 *d*	83.3 *d*	83.5 *d*	83.4 *d*	83.6 *d*	83.3 *d*	83.5 *d*
5″	72.4 *d*	72.4 *d*	69.2 *d*	69.2 *d*	72.4 *d*	72.2 *d*	72.3 *d*	72.3 *d*	69.2 *d*	69.2 *d*	72.3 *d*	72.3 *d*	69.2 *d*
6″	19.1 *d*	19.4 *d*	18.9 *q*	18.9 *q*	19.1 *d*	19.2 *d*	19.1 *d*	19.2 *d*	18.8 *q*	18.9 *q*	19.1 *q*	19.2 *q*	18.8 *q*
3″-OMe	57.8 *q*	58.0 *q*	59.2 *q*	59.2 *q*	57.8 *q*	57.8 *q*	57.8 *q*	57.8 *q*	59.3 *q*	59.2 *q*	57.5 *q*	57.5 *q*	59.3 *q*
	D-Thv	**D-Allme**	**D-Ole**	**D-Ole**	**D-Thv**	**D-Allme**	**D-Thv**	**D-Allme**	**D-Ole**	**D-Ole**	**D-Thv**	**D-Allme**	**D-Ole**
1‴	104.4 *d*	102.4 *d*	102.3 *d*	102.2 *d*	104.4 *d*	102.2 *d*	104.4 *d*	102.2 *d*	102.2 *d*	102.2 *d*	104.4 *d*	102.2 *d*	102.2 *d*
2‴	75.3 *d*	73.2 *d*	37.6 *t*	38.0 *t*	75.3 *d*	73.0 *d*	75.3 *d*	73.0 *d*	38.0 *t*	38.0 *t*	75.3 *t*	73.0 *d*	38.0 *t*
3‴	86.7 *d*	83.7 *d*	79.6 *d*	79.6 *d*	86.7 *d*	83.5 *d*	86.7 *d*	83.5 *d*	79.6 *d*	79.6 *d*	86.7 *d*	83.5 *d*	79.6 *d*
4‴	83.8 *d*	83.8 *d*	83.6 *d*	83.3 *d*	83.8 *d*	83.6 *d*	83.6 *d*	83.6 *d*	83.3 *d*	83.3 *d*	83.6 *d*	83.6 *d*	83.3 *d*
5‴	72.3 *d*	70.1 *d*	72.3 *d*	72.3 *d*	72.3 *d*	70.0 *d*	72.3 *d*	69.9 *d*	72.3 *d*	72.3 *d*	72.4 *d*	70.0 *d*	72.3 *d*
6‴	19.1 *q*	18.8 *q*	19.0 *q*	19.2 *q*	19.1 *q*	18.6 *q*	19.1 *q*	18.6 *q*	19.2 *d*	19.2 *d*	19.1 *d*	18.7 *q*	19.2 *q*
3‴-OMe	61.0 *q*	62.3 *q*	57.8 *q*	57.8 *q*	61.0 *q*	62.1 *q*	61.0 *q*	62.1 *q*	57.8 *q*	57.8 *q*	60.9 *q*	62.1 *q*	57.5 *q*
	D-Glc	**D-Glc**	**D-Thv**	**D-Allme**	**D-Glc**	**D-Glc**	**D-Glc**	**D-Glc**	**D-Allme**	**D-Allme**	**D-Glc**	**D-Glc**	**D-Allme**
1⁗	105.2 *d*	107.2 *d*	104.4 *d*	102.3 *d*	105.2 *d*	107.0 *d*	105.2 *d*	107.0 *d*	102.3 *d*	102.3 *d*	105.3 *d*	107.0 *d*	102.3 *d*
2⁗	76.2 *d*	76.2 *d*	75.3 *d*	73.0 *d*	76.2 *d*	75.8 *d*	76.2 *d*	75.8 *d*	73.0 *d*	73.0 *d*	76.2 *d*	76.0 *d*	73.0 *d*
3⁗	79.0 *d*	78.9 *d*	86.7 *d*	83.5 *d*	79.0 *d*	78.7 *d*	79.0 *d*	78.8 *d*	83.4 *d*	83.5 *d*	79.1 *d*	78.8 *d*	83.5 *d*
4⁗	72.3 *d*	72.5 *d*	83.6 *d*	83.6 *d*	72.3 *d*	72.2 *d*	72.4 *d*	72.2 *d*	83.6 *d*	83.6 *d*	72.3 *d*	72.2 *d*	83.6 *d*
5⁗	78.5 *d*	78.5 *d*	72.3 *d*	69.9 *d*	78.5 *d*	78.2 *d*	78.5 *d*	78.7 *d*	69.8 *d*	69.8 *d*	78.5 *d*	78.8 *d*	69.9 *d*
6⁗	63.3 *t*	63.5 *t*	19.1 *q*	18.2 *q*	63.3 *t*	63.3 *t*	63.4 *t*	63.3 *t*	18.6 *q*	18.2 *q*	63.5 *t*	63.3 *t*	18.7 *q*
3⁗-OMe			61.0 *q*	62.1 *q*					62.0 *q*	62.0 *q*			62.1 *q*
			D-Glc	**D-Glc**					**D-Glc**	**D-Glc**			**D-Glc**
1⁗′			105.0 *d*	107.0 *d*					107.0 *d*	107.0 *d*			107.0 *d*
2⁗′			76.2 *d*	75.8 *d*					75.8 *d*	75.8 *d*			75.9 *d*
3⁗′			79.1 *d*	78.8 *d*					78.8 *d*	78.8 *d*			78.8 *d*
4⁗′			72.4 *d*	72.2 *d*					69.8 *d*	72.2 *d*			72.2 *d*
5⁗′			78.5 *d*	78.7 *d*					78.8 *d*	78.8 *d*			78.7 *d*
6⁗′			63.5 *t*	63.3 *t*					63.5 *t*	63.3 *t*			63.3 *t*

[a] Ac: Acetyl; Bz: Benzoyl; D-Cym: D-Cymaropyranose; D-Ole: D-Oleandropyranose; D-Thv: D-Thevetopyranose; D-Allme: 6-deoxy-3-*O*-methyl-D-allopyranose; D-Glc: D-Glucopyranose.
[b] s: singlet; d: doublet; t: triple;: q: quartet.

Please cite this article in press as: Araya, J.J., et al. Verticillosides A–M: Polyoxygenated pregnane glycosides from *Asclepias verticillata* L. Phytochemistry (2012), doi:10.1016/j.phytochem.2012.02.019

Table 2
^{1}H NMR (500 MHz, C_5D_5N) spectroscopic data for compounds **1–5**.[a,b]

