Pergamon

Tetrahedron 57 (2001) 9549–9554

TETRAHEDRON

Structure and stereochemistry of a novel bioactive sphingolipid from a *Calyx* sp.

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Received 10 August 2001; accepted 4 October 2001

Abstract—Bioassay-directed fractionation of a sponge of the genus *Calyx* using a yeast bioassay for DNA-damaging agents yielded the novel sphingolipid calyxoside (**1**) as the major bioactive constituent. The structure of **1** was assigned as 1,3,26-trihydroxy-2,27-diaminooctacosan-18-one-1-β-D-glucoside by ^{1}H- and ^{13}C NMR, DEPT, DQCOSY, HMQC, and HMBC spectra. The carbonyl group was located at C-18 by analysis of the EI-MS fragmentation of the amino derivative of its aglycone pentaacetate. Its absolute configuration was determined as 2*S*,3*R*,26*S*,27*S* by analysis of the ^{1}H NMR and CD spectra of its aglycone pentabenzoate. © 2001 Published by Elsevier Science Ltd.

1. Introduction

In continuation of our search for novel natural products with potential as anticancer agents,[1–4] a methanol extract of a *Calyx* sp. was examined and found to show reproducible and selective bioactivity in our yeast assay for DNA-damaging agents,[1] and it was thus selected for bioassay-directed fractionation. Sponges of the genus *Calyx*, belonging to the family *Oceanapiidae*, have been the source of a number of interesting compounds. They have been reported to contain novel steroids with acetylene or cyclopropene groups in their side chains,[5,6] diketopiperazines with antibiotic and phytotoxic properties,[7] unique diphenyloxolanes,[8] pyridinetetradecanamines[9] and 11-methylpentadecanoic acid.[10]

2. Results and discussion

Partition of a CH_2Cl_2/methanol extract of the sponge between aqueous MeOH and *n*-hexane, followed by partition of the aqueous MeOH fraction with CH_2Cl_2, yielded a bioactive aqueous MeOH fraction with an IC_{12} of 148 μg mL^{-1} in the rad52Y yeast assay. Purification of this fraction by repeated chromatography on MCI gel (a porous polystyrene gel) with elution with 50% aqueous MeOH and monitoring of the fractions by bioassay and by ^{1}H NMR spectroscopy yielded the novel bioactive compound **1**, designated calyxoside.

Calyxoside (**1**) gave a positive ninhydrin test and did not show any absorption above λ 210 nm in its UV spectrum, indicating the presence of amino group(s) and the absence of any conjugation. Its molecular formula was $C_{34}H_{69}N_2O_9$, as deduced from HRFAB-MS, and this was confirmed by HRFAB-MS of its octaacetate derivative **2**. Its ^{13}C NMR and DEPT spectra in combination with its molecular formula indicated the presence of a terminal methyl group (δ_H 1.29 ppm (d) and δ_C 16.0 ppm), a ketone group (δ_C 214.3 ppm), an acetal group (δ_C 104.0 ppm and δ_H 4.35 (d, *J*=7.63 Hz), two oxygenated methylenes, six oxygenated methines and two methines with amino groups. The remaining carbons and hydrogens were assigned to a long alkyl chain based on the overlapping signals for methylene protons and the corresponding carbon signals.

Because of the overlap of the terminal methyl signal with methylene signals at higher field and the overlap of some signals in the δ 3–4.2 ppm region of the ^{1}H NMR spectrum of **1** (Table 1), calyxoside was converted to its octaacetate **2**, and the NMR spectra of **2** were used for structural analysis. The ^{13}C NMR spectrum of **2** in $CDCl_3$ showed signals for a ketone group (δ_C 211.7 ppm) and eight acetyl groups (δ_C 169.4, 169.5, 169.5, 169.7, 170.1, 170.8, 170.6, and 171.1; δ_H 1.96, 1.96, 1.98, 2.00, 1.02, 2.03, 2.06, and 2.07). Its DQCOSY and HMBC spectral data (Table 1) indicated the presence of three spin systems. The signals at δ_C 100.6 ppm (d) and δ_H 4.44 ppm (d, *J*=7.9 Hz), δ_C 71.3 ppm (d) and δ_H 4.92 (dd, *J*=7.9, 9.6 Hz), δ_C 72.6 ppm (d) and δ_H 5.17 (dd, *J*=9.6, 9.9 Hz), δ_C 68.2 ppm (d) and δ_H 5.04 (dd, *J*=9.9, 9.5 Hz), δ_C 71.8 ppm (d) and δ_H 3.68 (dd, m), δ_C 61.8 ppm (t) and δ_H 4.22 (dd, *J*=12.1, 4.1 Hz), and δ_H 4.13 (dd, *J*=12.1, 2.3 Hz) suggested the presence of a glucose moiety. The groups $CH_3CH(NHCOCH_3)CH(OCOCH_3)CH_2$– and $ROCH_2CH(NHCOCH_3)CH(OCOCH_3)CH_2$–, were assigned to two additional spin

