

N-Carboxamido-staurosporine and Selina-4(14),7(11)-diene-8,9-diol, New Metabolites from a Marine *Streptomyces* sp.[†]

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Abstract In our screening of micro-organisms for novel bioactive natural products, a new staurosporinone, *N*-carboxamido-staurosporine (**1c**), and a new sesquiterpene, (5*S*,8*S*,9*R*,10*S*)-selina-4(14),7(11)-diene-8,9-diol (**2a**), were isolated from the culture broth of the marine-derived *Streptomyces* sp. QD518. Their structures were determined by spectroscopic methods and by comparison of the NMR data with those of structurally related known natural products, which were isolated from the same strain.

Keywords marine streptomycete, staurosporine, selinane sesquiterpene

Introduction

The marine environment is a rich source of both biological and chemical diversity and delivers unique chemical compounds with the potential for industrial development as pharmaceuticals, cosmetics, nutritional supplements, molecular probes, fine chemicals, and agrochemicals [1]. In our search for bioactive substances from marine origin, the crude extract of the *Streptomyces* sp. isolate QD518 from the Jiaozhou Bay of Qindao, China, exhibited strong activity against *Escherichia coli*, *Streptomyces viridochromogenes*, and *Mucor miehei*. In the chemical screening, the extract showed fluorescent (366 nm) and UV

absorbing (254 nm) bands, which turned light green on spraying with anisaldehyde/sulphuric acid, while others turned red. Work-up of a 25-liter shaker culture led to the isolation of *N*-carboxamido-staurosporine (**1c**) and a selinane sesquiterpene **2a**. Beside these new metabolites, 14 known compounds belonging to 4 groups were isolated: 8 indole derivatives, namely 4-chloro-5-(3'-indolyl)-oxazole [2], 5-(3'-indolyl)-oxazole [2], 3-(hydroxyacetyl)-indole [3], indole-3-acetonitril [4], indole-3-carboxylic acid [5], 3-indolyl-ethanol [6], 3-indolylacrylamide [7], and acetyl- β -carbolin [8]; 4 benzene derivatives: vanillic acid, anthranilic acid, *m*-hydroxybenzyl-alcohol, chartreusin [9], a quinone, celastramycin B [10], and polyhydroxybutyric acid (sPHB) [11]. The known compounds were identified by substructure searches in AntiBase [12]. Here we want to report the taxonomy of the producing strain and the isolation, structure elucidation and biological activity of the new metabolites.

Results and Discussion

A 25-liter culture of *Streptomyces* sp. QD518 in meat extract medium delivered a dark brown culture broth, which was worked up in the usual manner. The resulting crude extract was separated by a sequence of chromatographic steps (Fig. 3).

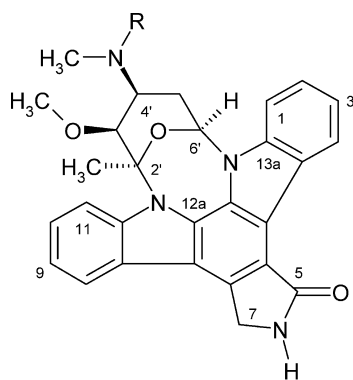
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1a: R = H
1b: R = CHO
1c: R = CONH₂

Compound **1a** gave an intensively blue fluorescent (366 nm) spot on TLC, which turned greenish on spraying with anisaldehyde/sulphuric acid. By the characteristic ¹H NMR and UV data and the molecular mass (*m/z* 467, [M+H]⁺ by (+)-ESI), it was easily identified as staurosporine (**1a**), which was previously isolated from *Streptomyces staurosporeus* Awaya (AM-2282) [13].

Due to the typical UV spectrum, a more polar compound from the same fraction seemed to be a staurosporine derivative as well. The molecular formula C₂₉H₂₆N₄O₄ (deduced from (+)-ESI HRMS) and comparison of the NMR data led to its identification as *N*-formyl-staurosporine (**1b**) [14, 15]. The doublet-like splitting of the *N*-methyl signal in the ¹H NMR spectrum is due to the *syn* and *anti* conformations of the formyl group [15].