Atom	**1**	*J* (Hz)	2	*J* (Hz)	**3**	*J* (Hz)	**4**	*J* (Hz)	**5**	*J* (Hz)
1	1.82 *m**	14.3, 13.4, 3.9	1.78 *m*	14.2, 13.5, 3.8	1.79 *m*	13.9, 13.2, 3.9	1.80 *m*	13.8, 13.3, 3.9	1.94 *m*	13.3, 13.2, 3.7
	1.12 *ddd*		1.08 *ddd*		1.09 *ddd*		1.09 *ddd*		1.19 *ddd*	
2	2.10 *m*		2.08 *m*		2.08 *m*		2.08 *m*		2.13 *m*	
	1.82 *m*		1.80 *m*		1.81 *m*		1.80 *m*		1.86 *m*	
3	3.86 *m*		3.85 *m*		3.85 *m*		3.85 *m*		3.91 *m*	
4	2.56 *br dd*	12.8, 4.6	2.54 *br dd*	12.9, 4.6	2.56 *br dd*	12.5, 4.5	2.54 *br dd*	13.0, 4.6	2.60 *br dd*	12.7, 4.8
	2.45 *m*		2.41 *m*		2.44 *m*		2.43 *m*		2.50 *m*	
6	5.31 *m*		5.29 *m*		5.28 *m*		5.29 *m*		5.40 *m*	
7	2.47 *m*		2.45 *m*		2.47 *m*		2.47 *m*		2.50 *m*	
	2.33 *m*		2.32 *m*		2.32 *m*		2.32 *m*		2.38 *m*	
9	1.72 *m*		1.69 *m*		1.70 *m*		1.71 *m*		1.66 *m*	
11	2.24 *ddd*	12.7, 12.3, 12.0	2.22 *ddd*	12.9, 12.6, 12.4	2.24 *m*		2.23 *ddd*	12.5, 12.3, 12.2	2.54 *m*	
	2.10 *m*		2.08 *m*		2.09 *m*		2.09 *m*		2.01 *m*	
12	4.99 *dd*	12.1, 4.1	4.97 *dd*	11.6, 3.9	4.98 *m*		4.98 *dd*	12.2, 4.2	3.92 *m*	
15	2.12 *m*		2.10 *m*		2.10 *m*		2.11*m*		2.06 *m*	
16	3.25 *m*		3.23 *m*		3.23 *m*		3.23 *m*		2.01 *m*	
	2.02 *m*		2.01 *m*		2.03 *m*		2.02 *m*			
18	1.94 *s*		1.94 *s*		1.94 *s*		1.94 *s*		1.98 *s*	
19	1.35 *s*		1.34 *s*		1.34 *s*		1.34 *s*		1.43 *s*	
20										
21	2.08 *s*		2.08 *s*		2.48 *s*		2.48 *s*		1.54 *d*	6.4
	12-Ac		**12-Ac**		**12-Ac**		**12-Ac**			
	2.49 *s*		2.48 *s*		2.08 *s*		2.08 *s*			
	D-Cym		**D-Cym**		**D-Cym**		**D-Cym**		**D-Cym**	
1′	5.29 *br d*	9.1	5.26 *br d*	9.4	5.29 *m*		5.29 *m*		5.29 *dd*	9.5, 1.7
2′	2.32 *m*		2.28 *m*		2.32 *m*		2.31 *m*		2.32 *m*	
	1.90 *ddd*	13.2, 9.8, 2.6	1.88 *ddd*	13.5, 9.6, 2.4	1.92 *m*		1.92 *m*		1.90 *m*	
3′	4.05 *m*		4.02 *m*		4.10 *m*		4.10 *m*		4.05 *m*	
4′	3.49 *dd*	9.6, 2.7	3.47 *dd*	9.5, 2.7	3.52 *m*		3.52 *m*		3.49 *dd*	9.5, 2.7
5′	4.23 *m*		4.22 *m*		4.24 *m*		4.23 *m*		4.25 *m*	
6′	1.44 *d*	6.1	1.43 *d*	6.2	1.40 *d*	6.0	1.38 *d*	6.2	1.44 *d*	6.0
3′-OMe	3.56 *s*		3.55 s		3.58 *s*		3.62 *s*		3.56 s	
	D-Ole		**D-Ole**		**D-Cym**		**D-Cym**		**D-Ole**	
1″	4.70 *dd*	9.7, 1.6	4.67 *dd*	9.6, 1.7	5.13 *dd*	9.7, 1.6	5.12 *dd*	9.6, 1.5	4.70 *dd*	9.7, 1.7
2″	2.50 *m*		2.46 *m*		2.32 *m*		2.31 *m*		2.50 *m*	
	1.78 *m*		1.73 *m*		1.82 *m*		1.81 *m*		1.78 *m*	
3″	3.59 *m*		3.56 *m*		4.02 *m*		4.00 *m*		3.59 *m*	
4″	3.62 *dd*	9.0, 8.8	3.56 *m*		3.44 *dd*	9.6, 2.6	3.42 *dd*	9.6, 2.6	3.62 *m*	
5″	3.56 *m*		3.54 *m*		4.20 *m*		4.17 *m*		3.56 *m*	
6″	1.68 *d*	6.1	1.61 *d*	5.4	1.39 *d*	6.0	1.37 *d*	6.2	1.68 *d*	6.0
3″-OMe	3.49 *s*		3.53 *s*		3.63 *s*		3.55 *s*		3.49 *s*	
	D-Thv		**D-Allme**		**D-Ole**		**D-Ole**		**D-Thv**	
1‴	4.90 *d*	7.9	5.27 *d*	8.1	4.68 *dd*	9.7, 1.5	4.67 *dd*	9.7, 1.4	4.90 *d*	7.9
2‴	3.93 *dd*	9.0, 7.9	3.83 *m*		2.49 *m*		2.48 *m*		3.93 *m*	
					1.77 *m*		1.73 *m*			
3‴	3.71 *dd*	9.2, 9.0	4.50 *dd*	2.6, 2.5	3.56 *m*		3.57 *m*		3.71 *dd*	8.9, 8.9
4‴	3.89 *dd*	9.3, 9.2	3.76 *dd*	9.5, 2.2	3.62 *m*		3.57 *m*		3.89 *m*	
5‴	3.77 *dq*	9.3, 6.1	4.29 *m*		3.57 *m*		3.55 *m*		3.77 *m*	9.4, 6.2
6‴	1.78 *d*	6.1	1.66 *d*	6.2	1.68 *d*	6.0	1.61 *d*	5.5	1.78 *d*	6.1
3‴-OMe	3.96 *s*		3.84 s		3.52 *s*		3.53 *s*		3.96 *s*	
	D-Glc		**D-Glc**		**D-Thv**		**D-Allme**		**D-Glc**	
1⁗	5.16 *d*	7.8	5.00 *d*	8.2	4.89 *d*	7.8	5.28 *m*		5.16 *d*	7.8
2⁗	4.07 *m*		4.05 *dd*	8.6, 8.5	3.93 *m*		3.85 *m*		4.07 *m*	
3⁗	4.26 *m*		4.28 *m*		3.71 *dd*	9.0, 8.8	4.50 *dd*	2.7, 2.6	4.26 *m*	
4⁗	4.26 *m*		4.24 *m*		3.89 *m*		3.77 *dd*	9.5, 2.4	4.26 *m*	
5⁗	4.00 *ddd*	8.6, 5.4, 2.9	4.02 *m*		3.76 *m*		4.30 *m*		4.00 *ddd*	8.6, 5.4, 3.0
6⁗	4.56 *dd*	11.5, 2.1	4.57 *dd*	11.5, 2.0	1.79 *d*	6.1	1.67 *d*	6.2	4.56 *dd*	11.5, 2.7
	4.38 *dd*	11.5, 5.3	4.41 *dd*	11.5, 5.3					4.38 *dd*	11.5, 5.6
3⁗-OMe					3.96 *s*		3.84 *s*			
					D-Glc		**D-Glc**			
1⁗′					5.16 *d*	7.8	5.01 *d*	7.9		
2⁗′					4.06 *m*		4.06 *m*			
3⁗′					4.26 *m*		4.28 *m*			
4⁗′					4.25 *m*		4.25 *m*			
5⁗′					4.01 *m*		4.02 *m*			
6⁗′					4.56 *br d*	11.7	4.58 *br d*	11.5		
					4.40 *br d*	11.7	4.40 *br d*	11.5		

[a] Ac: Acetyl; D-Cym: D-Cymaropyranose; D-Ole: D-Oleandropyranose; D-Thv: D-Thevetopyranose; D-Allme: 6-deoxy-3-*O*-methyl-D-allopyranose; D-Glc: D-Glucopyranose.
[b] s: singlet; d: doublet; t: triplet; q: quartet; m: multiplet; br: broad.