Keywords: sphingolipid; calyxoside; structure and stereochemistry; bioactivity.

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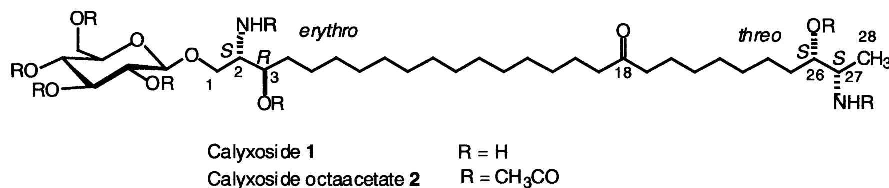
PII: S0040-4020(01)00958-9

Table 1. ^{1}H- and ^{13}C NMR data for calyxoside **1**, its octaacetyl derivative **2**, and oceanapiside **6**



Atom no.	Calyxoside **1** (in CD_3OD)		Calyxoside octaacetate **2** (in $CDCl_3$)				**6**
	δ_C	δ_H (mult. J Hz)	δ_C	δ_H (mult. J Hz)	DQCOSY	HMBC (C to H)	δ_C
1	66.7	4.02 (dd, 11.5, 8.7) 3.86 (dd, 11.5, 3.7)	67.1	3.88 (dd, 10.3, 3.9) 3.59 (dd, 10.3, 4.5)	H-2	H-1′, H-2	58.9
2	56.7	3.40 (m)	50.8	4.19 (m)	H-1a, H-1b, H-3	H-1a, H-1b, H-3	57.2
3	70.1	3.84 (m)	74.1	4.83 (m)	H-2, H-4	H-1a, H-1b, H-2	80.1
4	34.1	1.48 (m)	31.5[a]	1.51 (m)	H-3	H-2	33.1
5	27.0						26.7
6–9							
10							43.4
11							214.3
12							43.4
13–15							
16	24.8		29.6	1.51 (m)	H-17		
17	43.4	2.43 (t, 7.2)	42.7[a]	2.35 (t, 7.5)	H-16		
18	214.3		211.7			H-17, H-19	
19	43.5	2.43 (t, 7.2)	42.8[a]	2.35 (t, 7.5)	H-20		
20			29.6	1.51 (m)	H-21		
21–23							
24	26.2						26.2
25	34.5	1.42 (m)	31.4[a]	1.51 (m)	H-26	H-27	34.5
26	73.1	3.46 (m)	76.4	4.83 (m)	H-25, H-27	H-28	73.1
27	53.5	3.12 (m)	47.8	4.19 (m)	H-26, H-28	H-25	53.5
28	16.9	1.26 (d)	18.4	1.07 (d, 6.9)	H-27	H-26	16.0
1′	104.5	4.35 (d, 7.6)	100.6	4.44 (d, 7.9)	H-2′	H-1a, H-1b, H-3′	104.5
2′	74.8	3.25 (m)	71.3	4.92 (dd, 7.9, 9.6)	H-1′, H-3′	H-1′, H-3′, H-4’	74.8
3′	77.7	3.32 (m)	72.6	5.17 (dd, 9.6, 9.9)	H-2′, H-4	H-1′, H-4′	77.7
4′	71.4	3.29 (m)	68.2	5.04 (dd, 9.9, 9.5)	H-3′, H-5′	H-3′, H-6a′, H-6b′	71.6
5′	78.0	3.40 (m)	71.8	3.68 (m)	H-4′, H-6a′, H-6b′	H-1′, H-4′, H-6a′, H-6b′	78.1
6′	62.4	3.66 (dd, 11.8, 5.7) 3.89 (dd, 11.8, 3.7)	61.8	4.22 (dd, 12.1, 4.7) 4.13 (dd, 12.1, 2.3)	H-5′	H-4′	62.6
2-NH				6.09 (d, 8.9)	H-2		
27-NH				5.57 (d, 9.3)	H-27		
			[b]	[c]			

[a] These values may be interchanged.
[b] δ_C 169.4, 169.5, 169.7, 170.1, 170.6, 170.8, and 171.1.
[c] δ_H 1.96 (s), 1.96 (s), 1.98 (s), 2.00 (s), 2.02 (s), 2.03 (s), 2.06 (s), and 2.07 (s).

systems, consistent with the two terminal sequences of a long chain sphingolipid type of compound.