A third staurosporine **1c** was obtained as colourless crystals by preparative HPLC. It exhibited similar UV data in MeOH with characteristic absorption maxima [16] at 243, 292, 318, 333, 354 and 372 nm, suggesting also the presence of the indolo[2,3-*a*]carbazole chromophore. The ¹H NMR spectrum in DMSO-*d*₆ was very similar to that of **1b** with an additional 2H singlet at δ 6.02 due to an NH₂ group instead of the aldehyde signal at δ 8.24 in **1b**. The (+)-ESI mass spectrum indicated a *pseudo* molecular ion at *m/z* 532 [M+Na]⁺, and ESI HRMS delivered the molecular formula C₂₉H₂₇N₅O₄, which possesses one nitrogen atom more than **1a** and **1b**, due to an additional NH₂ group. The ¹³C NMR spectrum indicated the presence of 29 carbon signals as demanded by the formula and displayed also two carbonyl groups (δ 171.8 and 158.7) as that of **1b**. According to the APT and HMBC spectra with a correlation between the *N*-methyl and the carbonyl signal at δ 158.7 among others (Figure 1), the expected NH₂ group had replaced the aldehyde hydrogen in the formyl residue, which identified this compound as the new *N*-carboxamido-

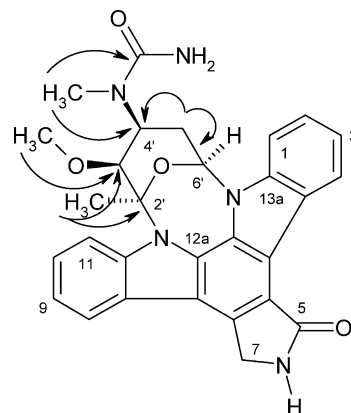


Fig. 1 Selected HMBC correlations of the sugar part of carboxamidostaurosporine (**1c**).

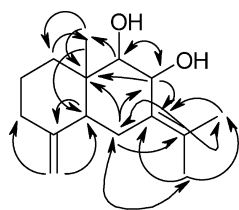
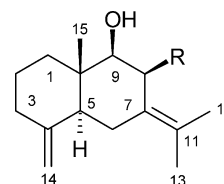
staurosporine (**1c**). Interestingly, this substitution caused a very strong downfield shift of 4'-H from δ 3.18 in **1b** to δ 4.84 in **1c**, which seems to indicate an anisotropy effect due to the amide carbonyl.

With respect to the tremendous number of staurosporines listed in the Chemical Abstracts, it was very unexpected that less than 10 urea derivatives like **1c** are known, none of them being natural [17].

Compound **2a** was isolated as a colourless oil, which gave no UV absorption on TLC and showed a violet colour after spraying with anisaldehyde/sulphuric acid. The ¹H NMR spectrum exhibited three 1H doublets at δ 4.81, 4.79 and 3.24; at high field, multiplets between δ 1.5~1.7, a ddd signal at δ 1.20 and three methyl signals (δ 1.80 d, 1.75 d, 0.99 s) appeared. Four of the 15 signals in the ¹³C NMR spectrum were olefinic, two were oxymethines. One of the five methylene signals (δ 106.4) was olefinic and due to an exo methylene group. EI MS indicated a molecular weight of *m/z* 236, and EI HRMS afforded the molecular formula C₁₅H₂₄O₂. The HMBC data exhibited a correlation between both the exo methylene 14-H₂ and the 15-methyl to the methine carbon C-5. In addition, the 15-methyl, 6-H₂ and the 8-H signals indicated couplings with the quaternary carbon C-10. Further correlations from the 12/13-methyl signals and 8-H to C-7 and H,H COSY signals between 5/6-H and 8/9-H delivered among others the sesquiterpene structure of selina-4(14),7(11)-diene-8,9-diol (**2a**, see Fig. 2). Structure **2a** was finally confirmed and all other alternatives were excluded by interpreting the 2D data with the structure elucidation program COCON [18], which delivered **2a** as the only solution agreeing with the COSY and HMBC data. Irradiation into the well-separated signal of 9-H (δ 3.24) showed a positive NOE on the signals at δ 4.79 (8-H), 1.70 (5-H) and 1-H_α (δ 1.20), indicating the *cis*-orientation of these protons. There was no interaction

