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Cytotoxic, anti-proliferative and antimicrobial furanoditerpenoids from *Stuhlmania moavi*

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ABSTRACT

The furanoditerpenoids voucapane, voucapane-6α,7α-diol, voucapane-18,19-diol and 18-hydroxyvoucapan-19-al were isolated from the cytotoxic stem and root bark extracts of *Stuhlmania moavi* Verdc. (Ceasalpiniaceae) and their structures established based on analysis of spectroscopic data. The compounds exhibited anti-proliferative, cytotoxic, antibacterial and antifungal activities, 18-hydroxyvoucapan-19-al showing the highest anti-proliferative and cytotoxic properties. Voucapane-18,19-diol was only mildly active but the activity was enhanced for its 18,19-di-(4-methyl)-benzenesulphonate. Some of these results thus corroborate the traditional medicinal uses of the crude extracts for the treatment of skin infections.

1. Introduction

In East Africa several plant species of the family Caesalpiniaceae are used as sources of traditional medicines for the treatment of malaria (Kokwaro, 1976; Chin et al., 2006), sleeping sickness (Freiburghaus et al., 1998) and inflammatory conditions (Dalziel, 1936). Among such plant species is *Stuhlmania moavi* that grows in Tanzania, and whose extracts are used for the treatment of skin infections. This, and the recent isolation of antitrypanosomal, antimosquito and other compounds from some Caesalpiniaceae species (Freiburghaus et al., 1998; Chin et al., 2006; Kihampa, 2008) prompted us to analyze the crude *S. moavi* root and stem bark extracts for anti-proliferative, cytotoxic and antimicrobial natural products, as part of our ongoing investigations for bioactive and other constituents of the less common Tanzanian indigenous plant species (Baraza et al., 2008), some of which are considered to be threatened by extinction. We now hereby report the isolation, structural determination, and bioactivities of the furanoditerpenoids voucapane (**1**), voucapane-6α,7α-diol (**2**), voucapane-18,19-diol (**3**) and 18-hydroxyvoucapan-19-al (**4**).

2. Results and discussion

Repeated chromatography of the root and stem bark extracts yielded the metabolites **1**–**4** whose ^{1}H and ^{13}C NMR spectra (Tables 1 and 2), and other spectroscopic data compared well with those reported for **1** and other related furanoditerpenoids (Mendoza et al., 2003; Cheenpracha et al., 2006). Furthermore, the ^{1}H and ^{13}C NMR spectra of **2** indicated two of the six methylene groups in compound **1** to be hydroxylated in **2** (δ_H 3.45, *dd*, J = 10.8, 3.3 Hz, and 4.31, *dd*, J = 3.3, 1.5 Hz; δ_C 73.9 and 70.6; IR, ν_{O-H} = 3433 cm^{-1}), whose C-6 and C-7 positions were established based on the fact that apart from the mutual coupling of 3.3 Hz, each of the two carbinol methine protons also coupled with only one other vicinal proton. This could be achieved only if C-6 and C-7 were the carbinol methines, as in structure **2**, where the observed $^{3}J_{5,6}$, $^{3}J_{6,7}$ and $^{3}J_{7,8}$ values of 10.8, 3.3 and 1.5 Hz indicated *trans* (diaxial) and *cis* (equatorial/axial) H-5/H-6 and H-7/H-8, respectively, and hence H-6/H-7 being *cis* (axial/equatorial). This led to H-5/H-6, H-6/H-7 and H-7/H-8 dihedral angle to be *ca.* 160°, 75° and 90°, respectively (Becker, 1980), which was consistent with the observed coupling constants. For similar compounds where the 6-OH and 7-OH relative stereochemistry is α and β the $J_{5,6}$, $J_{6,7}$ and $J_{7,8}$ values would be 11.1, 8.7 and 10.5 Hz, respectively (Amtonio and Luiz, 1996). The 6-OH and 7-OH relative stereochemistry in **2** was further indicated from the observed H-6/20-CH_3 and H-7/H-8 NOE interactions (Fig. 1).