When **1** was heated with 1% HCl in 50% aqueous MeOH, it was hydrolyzed to glucose and the aglycone calyxinin (**3**). The water-soluble fraction of the hydrolyzate was treated with acetic anhydride and pyridine to convert it to its pentaacetate, which was identified as glucose pentaacetate by GC–MS comparison with authentic material. The chemical shifts and coupling constants of the sugar moiety in **1** and **2** confirmed the assignment of the sugar as glucose, and the coupling constant of the anomeric proton ($J_{1',2}$=7.6 Hz in **1** and $J_{1',2}$=7.9 Hz in **2**) indicated that calyxoside is a β-glucoside. The long range correlation between H-1′ (δ_H 4.35 ppm, d, J=7.6 Hz) and C-1 (δ_C 66.7 ppm, t) in the HMBC spectrum of **1** and the long range correlations between H-1′ (δ_H 4.44 ppm, d, J= 7.9 Hz) and C-1 (δ_C 67.1 ppm, t) and H-1 (δ_H 3.88 ppm, dd, J=10.30, 3.89 Hz, and δ_H 3.59 ppm, dd, J=10.30, 4.5 Hz) with C-1′ (δ_C 100.6 ppm, t) in the HMBC spectrum of calyxoside octaacetate **2** indicated that glucosylation was on C-1. The assignment of the D stereochemistry to the glucose moiety was based on the hydrolysis of calyxoside by almond β-glucosidase, specific for β-D-glucosides or

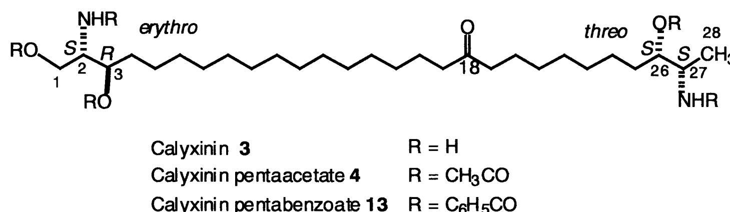


Table 2. Partial ^{1}H NMR data of the α-termini of compounds **8**, **13**, and model compounds

α-Terminus	Chemical shifts (ppm)				*J*-values (Hz)	
	δ_{C2-NH}	δ_{3-H}	δ_{2-H}	δ_{1-H}	J_{NH-H-2}	$J_{H-2-H-3}$
Model compound **11** (*erythro*)[a]	7.10	5.41	4.87	4.61, 4.65	8.7	4.1
Oceanin perbenzoate **8** (*erythro*)[a]	7.08	5.38	4.88	4.61, 4.64	8.6	3.8
Model compound **12** (*threo*)[a]	6.63	5.56	4.89	4.48, 4.57	9.2	4.8
Calyxinin pentabenzoate **13** (*erythro*)	7.10	5.38	4.88	4.62, 4.66	8.7	4.1

[a] See Ref. 15.

β-D-galactosides,[11] confirming that **1** is a sphingolipid 1-β-D-glucoside.

Treatment of the aglycone calyxinin (**3**) with acetic anhydride in pyridine converted it to calyxinin pentaacetate (**4**), which had the molecular composition $C_{38}H_{68}N_2O_9$ (HRFAB-MS), indicating that the aglycone **3** contains 28 carbons. The ^{1}H- and ^{13}C NMR spectra of **4** confirmed the presence of a carbonyl group and the terminal functional groups. This evidence thus established the structure of calyxoside as an unusual glucosylated ketosphingolipid, but did not serve to identify the location of the carbonyl group or the stereochemistry of the two aminoalcohol end units.

Two ketosphingolipids have been reported previously, rhizochalin (**5**),[12,13] a sphingolipid 3-galactoside, and oceanapiside (**6**),[14] a sphingolipid 3-β-D-glucoside. The ^{13}C NMR spectrum of calyxoside was very similar to the literature spectrum of oceanapiside except for the values of δ_{C-1}, δ_{C-2} and δ_{C-3}, consistent with glucosylation at C-1 in **1** and at C-3 in **6**.