Please cite this article in press as: Araya, J.J., et al. Verticillosides A–M: Polyoxygenated pregnane glycosides from *Asclepias verticillata* L. Phytochemistry (2012), doi:10.1016/j.phytochem.2012.02.019

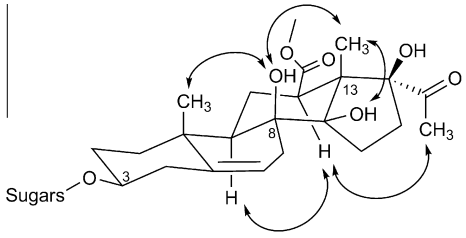


Fig. 3. Observed ROE couplings of compound **1**.

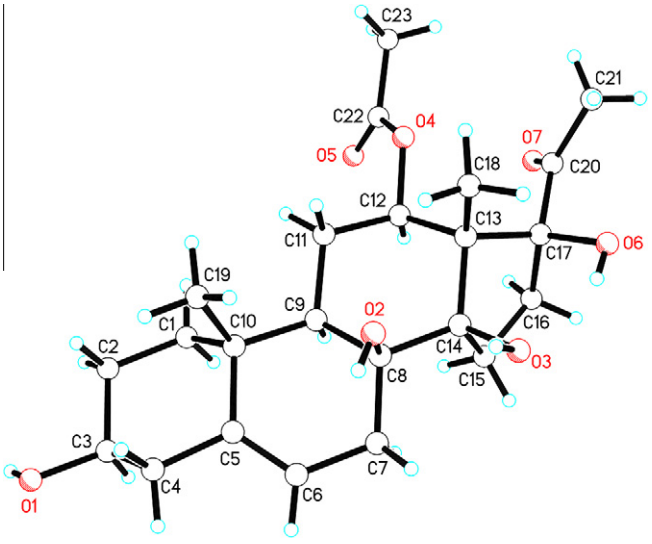


Fig. 4. X-ray structure of metaplexigenin (**1a**) obtained by hydrolysis of **1**.

proton of oleandrose H-1″ (δ 4.67) and C-4′ (δ 84.0); anomeric proton of 6-deoxy-3-*O*-methyl allopyranose H-1‴ (δ 5.27) and C-4″ (δ 83.7); and anomeric proton of glucose H-1‴ (δ 5.00) and C-4″ (δ 83.8). As a result, the structure of **2** was assigned to be metaplexigenin 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-(6-deoxy-3-*O*-methyl)-D-allopyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranose.

Verticillosides C (**3**) and D (**4**) were both isolated as white, amorphous powders. The HRMS of **3** and **4** showed very similar $[M+Na]^+$ ions at *m*/*z* 1199.5857 and 1199.5845, respectively, suggesting that both shared the same molecular formula $C_{57}H_{92}NaO_{25}$ (calcd 1199.5825). The aglycone structure in the two compounds was established to be metaplexigenin (*vide supra*). However, five anomeric protons were identified in the ^{1}H NMR spectroscopic (**3**: δ 5.29, 5.16, 5.13, 4.89 and 4.68; **4**: δ 5.29, 5.28, 5.12, 5.01 and 4.67) indicating that each compound contained five sugar units. Only one proton signal corresponding to H-1⁗ (δ 4.89 in **3**, and δ 5.28 in **4**) showed a significant difference when the ^{1}H NMR spectrum of **3** was compared to that of **4**. The ^{1}H,^{1}H DQFCOSY and ^{1}H,^{1}H-TOCSY and HSQC-TOCSY spectra assisted significantly with the identification for the spin systems of each sugar as well as with the assignment of all the ^{1}H and ^{13}C signals (Tables 1 and 2). Two units of cymarose and single units of oleandrose, thevetose, and glucose were identified in **3**. On the other hand, the sugars present in **4** were found to consist of two units of cymarose and single units of oleandrose, 6-deoxy-3-*O*-methyl allopyranose, and glucose. As previously described, HMBC correlations allowed for the establishment of the sugar sequence in **3**: cymarose I anomeric proton H-1′ (δ 5.29) and C-3 (δ 78.0); cymarose II anomeric proton H-1″ (δ 5.13) and C-4′ (δ 83.7); oleandrose anomeric proton H-1‴ (δ 4.68) and C-4″ (δ 83.5); thevetose anomeric proton H-1⁗ (δ 4.89) and C-4‴ (δ 83.6); and glucose anomeric proton H-1⁗ (δ 5.16) and C-4‴ (δ 83.6). In a comparable manner, the HMBC of **4** showed correlations between cymarose I anomeric proton H-1′ (δ 5.29) and C-3 (δ 78.0); cymarose II anomeric proton H-1″ (δ 5.12) and C-4′ (δ 83.7); oleandrose anomeric proton H-1‴ (δ 4.67) and C-4″ (δ 83.5); 6-deoxy-3-*O*-mehtyl allopyranose anomeric proton H-1⁗ (δ 5.28) and C-4‴ (δ 83.3); and glucose anomeric proton H-1⁗ (δ 5.01) and C-4‴ (δ 83.6). Hence, **3** was determined to be metaplexigenin 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-thevetopyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranose. Compound **4** was established to be metaplexigenin 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-(6-deoxy-3-*O*-methyl)-D-allopyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-cymaropyranose.

Verticilloside E (**5**), isolated as a white amorphous powder, showed a calculated molecular formula of $C_{48}H_{80}NaO_{21}$ based on the observed HRMS ion $[M+Na]^+$ at *m*/*z* 1015.5098 (calcd 1015.5090). The observation of two singlet methyl groups (δ 1.98, 1.43), a methyl doublet (δ 1.54), and an olefinic proton (δ 5.40) in the ^{1}H NMR spectrum as well as two olefinic carbons (δ 139.5 *s* and 120.2 *d*) in the ^{13}C NMR suggested the presence of a 20-hydroxy-pregn-5-ene skeleton. Using ^{1}H,^{1}H COSY, HSQC and HMBC spectra, ^{1}H and ^{13}C signals in the pregnane skeleton could be assigned unambiguously (Tables 1 and 2) and allowed for the identification of the aglycone as sarcostin (**5a**). Relative orientation of the hydroxy group at position C-17 was determined as beta based on the X-ray structure of the aglycone (**5a**) obtained by acid hydrolysis of **5** (Fig. 5). The ^{1}H and ^{13}C NMR spectroscopic data were in good agreement with previously reported data for sarcostin (Li et al., 2008). In addition, **5** showed four anomeric protons in the ^{1}H NMR spectrum (δ 5.29, 5.16, 4.90, and 4.70) indicating the presence of four sugars attached to the aglycone. The sugars were found to be the same as those present in **1** based on their ^{1}H and ^{13}C NMR data (Tables 1 and 2). Consequently, **5** was elucidated as sarcostin 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-thevetopyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranose. Moreover, verticilloside F (**6**) presented the same calculated molecular formula as **5** ($C_{48}H_{80}NaO_{21}$) based on the experimental HRMS ion $[M+Na]^+$ at *m*/*z* 1015.5153 (calcd 1015.5090). The ^{1}H and ^{13}C NMR signals of the aglycone portion of **5** and **6** were almost superimposable, indicating that **6** also had sarcostin as the pregnane core. On the other hand, after full assignment of the ^{1}H and ^{13}C NMR signals using 2D NMR spectra, the four sugars present in **6** were found to be the same as those present in compound **2** by means of careful comparison of their NMR data (Tables 1–3). Consequently, **6** was defined to be sarcostin 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-(6-deoxy-3-*O*-methyl)-D-allopyranosyl-(1 → 4)-β-oleandropyranosyl-(1 → 4)-β-D-cymaropyranose.

