Antibacterial and Cytotoxic Activity of Prenylated Bicyclic Acylphloroglucinol Derivatives from *Hypericum amblycalyx*

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Two new bicyclic acylphloroglucinol derivatives, hypercalyxone A (1-[5,7-dihydroxy-2-methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-chroman-8-yl]-2-methyl-propan-1-one, **1**) and B (1-[5,7-dihydroxy-2-methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-chroman-8-yl]-2-methyl-butan-1-one, **2**), have been isolated from the petroleum ether extract of the aerial parts of *Hypericum amblycalyx*, together with two further compounds (1-[5,7-dihydroxy-2-methyl-2-(4-methyl-pent-3-enyl)-chroman-8-yl]-2-methyl-propan-1-one, **3** and 1-[5,7-dihydroxy-2-methyl-2-(4-methyl-pent-3-enyl)-chroman-8-yl]-2-methyl-butan-1-one, **4**), which have been described only as semi-synthetic products. In addition, the known triterpene lup-20(29)-en-3-one was obtained. Structure elucidation was based on 1D and 2D NMR studies, as well as on data derived from mass spectrometry. The four acylphloroglucinol derivatives were evaluated for their cytotoxic and antibacterial activity. All compounds showed moderate cytotoxic activity against KB and Jurkat T cancer cells. Especially compounds **3** and **4** exhibited a strong antibacterial activity against different Gram-positive strains.

Key words: Hypericum amblycalyx, Acylphloroglucinols, Antibacterial Activity

Introduction

In our continuing search for biologically active metabolites from *Hypericum* species (Winkelmann *et al.*, 2000, 2001a, b; Heilmann *et al.*, 2002) we report here on the first phytochemical investigation of *Hypericum amblycalyx* Coust. & Gand., an endemic plant of east Crete (Turland *et al.*, 1993). Using cytotoxicity against KB cancer cells as a lead, two new phloroglucinol derivatives (**1**, **2**) were isolated from the aerial parts of the plant, together with two additional derivatives (**3**, **4**) previously only known as semi-synthetic products. In addition, the known triterpene lup-20(29)-en-3-one (**5**) was isolated (Dantanaraya *et al.*, 1982). The antibacterial and cytotoxic activity of the obtained phloroglucinol derivatives was characterized in different cellular assays.

Materials and Methods

Plant material

The aerial parts of *Hypericum amblycalyx* Coust. & Gand. (Guttiferae) were collected at Kavousi Hierapetra (Crete, alt. 140 m, limestone cliffs). The plant was identified by Dr. Z. Kypriotakis. A voucher specimen is deposited at the Herbarium of Technological Education Institute, School of Agricultural Production, Lab. of Taxonomy and Management of Wild Flora (Heraclion-Crete, Greece) with the identification number 9471/14-9-01.

General experimental procedures

Optical rotations were recorded using a Perkin-Elmer 241 polarimeter with methanol as solvent.

UV spectra were obtained in ethanol on an UVIKON 930 spectrophotometer. ^{13}C NMR spectra were measured at 295 K on a Bruker AMX-300 spectrometer (operating at 300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C). ^{1}H, [^{1}H, ^{1}H]-COSY, 500 ms NOESY, [^{13}C, ^{1}H]-HMBC/HSQC experiments were recorded at 295 K on a Bruker DRX-500 spectrometer (operating at 500.13 MHz for ^{1}H, and 125.77 MHz for ^{13}C). The spectra were measured in $CDCl_3$ and referenced against residual non-deuterated solvent $C\underline{H}Cl_3$ (^{1}H δ 7.27 ppm) and $\underline{C}DCl_3$ (^{13}C δ 77.0 ppm). Direct electron impact mass spectra were measured on a micromass VG-TRIBRID double-focusing mass spectrometer at 70 eV, and HRMALDI-MS on a IonSpec-Ultima-FTMS spectrometer (Ion Spec, Lake Forest, USA) with 2,5-dihydroxybenzoic acid (DHB) as matrix. HPLC separations were performed with a Merck-Hitachi L6200A Intelligent Pump connected to a Rheodyne 7125 Injector, a Merck-Hitachi L-4250 UV/vis detector, a Merck D-2500 Chromato-Integrator and a Knauer HPLC column (Spherisorb S5 ODS II, 5 μm, 250 × 8 mm). Silica gel 60 for column chromatography, particle size 40–63 μm (Merck), was used for vacuum liquid chromatography (column 22 × 7 cm). Silica gel 60 F_{254} precoated aluminum sheets (0.2 mm, Merck) and RP-18 F_{254} precoated sheets (0.25 mm, Merck) were used for TLC controls. All solvents used were of HPLC grade.