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Table 1
^{1}H NMR spectral data for the furanoditerpenoids **2**–**4**.

H	δ (**2**)	J (Hz)	δ (**3**)	J (Hz)	δ (**4**)	J (Hz)
1	1.02–2.01	*m*	0.97	*m*	1.05	*m*
			1.55	*m*	1.76	*m*
2	1.02–2.01	*m*	1.40	*m*	1.52	*m*
			1.46	*m*	1.60	*m*
3	1.02–2.01	*m*	1.30	*m*	1.41	*m*
			1.59	*m*	1.76	*m*
5	1.46	*d*, 11.4	1.37	*dd*, 11.7, 2.3	1.42	*dd*, 12.0, 2.5
6	3.45	*dd*, 10.8, 3.3	1.18	*m*	1.26	*m*
			1.59	*m*	2.32	*m*
7	4.31	*dd*, 3.3, 1.5	1.27	*m*	1.62	*m*
			1.62	*m*	1.90	*m*
8	1.69	*m*	1.65	*m*	1.80	*m*
9	1.69	*m*	1.42	*m*	1.52	*m*
11	2.46	*dd*, 17.7, 10.0	2.25	*dd*, 17.1,9.0	2.33	*dd*, 16.7, 10.5
	2.59	*dd*, 17.7, 7.0	2.45	*dd*, 17.1,7.8	2.59	*dd*, 16.7, 6.7
14	3.04	*m*	2.53	*m*	2.61	*m*
15	6.20	*d*, 2.0	6.23	*d*, 1.8	6.16	*d*, 2.0
16	7.21	*d*, 2.0	7.35	*d*, 1.8	7.20	*d*, 2.0
17	1.07	*d*, 6.9	0.89	*d*, 6.9	0.96	*d*, 7.5
18	1.27	*s*	3.32	*dd*, 10.8,5.6	3.87	*d*, 10.8
			3.24	*dd*, 10.8,5.6	3.49	*d*, 10.8
19	1.20	*s*	3.60	*dd*, 11.1,5.1	9.90	*s*
			3.28	*dd*, 11.1,5.1		
20	0.99	*s*	0.82	*s*	0.79	*s*
18-OH			4.28	*d, 5.6*		
19-OH			4.17	*d, 5.1*		

Table 2
^{13}C NMR chemical shifts (δ values) for the furanoditerpenoids **2**–**4.**

C	**2**	**3**	**4**	C	**2**	**3**	**4**
1	42.5	38.8	38.6	11	21.7	21.9	22.7
2	18.8	17.8	18.0	12	149.2	148.9	149.3
3	43.5	31.3	31.3	13	121.6	122.2	122.3
4	34.1	41.9	53.7	14	33.8	31.0	31.4
5	54.6	47.4	51.0	15	109.6	109.7	109.5
6	73.9	28.8	29.1	16	140.5	140.6	140.5
7	70.6	20.8	21.4	17	17.8	17.6	17.4
8	37.9	35.1	35.5	18	27.3	66.5	67.3
9	44.3	45.2	44.4	19	24.7	61.7	205.8
10	37.3	36.6	37.4	20	17.3	15.1	15.0

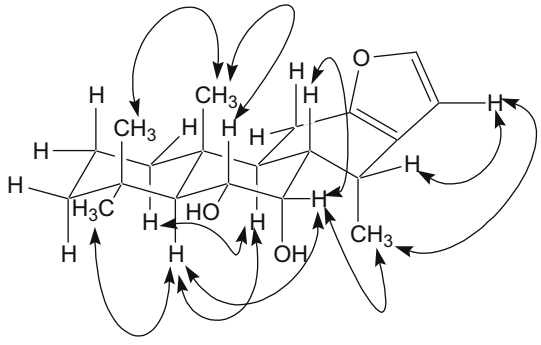

Fig. 1. Important NOE correlations for voucapane-6α,7α-diol (**2**).