Table 1 ^{13}C (150 MHz) and ^1H NMR (300 MHz, J in Hz) data of **1c** in $\text{DMSO}-d_6$

No.	C	^1H	No.	C	^1H
1	108.9	7.68 (d, 8.2)	10	124.8	7.47 (t br, 7.2)
2	125.0	7.47 (t br, 7.2)	11	113.6	8.05 (d, 8.6)
3	119.3	7.28 (t, 7.5)	11a	138.9	—
4	125.6	9.28 (d, 7.5)	12a	129.1	—
4a	122.6	—	12b	125.0	—
4b	115.2	—	13a	136.2	—
4c	132.5	—	2'	94.8	—
5	171.8	—	3'	84.0	4.22 (s br)
NH	—	8.55 (s)	4'	48.3	4.84 (m)
7	45.4	5.00 (s)	5	27.4	2.18 (m), 2.63 (m)
7a	119.3	—	6	82.4	7.00 (t, 6.7)
7b	114.0	—	Me	29.3	2.34 (s)
7c	123.7	—	OMe	60.1	2.77 (s)
8	121.3	8.00 (d, 7.7)	NMe	30.1	2.63 (s)
9	120.1	7.34 (t, 7.6)	CONH ₂	158.7	6.02 (s)

**Fig. 2** HMBC (\rightarrow) and selected H,H COSY (\leftrightarrow) correlations of **2a**; two of the latter are 5J couplings.**2a:** R = β -OH**2b:** R = H

with the 15-Me signal, and also irradiation into the signal of the latter did not deliver further information. In the dibenzoate, the 15-Me signal was, however, shifted to down-field ($\delta\Delta$ 0.29), which indicated clearly a *cis* orientation of both 15-Me and 9-OH as in all other selinanes.

As we observed a positive Cotton effect in the dibenzoate, a (+)-helical orientation of both ester groups can be expected. With the assumption of a chair conformation, this results in (*5S,8S,9R,10S*) as the absolute configuration of **2a**.

Selina-4(14),7(11)-diene-8,9-diol (**2a**) belongs to the group of selinane/eudesmane sesquiterpenes, which are common metabolites of plants (including algae), sponges and fungi. In bacteria, sesquiterpenoids are rare in general. Pentalenolactone was one of the first examples [19], and a few less polar terpenes have been found now as odour components in actinomycetes [21] and streptomycetes [20]. Only one selinane, selinadienol (**2b**) from *Streptomyces fradiae* IMRU3535, has been described from bacteria so far [21].

Biological Activities

Staurosporine (**1a**) possesses inhibitory activity against fungi and yeasts (but no significant activity on bacteria) [22], strong antihypertensive activity and shows pronounced inhibition of a number of experimental tumours [23]. Additionally, staurosporine (**1a**) and analogues are important biochemical tools [24] due to their potent inhibition of protein kinase C and platelet aggregation [25].

Compounds **1a**, **1b**, **1c** and **2a** were tested against *Staphylococcus aureus*, *Bacillus subtilis*, *Streptomyces viridochromogenes* (Tü 57) and *Escherichia coli*, the fungi *Mucor miehei* and *Candida albicans*, and the microalgae *Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus subspicatus*. In the agar diffusion test, the sesquiterpene **2a** showed no biological activity at a concentration of 80 μg /paper disk. Compounds **1a** and **1b** were measured at a concentration of 80, **1c** at 40 μg /disk; they exhibited no activity against the bacteria, and the phycotoxic activity of **1b** and **1c** were lower than that of **1a**. Only **1c** showed a weak but selective activity against *Streptomyces viridochromogenes* (Table 2).