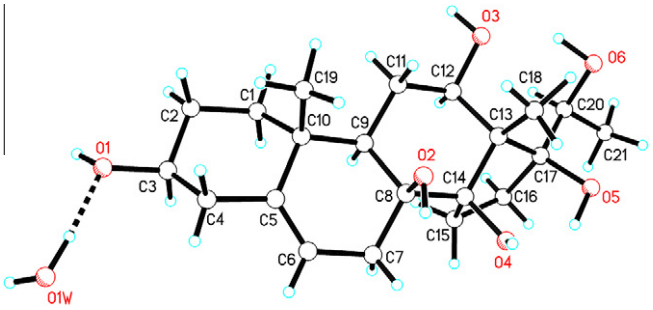


Fig. 5. X-ray structure of sarcostin (**5a**) obtained by hydrolysis of **5**.

Table 3
^{1}H NMR (500 MHz, C_5D_5N) spectroscopic data for compounds **6**–**10**.[a,b]

Atom	**6**	*J* (Hz)	**7**	*J* (Hz)	**8**	*J* (Hz)	**9**	*J* (Hz)	**10**	*J* (Hz)
1	1.93 *m*	13.4, 13.2, 3.5	1.89 *m*	13.4, 13.2, 3.6	1.89 *m*	13.8, 13.7, 3.5	1.90 *m*	13.8, 13.7, 3.5	1.92 *m*	13.5, 13.1, 3.8
	1.18 *ddd*		1.14 *ddd*		1.13 *ddd*		1.13 *dd*		1.20 *ddd*	
2	2.13 *m*		2.12 *m*		2.11 *m*		2.12 *m*		2.13 *m*	
	1.86 *m*		1.87 *m*		1.86 *m*		1.85 *m*		1.86 *m*	
3	3.90 *m*		3.89 *m*		3.89 *m*		3.89 *m*		3.90 *m*	
4	2.60 *br dd*	12.6, 4.5	2.59 *br d*	12.6, 4.6	2.58 *br dd*	12.8, 4.7	2.59 *br dd*	12.8, 4.6	2.61 *br dd*	12.6, 3.6
	2.49 *m*		2.48 *m*		2.47 *m*		2.49 *m*		2.50 *m*	
6	5.41 *m*		5.38 *m*		5.36 *m*		5.36 *m*		5.39 *m*	
7	2.48 *m*		2.49 *m*		2.49 *m*		2.50 *m*		2.49 *m*	
	2.37 *m*		2.37 *m*		2.37 *m*		2.38 *m*		2.38 *m*	
9	1.65 *m*		1.64 *m*		1.64		1.65 *m*		1.67 *m*	
11	2.53 *m*		2.54 *m*		2.46 *m*		2.47 *m*		2.54 *m*	
	2.00 *m*		1.90 *m*		1.90 *m*		1.92 *m*		2.01 *m*	
12	3.92 *m*		3.95 *m*		3.96 *m*		3.96 *m*		3.91 *m*	
15	2.06 *m*		2.13*m*		2.14 *m*		2.15 *m*		2.07 *m*	
16	2.00 *m*		3.39 *m*		3.40 *m*		3.41 *dd*	9.8, 2.7	2.01 *m*	
			2.13 *m*		2.12 *m*		2.03 *m*			
18	1.97 *s*		2.02 *s*		2.02 *s*		2.02 *s*		1.98 *s*	
19	1.43 *s*		1.42 *s*		1.41 *s*		1.41 *s*		1.43 *s*	
20									4.49 *q*	6.4
21	1.54 *d*	6.4	2.60 *s*		2.65 *s*		2.66 *s*		1.54 *d*	6.4
	D-Cym		**D-Cym**		**D-Cym**		**D-Cym**		**D-Cym**	
1′	5.30 *dd*	9.6, 1.7	5.30 *dd*	9.5, 1.6	5.30 *dd*	9.4, 1.6	5.30 *dd*	9.6, 1.6	5.32 *dd*	9.5, 1.60
2′	2.30 *m*		2.32 *m*		2.30 *m*		2.33 *m*		2.33 *m*	
	1.90 *m*		1.89 *m*		1.89 *m*		1.92 *m*		1.92 *m*	
3′	4.03 *m*		4.05 *m*		4.03 *m*		4.10 *m*		4.11 *m*	
4′	3.48 *dd*	9.6, 2.7	3.48 *dd*	9.6, 2.7	3.48 *dd*	9.5, 2.6	3.52 *m*		3.52 *m*	
5′	4.23 *m*		4.23 *m*		4.23 *m*		4.24 *m*		4.25 *m*	
6′	1.43 *d*	6.2	1.42 *d*	6.2	1.41 *d*	6.2	1.38 *d*	6.2	1.38 *d*	6.2
3′-OMe	3.56 *s*		3.58 *s*		3.55 *s*		3.63 *s*		3.63 *s*	
	D-Ole		**D-Ole**		**D-Ole**		**D-Cym**		**D-Cym**	
1″	4.67 *dd*	9.7, 1.6	4.69 *dd*	9.7, 1.6	4.68 *dd*	9.6, 1.6	5.12 *dd*	9.6, 1.5	5.11 *dd*	9.7, 1.5
2″	2.47 *m*		2.48 *m*		2.40 *m*		2.33 *m*		2.32 *m*	
	1.74 *m*		1.75 *m*		1.74 *m*		1.88 *m*		1.81 *m*	
3″	3.56 *m*		3.57 *m*		3.56 *m*		4.02 *m*		4.00 *m*	
4″	3.56 *m*		3.60 *m*		3.56 *m*		3.43 *dd*	9.8, 2.7	3.43 *dd*	9.6, 2.6
5″	3.53 *m*		3.76 *dq*	9.4, 6.1	3.54 *m*		4.18 *dq*	9.8, 6.2	4.17 *dq*	9.6, 6.2
6″	1.60 *d*	5.8	1.68 *d*	6.0	1.61 *d*	5.8	1.38 *d*	6.2	1.38 *d*	6.2
3″-OMe	3.53 *s*		3.51 *s*		3.52 *s*		3.55 *s*		3.56 s	
	D-Allme		**D-Thv**		**D-Allme**		**D-Ole**		**D-Ole**	
1‴	5.28 *d*	8.0	4.89 *d*	7.9	5.27 *d*	8.0	4.68 *dd*	9.8, 1.5	4.67 *dd*	9.6, 1.4
2‴	3.84 *m*		3.92 *m*		3.83 *m*		2.48 *m*		2.48 *m*	
							1.74 *m*		1.74 *m*	
3‴	4.50 *dd*	2.7, 2.7	3.71 *dd*	9.0, 9.0	4.50 *dd*	2.7, 2.7	3.58 *m*		3.57 *m*	
4‴	3.76 *dd*	9.6, 2.4	3.89 *dd*	9.0, 8.8	3.77 *dd*	9.6, 2.4	3.57 *m*		3.58 *m*	
5‴	4.29 *m*		3.55 *m*		4.29 *m*		3.55 *m*		3.54 *m*	
6‴	1.67 *d*	6.2	1.78 *d*	6.1	1.66 *d*	6.2	1.62 *d*	5.5	1.60 *d*	5.4
3‴-OMe	3.85 *s*		3.95 *s*		3.84 *s*		3.53 *s*		3.53 *s*	
	D-Glc		**D-Glc**		**D-Glc**		**D-Allme**		**D-Allme**	
1⁗	5.01 *d*	7.7	5.16 *d*	7.8	5.01 *d*	7.8	5.27 *d*	8.1	5.28 *d*	8.1
2⁗	4.06 *dd*	8.8, 8.4	4.05 *m*		4.06 *m*		3.85 *m*		3.86 *m*	
3⁗	4.28 *m*		4.26 *m*		4.27 *m*		4.51 *dd*	2.7, 2.7	4.51 *dd*	2.7, 2.7
4⁗	4.25 *m*		4.25 *m*		4.25 *m*		3.77 *dd*	9.5, 2.5	3.77 *dd*	9.6, 2.4
5⁗	4.00 *m*		3.99 *m*		4.04 *ddd*	8.9, 5.4, 2.4	4.26 *m*		4.30 *m*	
6⁗	4.58 *dd*	11.6, 2.4	4.55 *dd*	11.4, 2.4	4.58 *br d*	11.8	1.67 *d*	6.2	1.68 *d*	6.2
	4.41 *dd*	11.6, 5.4	4.39 *dd*	11.4, 5.5	4.41 *dd*	11.8, 5.9				
3⁗-OMe							3.85 *s*		3.86 s	
							D-Glc		**D-Glc**	
1⁗′							5.02 *d*	8.0	5.02 *d*	8.2
2⁗′							4.07 *m*		4.06 *dd*	8.6, 8.2
3⁗′							4.29 *m*		4.29 *m*	
4⁗′							4.30 *m*		4.26 *m*	
5⁗′							4.05 *m*		4.03 *ddd*	9.0, 5.6, 2.5
6⁗′							4.58 *dd*	11.5, 5.4	4.58 *dd*	11.6, 2.5
							4.41 *dd*	11.5, 2.5	4.42 *dd*	11.6, 5.6

