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VANICOSIDES A AND B, PROTEIN KINASE C INHIBITORS FROM *POLYGONUM PENSYLVANICUM*

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ABSTRACT.—Two new protein kinase C inhibitory glycosides, vanicosides A [**1**] and B [**2**] were isolated from *Polygonum pensylvanicum* (Polygonaceae) by chromatographic techniques guided by bioassays for PKC inhibition. The structures of these glycosides were established primarily by analysis of ^{1}H- and homonuclear correlated nmr and high-resolution fab mass spectra of **1** and **2** and the derived octaacetate, **3**. Both glycosides were found to have *p*-coumaryl esters at the 1, 3, and 6 carbons of sucrose and a feruloyl ester at the 6′ carbon. Vanicoside A [**1**] also had an acetate group at the 2′ carbon.

Various members of the *Polygonum* genus, a member of the Polygonaceae, have been of interest for several years due to the different classes of natural products isolated from these relatively common species. Included among the classes of compounds isolated from *Polygonum* species are flavonoids (1–3), chalcone derivatives (3,4), anthraquinones (5), drimane-type sesquiterpenes related to warburganal (6,7), and glycosides (8). Although many of the reported studies did not employ activity-guided fractionation procedures, several of the compounds isolated in these investigations have also been reported to have biological activity. Warburganal, from *P. hydropiper*, was demonstrated to have cytotoxic activity (6), 5,7-dihydroxychromone, from *P. lapathifolium*, was reported to inhibit germination in velvetleaf seeds (9), and emodin, from *P. cuspidatum*, was found to be a protein tyrosine kinase inhibitor (5).

Extracts of *Polygonum pensylvanicum* L. have been screened for various types of biological activity over the past twenty years including cytotoxicity, but have not previously shown significant activity. However, the availability of several new bioassays prompted the reexamination of several plants, including *P. pensylvanicum*. Screening of an EtOH extract of *P. pensylvanicum* for activity in a protein kinase C inhibition assay gave significantly positive results. Protein kinase C (PKC) is a Ca^{2+} and phospholipid-dependent protein kinase which is involved in signal transduction, cellular proliferation, and cellular differentiation (10–14). PKC is activated by diacylglycerol and the tumor-promoting phorbol esters, and it has been suggested that the uncontrolled production of an active form of PKC may promote carcinogenesis. Some naturally occurring compounds, such as verbascoside (14) and 11-hydroxystaurosporine (15), which inhibit PKC activity have also demonstrated antineoplastic activity, indicating that a PKC inhibition assay should be useful in the search for new antineoplastic agents from natural sources. PKC has also been implicated in the trans- activation of HIV-1, and depletion of PKC reduces HIV-1 activation without affecting the synthesis of *tat* protein (16–18). Consequently, new inhibitors of PKC may also provide new leads for the treatment of HIV.

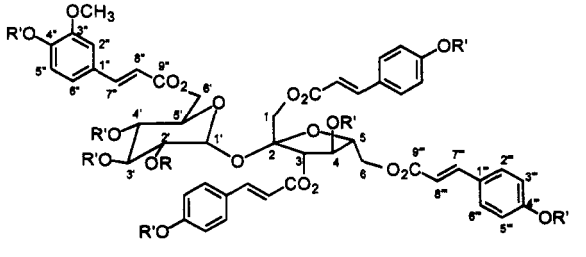
RESULTS AND DISCUSSION

As noted above, the EtOH extract of *P. pensylvanicum* demonstrated significant PKC inhibitory activity with an IC_{50} of 38 μg/ml. An attempt to partition the extract between CH_2Cl_2 and H_2O resulted in the bulk of the extract remaining insoluble in either layer, and the activity was concentrated in this insoluble material. The interfacial layer was then partitioned between H_2O and EtOAc, and, although the EtOAc layer contained some active material, the major active fraction was again the insoluble material.