Compounds **1b**, **1c** and **2a** were tested *in vitro* for anticancer activity in a panel of 37 human tumor cell lines derived from solid human tumors comprising bladder, central nervous system, colon, gastric, head and neck, lung, mammary, ovarian, pancreatic, prostate and renal cancers, as well as cell lines established from human melanoma, pleuramesothelioma and the uteri body. Compound **1c** was found to be the most potent substance, exhibiting a mean IC₅₀ value of 0.016 $\mu\text{g/ml}$ and a mean IC₇₀ value of 0.17 $\mu\text{g/ml}$ (Table 3). Importantly, **1c** showed a high tumor selectivity score (criterion for a selective inhibition of a cell line: individual IC₇₀ values smaller than 1/3 of mean IC₇₀ value), effecting selective activity in 10 out of 37 cell lines (Table 4). As well as compound **1c**, **1b** displayed marked activity, exhibiting a mean IC₅₀ value of 0.063 $\mu\text{g/ml}$ and a mean IC₇₀ value of 0.35 $\mu\text{g/ml}$. Selective activity was determined in 6 out of 37 cell lines (16%). The terpene **2a** was inactive in all cell lines up to a concentration of 10 $\mu\text{g/ml}$. In conclusion, compounds **1c** and **1b** should be considered as candidate compounds for further profiling as anticancer drugs.

Experimental

Material and methods were used as described previously [26]. Rf values were measured on silica gel with CH₂Cl₂/

Table 2 Inhibition diameters in the agar diffusion test of the staurosporines **1a**, **1b** at concentrations of 80 and **1c** at 40 μg /paper disk

	CA	SV	MM	CV	CS	SS
1a	11	0	20	33	30	30
1b	0	0	11	14	18	14
1c	0	11	0	14	16	15

CA, *Candida albicans*; SV, *Streptomyces viridochromogenes*; MM, *Mucor miehei*; CV, *Chlorella vulgaris*; CS, *Chlorella sorokiniana*; SS, *Scenedesmus subspicatus*.

7% MeOH, if not stated otherwise.

Taxonomy of the Producing Strain

The strain M518 has been derived from a sediment of Jiaozhou Bay in China. It was isolated on Gause's starch medium with incubation at 28°C. The pure culture was maintained on Gause's starch agar medium with K₂Cr₂O₇ at 4°C. The strain forms a brown vegetative mycelium and a brown-violet aerial mycelium. The substrate mycelium does not have transverse septa and no fragments, the aerial mycelium has few branches. The strain forms straight sporophores. The spores are long-oval with smooth surface. Melanin is not produced on tyrosine agar, and water-soluble pigments are not produced on other media. The strain can utilize starch, glucose and esculin as carbon source. The strain produces no pyocyanine and fluorochrome and does not peptonize or coagulate milk. Gelatine is not degraded and hydrogen sulphide is not produced. The strain is catalase positive, lipase positive and nitrate reductase negative. Due to its physiology and morphological features as well as the 16S rRNA (GenBank accession number DQ184649), the strain can be assigned to the genus *Streptomyces*. The strain is deposited in the culture collection of marine actinomycetes at the Institute of Oceanology, Chinese Academy of Sciences, Nanhai Road 7, 266071 Qingdao, China.

Meat Extract Medium

10 g glucose, 2 g peptone (Fluka), 1 g yeast extract (Marcor) and 1 g meat extract (Fluka) were dissolved in a mixture of 500 ml tap water and 500 ml artificial sea water,

Table 4 Antitumor-selectivity corresponding to the number of cell lines with individual IC₇₀ \leq 1/3 (mean IC₇₀)/total

Compound	Total selectivity	% Selectivity
1b	6/37	16
1c	10/37	27
2a	0/37	0

Table 3 Mean antitumor activity of **1b**, **1c**, and **2a** in a panel of 37 cell lines

Compound	Mean IC ₅₀ [$\mu\text{g/ml}$]	Mean IC ₇₀ [$\mu\text{g/ml}$]	Mean IC ₉₀ [$\mu\text{g/ml}$]
<i>N</i> -Formyl-staurosporine (1b)	0.063	0.348	2.68
<i>N</i> -Carboxamido-staurosporine (1c)	0.016	0.171	2.35
Sesquiterpene 2a	>10	>10	>10

the pH was adjusted to 7.8 with 2 N NaOH and sterilized at 121°C for 33 minutes.