[a] D-Cym: D-Cymaropyranose; D-Ole: D-Oleandropyranose; D-Thv: D-Thevetopyranose; D-Allme: 6-deoxy-3-*O*-methyl-D-allopyranose; D-Glc: D-Glucopyranose.
[b] s: singlet; d: doublet; t: triplet; q: quartet; m: multiplet; br: broad.

Please cite this article in press as: Araya, J.J., et al. Verticillosides A–M: Polyoxygenated pregnane glycosides from *Asclepias verticillata* L. Phytochemistry (2012), doi:10.1016/j.phytochem.2012.02.019

Table 4
^{1}H NMR (500 MHz, C_5D_5N) spectroscopic data for compounds **11**–**13**.[a,b].

Atom	**11**	*J* (Hz)	**12**	*J* (Hz)	**13**	*J* (Hz)
1	1.80 *m*	14.1, 13.8, 3.6	1.81 *m*	14.1, 13.8, 3.4	1.80 *m*	14.1, 13.5, 3.6
	1.13 *ddd*		1.12 *ddd*		1.12 *ddd*	
2	2.10 *m*		2.10 *m*		2.09 *m*	
	1.82 *m*		1.82 *m*		1.84 *m*	
3	3.89 *m*		3.89 *m*		3.89 *m*	
4	2.60 *br dd*	13.1, 4.4	2.59 *br dd*	13.0, 4.5	2.59 *br dd*	13.0, 4.3
	2.47 *m*		2.47 *m*		2.47 *m*	
6	5.40 *m*		5.39 *m*		5.38 *m*	
7	2.52 *m*		2.51 *m*		2.51 *m*	
	2.40 *m*		2.40 *m*		2.39 *m*	
9	1.82 *m*		1.82 *m*		1.81 *m*	
11	2.53 *m*		2.53 *m*		2.52 *m*	
	2.19 *m*		2.19 *m*		2.19 *m*	
12	5.40 *m*		5.40 *m*		5.40 *m*	
15	2.14 *m*		2.14 *m*		2.13 *m*	
16	1.99 *m*		1.99 *m*		1.98 *m*	
18	2.24 *s*		2.24 *s*		2.24 *s*	
19	1.37 *s*		1.37 *s*		1.36 *s*	
20	4.12 *dq*	6.1, 6.1, 6.1, 5.3	4.12 *dq*	6.2, 6.2, 6.2, 5.6	4.12 *m*	
21	1.29 *d*	6.1	1.29 *d*	6.2	1.29 *d*	6.1
	12-Bz		**12-Bz**		**12-Bz**	
	8.59 *br d*	8.2	8.59 *br d*	8.2	8.59 *br d*	8.2
	7.41 *br dd*	8.2, 7.6	7.41 *br dd*	8.2, 7.6	7.41 *br dd*	8.2, 7.6
	7.50 *m*		7.50 *m*		7.50 *m*	
	D-Cym		**D-Cym**		**D-Cym**	
1′	5.31 *dd*	9.7, 1.5	5.30 *dd*	9.7, 1.5	5.30 *dd*	9.6, 1.6
2′	2.33 *ddd*	13.5, 2.9, 1.5	2.31 *m*		2.32 *m*	
	1.90 *m*		1.89 *m*		1.90 *m*	
3′	4.06 *m*		4.04 *m*		4.10 *m*	
4′	3.50 *m*		3.49 *dd*	9.6, 2.4	3.53 *m*	
5′	4.25 *m*		4.24 *m*		4.24 *m*	
6′	1.45 *d*	6.2	1.44 *d*	6.2	1.38 *d*	6.2
3′-OMe	3.59 *s*		3.56 *s*		3.63 *s*	
	D-Ole		**D-Ole**		**D-Cym**	
1″	4.70 *dd*	9.6, 1.6	4.68 *dd*	9.6, 1.6	5.12 *dd*	9.4, 1.3
2″	2.49 *m*		2.49 *m*		2.31 *m*	
	1.78 *m*					
			1.75 *m*		1.81 *m*	
3″	3.56 *m*		3.57 *m*		4.00 *m*	
4″	3.63 *m*		3.56 *m*		3.41 *dd*	9.6, 2.5
5″	3.56 *m*		3.54 *m*		4.17 *m*	
6″	1.69 *d*	6.0	1.62 *d*	5.9	1.38 *d*	6.2
3″-OMe	3.52 *s*		3.54 *s*		3.55 *s*	
	D-Thv		**D-Allme**		**D-Cym**	
1‴	4.89 *d*	7.9	5.28 *d*	7.9	4.67 *dd*	9.6, 1.2
2‴	3.92 *m*		3.85 *m*		2.48 *m*	
					1.73 *m*	
3‴	3.72 *dd*	9.0, 8.9	4.50 *dd*	2.7, 2.7	3.56 *m*	
4‴	3.89 *dd*	9.4, 9.0	3.76 *m*		3.57 *m*	
5‴	3.76 *dq*	9.4, 6.1, 6.1, 6.1	4.31 *m*		3.55 *m*	
6‴	1.67 *d*	6.1	1.68 *d*	6.4	1.61 *d*	5.3
3‴-OMe	3.84 *s*		3.85 *s*		3.53 *s*	
	D-Glc		**D-Glc**		**D-Allme**	
1⁗	5.16 *d*	7.7	5.01 *d*	7.7	5.28 *d*	8.0
2⁗	4.06 *m*		4.07 *m*		3.85 *m*	
3⁗	4.26 *m*		4.28 *m*		4.50 *dd*	2.6, 2.4
4⁗	4.26 *m*		4.26 *m*		3.76 *dd*	9.6, 2.4
5⁗	4.00 *m*		4.04 *m*		4.29 *m*	
6⁗	4.55 *br d*	11.5	4.58 *br d*	11.5	1.68 *d*	6.2
	4.40 *br d*	11.5	4.41 *br d*	11.5		
3⁗-OMe					3.85 *s*	