Extraction of the interfacial material with several different solvents did not give significant purification, but did remove much of the activity from material that was persistently insoluble. In particular, extraction with hot Me_2CO seemed to be the most effective for this trituration. A small portion of the Me_2CO-soluble fraction was subjected to prep. tlc to give small amounts of two relatively pure compounds which demonstrated significant inhibition of PKC activity. The remainder of the Me_2CO-soluble fraction was then subjected to column chromatography over Si gel and eluted with a gradient of MeOH in EtOAc. This served to concentrate the active material into three fractions. Guided by the reference samples of the two active principles and by PKC inhibitory activity, the three active fractions were subjected to several column chromatographic and prep. tlc separations to isolate additional amounts of the active principles. The two pure compounds, which have been named vanicoside A [**1**] and vanicoside B [**2**], showed inhibition of PKC with IC_{50} values of 44 μg/ml and 31 μg/ml, respectively.

Vanicosides A and B were isolated as white, amorphous solids that resisted all attempts at crystallization. Initial ^{1}H-nmr studies of the two compounds showed that both were similar in structure with several aromatic moieties and the apparent presence of one or more carbohydrate rings, but not many other functional groups. The ir spectrum of each compound showed a large hydroxyl absorbance and a broad carbonyl absorbance at ca. 1695 cm^{-1}. The ir spectrum of vanicoside A also showed a carbonyl absorbance at 1719 cm^{-1}, suggesting the presence of at least two different types of carbonyl groups. Low-resolution fabms of the individual compounds indicated a molecular weight of 998 mass units for **1** and 956 mass units for **2**, suggesting that the two compounds differed by an acetate unit. There was indeed a single acetate methyl present in the ^{1}H-nmr spectrum of vanicoside A [**1**] at 2.06 ppm, but no acetate methyl visible in the spectrum of vanicoside B [**2**]. Acetylation of both compounds in individual experiments gave the identical octaacetate derivative, **3**, confirming that vanicosides A and B were identical in basic structure, differing only by the acetate moiety. Fabms of the octaacetate gave a molecular weight of 1292 mass units, indicating the addition of seven acetyl groups to vanicoside A [**1**] and eight to vanicoside B [**2**], which served to confirm the molecular weights of the parent compounds.

The ^{1}H-nmr spectra (Table 1) of both compounds were complex in the region between 6.3 and 7.8 ppm, which integrated for twenty-three protons in each case. The major part of the initial structure elucidation was carried out using the spectra of vanicoside B [**2**], since it was isolated in larger quantities. Apparent in this region of the spectrum were four sets of coupled doublets at 6.38 and 7.62 ppm, 6.42 and 7.69 ppm, 6.43 and 7.75 ppm, and 6.58 and 7.65 ppm. [Coupling pairs were determined from examination of the homonuclear correlated (COSY) nmr spectrum.] The coupling constants for each set of doublets was ca. 16 Hz, and each doublet integrated for one proton. These patterns suggested the presence of four trans-double bonds, each conjugated to a carbonyl group.



1 R=$COCH_3$; R′=H
2 R=R′=H
3 R=R′=$COCH_3$

TABLE 1. ^{1}H- (J in Hz) and ^{13}C-Nmr Chemical Shifts (in ppm) for **1**, **2**, and **3**.[a]

