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

Shao Jie Wu, Serge Fotso, Fuchao Li, Song Qin, Gerhard Kelter, Heinz H. Fiebig, Hartmut Laatsch

Received: May 9, 2006 / Accepted: May 19, 2006
© Japan Antibiotics Research Association

**Abstract** In our screening of micro-organisms for novel bioactive natural products, a new staurosporinone, N-carboxamido-staurosporine (1c), and a new sesquiterpene, (5S,8S,9R,10S)-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 streptomycte, 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-indoly lacrylamide [7], and acetyl-β-carbolin [8]; 4 benzene derivatives: vanillic acid, anthranilic acid, m-hydroxybenzyl-alcohol, chartreusin [9], a quinone, celastracycin 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).

---

**H. Laatsch** (Corresponding author), S. J. Wu, S. Fotso: Department of Organic and Biomolecular Chemistry, University of Göttingen, Tammanstrasse 2, D-37077 Göttingen, Germany, E-mail: HLAATSC@gwdg.de

S. J. Wu, F. Li, S. Qin: Institute of Oceanology, Chinese Academy of Sciences, No. 7 Nanhai Road, Qingdao 266071, P.R. China

S. J. Wu, F. Li: Graduate School of Chinese Academy of Science, Beijing, 100049, P.R. China

G. Kelter, H. H. Fiebig: OncoTest GmbH, Am Flughafen 12-14, D-79108 Freiburg, Germany

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

Due to the typical UV spectrum, a more polar compound from the same fraction seemed to be a staurosorinone derivative as well. The molecular formula C$_{21}$H$_{19}$N$_{2}$O$_{4}$ (deduced from (+)-ESI HRMS) and comparison of the NMR data led to its identification as 3-formylstaurosorinone (1b) [14, 15]. The doublet-like splitting of the N-methyl signal in the $^1$H NMR spectrum is due to the *syn* and *anti* conformations of the formyl group [15].

A third staurosorinone 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 $^1$H NMR spectrum in DMSO-$d_6$ was very similar to that of 1b with an additional 2H singlet at $\delta$ 6.02 due to an NH$_2$ group instead of the aldehyde signal at $\delta$ 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 C$_{25}$H$_{21}$N$_{2}$O$_{4}$, which possesses one nitrogen atom more than 1a and 1b, due to an additional NH$_2$ group. The $^{13}$C NMR spectrum indicated the presence of 29 carbon signals as demanded by the formula and displayed also two carbonyl groups ($\delta$ 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 $\delta$ 158.7 among others (Figure 1), the expected NH$_2$ group had replaced the aldehyde hydrogen in the formyl residue, which identified this compound as the new N-carboxamido-staurosorinone (1c). Interestingly, this substitution caused a very strong downfield shift of 4'-H from $\delta$ 3.18 in 1b to $\delta$ 4.84 in 1c, which seems to indicate an anisotropy effect due to the amide carbonyl.

With respect to the tremendous number of staurosorinones 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 $^1$H NMR spectrum exhibited three 1H doublets at $\delta$ 4.81, 4.79 and 3.24; at high field, multiplets between $\delta$ 1.5-1.7, a ddd signal at $\delta$ 1.20 and three methyl signals ($\delta$ 1.80 d, 1.75 d, 0.99 s) appeared. Four of the 15 signals in the $^{13}$C NMR spectrum were olefinic, two were oxymethines. One of the five methylene signals ($\delta$ 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 C$_{15}$H$_{26}$O$_{5}$. The HMBC data exhibited a correlation between both the exo methylene 14-H$_{2}$ and the 15-methyl to the methine carbon C-5. In addition, the 15-methyl, 6-H$_{2}$ 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$_{8}$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 ($\delta$ 3.24) showed a positive NOE on the signals at $\delta$ 4.79 (8-H), 1.70 (5-H) and 1-H$_{8}$ ($\delta$ 1.20), indicating the cis-orientation of these protons. There was no interaction
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 (Δδ 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
Stauroporine (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, stauroporine (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 phytotoxic 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 µg/ml and a mean IC₇₀ value of 0.17 µg/ml (Table 3). Importantly, 1c showed a high tumor selectivity score (criterion for a selective inhibition of a cell line: individual IC₅₀ 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 µg/ml and a mean IC₇₀ value of 0.35 µg/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 µg/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 staurosponine 1a, 1b at concentrations of 80 and 1c at 40 µg/paper disk

<table>
<thead>
<tr>
<th></th>
<th>CA</th>
<th>SV</th>
<th>MM</th>
<th>CV</th>
<th>CS</th>
<th>SS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>11</td>
<td>0</td>
<td>20</td>
<td>33</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>1b</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>14</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>1c</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>14</td>
<td>16</td>
<td>15</td>
</tr>
</tbody>
</table>

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

### Table 4  Antitumor-selectivity corresponding to the number of cell lines with individual IC₅₀ = 1/3 (mean IC₇₀)/total