(*continued on next page*)

Please cite this article in press as: Araya, J.J., et al. Verticillosides A–M: Polyoxygenated pregnane glycosides from *Asclepias verticillata* L. Phytochemistry (2012), doi:10.1016/j.phytochem.2012.02.019

Table 4 (*continued*)

Atom	**11**	*J* (Hz)	**12**	*J* (Hz)	**13**	*J* (Hz)
					D-Glc	
1′′′′′					5.01 *d*	7.8
2′′′′′					4.05 *m*	
3′′′′′					4.28 *m*	
4′′′′′					4.25 *m*	
5′′′′′					4.00 *m*	
6′′′′′					4.59 *br d*	11.5
					4.42 *m*	

[a] Bz: Benzoyl; D-Cym: D-Cymaropyranose; D-Ole: D-Oleandropyranose; D-Thv: D-Thevetopyranose; D-Allme: 6-deoxy-3-*O*-methyl-D-allopyranose; D-Glc: D-Glucopyranose.
[b] s: singlet; d: doublet; t: triplet; q: quartet; m: multiplet; br: broad.

Verticillosides G (**7**) and H (**8**) displayed a HRMS ion $[M+Na]^+$ at *m*/*z* 1013.4913 and 1013.4921, respectively suggesting the same molecular formula of $C_{48}H_{78}NaO_{21}$ (calcd 1013.4933) for both compounds. As in the case of compounds **1**–**4**, three methyl singlets were observed in the ^1H NMR (**7**: δ 2.60, 2.02, 1.42; **8**: δ 2.65, 2.02, 1.41); however, the acetyl ^1H and ^{13}C signals were missing in **7** and **8**. Following a similar analysis of the ^1H, ^{13}C and 2D NMR spectroscopic data as described before, the aglycones present in the two compounds were identified to be 12-*O*-deacylmetaplexigenin and the signal assignments were in good agreement with previously reported values (Warashina and Noro, 2000). The ^1H and ^{13}C signals were totally assigned for the sugar portion using 2D NMR spectra (Tables 1 and 3). While the sugars in **7** were found to be the same as in **1**, the sugars in **8** were identical to those present in **2** based on their NMR data comparison. The structures of **7** and **8** were determined to be 12-*O*-deacylmetaplexigenin 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-thevetopyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranose and 12-*O*-deacylmetaplexigenin 3-*O*-β-D-glucopiranosyl-(1 → 4)-β-D-thevetopyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranose, respectively.

Verticilloside I (**9**) showed a HRMS ion $[M+Na]^+$ at *m*/*z* 1159.5904 indicating a molecular formula of $C_{55}H_{92}NaO_{24}$, (calcd 1159.5876). Complete assignment of ^1H and ^{13}C NMR signals using 2D NMR spectroscopic showed that the aglycone corresponded to 12-*O*-deacylmetaplexigenin. The five sugars present in **9** were identical to those found in **4** and their ^1H and ^{13}C sugar signals were almost identical (Tables 1 and 3). Hence, structure of **9** was confirmed to be 12-*O*-deacylmetaplexigenin 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-(6-deoxy-3-*O*-methyl)-D-allopyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranose. Likewise, verticilloside J (**10**) had a molecular formula of $C_{55}H_{90}NaO_{24}$ on the basis of the observed HRMS ion $[M+Na]^+$ at *m*/*z* 1057.5734 (calcd 1157.5720). Following the complete assignment of ^1H and ^{13}C NMR signals aided by 2D NMR data, the aglycone was found to be sarcostin and the sugars the same as those present in **4** (Tables 1 and 3). Hence, **10** was determined as sarcostin 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-(6-deoxy-3-*O*-methyl)-D-allopyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranose respectively.

Verticillosides K (**11**) and L (**12**) displayed very similar $[M+Na]^+$ ions at *m*/*z* 1119.5337 and 1119.5364, respectively, thus sharing the same calculated molecular formula of $C_{55}H_{84}NaO_{22}$ (calcd 1119.5352). Unlike previously described compounds, ^1H NMR spectrum of **11** showed aromatic signals corresponding to a benzoyl group (δ 8.59, 7.50, and 7.41) and further confirmed by ^{13}C NMR resonances (δ 167.0, 133.6, 132.1, 130.8, and 129.2). The benzoyl group was found to be attached to position 12 by means of the observed HMBC correlation between H-12 (δ 5.40) and the benzoyl carbonyl signal (δ 167.0). The aglycone was established as 12-*O*-benzoylsarcostin based on the HMBC correlations between the proton signal δ 2.24 (H-18) with carbon resonances of δ 57.8 (C-13), 75.7 (C-12), 89.0 (C-14), and 89.2 (C-17); and between proton signal δ 1.37 (Me-19) and carbon resonances of δ 39.2 (C-1), 139.5 (C-5), 44.5 (C-9), and 37.7 (C-10). The experimental NMR spectroscopic data (Tables 1 and 4) of the aglycone were in good agreement with previously reported values (Gan et al., 2008). The ^1H NMR and ^{13}C NMR values of the aglycone in compound **12** were almost identical to those just described for **11**, indicating that the two compounds shared the same aglycone (Tables 1 and 4). Furthermore, following full assignment of ^1H and ^{13}C NMR data using 2D NMR spectra, close comparison of their spectra revealed that the sugars present in **11** and **12** were identical to those present in **1** and **2**, respectively. Consequently, the structure of **11** was established as 12-*O*-benzoylsarcostin 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-thevetopyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranose, and structure of **12** as sarcostin 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-(6-deoxy-3-*O*-methyl)-D-allopyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranose.

Finally, verticilloside M (**13**) had a HRMS $[M+Na]^+$ ion at *m*/*z* 1263.6037 (calcd 1263.6138) suggesting a molecular formula of $C_{62}H_{96}NaO_{25}$. By closed comparison of the 1D and 2D NMR spectra, the aglycone of **13** was identified to be 12-*O*-benzoylsarcostin, as in compound **11** (Tables 1 and 4). The sugar moiety, however, contained the same sequence of five units as those described for compound **4**. Hence, compound **13** was identified as 12-*O*-benzoylsarcostin 3-*O*-β-D-glucopyranosyl-(1 → 4)-β-(6-deoxy-3-*O*-methyl)-D-allopyranosyl-(1 → 4)-β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranose.

Cytotoxicity of the isolates (**1**–**13**) was tested against the paired breast cell lines Hs578T (cancer) and Hs578Bst (normal); however, no toxicity was observed in the experimental concentration range of 0.2–50 μM (see Supplementary Data).

3. Conclusions

The present work reports the isolation and chemical characterization of 13 new polyoxygenated glycosides from *A. verticillata*. The structures of these compounds were established on the basis of spectroscopic data, including 800 MHz 2D NMR experiments. Furthermore, relative stereochemistry for two compounds (**1** and **5**) was confirmed by means of X-ray crystallography. Our investigation shows the diversity of secondary metabolites present within *Asclepias*, a genus widely distributed in the US Midwest flora. Although the isolates did not show significant cytotoxicity against the tested cell lines, evaluation against other biological targets is underway and will be reported in due course.