Fermentation and Isolation

The marine *Streptomyces* sp. QD518 was cultivated in a 25-liter scale on meat extract medium at 28°C for 7 days on a linear shaker (110 rpm). The culture broth was mixed with *ca.* 1.5 kg Celite and filtered under pressure. The water phase was extracted with a XAD-16 column (4×140 cm), the resin washed with distilled water and eluted with methanol, while the mycelium was extracted firstly with ethyl acetate and then acetone. Both extracts were combined, evaporated to dryness and separated by chromatography on silica gel using a CH₂Cl₂/MeOH gradient to afford four fractions A~D. Fraction A contained fatty acids and was discarded. Fraction B delivered on Sephadex LH-20 (CH₂Cl₂/50%MeOH) three sub-fractions B₁, B₂, B₃, which were further purified by PTLC (CH₂Cl₂/3% MeOH). Fraction B₁ delivered the sesquiterpene **2a** (4 mg) and an olefin (5 mg), which could not be obtained pure due to rapid decomposition. Fractions B₂ and B₃ gave 8 known compounds, 1-acetyl-β-carbolin (Rf=0.63, 2.5 mg), celastramycin B (1.5 mg), vanillic acid (3.5 mg), anthranilic acid (Rf=0.29, 2 mg), *p*-hydroxybenzyl-alcohol (2 mg), salicylic alcohol (3 mg), 3-(2-hydroxyethyl)-indole (6 mg) and 3-indoleacrylamide (3 mg). Chromatography of fraction C using Sephadex LH-20 (MeOH), PTLC (DCM/5% MeOH) and HPLC (H₂O/MeCN gradient) delivered 5 known compounds, namely 4-chloro-5-(3'-indolyl)-oxazole (Rf=0.63, 28 mg), 5-(3'-indolyl)-oxazole (Rf=0.43, 2 mg), 3-(hydroxyacetyl)-indole (Rf=0.34, 5 mg), indole-3-acetonitrile (Rf=0.43, 2 mg), and indole-3-carboxylic acid (Rf=0.23). Trituration of fraction D with methanol delivered polyhydroxy butyric acid (PHB, 5 mg) as insoluble material. The soluble part yielded on Sephadex LH-20 (CH₂Cl₂/50% MeOH) the fractions D₁, D₂, D₃. PTLC followed by preparative HPLC of fraction D₁ gave chartreusin (2 mg). Purification of fraction D₂ on Sephadex LH-20 (MeOH) delivered sub-fractions D₂₁ and D₂₂. Sub-fraction D₂₂ yielded fine crystals of staurosporine (**1a**, 25 mg, Rf=0.37) in MeCN. The mother liquor was separated by HPLC and delivered *N*-formylstaurosporine (**1b**, Rt=16.23 minutes, 2 mg, Rf=0.31) and *N*-carboxamido-staurosporine (**1c**, Rt=17.44 minutes, 4 mg).

N-Carboxamido-staurosporine (**1c**)

Light yellow solid, Rf=0.46; UV (MeOH): λ_{max} (log ε)=372 (3.30), 354 (3.25), 333 (3.43), 318 (3.43), 292 (4.01), 243 (3.67) nm. –IR (KBr): ν=2855, 2929, 2359, 2344, 1633, 1458, 1385, 1316, 1282, 1120, 1018 cm⁻¹. NMR data see Table 1. (+)-ESI MS *m/z*