<table>
<thead>
<tr>
<th>Compound</th>
<th>Total selectivity</th>
<th>% Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1b</td>
<td>6/37</td>
<td>16</td>
</tr>
<tr>
<td>1c</td>
<td>10/37</td>
<td>27</td>
</tr>
<tr>
<td>2a</td>
<td>0/37</td>
<td>0</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean IC₅₀ [µg/ml]</th>
<th>Mean IC₇₀ [µg/ml]</th>
<th>Mean IC₉₀ [µg/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Formyl-staurosponine (1b)</td>
<td>0.063</td>
<td>0.348</td>
<td>2.68</td>
</tr>
<tr>
<td>N-Carboxamido-staurosponine (1c)</td>
<td>0.016</td>
<td>0.171</td>
<td>2.35</td>
</tr>
<tr>
<td>Sesquiterpene 2a</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

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 pycocyanine 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,
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 x 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 CH3Cl/MeOH gradient to afford four fractions A-D. Fraction A contained fatty acids and was discarded. Fraction B delivered on Sephadex LH-20 (CH3Cl/50%MeOH) three sub-fractions B1, B2, B3, which were further purified by PTLC (CH3Cl/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), antranilic 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 (CH3Cl/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, RF=0.1623 minutes, 2 mg, RF=0.01) and N-carboxamido-stauroporine (1c, RF=0.1744 minutes, 4 mg).

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

Light yellow solid, RF=0.46; UV (MeOH): λmax (log ε)=372 (3.30), 354 (3.25), 333 (3.43), 318 (3.43), 292 (4.01), 243 (3.67) nm. IR (KBr): ν=2947, 2915, 1633, 1458, 1385, 1316, 1282, 1120, 1018 cm⁻¹. NMR data see Table 1. (+)-ESI MS m/z (%): 532 ([M+Na]+, 100), 1041 ([M+2Na]+, 90). (+)-ESI HRMS: 510.213818 (510.213589 [M+H]+, calcd. for C25H28N4O4).

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

Colourless oil, RF=0.31 (CH3Cl/3% MeOH). UV (MeOH): λmax (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-H14), 4.79 (d, J=4.0 Hz, 1H, 2-H12), 4.60 (q, J=1.6 Hz, 1H, 14-H14), 3.24 (d, J=4.2 Hz, 1H, 9-H), 2.43, 2.14 (ABX JAB=JAX=14.3, JBX=2.9 Hz, 2H, 6-H), 2.32 (dm, 2H, 3-H), 1.95 (m, 1H, 1-H14), 1.80 (d, J=2.1 Hz, 3H, 12-H12), 1.75 (d, J=2.1 Hz, 3H, 13-H13), 1.70-1.50 (m, 3H, 2-H12, 5-H15), 1.20 (dd, 2×J=12.7, 5.4 Hz, 1H, 1-H14), 0.99 (s, 3H, 15-H15). 13C NMR (CDCl3, 150 MHz): δ=149.6 (C14), 130.0 (C4), 129.9 (C5), 106.4 (CH1-14), 80.2 (CH9), 70.3 (CH8), 48.0 (CH5), 40.8 (C9), 38.2 (CH1-14), 36.5 (CH2-3), 23.7 (CH3-6), 22.6 (CH2-2), 20.7 (CH12), 20.0 (CH13), 12.0 (CH5-15). 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 (100).
(SS,8S,9R,10S)-Selina-4(14),7(11)-diene-8,9-dibenoate
To a stirred solution of 4 mg 2a in 1 mL CH₂Cl₂, 0.5 mL pyridine, 1.5 mL benzyl alcohol 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, Rf = 0.68 (CH₂Cl₂). CD (MeOH): λ(θ) (θ) = 240 ( +2850), 224 (−24500) nm (H NMR (CDCl₃, 300 MHz): δ 8.18 (d, 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 (m, 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 (brq, J = 1.5 Hz, 1H, 14-H₂), 4.70 (brq, J = 1.5 Hz, 1H, 14-H₂), 2.58 (dd, J = 14.2, 4.2 Hz, 1H, 6-H), 2.38 (brt, J = 12.8 Hz, 2H, 3-H₂), 2.03 (m, 1H, 6-H), 1.70−1.50 (m, 4H, 1H-1H₂-2-H₂), 1.93 (d, J = 1.8 Hz, 3H, 12-H), 1.77 (d, J = 0.97 Hz, 3H, 13-H), 0.99 (s, 3H, 15-H), ESI MS m/z (%) = 467.0 ([M+Na⁺, 92]⁺), 91.1 ([2M+Na⁺, 54]⁺). ESI HRMS 467.21875 (467.21982 [M+Na⁺] calc'd for C₉₇H₆₀O₄Na).

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 IC₅₀ value of a cell line compared to the mean IC₅₀ 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).

Acknowledgments We thank R. Machinek and H. Frausendorf for NMR and mass spectra, and F. Lissy and A. Kohl for technical assistance. This work was supported by a grant from the International Bureau at DLR (CHN 01/326) and by the National High Technology Research and Development Program of China (863 Program, 2001AA624020), and the Key Innovative Project of CAS (KZCX3-SW-215).

References