The spectral features of **3** were closely related to those observed for **1** and **2**, except that instead of signals due to three tertiary methyl groups, both the ^{1}H and ^{13}C NMR spectra exhibited resonances for only one such group, and new signals due to hydroxymethylene groups appeared (Tables 1 and 2; IR, ν_{O-H} = 3295 cm^{-1}). This indicated that two of the tertiary methyl groups in **1** were hydroxylated in **3**. HMBC C/H interactions (Fig. 2) indicated that both the hydroxymethylene units were substituted at the same carbon atom. This was achievable only if both C-18 and C-19 were the hydroxylated carbons, as in structure **3**, which was further confirmed upon analysis of HMBC and NOE interactions (Figs. 2 and 3).

The spectroscopic data for compound **4** compared well with those observed for **3**, except that one of the two hydroxymethylene groups had been oxidized to an aldehyde unit (δ_H 9.90, 1H, *s*, CH=O and $\delta_{C=O}$ 205.8; IR, $\nu_{C=O}$ 1700 cm^{-1}), whose C-19 position was

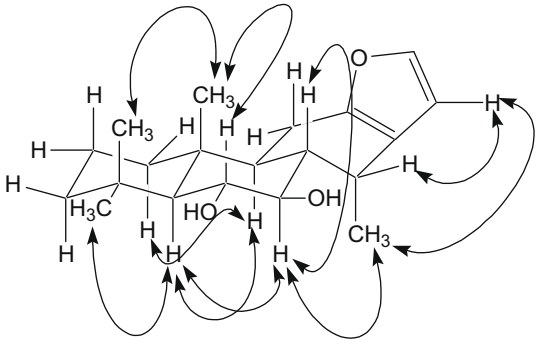

Fig. 2. Important H/C HMBC correlations for voucapane-18,19-diol (**3**).

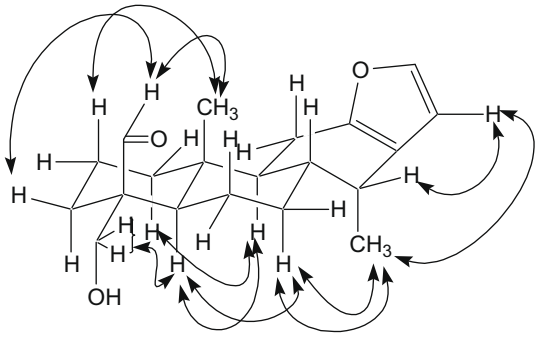

Fig. 3. Important NOE correlations for voucapane-18,19-diol (**3**).

deduced based on the observed H-19/H-10 and H-5/H-18 NOE and HMBC interactions (Figs. 4 and 5), which also further confirmed the structure.

Although furanoditerpenoids similar to **1**–**4** have previously been reported from various sources including the genus *Caesalpinia* (Cheenpracha et al., 2006; Ragasa et al., 2002; Lyder et al., 1998; Peter and Tinto, 1997), the co-existence of a series of such compounds as C-18 and C-19 variously oxidized metabolites like compounds **1**–**4** is unprecedented.

The crude extracts when evaluated for possible cytotoxicity in the brine shrimp test (Meyer et al., 1982) showed potent activity (Table 3), the efficacy being comparable to that shown by the standard cytotoxic agent Cyclophosphamide. This prompted us to analyze the isolated furanoditerpenoids for possible cytotoxicity and anti-proliferative activity. The results given in Table 4 from the *in vitro* anti-proliferative and cytotoxic assay of the furanoditerpenoids **3** and **4** against L-929, K-562, and HeLa cell lines respectively showed different levels of mild activity as compared to the standard anticancer agents Taxol®, Colchicine and Camptothecin. The difference in anti-proliferative and cytotoxic potency of the furanoditerpenoids **3** and **4** suggested the effect of the aldehyde group in enhancing the activity. The 18,19-diacetoxy and 18,19-dibenzoyloxy derivatives **5** and **6** of **3** showed diminished activity compared to **3**, while the activity of the 18,19-di-(4-methyl)-benzenesulphonate **7** was higher than that of **3** for all the cell lines, indicating the sulphonate group to be a better activity enhancer as compared to the acetoxy and benzoyloxy moieties. However, overall all compounds **3**–**7** were less active than any of the anticancer agents used as standards, the most active compounds being 100 times less active than the standards, hence making the isolated compounds and their derivatives to be unsuitable as candidates for anticancer agents development.