Extraction and isolation

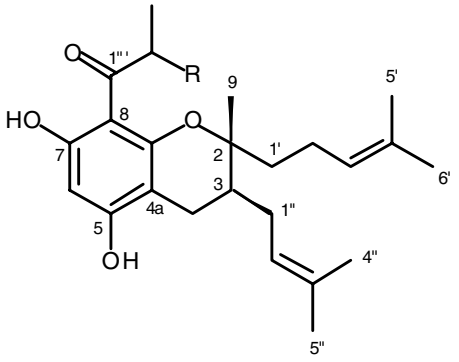
Air-dried and powdered aerial parts of *Hypericum amblycalyx* (800 g) were extracted successively with petroleum ether 30–60, diethyl ether, methanol, and 1:1 (v/v) methanol-water mixture, to afford 10 g of petroleum ether-soluble material after removal of solvent under vacuum. The petroleum ether extract was applied to VLC over silica gel (40–60 μm). Elution with hexane containing increasing amounts of ethyl acetate and final washing with methanol yielded 52 fractions (≈ 150 ml each). Based on TLC similarities, identical fractions were combined to give a total of 11 fractions. Fraction 4 (720 mg, eluted with hexane-ethyl acetate approx. 85:15 (v/v)), showing cytotoxicity against KB cells at 25 μg/ml, was subjected to further purification by RP-HPLC. Using a gradient of MeOH-H_2O-TFAA 30:70:0.5, as solvent A and MeOH-TFAA 100:0.5 (v/v), as solvent B (0′ to 15′ linear, from 100% A to 60% A; 15′ to 30′ linear, from 60% A to 50% A; 30′ to 40′ linear, from 50% A to 25% A; 40′ to 50′ 25% A; 50′ to 60′ linear, from 25% A to 0% A, flow 2 ml/min), and final washing with 100% B (flow 2 ml/min) compounds **1** (4.5 mg, eluted after 51.4′) and **2** (4.0 mg, eluted after 53.4′) were isolated as pure yellow oils. Applying the same gradient to the VLC fraction 5, (560 mg, eluted with hexane-ethyl acetate approx. 80:20 (v/v)), also cytotoxic against KB cells at 25 μg/ml, the phloroglucinol derivatives **3** (6.4 mg, eluted after 37.6′) and **4** (17.2 mg, eluted after 40.0′) were obtained as pure yellow oils. From VLC fraction 1 the known triterpene lup-20(29)-en-3-on (**5**) (2.3 mg) was isolated by VLC separation and RP-HPLC purification.

Hypercalyxone A: (1-[5,7-dihydroxy-2-methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-chroman-8-yl]-2-methyl-propan-1-one, **1**): yellow oil (4.5 mg); $C_{25}H_{36}O_4$; $[\alpha]_D^{22}$ + 2° (*c* 0.10, MeOH); UV (EtOH) λ_{max} (log ε) 294 (4.1) nm; ^{1}H and ^{13}C NMR spectral data, see Tables I and II; DEI-MS (CH_2Cl_2) *m/z* 400 $[M]^+$ (5), 357 $[M\text{-}C_3H_7]^+$ (8), 315 (15), 209 (64), 165 (59); HRMALDI-MS (pos.) 423.2502 $[M+Na]^+$ (calcd. 423.2511).

Hypercalyxone B: (1-[5,7-dihydroxy-2-methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-chroman-8-yl]-2-methyl-butan-1-one, **2**): yellow oil (4.0 mg); $C_{26}H_{38}O_4$; $[\alpha]_D^{22}$ – 1° (*c* 0.10, MeOH); UV (EtOH) λ_{max} (log ε) 295 (4.3) nm; ^{1}H and ^{13}C NMR spectral data, see Tables I and II; DEI-MS (CH_2Cl_2) *m/z* 414 $[M]^+$ (12), 357 $[M\text{-}C_4H_9]^+$ (15), 329 (29), 223 (100), 165 (86); HRMALDI-MS (pos.) 437.2658 $[M+Na]^+$ (calcd. 437.2668).