In agreement with previous studies on oceanapiside, direct attempts to determine the position of the carbonyl group in **1** or **2** by mass spectrometry were unsuccessful, since fragmentation was dominated by cleavages α to the amino groups and no ions could be assigned with confidence to cleavages α to the carbonyl group. The position of the carbonyl group in rhizochalin was assigned by conversion of its acetate to a mixture of esters by Baeyer–Villiger oxidation followed by EI-MS, while its location in oceanapiside was assigned by MALDI MS/MS on a deuterated sample which took more than two months to prepare. Neither of these approaches seemed attractive, and we elected to convert the carbonyl group of calyxinin pentaacetate (**4**) to an amino group to facilitate cleavage α to the erstwhile carbonyl group. Compound **4** was thus converted to 18-aminocalyxinin pentaacetate (**7**) by reductive amination with lithium cyanoborohydride in the presence

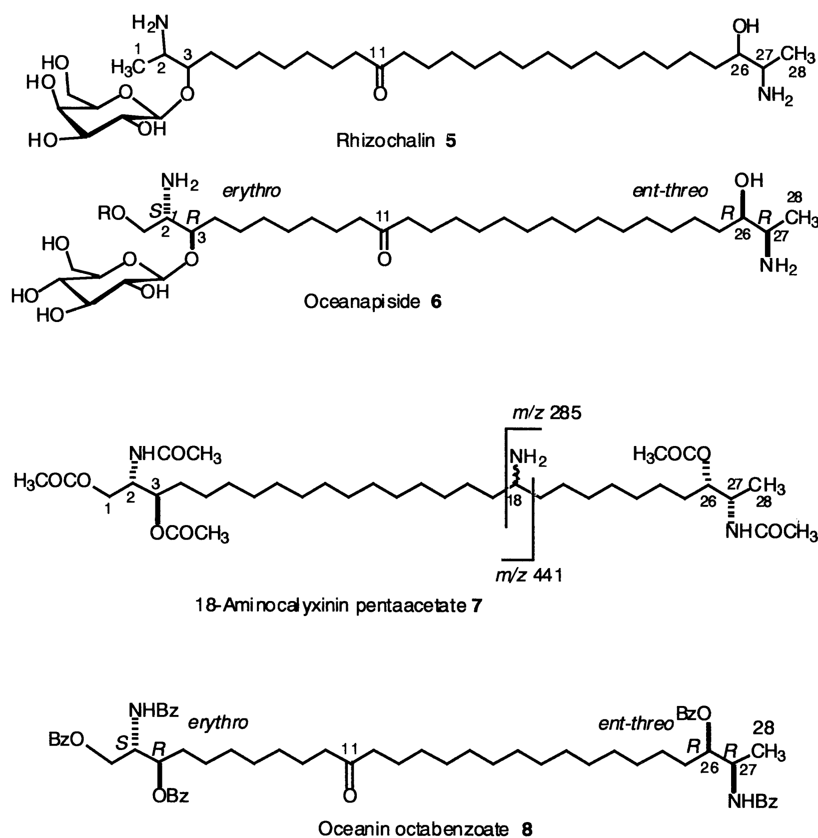


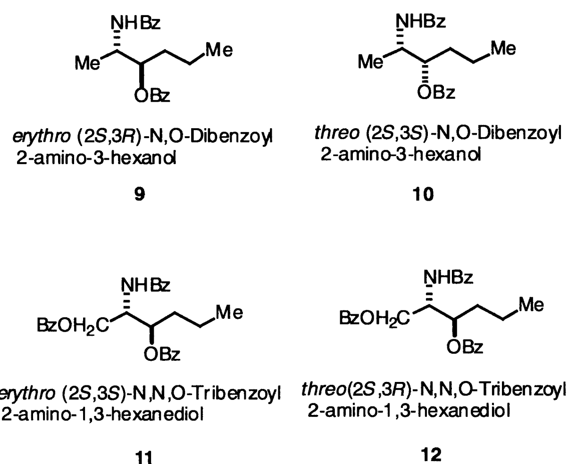
Table 3. Partial ^{1}H NMR data of the ω-termini of compounds **8**, **13**, and model compounds