These results corroborate the previously reported moderate cytotoxicity of furanoditerpeneoids similar to **1**–**4** against a series of cell line panels (Liu et al., 2006; Kanokmedhakul et al., 2006), suggesting that the furanoditerpenoid skeleton is not a suitable pharmacophore in anticancer drug research.

In order to establish the possible antibacterial and antifungal activity of the isolated metabolites that would corroborate the traditional use of the crude extracts for the treatment of skin infections, compounds **1**–**4** were assayed for these activities *in vitro* against the standard bacteria species *Bacillus subtilis* [ATTC 6633 (IMET NA)], *Staphylococcus aureus* [SG511 (IMET 10760)], *Escherichia coli* (SG 458), *Pseudomonas aeruginosa* (K 799/61) and *Mycobacterium vaccae* (IMET 10670), and against the fungal species *Sporobolomyces salmonicolor* (SBUG 549), *Candida albicans* (BMSY 212) and *Penicillium notatum*. Generally, all the furanoditerpenoids showed better activity against the bacteria than the fungal strains (Table 5). Compound **2** exhibited the highest activity against *M. vaccae*, while **4** was highly potent against *B. subtilis*. The furanoditerpenoid **3** showed both antibacterial and antifungal activity. Compared with the standard antibacterial and antifungal agents Ciprofloxacin and Amphotericin respectively, the natural products **1**–**4** were less active, except **2** that showed a slightly higher activity against *M. vaccae* compared to Amphotericin.

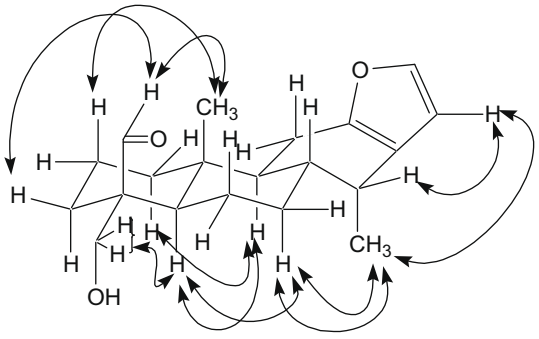

Fig. 4. Important NOE correlations for 18-hydroxyvoucapan-19-al (**4**).

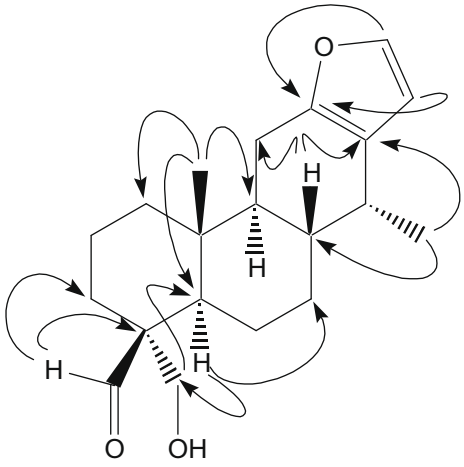

Fig. 5. Important HMBC correlations for 18-hydroxyvoucapan-19-al (**4**).

Table 3
Toxicity (LC_{50} in μg/ml) of *S. moavi* crude extracts in the brine shrimp test.

Extract	Root bark	Stem bark
Pet ether	34.88 (23.39, 48.47)	18.28 (5.60, 32.36)
Dichloromethane	14.42 (9.43, 19.52)	19.84 (13.19, 26.78)
Methanol	16.34 (6.02, 27.25)	61.93 (38.26, 99.11)

Cyclophosphamide used as standard toxic agent [LC_{50} = 16.33 (10.60, 25.15) μg/ml]; values in parentheses represent 95% lower and upper confidence limits.