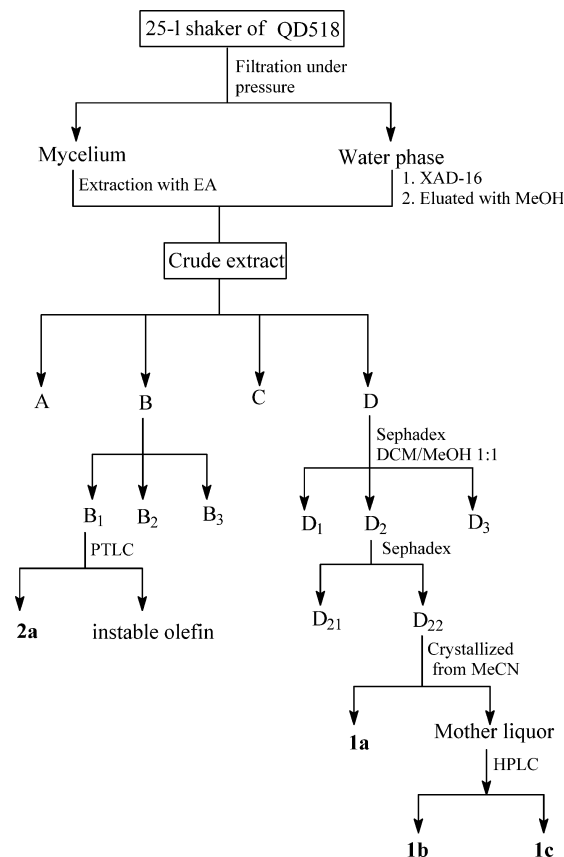


Fig. 3 Work up scheme for the marine *Streptomyces* sp. isolate QD518.

(%)=532 ([M+Na]⁺, 100), 1041 ([M+2Na]⁺, 90). (+)-ESI HRMS: 510.213818 (510.213589 [M+H]⁺, calcd. for C₂₉H₂₈N₅O₄).

(5*S*,8*S*,9*R*)-Selina-4(14),7(11)-diene-8,9-diol (**2a**)

Colourless oil, Rf=0.31 (CH₂Cl₂/3% MeOH). UV (MeOH): λ_{max} (log ε)=253 (3.51), 204 (4.78) nm. IR (KBr): ν=3650, 2928, 2361, 2343, 1636, 1437, 1385, 1107 cm⁻¹. ¹H NMR (CDCl₃, 300 MHz): δ 4.81 (q, *J*=1.7 Hz, 1H, 14-H_A), 4.79 (d, *J*=4.0 Hz, 1H, 8-H), 4.60 (q, *J*=1.6 Hz, 1H, 14-H_B), 3.24 (d, *J*=4.2 Hz, 1H, 9-H), 2.43, 2.14 (ABX *J*_{AB}=*J*_{AX}=14.3, *J*_{BX}=2.9 Hz, 2H, 6-H₂), 2.32 (dm, 2H, 3-H₂), 1.95 (m, 1H, 1-H_β), 1.80 (d, *J*=2.1 Hz, 3H, 12-H₃), 1.75 (d, *J*=2.1 Hz, 3H, 13-H₃), 1.70~1.50 (m, 3H, 2-H₂, 5-H), 1.20 (ddd, 2×*J*=12.7, 5.4 Hz, 1H, 1-H_α), 0.99 (s, 3H, 15-H₃). ¹³C NMR (CDCl₃, 150 MHz): δ=149.6 (C_q-4), 130.0 (C_q-7*), 129.9 (C_q-11*), 106.4 (CH₂-14), 80.2 (CH-9), 70.3 (CH-8), 48.0 (CH-5), 40.8 (C_q-10), 38.2 (CH₂-1), 36.5 (CH₂-3), 23.7 (CH₂-6), 22.6 (CH₂-2), 20.7 (CH₃-12), 20.0 (CH₃-13), 12.0 (CH₃-15). EI MS *m/z* (%)=236.2 (M⁺, 100), 221.2 (18), 203.2 (24), 189.2 (35), 175.2 (35), 147.1 (25), 133.1 (30), 124.1

(48), 109.1 (58), 95.1 (40), 81.1 (32), 69.1 (25), 55.1 (26), 41.0 (37). EI HRMS: 236.17761 (236.177608 [M]⁺ calcd. for C₁₅H₂₄O₂).