ω-Terminus	Chemical shifts (ppm)				*J*-values (Hz)	
	$\delta_{C27\text{-}NH}$	$\delta_{26\text{-}H}$	$\delta_{27\text{-}H}$	$\delta_{28\text{-}H}$	$J_{NH\text{-}H\text{-}27}$	$J_{H\text{-}26\text{-}H\text{-}27}$
Model compound **9** (*erythro*)[a]	6.97	5.24	4.46	0.97	7.8	2.6
Model compound **10** (*threo*)[a]	6.39	5.24	4.53	0.94	9.0	5.2
Oceanin perbenzoate **8** (*ent-threo*)[a]	6.38	5.21	4.53	1.28	8.9	5.2
Calyxinin pentabenzoate **13** (*threo*)	6.38	5.21	4.53	1.29	9.0	5.3

[a] See Ref. 15.

of ammonium acetate and molecular sieves. The predominant fragment ions of **7** at *m/z* 441 ($C_{24}H_{45}N_2O_5$) and 285 ($C_{15}H_{28}N_2O_3$), corresponding to cleavage of the C-18/C-19 and C-17/C-18 bonds, respectively, indicated that the amino group was located on C-18. The carbonyl group in **1** could thus be assigned to the C-18 position.

The stereochemistry of calyxoside remained to be assigned. Fortunately the absolute configuration of oceanapiside (**6**) was recently determined by a careful analysis of the ^{1}H NMR and CD spectra of its perbenzoyl aglycone (**8**) in comparison with the corresponding spectra of the synthetic model compounds **9–12**.[15] In this work it was observed that the CD spectrum of the aglycone perbenzoate **8** was the sum of the expected local CD spectra at each terminus, since the 22 carbons separating the termini greatly diminished intramolecular exciton coupling and intramolecular effects were negligible due to the high dilution of sample (10^{-4}–10^{-5} M) used in the determination.



We elected to use this approach to assign the stereochemistry of calyxoside. Calyxinin pentabenzoate **13** was thus prepared, and its ^{1}H NMR spectrum was compared with those of the model compounds **9–12** as well as that of oceanin octabenzoate **8**, as shown in Tables 2 and 3. The *threo* and *erythro* configurations at the α- and ω-termini could be differentiated by comparison of their chemical shifts and coupling constants with those of the model compounds **9–12**. The similarities of $\delta_{C\text{-}2,NH}$, $J_{NH\text{-}H\text{-}2}$, and $J_{H\text{-}2\text{-}H\text{-}3}$ as well as of $\delta_{H\text{-}27}$, $J_{NH\text{-}H\text{-}27}$, and $J_{H\text{-}26\text{-}H\text{-}27}$ of **13** with the corresponding values of **8** and of the appropriate model compounds indicated that calyxinin and calyxoside have the same relative configuration as oceanin, with the *erythro* configuration at the α-terminus and the *threo* configuration at the ω-terminus.

The CD spectrum of calyxinin pentabenzoate **13** showed $\Delta\varepsilon$ values of 25.06 at 237 nm and 10.40 at 220 nm. Comparison with the data in Table 4 indicated that the absolute configuration of calyxinin pentabenzoate is (2*S*,3*R*) at the α-terminus and (26*S*,27*S*) at the ω-terminus. Calyxoside thus differs in absolute configuration from oceanapiside, which is (2*S*,3*R*) at the α-terminus but (26*R*,27*R*) at the ω-terminus.

It could thus be concluded that calyxoside **1** is a new natural product with the structure (2*S*,3*R*,26*S*,27*S*)-2,27-diamino-1,3,26-trihydroxynonacosan-18-one-1-β-D-glucoside. Its structure is unusual in being only the sixth member of a small class of 'two-headed' sphingolipids. In addition to the keto compounds rhizochalin[12] and oceanapiside[14,15] previously mentioned, the other members include the polyenes leucettamols A and B[16] and BRS1.[17]

Calyxoside (**1**) was tested against the RS322 (rad52), the RS188N (RAD^+) and RS321(rad52.top1) yeast strains.