Table 4
Anti-proliferative and cytotoxic activity of compounds **3**–**7** against L-929, K-562, and HeLa cell lines.

Compound	Anti-proliferative activity (μg/ml)		Cytotoxicity (μg/ml)
	L-929 (GI_{50})	K-562 (GI_{50})	HeLa (CC_{50})
3	14.7	13.9	20.3
4	5.4	9	10.8
5	34.2	38.7	36.2
6	>50	>50	>50
7	10.2	8.4	12.3
Taxol®	0.1	0.01	0.01
Colchicine	0.9	0.02	0.006
Camptothecin	0.02	0.002	0.2

The results indicated that the functional groups of the parent furanoditerpenoids were important for the exerted antibacterial activity against *B. subtilis*, as the least functionalized diterpenoid **1** was also the least active. Furthermore, the diterpene diols **2** and **3** exhibited moderate activity, which indicated that the presence and appropriate position of the hydroxyl groups was essential for enhanced activity. The furanoditerpenoid **4,** which is a structural analogue of **3** but having one of the hydroxyl groups oxidized to an aldehyde moiety, was more active against *B. subtilis* than the other furanoditerpenoids **1**–**3**. This suggested the importance of an electrophilic site (aldehyde carbon) for enhanced *B. subtilis* activity.

In order to evaluate the effect of the hydroxyl as well as other substituents on the activity of the voucapanoids **1**–**4**, the 18,19-diacetoxy, 18,19-dibenzoyloxy and 18,19-di-(4-methyl)benzenesulphonate derivatives **5**–**7** of **3** were assayed against the bacteria and fungal strains. All the compounds were inactive

Table 5
Zones of inhibition (mm) for the furanoditerpenoids **1**–**4** and derivatives **5**–**7** of **2** against bacterial and fungal strains.

Compound	Bacterial and fungal strains, and zones of inhibition							
	B1	B3	B4	B9	M4	H4	H8	P1
1	12.0 ± 0.6	11.0 ± 0.3	0	0	16.0p ± 1.0	0	0	0
2	13.0 ± 0.3	12.0 ± 0.6	0	0	24.0p ± 1.2	0	0	0
3	16.0p ± 1.0	14.5p ± 1.1	0	0	14.5 ± 1.0	13.0p ± 0.8	12.0p ± 0.7	14.0 ± 1.0
4	20.5 ± 1.1	0	0	0	17.0p ± 1.3	0	0	0
5	13.0 ± 0.8	12.0 ± 0.2	0	0	0	0	0	14.0 ± 0.6
6	0	0	0	0	0	0	0	13.0 ± 0.8
7	0	0	0	0	0	0	0	13.0 ± 0.4
Ciprofloxacin (5 mg/ml)	28.0 ± 1.0	18.0 ± 1.0	23.0/32.0p ± 1.0	22.0/270p ± 1.0	22.0p ± 1.0	–	–	–
Amphotericin (10 mg/ml)	–	–	–	–	–	14.0 ± 1.0	20.0 ± 1.0	18.0 ± 1.0

B1 = *Bacillus subtilis* [ATTC 6633 (IMET NA)]; B3 = *Staphylococcus aureus* [SG511 (IMET 10760)]; B4 = *Escherichia coli* (SG 458); B9 = *Pseudomonas aeruginosa* (K 799/61); M4 = *Mycobacterium vaccae* (IMET 10670); H4 = *Sporobolomyces salmonicolor* (SBUG 549); H8 = *Candida albicans* (BMSY 212); P1 = *Penicilium notatum*; each value represents mean ± SD (n = 3); p = partial inhibition.

Table 6
MIC (mg/ml) values for furanoditerpenoid **2** against some bacterial strains.