4. Experimental section

4.1. General experimental procedures

Melting points were recorded with an OptiMelt automatic apparatus. IR spectra were obtained with a Thermo Nicolet Avatar

380 FT-IR. UV–Vis spectra were acquired in a Varian Cary 50 Scan; ^{1}H NMR, ^{13}C NMR and two dimensional spectra were acquired with a Bruker Avance AV-III 500 with a dual carbon/proton cryoprobe. Some NMR experiments were conducted in a Bruker Unity 800 MHz NMR with direct detection probe. HRMS were conducted with a LCT Premier Waters corp. (Milford, MA). Semi-preparative HPLC was conducted using an Agilent 1200 HPLC system with a Phenomenex Luna C18 column (5 μm, 250 × 10 mm), flow rate of 4.5 mL/min (approx. 160 bar), injection volume of 50 μL (ca. 10 mg sample), and UV detection using diode array. Preparative HPLC separations were done using an Agilent 1100 HPLC system with a Phenomenex Luna C18 column (5 μm, 250 × 21 mm), flow rate of 35 mL/min (aprox. 35 bar), injection volume of 800 μL (ca. 100 mg sample), and UV detection using multi-wavelength detector.

4.2. Plant Material

Above-ground biomass of *A. verticillata* was collected in July 10th of 2009 by Loring and Long at the Dog Leg Prairie of the Nelson Environmental Study Area, Lawrence, Kansas (latitude: 39.0550°; longitude: 95.1967°). Botanical identification was performed by Kelly Kindscher and a voucher specimen was deposited in the R.L. McGregor Herbarium (Collection number H. Loring 3559).

4.3. Plant extraction and isolation

A. verticillata fresh biomass (5.5 kg) was left to dry at room temperature. The dry material (1.2 kg) was then ground to a fine powder and extracted four times with a mixture of MeOH and CH_2Cl_2 (1:1, v/v) at room temperature. The organic solvents (c.a. 20 L) were removed under reduced pressure to afford the crude extract (138 g) (11.5% w/w of dry weight). The organic extract was suspended in MeOH:H_2O 9:1 (1 L) and extracted with hexanes (500 mL × 3) to give a hexanes fraction. Then, MeOH was removed from the aqueous layer under reduced pressure, with the volume adjusted to 500 mL with distilled H_2O. This was then successively extracted with CH_2Cl_2 (500 mL × 3) and n-BuOH (500 mL × 3) to give $CHCl_2$ and n-BuOH fractions respectively. The n-BuOH extract (41 g) was suspended in H_2O (500 mL) and adsorbed in a MCI gel (500 g) column, washed with H_2O (2 L), and then eluted with mixtures of H_2O–MeOH (9:1 → 0:100, v/v) starting with 10% step increments (2 L each fraction) to afford a total of 10 fractions (1–10). Fractions 6–9 were purified as follows: first Sephadex LH-20 (500 g) CC using MeOH as eluent, followed by silica gel CC using $CHCl_3$:MeOH 95:5 (v/v) or $CHCl_3$:MeOH 90:10 (v/v) as mobile phase, and finally semi-preparative or preparative HPLC chromatography using mixtures of CH_3CN–H_2O for elution. A total of 13 pregnane glycosides were isolated and chemically elucidated using ^{1}H NMR, ^{13}C NMR, 2D NMR, IR, UV, and HRMS.

4.4. Verticilloside A (**1**)

Amorphous white powder; mp 164.9–166.9 °C; $[\alpha]_D^{25}$ = −14.3 (c. 0.412, MeOH); IR ν_{max} (film) cm^{-1}: 3366.6 (OH), 1706.0 (C=O), 1635.8 (C=O), 1158.4 (C–O), 1059.8 (C-O); UV_{max} 206.9, 277.5; HRMS *m/z*: 1055.5276 [M+Na]$^+$ (1055.5039 calcd for $C_{50}H_{80}NaO_{22}$) for ^{1}H and ^{13}C NMR spectroscopic data see Tables 1 and 2.

4.5. Verticilloside B (**2**)

Amorphous white powder; mp 169.8–172.7 °C; $[\alpha]_D^{25}$ = −5.0 (c. 0.735, MeOH); IR ν_{max} (film) cm^{-1}: 3394.8 (OH), 1708.6 (C=O), 1635.0 (C=O), 1058.7 (C–O), 1035.2 (C–O); UV_{max} (nm, MeOH): 208.3, 267.9; HRMS *m/z*: 1052.5271 [M+Na]$^+$ (1055.5039 calcd for $C_{50}H_{80}NaO_{22}$) for ^{1}H and ^{13}C NMR spectroscopic data see Tables 1 and 2.

4.6. Verticilloside C (**3**)

Amorphous white powder; mp 169.0–170.5 °C; $[\alpha]_D^{25}$ = +6.6 (c. 0.378, MeOH); IR ν_{max} (film) cm^{-1}: 3389.7 (OH), 1708.7 (C=O), 1644.8 (C=O), 1156.2 (C–O), 1059.8 (C-O); UV_{max} (nm, MeOH): 206.9, 274.1; HRMS *m/z*: 1199.5857 [M+Na]$^+$ (1199.5825 calcd for $C_{57}H_{92}NaO_{25}$); for ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

4.7. Verticilloside D (**4**)

Amorphous white powder; mp 171.1–175.2 °C; $[\alpha]_D^{25}$ = +31.8 (c. 1.63, MeOH); IR ν_{max} (film) cm^{-1}: 3388.7 (OH), 1703.8.0 (C=O), 1642.2 (C=O), 1148.3, 1054.7; UV_{max} (nm, MeOH): 207.0, 282.0; HRMS *m/z*: 1199.5845 [M+Na]$^+$ (1199.5825 calcd for $C_{57}H_{92}NaO_{25}$) for ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

4.8. Verticilloside E (**5**)

Amorphous white powder; mp 160.1–162.6 °C; $[\alpha]_D^{25}$ = +17.5 (c. 0.479, MeOH); IR ν_{max} (film) cm^{-1}: 3378.3 (OH), 1150.1 (C–O), 992.8 (C–O); UV_{max} (nm, MeOH): 207.0, 267.0; HRMS *m/z*: 1015.5098 [M+Na]$^+$ (1015.5090 calcd for $C_{48}H_{80}NaO_{21}$); ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 2.

4.9. Verticilloside F (**6**)

Amorphous white powder; mp 165.8–167.6 °C; $[\alpha]_D^{25}$ = +21.7 (c. 0.974, MeOH); IR ν_{max} (film) cm^{-1}: 3385.3 (OH), 1152.8 (C–O), 996.3 (C–O); UV_{max} (nm, MeOH): 207.2, 272.5; HRMS *m/z*: 1015.5153 [M+Na]$^+$ (1015.5090 calcd for $C_{48}H_{80}NaO_{21}$); for ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 3.

4.10. Verticilloside G (**7**)

Amorphous white powder; mp 157.6–159.8 °C; $[\alpha]_D^{25}$ = +13.6 (c. 0.366, MeOH); IR ν_{max} (film) cm^{-1}: 3385.3 (OH), 1685.8 (C=O), 1152.8 (C–O), 996.3 (C-O); UV_{max} (nm, MeOH): 207.1, 282.0; HRMS *m/z*: 1013.4913 [M+Na]$^+$ (1013.4933 calcd for $C_{48}H_{78}NaO_{21}$) for ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 3.

4.11. Verticilloside H (**8**)

Amorphous white powder; mp 163.1–164.3 °C; $[\alpha]_D^{25}$ = +12.3 (c. 0.674, MeOH); IR ν_{max} (film) cm^{-1}: 3425.2 (OH), 1696.5 (C=O), 1152.1 (C–O), 997.4 (C–O); UV_{max} (nm, MeOH): 206.1, 280.7; HRMS *m/z*: 1013.4921 [M+Na]$^+$ (1013.4933 calcd for $C_{48}H_{78}NaO_{21}$) for ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 3.