(5*S*,8*S*,9*R*,10*S*)-Selina-4(14),7(11)-diene-8,9-dibenzoate

To a stirred solution of 4 mg **2a** in 1 ml CH₂Cl₂, 0.5 ml pyridine, 1.5 ml benzoyl chloride and 10 mg DMAP were added. The reaction mixture was stirred for 12 hours at room temperature, then poured into water followed by extraction with ethyl acetate and chromatography on silica gel/CH₂Cl₂. The obtained monobenzoate was again benzoylated and worked up in the same manner to deliver 1.5 mg of **2a** dibenzoate as a colourless oil, R_f=0.68 (CH₂Cl₂). CD (MeOH): λ_{ext} ([θ]²⁵)=240 (+2850), 224 (−24500) nm ¹H NMR (CDCl₃, 300 MHz): δ 8.18 (dd, *J*=1.3, 8.4 Hz, 2H, Ar-H), 8.02 (dd, *J*=1.3, 8.4 Hz, 2H, Ar-H), 7.94 (dd, *J*=1.3, 8.4 Hz, 2H, Ar-H), 7.69 (tt, *J*=7.5, 1.3 Hz, 1H, Ar-H), 7.54 (m, 1H, Ar-H), 7.44 (m, 1H, Ar-H), 7.34 (brt, *J*=7.9 Hz, 1H, Ar-H), 6.49 (d, *J*=3.9 Hz, 1H, 8-H), 5.03 (d, *J*=4.0 Hz, 1H, 9-H), 4.89 (br q, *J*=1.5 Hz, 1H, 14-H_A), 4.70 (brq, *J*=1.5 Hz, 1H, 14-H_B), 2.58 (dd, *J*=14.2, 4.2 Hz, 1H, 6-H_a), 2.38 (brt, *J*=12.8 Hz, 2H, 3-H₂), 2.03 (m, 1H, 6-H_b), 1.70~1.50 (m, 4H, 1-H₂, 2-H₂), 1.93 (d, *J*=1.8 Hz, 3H, 12-H₃), 1.77 (d, *J*=0.97 Hz, 3H, 13-H₃), 0.99 (s, 3H, 15-H₃). ESI MS *m/z* (%)=467.0 ([M+Na, 92]⁺), 911.1 ([2M+Na, 54]⁺). ESI HRMS 467.21875 (467.21982 [M+Na]⁺ calcd. for C₂₉H₃₂O₄Na).

Cytotoxicity and Proliferation Assay

Anti-tumor activity of the compounds **1b**, **1c** and **2a** was tested in a monolayer cytotoxicity and proliferation assay using human tumor cell lines as described previously [27]. Briefly, the number of viable cells after 4 days of incubation with a test compound was determined using propidium iodide fluorescence as a read-out. Antitumor activity including the induction of apoptosis and the inhibition of cell proliferation was recorded as a reduction of the viable cell number relative to control wells and expressed as T/C (test/control) value. The requirement of antitumor activity was a T/C value of <30%. Tumor selectivity was defined as a 3-fold lower individual IC₇₀ value of a cell line compared to the mean IC₇₀ value over the 37 cell line panel. Compounds were tested in triplicate in a panel of 37 human tumor cell lines at five different concentrations ranging from 0.001 μg/ml up to 10 μg/ml (**2a**), and 0.0003 μg/ml up to 3 μg/ml (compounds **1b** and **1c**), respectively. Twenty-four out of the 36 test cell lines had been established from patient-derived tumor xenografts growing in nude mice as described by Roth *et al.* 1999 [28]. The origin of the donor xenografts was described by Fiebig *et al.* 1992 [29]. The remaining 12 cell lines were

kindly provided by the US National Cancer Institute or purchased from the American Type Culture Collection (Rockville, MD, USA).

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