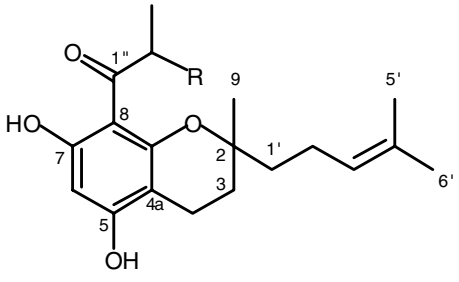
1-[5,7-dihydroxy-2-methyl-2-(4-methyl-pent-3-enyl)-chroman-8-yl]-2-methyl-propan-1-one (**3**): yellow oil (6.4 mg); $C_{20}H_{28}O_4$; $[\alpha]_D^{22}$ + 16° (*c* 0.10, MeOH); UV (EtOH) λ_{max} (log ε) 294 (4.2) nm; ^{1}H and ^{13}C NMR spectral data, see Tables I and II; DEI-MS (CH_2Cl_2) *m/z* 332 $[M]^+$ (17), 289 (68), 247 (23), 165 (100); HRMALDI-MS (pos.) 355.1877 $[M+Na]^+$ (calcd. 355.1885).

1-[5,7-dihydroxy-2-methyl-2-(4-methyl-pent-3-enyl)-chroman-8-yl]-2-methyl-butan-1-one (**4**)*:* yellow oil (17.2 mg); $C_{21}H_{30}O_4$; $[\alpha]_D^{22}$ + 14° (*c* 0.10, MeOH); UV (EtOH) λ_{max} (log ε) 295 (4.3) nm; ^{1}H and ^{13}C NMR spectral data, see Tables I and II; DEI-MS



1 R = CH_3 1-[5,7-dihydroxy-2-methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-chroman-8-yl]-2-methyl-propan-1-one

2 R = CH_2CH_3 1-[5,7-dihydroxy-2-methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-chroman-8-yl]-2-methyl-butan-1-one



3 R = CH_3 1-[5,7-dihydroxy-2-methyl-2-(4-methyl-pent-3-enyl)-chroman-8-yl]-2-methyl-propan-1-one

4 R = CH_2CH_3 1-[5,7-dihydroxy-2-methyl-2-(4-methyl-pent-3-enyl)-chroman-8-yl]-2-methyl-butan-1-one

Fig. 1. Structures of the isolated phloroglucinols (**1–4**).

(CH_2Cl_2) *m/z* 346 $[M]^+$ (14), 289 (61), 261 (21), 165 (100); HRMALDI-MS (pos.) 369.2038 $[M+Na]^+$ (calcd. 369.2042).

Cytotoxicity assay

The cytotoxicity of all fractions and pure compounds was determined in a KB (HeLa cells, ATCC CCL17), a Caco-2 (ATCC), a myosarcoma (372/9/1993, Düsseldorf Chir.) and a Jurkat T tumor cell (ATCC TIB-152) assay. The tests against KB, Caco-2, and Jurkat T cells were performed as described in (Heilmann *et al.*, 2001; Gertsch *et al.*, 2002; Jager *et al.*, 2002). Fractions were tested at 25 and 50 μg/ml, pure compounds in a concentration range between 0.1 and 20 μg/ml. Before using the myosarcoma cells for cytotoxicity tests, the tumor cells were intensively characterized by histology, histochemistry and immunohistochemistry (*e.g.* antibody testing against desmine, vimentine, cytoceratine, integrine etc.) as an epitheloid myosarcom (ID-Nr. 372/9/1993). The cells were cultivated and passaged from primary cultures and contain more than 95% of epithelial cancer cells (Schulte *et al.*, 2000). 4000 cells of the ninth passage were seeded in each well of a 96-well plate (medium: 1:1 mixture of Dulbeccos Modified Eagle medium, and Nutrient mixture F-12 (HAM with L-glutamine), Gibco^{TM}) containing 10% fetal calf serum (inactivated for 30 min at 57° C). After attachment (37 °C, 95% humidity and 3% CO_2) cells were supplemented with the test substances. Total assay volume was 150 μl. For quantification 10 μl of WST-1 (4-(3-[4-iodophenyl]-2-[4-nitrophenyl]-2H-5-tetrazolio)-1,3-benzene disulfonate) solution (Roche) per well was added 69 h after supplementation of the test compounds and incubated for further 3 h at 37 °C. Absorption was measured at a wavelength of 450 nm and a reference wavelength of 690 nm using a microplate reader. Every