Compound	Bacteria strains					
	B1	B3	M2	M3	M4	M5
2	>100	50	25	6.25	6.25	12.5
Ciprofloxacin	<0.05	0.2	1.6	<0.05	<0.05	<0.05

against *B. subtilis*, *S. aureus*, *S. salmonicolor*, *C. albicans* and *M. vaccae*, strains that were originally susceptible to the parent compound **3** (Table 5). However, derivative **5** exhibited the same level of moderate activity as **3** against *B. subtilis* as well as against *S. aureus*. The level of activity of **5**–**7** against the fungus *P. notatum* was similar to that observed for **3**, hence indicating no influence on the antifungal activity on replacement of the hydroxyl groups in **3** to form **5**–**7**.

The minimum (bacterial) inhibitory concentration (MIC) was determined for the more active compound **2.** The results (Table 6) indicated the compound to be weakly active compared to the standard antibacterial agent Ciprofloxacin.

Despite the weak antibacterial and antifungal activities, the results tend to corroborate the traditional use of the crude extracts for the treatment of skin infections. However, since the bioactivities exerted by the crude plant extracts as used in traditional medicines could be influence by many factors, including synergism of the constituents, it would be important to carry out further bioassays of combination formulations of the isolated furanoditerpenoids, which could not be undertaken in these studies due to paucity of the available samples.

3. Experimental

3.1. General remarks

CC: Silica gel 60 (Merck 230–400 mesh, pet ether/EtOAc gradient elution) and Sephadex® LH-20 (Pharmacia, MeOH/$CHCl_3$); TLC: Kieselgel 60 F_{254} (Merck) pre-coated on plastic plates (0.20 mm), visualization by UV/VIS and anisaldehyde spray (Stahl, 1969); m.p. (uncorrected): Electrothermal 9100; UV and IR spectra: Hitachi 200-20, and JASCO FT/IR-4100 spectrophotometers respectively; optical rotation: JASCO P-1020 polarimeter; ^{1}H NMR (300 and 500 MHz) and ^{13}C NMR (75 MHz): Bruker DRX-300 spectrometer in $CDCl_3$, internal standard TMS (^{1}H NMR) and solvent signal (^{13}C NMR); electron spray ionization mass spectra (ESIMS): HP 5990/5988A mass spectrometer at 70 eV; high resolution electron spray ionization mass spectra (HRESIMS): JEOL JMS-HX 110 mass spectrometer.

3.2. Plant materials

The root and stem barks were collected in October 2004 from Kwedijela forest in Handeni District in Tanzania. The plant species was authenticated at the Herbarium of the Department of Botany, University of Dar es Salaam where a voucher specimen No. FMM 3326 is preserved.

3.3. Extraction and isolation

The air-dried and powdered plant materials were extracted sequentially at room temperature with pet ether, CH_2Cl_2 and MeOH (each 2 × 72 h). The pet ether extract when fractionated by vacuum liquid chromatography (VLC, pet ether/EtOAc gradient elution) and then separated by repeated CC yielded compounds **1** and **2**. The CH_2Cl_2 and MeOH extracts on similar work up gave the furanoditerpenoids **3** and **4**.

3.3.1. Voucapane-6α,7α-diol (**2**)

White crystals; m.p. 95–98 °C; yield, 7.0 mg; spray – pink; $[\alpha]_D^{20}$ + 53.5° (c, 0.22, MeOH); IR, ν_{max} 3433, 1459, 1366, 1205, 1118, 1060 and 1040 cm^{-1}; HRESIMS, *m/z* 317.2137{[M−H]$^+$, calc. for $C_{20}H_{30}O_3$: 318.2117}; ESIMS, *m/z* (% rel. int.) 319 ([M+H]$^+$, 92), 301 (71), 283 (100), 267 (59), 239 (29), 237 (23), 219 (25), 209 (24), 199 (21), 173 (26), 171 (16), 159 (26), 157 (18), 143 (10), 133 (19), 119 (11) and 109 (9); ^{1}H and ^{13}C NMR: Tables 1 and 2.