Table 4. CD spectral values (MeOH) for compounds **13**, **8**, and model compounds

Compound	α-Terminus stereochemistry	ω-Terminus stereochemistry	$\Delta\varepsilon_1$(237 nm)	$\Delta\varepsilon_2$(220 nm)	*A* value ($\Delta\varepsilon_1-\Delta\varepsilon_2$)
Calyxinin pentabenzoate **13**	*erythro*	*threo*	−5.06	+0.40	−5.46
Oceanin octabenzoate **8**[a]	*erythro*	*ent-threo*	−9.61	+7.12	−16.73
12+**10**[a]	*threo*	*threo*	−0.57	−3.19	+2.62
11+**10**[a]	*erythro*	*threo*	−5.13	+0.73	−5.86
12+**9**[a]	*threo*	*erythro*	−9.19	+1.92	−11.11
11+**9**[a]	*erythro*	*erythro*	−13.75	+5.85	−19.60
12+*ent*-**10**[a]	*threo*	*ent-threo*	−6.57	+3.86	−10.43
11+*ent*-**10**[a]	*erythro*	*ent-threo*	−11.13	+7.86	−18.99
10+*ent*-**9**[a]	*threo*	*ent-erythro*	+2.06	−1.25	+3.31
11+*ent*-**9**[a]	*erythro*	*ent-erythro*	−5.63	+2.67	−8.30

[a] See Ref. 15.

Compound **1** had IC_{12} values of 62 and 36 μg mL^{-1} in RS321 and RS322, respectively, but was inactive (IC_{12}>1000 μg mL^{-1}) in the repair-proficient strain RS188N. These data are characteristic of a selective DNA-damaging agent which does not act as a topoisomerase I or topoisomerase II inhibitor. Compound **1** was also tested for cytotoxicity in several mammalian cell lines, with the following results (cell line: IC_{50} value, μg mL^{-1}): HFF, 20; MRC-5, 20, SW480, 5.0, HT-29, 10, Saos-2, 5.0, DLD-1, 5.0, H460, 3.0. These data indicate that **1** has a relatively weak cytoxicity, without any strong selectivity for any cell line; it is thus unlikely to be an attractive candidate for further development based on this activity profile.

3. Experimental

3.1. General experimental procedures

The CD spectrum was recorded on a JASCO J720 Spectropolarimeter. NMR spectra were recorded on a Varian Unity 400 NMR instrument at 399.951 MHz for ^{1}H and 100.578 MHz for ^{13}C, using standard Varian pulse sequence programs. Low resolution mass spectra were taken on a VG 7070 E-HF at VPI&SU. High resolution mass spectra were obtained on a Kratos MS50 instrument at the Nebraska Center for Mass Spectrometry. MCI gel (75–150 μm particle size) was obtained from Mitsubishi Chemicals. Other conditions were as previously described.[1]

3.2. Yeast bioassay

The experimental methods utilized in the *Saccharomyces cerevisieae* bioassay using DNA repair deficient mutants were performed as previously described.[18] The IC_{12} values refer to the concentration in μg mL^{-1} required to produce a zone of inhibition 12 mm diameter around a 100 μL well during a 48 h incubation period at 37°C.

3.3. Materials

Calyx sp. **2** (Oceanapiidae) (SCLID 41689/88) was collected by Dr Patrick Colin, Coral Reef Research Foundation, Palau, in Sulawesi in May 1993. A voucher specimen, number 0CDN1359, was deposited at the Smithsonian Institution. The taxonomic assignment was made by Dr Michelle Kelly, NIWA, NZ. The material was extracted with CH_2Cl_2/MeOH at the NCI Frederick Research and Development Center, Frederick, MD to yield extract C011149, also known as SCLID 41688.

3.4. Isolation of calyxoside

The MeOH extract SCLID 41688 (3.36 g, IC_{12} 625 μg mL^{-1} in the RS322 (Rad52Y) assay and IC_{12} 1250 μg mL^{-1} in the RS321 assay) was partitioned between *n*-hexane and MeOH/H_2O (60:40) and this was then diluted to MeOH/H_2O (50:50) and extracted with CH_2Cl_2. The resulting aqueous MeOH fraction (1.17 g) was bioactive with IC_{12} 148 μg mL^{-1} in the RS322 assay and IC_{12} 125 μg mL^{-1} in the RS321 assay, and the *n*-hexane and CH_2Cl_2 fractions were inactive in this assay. The bioactive aqueous MeOH fraction (1.1682 g) was subjected to cc on MCI gel (30 g) with 50% aqueous MeOH; 33 fractions of 10 mL each were collected. Fractions 13–17 were bioactive and gave typical lipid signals in their ^{1}H NMR spectra. After repeated cc on MCI gel with 50% aqueous MeOH, calyxoside (**1**, 98 mg) was obtained from fractions 13–15. It had IC_{12} values of 36.2 μg mL^{-1} against RS322 and 62.5 μg mL^{-1} against RS321. None of the fractions nor calyxoside (**1**) showed any activity against the wild-type RS188 yeast strain.