Table I. ^{13}C NMR data of compounds **1–4** (in $CDCl_3$, 295 K).

C	**1**	**2**	**3**	**4**
2	81.4	81.3	78.7	78.3
3	36.0	35.9	29.1	29.1
4	21.8	21.7	16.1	16.1
4a	100.0	100.1	99.7	99.9
5	159.7	159.7	159.9	160.1
6	95.5	95.5	95.4	95.4
7	165.2	165.1	165.2	165.0[a]
8	105.4	106.0	105.4	106.0
8[a]	156.7	156.8	156.9	157.0
9	20.0	20.2	23.8	23.9[a]
1′	39.4	39.3	39.8	39.6[a]
2′	21.8	21.7	22.6	22.5
3′	123.7	123.6	123.6	123.6
4′	132.1	132.1	132.1	132.1
5′	17.6	17.7	17.6	17.6
6′	25.7	25.7	25.7	25.7
1″	29.0	29.0	210.6	210.6[a]
2″	121.7	121.7	39.8	45.8[a]
3″	133.5	133.5	19.2	26.4[a]
4″	17.9	17.9	19.7	11.8[a]
5″	25.9	25.9		16.6[a]
1‴	210.5	210.4		
2‴	39.2	45.7		
3‴	19.2	26.9		
4‴	19.8	11.8		
5‴		16.6		

[a] Signals broadened or even doubled due to the presence of an epimer.

Table II. ^{1}H NMR data of compounds **1–4** (δ ppm; m; *J* Hz)[a].

H	1	2	3	4[c]
3	1.92 (m)	1.92 (m)	1.78 (m) 1.88 (m)	1.83 (2H, m)
4	2.14 (dd, 10.3, 16.3) 2.73 (dd, 5.5, 16.3)	2.14 (dd, 10.2, 16.3) 2.73 (dd, 5.5, 16.3)	2.61 (2H, m)	2.59 (2H, m)
6	5.95 (s)	5.95 (s)	5.96 (s)	5.97 (s)
9	1.24 (3H, s)	1.25 (3H, s)	1.36 (3H, s)	1.36 (3H, s)
1′	1.82 (2H, m)[b]	1.82 (2H, m)[b]	1.72 (t, 8.4)	1.71 (2H, m)[b]
2′	2.13 (2H, m)[b]	2.14 (2H, m)[b]	2.10 (m)	2.09 (2H, m)[b]
3′	5.11 (bt, 7.2)	5.11 (bt, 6.5)	5.11 (bt, 6.4)	5.10 (m)
5′	1.62 (3H, s)	1.62 (3H, s)	1.62 (3H, s)	1.61 (3H, s)
6′	1.71 (3H, s)	1.70 (3H, s)	1.70 (3H, s)	1.69 (3H, bs)
1″	1.82 (m)[b] 2.25 (m)	1.82 (m)[b] 2.24 (m)		
2″	5.18 (bt, 7.2)	5.18 (bt, 6.6)	3.87 (sept, 6.7)	3.77 (m)
3″			1.18 (3H, d, 6.7)	1.43 (m)[b] 1.83 (m)[b]
4″	1.63 (3H, s)	1.63 (3H, s)	1.18 (3H, d, 6.7)	0.90 (3H, bt, 7.4)
5″	1.75 (3H, s)	1.74 (3H, s)		1.16 (3H, d, 6.8)
2‴	3.87 (sept, 6.8)	3.79 (sext, 6.7)		
3‴	1.17 (3H, d, 6.8)	1.41 (m) 1.82 (m)[b]		
4‴	1.18 (3H, d, 6.8)	0.90 (3H, t, 7.4)		
5‴		1.15 (3H, d, 6.7)		
OH	13.87 s	13.87 s	13.96 s	14.06 s

[a] Spectra measured at 500 MHz, 295 K, in $CDCl_3$;
[b] multiplicity not determined due to overlapping signals;
[c] signals broadened or even doubled due to the presence of an epimer.

test was performed in hexaplicates (n = 6) at eight different concentrations. Maximal observed standard deviation was about 10% (absolute). Positive control measurements were performed with aculeatin A (Heilmann *et al.*, 2000, IC_{50} 0.3 μg/ml).