3.3.2. Voucapane-18,19-diol (**3**)

White crystals; m.p. 206–207 °C; yield, 484 mg; spray – pink; $[\alpha]_D^{20}$ + 49.0° (c, 0.22, MeOH); UV, λ_{max}^{MeOH} (log ε) 213 (3.79) nm; IR, ν_{max} 3295, 1595, 1506, 1194, 1139, 1085 and 1059 cm^{-1}; HRESIMS, *m/z* 317.2133 {[M−H]$^+$, calcd for $C_{20}H_{29}O_3$: 317.2117}; ESIMS, *m/z* (% rel. int.) 319 ([M+H]$^+$, 20), 301 (54), 283 (100), 271 (38), 265 (78), 255 (44), 253 (36), 239 (36), 237 (27), 201 (26), 199 (47), 189 (70), 185 (31), 173 (24), 159 (22), 147 (34), 145 (30), 133 (27), 121 (13) and 109 (7); ^{1}H and ^{13}C NMR: Tables 1 and 2.

3.3.3. 18-Hydroxyvoucapan-19-al (**4**)

White crystals; m.p. 185–187 °C; yield, 8 mg; spray – pink; $[\alpha]_D^{20}$ + 37.1° (c, 0.22, MeOH); IR, ν_{max} 3491, 2872, 1700, 1652, 1507, 1194, 1134, 1098, 1069, 1043 and 1033 cm^{-1}; HRESIMS, *m/z* 339.1933 {[M+Na]$^+$, calcd for $C_{20}H_{28}O_3Na$: 339.1936}; ESIMS *m/z* (% rel. int.) 316 ([M+H]$^+$), 298 (50), 283 (100), 267 (17), 255 (42) and 240 (30); ^{1}H and ^{13}C NMR: Tables 1 and 2.

3.3.4. 18,19-Dibenzoyloxyvoucapane (**5**)

A solution of compound **3** (60 mg, 0.15 mmol), benzoyl chloride (75 mL, 0.72 mmol), and a catalytic amount of DMAP in CH_2Cl_2

(40 mL) was stirred at room temperature for 24 h and the resulting mixture diluted with CH_2Cl_2 (60 mL), then washed sequentially with 2 M HCl (2 × 30 mL), saturated $NaHCO_3$ (2 × 20 mL), and saturated NaCl (30 mL). Workup of the organic layer and then CC gave **5** (50.1 mg, 83.5%); m.p. 163–165 °C, $[\alpha]_D^{20}$ + 13.6° (*c* 0.212, $CDCl_3$); spectral properties in agreement with the structure.

*3.3.5. Voucapane-18,19-di-(4-methyl)benzenesulphonate (**6**)*

Compound **6** was synthesized from **3** and 4-methylbenzenesulphonyl chloride as described for **5**. Yield, 52.5 mg (87.5%), m.p. 169–171 °C; $[\alpha]_D^{20}$ + 25.5° (*c* 0.201, $CDCl_3$); spectral properties in agreement with the structure.

*3.3.6. 18,19-Di-acetoxyvoucapane (**7**)*

Compound **7** was synthesized from **3** and acetic anhydride as described for **5**. Yield, 54 mg (90%); $[\alpha]_D^{20}$ + 30.6° (*c* 0.212, $CDCl_3$); spectral properties in agreement with the structure.

3.4. Brine shrimp test

The crude extracts were assayed in the brine shrimp test (BST) in artificial seawater and DMSO according to standard procedures (Meyer et al., 1982) and Cyclophosphamide was used as the standard toxic agent. LC_{50} values (the concentration required to kill 50% of the shrimp larvae) were determined using Probit analysis (Finney, 1971).