C	1		2		3	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	4.25, 4.6 m	63.80	4.2, 4.7 m	64.14	4.25 m	62.96
2	—	102.12	—	101.96	—	104.54
3	5.61 d (8.4)	77.64	5.69 d (8.5)	77.52	5.74 d (5.9)	77.25
4	4.65 m	72.65	4.8 m	72.77	5.57 m	75.98
5	4.3 m	79.82	4.25 m	79.79	4.5 m	79.85
6	4.6 m	64.55	4.6 m	64.54	4.5 m	64.55
1′	5.66 d (3.6)	89.18	5.58 d (3.7)	91.57	5.83 d (3.3)	90.75
2′	4.69 m	73.01	3.51 dd (3.7,9.7)	71.64	4.95 dd (3.4,10.5)	70.63
3′	3.9 m	70.79	3.75 dd (9.7,9.4)	70.80	5.51 dd (10.5,9.7)	70.05
4′	3.51 dd (9.4,9.4)	70.66	3.40 dd (9.5,9.6)	73.90	5.09 dd (9.7,9.7)	69.17
5′	4.3 m	70.79	4.35 m	70.86	4.5 m	69.27
6′	4.2, 4.35 m	64.49	4.2–4.4 m	64.35	4.5 m	64.65
1″, 1‴	—	125.69, 125.77, 125.84, 126.38	—	125.69, 125.78, 126.37	—	132.29, 132.33, 133.78
2″	7.33 d (1.8)	110.08	7.37 d (1.8)	110.09	7.40 d (1.5)	112.18
3″	—	148.97[b]	—	148.94[b]	—	153.12[b]
4″	—	147.62[b]	—	147.59[b]	—	152.22[b]
5″	6.81	114.94	6.84 d (8.1)	114.09	7.05 d (8.1)	123.70
6″	7.11 dd (1.8,8.2)	123.24	7.13 dd (1.8,8.2)	123.13	7.21 dd (1.5,8.1)	121.95
7″, 7‴	7.58, 7.62, 7.64, 7.72 ea d (16)	144.92, 145.10, 145.91	7.62, 7.65, 7.69, 7.75 ea d (16)	144.91, 144.79, 145.68	7.6–7.8 m	144.73, 144.95, 145.97
8″, 8‴	6.33, 6.40, 6.45, 6.53 ea d (16)	113.41, 113.87, 113.97, 114.53	6.38, 6.42, 6.43, 6.58 ea d (16)	113.55, 114.04, 114.65	6.51, 6.55, 6.61, 6.64 ea d (16)	117.34, 117.88, 117.99, 118.26
9″, 9‴	—	165.93, 165.96, 166.46, 166.64	—	165.85, 166.38, 166.61	—	165.55, 165.96, 166.38, 166.45
2‴, 6‴	7.45–7.65 m	130.02, 130.06, 130.31	7.49–7.60 m	129.96, 130.19	7.6–7.7 m	129.91, 130.17
3‴, 5‴	6.85–6.89 m	115.63, 115.64, 115.73	6.86–6.91 m	115.60	7.1–7.2 m	122.85
4‴	—	159.53, 159.63, 159.81	—	159.56, 159.77	—	165.55, 165.96, 166.38, 166.45
OCH_3	3.85 s	55.25	3.88 s	55.20	3.83 s	56.03
$COCH_3$	2.06 s	20.03	—	—	1.79, 1.90, 2.02, 2.04, 2.21, 2.22, 2.23 (2)	20.15, 20.20, 20.42, 20.60
$COCH_3$	—	170.12	—	—	—	168.44, 169.05, 169.73, 169.89, 170.26, 170.38

[a]In cases where some individual carbon resonances appear to be missing, these resonances are not sufficiently resolved from the resonances for analogous carbons at 75 MHz.

[b]These resonances may be interchanged.

The remaining fifteen protons could be attributed to aromatic moieties. One of the aromatic rings was clearly substituted in a 1,3,4-trisubstitution pattern, as seen from the COSY spectrum. A doublet of doublets (J=1.8, 8.2 Hz) at 7.13 ppm, which integrated for one proton, was coupled to a doublet (J=1.8 Hz) at 7.37 ppm, which also integrated for one proton, and to part of a multiplet at 6.83–6.91 ppm, which integrated for seven protons. The chemical shifts of the protons in this system suggested that the 2- and 6-protons of the aromatic ring were adjacent to an electron-withdrawing moiety, one of the

double bonds, while the 5-proton was adjacent to an electron-donating group, either a hydroxy moiety or the aromatic methoxy group noted as a three-proton singlet at 3.88 ppm. In the spectrum of the octaacetate derivative, **3** (Table 1), the 2-proton is seen at 7.4 ppm (d, J=1.5 Hz), the 6-proton at 7.21 ppm (dd, J=1.5, 8.1 Hz), and the 5-proton at 7.05 ppm (d, J=8.1 Hz). This downfield shift in the resonance for the 5-proton indicates that there was a hydroxy moiety at C-4 in the unacetylated compound **2**, and the lack of a significant shift in the position of the 2-proton indicates the presence of a methoxy group at C-3. Thus, these resonances must be due to a feruloyl ester moiety in the structure. The chemical shifts of the aromatic protons assigned to the feruloyl ester also corresponded well with analogous data from other natural products (19).

The remaining twelve aromatic protons could be seen as two coupled multiplets, each with six protons, at 6.86–6.91 ppm and 7.49–7.60 ppm. This suggested that there were three para-disubstituted aromatic rings, with one of the substituents being a hydroxy moiety and the other being one of the conjugated double bonds. The multiplet at 6.83–6.91 ppm shifted downfield to 7.10–7.17 ppm in the octaacetate derivative **3**, indicating that these resonances corresponded to the aromatic rings in three *p*-coumaryl esters.