4.12. Verticilloside I (**9**)

Amorphous white powder; mp 172.2–174.0 °C; $[\alpha]_D^{25}$ = +25.2 (c. 0.447, MeOH); IR ν_{max} (film) cm^{-1}: 3391.3 (OH), 1149.6 (C–O), 999.4 (C–O); UV_{max} (nm, MeOH): 206.1, 269.2; HRMS *m/z*: 1159.5904 [M+Na]$^+$ (1159.5876 calcd for $C_{55}H_{92}NaO_{24}$); for ^{1}H and ^{13}C NMR spectroscopic data, see Tables 1 and 3.

4.13. Verticilloside J (**10**)

Amorphous white powder; mp 170.9–172.1 °C; $[\alpha]_D^{25}$ = +13.1 (c. 0.288, MeOH); IR ν_{max} (film) cm^{-1}: 3455.0 (OH), 1154.8 (C–O), 1051.3 (C–O); UV_{max} (nm, MeOH): 206.0, 268.9; HRMS *m/z*:

Please cite this article in press as: Araya, J.J., et al. Verticillosides A–M: Polyoxygenated pregnane glycosides from *Asclepias verticillata* L. Phytochemistry (2012), doi:10.1016/j.phytochem.2012.02.019

1057.5734 $[M+Na]^+$ (1157.5720 calcd for $C_{55}H_{90}NaO_{24}$); for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 3.

4.14. Verticilloside K (**11**)

Amorphous white powder; mp 173.1–174.8 °C; $[\alpha]_D^{25}$ = +53.6 (c. 0.467, MeOH); IR ν_{max} (film) cm^{-1}: 3389.0 (OH), 3032.3 (Ar–H), 1732.5 (C=O), 1635.8 (C=O), 1155.5 (C–O), 977.7 (C–O); UV_{max} (nm, MeOH): 204.1, 226.1, 274.1; HRMS *m/z*: 1119.5337 $[M+Na]^+$ (1119.5352 calcd for $C_{55}H_{84}NaO_{22}$) for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 4.

4.15. Verticilloside L (**12**)

Amorphous white powder; mp 176.8–178.2 °C; $[\alpha]_D^{25}$ = +25.2 (c. 0.254, MeOH); IR ν_{max} (film) cm^{-1}: 3340.5 (OH), 3031.8 (Ar–H), 1698.8 (C=O), 1635.8 (C=O), 1153.4 (C–O), 978.5 (C–O); UV_{max} (nm, MeOH): 204.0, 226.0, 274.1; HRMS *m/z*: 1119.5364 $[M+Na]^+$ (1119.5352 calcd for $C_{55}H_{84}NaO_{22}$) for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 4.

4.16. Verticilloside M (**13**)

Amorphous white powder; mp 176.8–178.2 °C; $[\alpha]_D^{25}$ = +25.2 (c. 0.254, MeOH); IR ν_{max} (film) cm^{-1}: 3340.5 (OH), 3031.8 (Ar–H), 1698.8 (C=O), 1635.8 (C=O), 1153.4 (C–O), 978.5 (C–O); UV_{max} (nm, MeOH): 204.0, 226.0, 274.1; HRMS *m/z*: 1263.6037 $[M+Na]^+$ (1263.6138 calcd for $C_{62}H_{96}NaO_{25}$); for ^1H and ^{13}C NMR spectroscopic data, see Tables 1 and 4.

4.17. Acid hydrolysis of glycosides

Hydrolysis of glycosides was conducted as described elsewhere (Warashina and Noro, 2010). Acid hydrolysis of a mixture of **1**–**4** (50 mg) afforded the aglycone metaplexigenin (**1a**); mixtures of **5**, **6** and **10** (50 mg) produced sarcostin (**5a**); a mixture of **7**–**9** (50 mg) gave 12-*O*-deacylmetaplexigenin; and a mixture of **11**–**13** (50 mg) afforded 12-*O*-benzoylsarcostin (**11a**). Structure of the aglycones were established by spectroscopic methods and compared with previously reported data. From the combined aqueous layers the following sugars were purified and identified by comparison of spectroscopic and optical rotation (after 24 h equilibration time) data reported in literature:

D-Cymarose $[\alpha]_D^{25}$ = +50.1 (c. 0.1 H_2O); lit. + 51.6 (Tsukamoto et al., 1986).

D-Thevetoside $[\alpha]_D^{25}$ = +38.0 (c. 0.1 H_2O); lit. + 42.3 (Hamed et al., 2004).

D-Oleandrose $[\alpha]_D^{25}$ = −9.8 (c. 0.1 H_2O); lit. + 10.3 (Nakagawa et al., 1983).

(6-Deoxy-3-*O*-methyl)-D-allose $[\alpha]_D^{25}$ = +3.9 (c. 0.1 H_2O); lit. + 5 (Allgeier, 1968).

Glucose $[\alpha]_D^{25}$ = +50 (c. 0.1 H_2O); lit. + 56 (Jacobs and Craig, 1944).

4.18. X-ray structure determination

Crystallization of the aglycones metaplexigenin (**1a**) and sarcostin (**5a**) was carried out using mixtures of CH_2Cl_2, MeOH, and CH_3CN using slow evaporation technique. Then, the obtained crystals were submitted for X-ray diffraction determination (Figs. 4 and 5). The crystal structures have been deposited at the Cambridge Crystallographic Data Center and allocated the deposition number CCDC 840311 and CCDC 840314, respectively. These data can be obtained free of charge via supplementary crystallographic data via http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the CCDC, 12 Union Road, Cambridge CB2 1 EZ, UK; fax: +44 1223 336033; email: deposit@ccdc.cam.ac.uk). See also Supplementary Information for crystal data and structure refinement.

4.19. Cytotoxicity assay

Breast cancer cell line Hs578T and one normal breast Hs578Bst cell lines were seeded in separated 384-well plates (seeding density of 3000 cells per well, in a volume of 30 μL per well) and allowed to attach and grow overnight in a cell incubator. Then, compounds were added using a Lybcyte ECHO acoustic liquid handling instrument (eight concentrations in the range 0.2–50 μM) and plates were incubated for 72 h. Next, cell viability was determined adding 10 μL of CellTiter-Glo (CTG) reagent, shaking plates for 2 min followed by reading of luminescence after 15 min stabilizing period. Each dose-response curve was determined by triplicate. The data were normalized dividing by the median and IC_{50} calculation was done using GraphPad Prism software.

Acknowledgment

This study was supported by grant IND0061464 (awarded to B.N.T and K.K.) from the Kansas Bioscience Authority (KBA) and Center for Heartland Plant Innovations (HPI). The authors thank Q. Long and H. Loring, botanists at the University of Kansas or at the Kansas Biological Survey at the University of Kansas for assistance in plant collections and identifications; Dr. Justin Douglas (KU, Lawrence) for assistance in running the NMR experiments, Dr. Victor Day for conducting the X-ray crystallographic analyses (NSF grant CHE-0923449, to V. Day), and Dr. Huaping Zhang for his critical reading of this manuscript. J.A. thanks LASPAU-Fulbright program and the University of Costa Rica for financial support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2012.02.019.

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Please cite this article in press as: Araya, J.J., et al. Verticillosides A–M: Polyoxygenated pregnane glycosides from *Asclepias verticillata* L. Phytochemistry (2012), doi:10.1016/j.phytochem.2012.02.019