3.5. Anti-proliferative and cytotoxicity assays

The anti-proliferative assay was carried out as described in the literature (Dahse et al., 2001) using the cell lines K-562 (human chronic myeloid leukemia) and L-929 (mouse fibroblast) and the activity was expressed as GI_{50} values (concentration which inhibited cell growth by 50%). Cytotoxicity assay was carried out against HeLa cells and the activity expressed as GC_{50} values (concentration at which cells are destroyed by 50%; used partially in referring to the lysis of cells). For both the assays Taxol®, Colchicine and Camptothecin were used as the standard anticancer agents. The cells were incubated at 10 different concentrations of each of the target compounds. Cells of established suspended cell lines K-562 (DSM ACC 10) and adherent L-929 were cultured in the RPMI medium (Dahse et al., 2001). The adherent cells of L-929 were harvested at the logarithmic growth phase after trypsinization, using 0.25% trypsin in PBS containing 0.02% EDTA (Biochrom KG L2163). For each experiment with K-562, L-929, and HeLa cells approximately 10,000 cells were seeded with 0.1 mL RPMI 1640 (GIBCO BRL 21875-034) containing 25 mg/ml Gentamicin sulfate (BioWhittaker 17-528Z but without HEPES) per well of the 96-well microplates (K-562: NUNC 163320, L-929, HeLa: NUNC 167008). For the cytotoxicity assay, the HeLa cells were pre-inoculated for 48 h without the test substances.

The dilution of the compounds was carefully made on the monolayers of HeLa cells after the pre-incubation time. Cells of L-929, K-562 and HeLa in the presence of the respective test compounds were incubated for 72 h at 37 °C in a humidified atmosphere and 5% CO_2. Suspension cultures of K-562 in microplates were analyzed by an electronic cell analyzer system (CASY 1, SCHÄRFE, Reutlingen, Germany) using an aperture of 150 mm. The contents of each well (0.2 ml) in the microplate were diluted 1:50 with CAYSTON (SCHÄRFE). Every count/ml was automatically calculated from the arithmetic mean of three successive counts of 0.4 ml each. From the dose response curves the GI_{50} values were calculated using the computer program CASYSTAT. The GI_{50} value was defined as the 50% intersection line of the concentration–response curve, determined by the cell count/ml as compared to the control. The essential parameters for the estimation of growth inhibition and for changes in the diameter distribution curve were expressed as diagrams. The monolayer of the adherent L-929 and HeLa cells were fixed by glutaraldehyde and stained with a solution of methylene blue. After washing gently, the stain was eluted by 0.2 ml of 0.33 M HCl into the wells. The optical densities were measured at 660 nm in a microplate reader.

3.6. Antibacterial and antifungal assay

The agar diffusion method (Jorgensen et al., 1999; Joseph et al., 2006) was used in the assays against microorganisms obtained from the Hans Knöll Institute for Natural Product Research and Infection Biology (HKI), Jena in Germany, Ciprofloxacin (5 mg/mL) and Amphotericin (10 mg/mL) being used as the standard antibacterial and antifungal agents. Antibacterial and antifungal activity was expressed as the average diameter of inhibition zones.

The minimum inhibitory concentration (MIC) was determined for compound **2** in a serial microplate dilution assay against each test bacterial species (Elloff, 1998), with twofold serial dilution of the compound dissolved in DMSO, beyond the level where no inhibition of growth of the bacterial strains *B. subtilis* [ATTC 6633 (IMET NA)], *S. aureus* [SG511 (IMET 10760)], *Mycobacterium smegmatis* (SG987), *M. aurum* (SB66), *M. vaccae* (IMET 10670) and *M. fortuitum* was observed. Ciprofloxacin at a concentration of 100 mg/ml was used as the reference antibiotic and two wells were used as sterility and growth control, respectively, with the sterility control containing only Oxoid MH broth, while the negative growth control contained both MH broth as well as the test organism. The microplates were sealed and incubated at 37 °C and 100% relative humidity for 18 h. As an indicator of bacterial growth, 40 mL aliquots of a 0.2 mg/mL solution of *p*-iodonitrotetrazolium violet (INT) dissolved in water were added to the microplate wells and incubated at 37 °C for 30 min.

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