Analogous resonances to those discussed above were seen in the ^{1}H-nmr spectra of vanicoside A, as was expected from the mass spectral evidence. At this point, the data strongly indicated that **1** and **2** were related to hydropiperoside, a glycoside previously isolated from *P. hydropiper* which contains three *p*-coumaryl esters at the 1, 3, and 6 positions of the fructofuranose ring of sucrose (8). Examination of the resonances between 3.5 and 5.7 ppm in the ^{1}H-nmr, COSY, and ^{1}H-^{13}C- correlated (HETCOR) spectra of vanicoside B [**2**] confirmed the presence of a sucrose moiety. A one-proton doublet (J=3.7 Hz) at 5.58 ppm due to the anomeric proton of the glucopyranose ring was coupled to a doublet of doublets at 3.51 ppm (1H, J=3.7, 9.7 ppm), which was coupled in turn to another doublet of doublets at 3.75 ppm (1H, J=9.7, 9.5 Hz). This latter resonance was coupled to a third doublet of doublets at 3.40 ppm (1H, J=9.5, 9.5 Hz) which was further coupled to a multiplet due to a methine at 4.35 ppm. Also covered by the multiple resonances between 4.2 and 4.38 ppm was a methylene due to the 6′-protons on the pyranose ring of the sucrose moiety. The stereochemistry of the anomeric proton was determined to be β from the coupling constant.

The fructofuranose ring was defined by the 3-proton which was apparent as a doublet at 5.69 ppm (1H, 8.5 Hz). This proton was coupled to a multiplet at 4.8 ppm, which was in turn coupled to a multiplet at 4.3 ppm. Each of these multiplets was correlated to an appropriate methine carbon in the ^{13}C-nmr spectrum. The methine at 4.3 ppm was correlated in the COSY spectrum to a methylene resonance at 4.6 ppm due to the 6-protons of the furanose moiety. The 1-protons were found as two resonances at 4.2 and 4.7 ppm, correlated to each other but to no other proton.

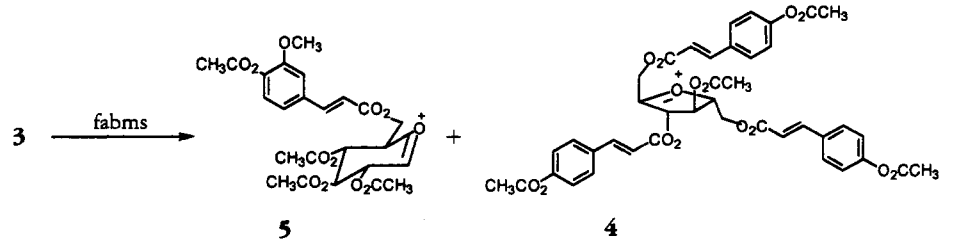
The resonances for the entire sucrose moiety were similar to those reported for hydropiperoside (8) with one notable exception, namely, the resonances for the 6′-methylene group were shifted downfield in the spectrum of vanicoside B [**2**] (4.2–4.38 ppm) from the values in the data reported for hydropiperoside (3.79 and 3.85 ppm). Even accounting for solvent differences, this shift indicated that one of the ester groups was located on the 6′-carbon. The locations of the remaining ester groups were determined by comparison of the spectra of **2** with the spectra of the octaacetate derivative **3**. In this comparison, there were significant downfield shifts for the protons on carbons 4, 2′, 3′, and 4′ in the ^{1}H-nmr spectrum of the octaacetate **3** from their positions in the spectrum of **2**. This indicated that there were hydroxyl groups on those carbons in **2**, and, as a

consequence, the remaining esters were located on carbons 1, 3, and 6 of the furanose ring.