3.4.1. Calyxoside 1. Colorless gel-like substance; $[\alpha]^{25}_{D}=-15.8$ (*c* 0.321, MeOH); IR (KBr, film) ν_{max} 3600–2900 (OH) and 1712 cm^{-1} (CO); ^{1}H NMR and ^{13}C NMR data, see Table 1. HRFAB-MS $(M+H)^+$ *m/z* 649.5024 $(M+H)^+$ ($C_{34}H_{69}N_2O_9$ calcd *m/z* 649.5037).

3.4.2. Calyxoside octaacetate 2. Calyxoside **1** (4 mg) was dissolved in acetic anhydride (0.2 mL) and pyridine (20 μL) and the mixture stirred for 12 h at room temperature. The product was worked up in the usual way to give calyxoside octaacetate (3 mg) as a colorless gel-like substance: $[\alpha]^{25}_{D}=-5.23$ (*c* 0.38, $CHCl_3$), ^{1}H-NMR ^{13}C-NMR, DQCOSY, and HMBC data, see Table 1; HRFAB-MS *m/z* 985.5820 $(M+H)^+$ (calcd for $C_{50}H_{85}N_2O_{17}$, *m/z* 985.5848).

3.4.3. Hydrolysis of calyxoside. A solution of calyxoside (**1**, 10.2 mg) was mixed with 1% HCl (2 mL) in 50% aq. MeOH solution. The reaction mixture was stirred at room temperature overnight. The MeOH was removed by evaporation and the reaction mixture diluted with H_2O (2 mL), neutralized with NH_4OH, and extracted with CH_2Cl_2. The aqueous solution was evaporated to dryness, and the residue was stirred in acetic anhydride (0.5 mL) and pyridine (3 drops) at room temperature overnight. After removing the reagents with N_2, the product was analyzed by GC–MS. The retention time of the major peak and its EI-MS spectrum were the same as those of an authentic standard of D-glucose pentaacetate.

Evaporation of the CH_2Cl_2 extract yielded the aglycone calyxinin (**3**) as a colorless gum-like substance: $[\alpha]^{25}_{D}=-2.17$ (*c* 0.23, MeOH); ^{1}H NMR δ ppm (Hz) in CD_3OD/$CDCl_3$: 1.06 (3H, t, J=6.6 Hz), 1.2–1.6, 2.44 (4H, t, J=7.21 Hz), 2.72 (2H, m), 3.21 (1H, m), 3.49 (4H, m), 3.72 (1H, m), and ^{13}C NMR δ ppm in CD_3OD/$CDCl_3$: 18.9, 24.6, 24.7, 26.4, 26.8, 30.0(0), 30.0(4), 30.3–30.5, 34.2, 34.4, 43.4, 52.2, 57.6, 63.8, 76.4.

3.4.4. Calyxinin pentaacetate 5. Calyxinin (5.67 mg) was mixed with acetic anhydride 0.2 mL and anhydrous pyridine (3 drops) and stirred overnight at room temperature. The reaction mixture was worked up in the usual way to give calyxinin pentaacetate (5 mg); ^{1}H NMR δ ppm (Hz) in $CDCl_3$ 1.10 (3H, d, J=6.80 Hz, 3×H-28), 1.2–1.7, 1.99 (3H, s, CH_3CO–), 2.00 (3H, s, CH_3CO–), 2.06 (3H, s, CH_3CO–), 2.07 (3H, s, CH_3CO–), 2.09 (3H, s, CH_3CO–), 2.37 (4H, t, J=7.41 Hz, 2×H-17 and 2×H-19), 4.06 (1H, dd, J=11.59, 3.92 Hz, H-1b), 4.20 (1H, m, H-27), 4.25 (1H, dd, J=11.59, 6.08 Hz, H-1a), 4.39 (1H, m, H-2), 4.85 (1H, m, H-26), 4.90 (1H, m, H-3), 5.53 (1H, d, J=8.86 Hz, –N*H*COCH$_3$), 5.89 (1H, d, J=9.27 Hz, –N*H*COCH$_3$); ^{13}C NMR δ ppm in $CDCl_3$ 18.5 (C-28), 28.8–31.5 (–CH_2– and

CH_3CO– signals), 42.7 (C-17 or C-19), 42.8 (C-17 or C-19), 47.1 (C-27), 50.5 (C-2), 62.6 (C-1), 74.0 (C-3), 76.4 (C-26), 169.4 (–C=O), 169.7 (–C=O), 170.9 (–C=O), 170.9 (–C=O), 171.0 (–C=O), 211.7 (C-18); HRFAB-MS *m/z* 697.4991 $(M+H)^+$ (calcd for $C_{38}H_{69}N_2O_9$, *m/z* 697.5003).