Antibacterial and antifungal assays

The Gram-positive bacteria *Bacillus cereus* (ATCC 10702), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), and *Micrococcus luteus* (ATCC 9341), the Gram-negative bacteria *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATTC 27853), and the fungus *Candida albicans* (H-29, ATCC 26790) were used as test organisms. The assays were carried out by the doubling dilution method using a modified procedure as published previously (Liu *et al.*, 1999; Winkelmann *et al.*, 2000).

Results and Discussion

A petroleum ether extract of the dried aerial parts of *Hypericum amblycalyx* was fractionated by VLC, followed by purification with C-18 reverse-phase HPLC, to give hypercalyxone A (**1**) and B (**2**) as yellow oils. HR-MALDI of compound **1** determined the $[M+Na]^+$ peak as 423.2502, establishing a molecular formula of $C_{25}H_{36}O_4$. Examination of the ^{1}H, ^{13}C/DEPT135, and 2D NMR spectra (HSQC, DQF-COSY, HMBC) indicated the presence of a 3-methyl-but-2-enyl (δ_C 29.0 C-1″, 121.7 C-2″, 133.5 C-3″, 17.9 C-4″, 25.9 C-5″) and a 4-methyl-pent-3-enyl (δ_C 39.4 C-1′, 21.8 C-2′, 123.7 C-3′, 132.1 C-4′, 17.6 C-5′, 25.7 C-6′) side-chain. Furthermore, signals common for a 2-methylpropionyl substituent (δ_C 210.5 C-1‴, 39.2 C-2‴, 19.2 C-3‴, 19.8 C-4‴) could be identified. The presence of a chroman ring system was indicated by five aromatic carbons (δ_C 100.0, 105.4, 156.7, 159.7, 165.2), three of them oxygen-substituted as indicated by their chemical shifts, one methine (C-3, δ_C 36.0), one methylene (C-4, δ_C 21.0) and finally one aliphatic quaternary carbon (C-2, δ_C 81.4), which is shifted downfield due to oxygen substitution. Its formation and the position of the substituents at the ring system, were determined by HMBC connectivities, which are summarized in Fig. 2. Particularly valuable information was obtained by HMBC correlations to the proton at δ_H 13.87, whose downfield shift is characteristic for a hydrogen-bonded hydroxyl proton. Since two regioisomers are possible, the

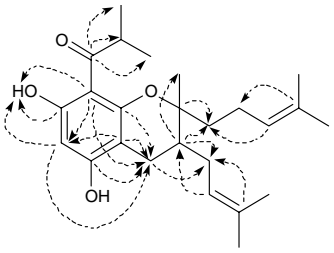


Fig. 2. Important HMBC correlations observed for hypercalyxone A (**1**).

orientation of the chroman ring has to be determined. The ether bridge can be formed by the hydroxyl group either in ortho or in para position to the 2-methylpropionyl substituent. However, a NOE correlation from H-2‴ to H_3-9 unambiguously proved the formation of the ether bridge in the *ortho* positition to the acyl substituent. The relative stereochemistry at position C-2 and C-3 has been determined by NOE correlations. A strong NOE was detected between H_3-9 (δ_H 1.24, s) and H_2-1″ (δ_H 2.25 and 1.82, each m) and only a weak one between H_3-9 and H-3 (δ_H 1.92, m), therefore indicating the methyl group at C-2 and the prenyl side chain at C-3 being on the same side of the bicyclic ring system. A coupling constant of 10.3 Hz between H-4ax (δ_H 2.14, dd, J = 10.3 and 16.3 Hz) and H-3 indicated a diaxial arrangement of H-4ax and H-3. Therefore, the 3-methyl-but-2-enyl side chain at C-3 has to be in an equatorial orientation.