The only remaining questions in the structure of **2** were the exact locations of the feruloyl ester and the three *p*-coumaryl esters. These were determined by examination of high-resolution fabms data of the octaacetate **3**. Two major fragments were obtained in the mass spectrum (Figure 1), one at *m/z* 769.2145 and one at *m/z* 507.1501. In these glycosides, fragmentation is expected to occur at the glycoside bond, as was seen in hydropiperoside (8), to give one fragment, **5**, from the pyranose ring and one, **4**, from the furanose ring. The first, higher mass fragment must be due to the furanose ring fragment **4**, inasmuch as it contains three of the nine carbon ester moieties. This fragment best matches a formula of $C_{41}H_{37}O_{15}$ (calcd 769.2132), indicating that this fragment must contain the three *p*-coumaryl esters. If the feruloyl ester had been part of this fragment, the ion would occur at *m/z* 799, but no ion was detected at this *m/z* value. Following a similar rationale for the other major fragment **5**, this fragment best matches a formula of $C_{24}H_{27}O_{12}$ (calcd 507.1502), indicating that the fragment contains the feruloyl ester. If the fragment contained a *p*-coumaryl ester instead of the feruloyl ester, the fragment would occur at *m/z* 477. Again, no ion was detected at *m/z* 477. These data confirm that the structure of vanicoside B is as shown in structure **2**, with the *p*-coumaryl esters in the furanose ring at carbons 1, 3, and 6 and the feruloyl ester in the pyranose ring at carbon 6′. Additional confirmation of this assignment came from the low-resolution fabms of both **1** and **2**, which showed a significant fragment ion at *m/z* 601, corresponding to **6**.

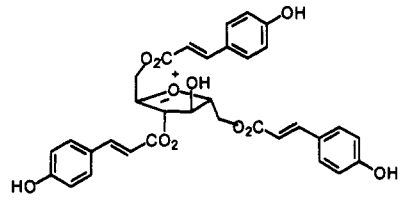
With the structure of vanicoside B [**2**] confirmed, determination of the structure of vanicoside A was relatively straightforward. The major question, insofar as both **1** and **2** gave the same octaacetate **3** and only differed by the presence of an acetate moiety in **1**, was the position of the acetate group in **1**. Examination of the ^{1}H-nmr and COSY spectra of **1** revealed that the principal difference was a downfield shift of the 2′-proton on the glucopyranose ring to 4.69 ppm in **1** from 3.51 ppm in **2**. There was also a slight downfield shift of the ^{13}C-nmr resonance for the 2′ carbon, 73.01 ppm in **1** versus 71.64 ppm in **2**. From these data, the acetate moiety in vanicoside A must be on the 2′ carbon of the glucopyranose ring, confirming the structure **1**.

Phenylpropanoid glycosides related to vanicosides A [**1**] and B [**2**] have been isolated from other plants in the families Polygalaceae (20), Rosaceae (21), Brassicaceae (22) and Liliaceae (23–26), as well as the previously noted hydropiperoside from *P. hydropiper* (Polygonaceae). Of these, the only biological activity reported was the growth inhibitory effects on lettuce seedlings of some feruloylsucrose principles from *Lilium longiflorum* (23). As noted previously, both vanicoside A [**1**] and vanicoside B [**2**] inhibited PKC activity at IC_{50} values of 44 μg/ml and 31 μg/ml, respectively. The octaacetate derivative **3** was inactive in the PKC inhibition assay (IC_{50}>50 μg/ml), indicating the requirement for free hydroxyl or phenol groups in these compounds to



5 4

FIGURE 1



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demonstrate activity. Both **1** and **2** also showed cytotoxicity against an MCF cell line at submicromolar dose levels. Other phenylpropanoid glycosides, most notably verbascoside (acteoside) (27), also show PKC inhibitory activity, and acteoside (verbascoside) has also demonstrated weak cytotoxicity (28). *P. pensylvanicum* contains other glycosides related to **1** and **2**, and efforts are currently underway to isolate these in quantities sufficient for structure elucidation and evaluation of PKC inhibitory activity.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Ir spectra were measured on a Perkin-Elmer Model 1600 Ftir spectrometer. Nmr spectra were recorded on a General Electric QE-300 spectrometer at 300 MHz (^{1}H) or 75 MHz (^{13}C) in Me_2CO-d_6 using residual Me_2CO as an internal standard. Low-resolution fabms were obtained at VCU and high-resolution fabms were obtained at the Midwest Center for Mass Spectrometry (partial support by the National Science Foundation, Biology Division, Grant No. DIR9017262) at the University of Nebraska. Analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN. PKC inhibition and MCF cytotoxicity assays were carried out at Sphinx Pharmaceutical Corporation in Durham, North Carolina.