3.4.5. Reductive amination of calyxinin pentaacetate 5 by lithium cyanoborohydride. Calyxinin pentaacetate (**5**, 4.13 mg, 0.0059 mmol) was mixed with molecular sieves (3.3 mg), NH_4OAc (12.2 mg, 0.052 mmol) in 1 mL absolute MeOH. A solution of $LiBH_3CN$ (0.33 mg, 0.0052 mmol) in absolute MeOH was added dropwise. The reaction mixture was stirred for 48 h at room temperature. After removal of most of the MeOH, the residue was taken up in H_2O (5 mL) and extracted with CH_2Cl_2 (4×5 mL). After washing with saturated NaCl solution and water and drying with anhydrous Na_2SO_4, the CH_2Cl_2 fraction was evaporated in vacuo to give 2.1 mg 18-aminocalyxinin pentaacetate (**7**): EI-MS *m/z* 696 $(M-1)^+$, 441 $(C_{24}H_{45}N_2O_5)^+$, 381, 285 $(C_{15}H_{28}N_2O_3)^+$, 225.

3.4.6. Calyxinin pentabenzoate 13. A mixture of calixinin (**3**, 2.25 mg, 4.6 μmol), DMAP (1 mg), benzoic acid (11.0 mg, 0.09 mmol), and EDC (16.5 mg, 0.09 mmol) in 1 mL CH_2Cl_2 was stirred at room temperature overnight. The product was purified by PTLC on Si gel with the solvent (CH_2Cl_2/EtOAc (10:1) to give 3.3 mg (71.1%) of calyxinin pentabenzoate (**13**): CD $\Delta\varepsilon=+0.40$ at λ 219 nm and $\Delta\varepsilon=-5.06$) at λ 235 nm (*c* 0.00021, MeOH), ^1H NMR δ ppm (Hz) in $CDCl_3$: 1.29 (3H, d, *J*=6.8 Hz, 3×H-28), 1.1–1.8, 1.75 (2H, m, H-25), 1.88 (1H, m, H-4b), 1.95 (1H, m, H-4a), 2.34 (4H, t, *J*=7.41 Hz, 2×H-17 and 2×H-19), 4.53 (1H, m, H-27), 4.63 (2H, m, 2×H-1), 4.88 (1H, m, H-2), 5.21 (1H, m, H-26), 5.38 (1H, m, H-3), 6.38 (1H, d, *J*=9.06 Hz, –N*H*$COCH_3$-27), 7.10 (1H, d, *J*=8.86 Hz, –N*H*$COCH_3$-2), 7.2–8.2 (aromatic proton signals), ^{13}C NMR δ ppm in $CDCl_3$ 18.5 (C-28), 23.7–30.0, 31.7 (C-25), 32.2 (C-4), 42.7 and 42.8 (C-17 and C19), 48.3 (C-27), 51.7 (C-2), 63.0 (C-1), 75.9 (C-3), 77.2 (C-26), 120–140 (aromatic carbon signals), 166.7 (–*C*OO–), 166.9 (–*C*OO–), 167.0 (–*C*OO–), 167.1 (2×–*C*OO–), 211.7 (C=O at C-18); HRFAB-MS *m/z* 1007.5768 (calcd for $C_{63}H_{78}O_9N_2+H$, *m/z* 1007.5786).

Acknowledgements

This work was supported by a National Cooperative Drug Discovery Group award to the University of Virginia (U19 CA 50771, Dr S. M. Hecht, Principal Investigator), and this support is gratefully acknowledged. We thank Dr Gordon Cragg, Dr David Newman, and the National Cancer Institute for making the extract of *Calyx* sp. available through the NCI Natural Products Repository. We also thank Mr Kim Harich and Mr William R. Bebout, Sr., (Virginia Polytechnic Institute and State University) and the Nebraska Center for Mass Spectrometry for low and high resolution mass spectra, respectively.

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