Compound **2** was elucidated by comparing its ^1H and ^{13}C chemical shifts with the corresponding data of hypercalyxone A (**1**). An increase of the molecular weight by 14 atomic mass units to *m/z* 414 $[M]^+$ in the DEI-MS, suggested the replacement of the 2-methylpropionyl substituent at C-8 by a 2-methylbutyryl side chain. This was confirmed by interpretation of the HSQC, HMBC, DQF-COSY and NOESY spectra and compound **2** was identified as 1-[5,7-dihydroxy-2-methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-chroman-8-yl]-2-methyl-butan-1-one and trivially named as hypercalyxone B (**2**).

Compounds **3** and **4** were isolated from a more polar fraction of the petroleum ether extract. Comparison of the 1D and 2D NMR spectra, as well as the mass spectra, of compound **3** with compound **1** and compound **4** with **2**, respectively, revealed unambiguously that the only difference was the absence of the prenyl residue at position 3 in the isolates **3** and **4**. Compounds **3** and **4** have never been isolated before from a natural source, and were only described as semi-synthetic products, obtained by acid-catalysed cyclization of monoprenylated acylphloroglucinol derivatives from *Helichrysum natalitium* (Bohlmann and Zdero, 1979). As only ^1H NMR and MS data were previously reported for **3** and **4**, the spectroscopic data are given in Tables I and II. We further report the isolation of the known compound lup-20(29)-en-3-one (**5**) (Dantanarayana *et al.*, 1982) which was identified by 1D and 2D NMR spectroscopy and verified by comparison with literature data.

The four acylphloroglucinol derivatives were tested for their cytotoxic potential against KB, Caco-2, myosarcoma and Jurkat T cells. Against KB cells, the new compounds **1** and **2** displayed IC_{50} values of 6.5 ± 0.78, and 7.0 ± 0.63 μg/ml, respectively. Since compounds **3** and **4** showed similar activity (IC_{50} 8.5 ± 0.38, and 6.2 ± 0.72 μg/ml), we conclude that the 3-methyl-but-2-enyl side chain in position 3 contributes not significantly to the cytotoxic effects. Furthermore, no notably different activity was observed between the compounds with a 2-methylpropionyl (**1**, **3**) or a 2-methylbutyryl (**2**, **4**) side chain. Very similar results were obtained by the cytotoxicity assays using Jurkat T and Caco-2 cells (Table III). All compounds were not active against myosarcoma cells up to 20 μg/ml. The compounds were active against *Bacillus cereus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Micrococcus luteus*. In particular, compounds **3** and **4** displayed a remarkable activity with MIC values in the range of the reference compound chloramphenicol (see Table III). Since **3** and **4** are significantly more active than **1** and **2** we conclude that the 3-methyl-but-2-enyl side chain in position 3 decreases the antibacterial activity. Furthermore, the compounds with a 2-methylpropionyl (**1**, **3**) side chain displayed a stronger activity than the corresponding ones with a 2-methylbutyryl (**2**, **4**) residue. In contrast, no activity was observed against the Gram-negative bacteria *Pseudomonas aeruginosa*, *Escherichia coli* and the fungus *Candida albicans* at 128 μg/ml.

Table III. Antibacterial and cytotoxic activities of compounds **1–4**; values represent the minimum inhibition concentration (MIC) in broth (in μg/ml) for antibacterial and IC_{50} (in μg/ml) for cytotoxic activity (n = 6).

Compound	Antibacterial activity (MIC)				Cytotoxicity (IC_{50})		
	B. cereus	*S. aureus*	*S. epidermidis*	*M. luteus*	KB	Caco-2	Jurkat
1	64	32	16	8 (20)[a]	6.5	15.7	9.9
2	64	32	32	32	7.0	15.3	10.4
3	8 (24.0)[a]	8 (24.0)[a]	2 (6.0)[a]	2 (6.0)[a]	8.5	20.0	9.2
4	32	16	2 (5.8)[a]	4 (11.6)[a]	6.2	15.6	8.3
Chloramphenicol	2 (6.2)[a]	8 (24.7)[a]	4 (12.4)[a]	2 (6.2)[a]			
Podophyllotoxin					0.005		

[a] MIC expressed in μM.

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