PLANT MATERIAL.—Stems, leaves, flowers, and fruits of *P. pensylvanicum* (PR-17541) were collected in Florida in October 1968, and were supplied by the Medicinal Plant Resources Laboratory, USDA, Beltsville, Maryland, where voucher specimens are preserved.

ISOLATION OF VANICOSIDES A [**1**] AND B [**2**].—Dried, ground stems, leaves, flowers, and fruits of *P. pensylvanicum* (1.03 kg) were percolated in a Soxhlet extractor with 8 liters of 95% EtOH for 24 h. The resulting extract was concentrated *in vacuo* to give a dark viscous tar (69.2 g). This material was partitioned between CH_2Cl_2 (3×500 ml) and H_2O (500 ml). The material that would not dissolve was removed by filtration and partitioned between EtOAc (3×500 ml) and H_2O (500 ml). The remaining undissolved material was again removed by filtration and extracted with hot Me_2CO (6×150 ml). The Me_2CO layers were combined and concentrated *in vacuo* to give 5.9 g of a dark brown solid. A portion (116 mg) of this material was subjected to prep. tlc over Si gel 60 developed with 5% MeOH in EtOAc to give 8 components, of which two were active. Using these active constituents as tlc reference samples, and guided by bioassays for PKC inhibition, the remaining Me_2CO-soluble material was subjected to cc over Si gel eluted with EtOAc followed by EtOAc containing increasing amounts of MeOH. Several fractions which eluted with 1–2% MeOH in EtOAc either showed activity or spots similar to the reference samples. These fractions were triturated with CH_2Cl_2 to remove colored impurities. The remaining pale tan solids were individually subjected to cc over Si gel eluted with a gradient of MeOH in CH_2Cl_2. Fractions eluting between 4% and 8% MeOH in CH_2Cl_2 contained the two active principles. These were subjected to prep. tlc on Si gel 60 developed with 10% MeOH in CH_2Cl_2 (4–7 developments) and identical bands were combined to give 44.1 mg of vanicoside A [**1**] and 110.6 mg of vanicoside B [**2**]. A 10-g portion of the EtOAc solubles from the EtOAc-H_2O partition was processed in a similar fashion to yield an additional 268.0 mg of **1** and 709.3 mg of **2**.

Vanicoside A [**1**].—Amorphous white solid; mp 161–163°; ir (KBr) ν max 3425 (br), 1719, 1701, 1690, 1631, 1602 cm^{-1}; ^{1}H and ^{13}C nmr, see Table 1; fabms *m/z* 999 $[M+H]^+$, 601.

Vanicoside B [**2**].—Amorphous white solid; mp 156–159°; ir (KBr) ν max 3401 (br), 1696 (br), 1631, 1601 cm^{-1}; ^{1}H and ^{13}C nmr, see Table 1; fabms *m/z* 963 $[M+Li]^+$, 601.

Acetylation of **2**.—A suspension of vanicoside B [**2**] (197.6 mg) in 1 ml of pyridine-Ac_2O (1:1) was stirred at room temperature for 24 h. The solid went into solution after ca. 5 min. The solution was poured into ice-H_2O (5 ml) and extracted with CH_2Cl_2 (3×15 ml). The combined CH_2Cl_2 layers were washed with 1 N HCl (3×10 ml), saturated NaCl (10 ml), dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to a

white foam. The foam was crystallized and recrystallized from CH_2Cl_2/Et_2O to give 224.3 mg of white crystals of the octaacetate **3**. (A 5.1 mg sample of **1** was treated in a similar fashion to give the identical octaacetate **3**.) This compound exhibited: mp 111–112°; ir (KBr) ν max 1760, 1754, 1713, 1637, 1595 cm^{-1}; ^1H and ^{13}C nmr, see Table 1; fabms *m/z* 1299 $[M+Li]^+$; 769, 507; hrfabms *m/z* 1299.3654 ($C_{65}H_{64}O_{28}$+Li req. 1299.3743), 769.2145 ($C_{41}H_{37}O_{15}$ req. 769.2132), 507.1502 ($C_{24}H_{27}O_{12}$ req. 507.1502); *anal.* calcd for $C_{65}H_{64}O_{28}\cdot 1/2\ H_2O$: C 59.95, H 5.03; found C 60.00, H 4.96.

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