

ORIGINAL ARTICLE

# Caboxamycin, a new antibiotic of the benzoxazole family produced by the deep-sea strain *Streptomyces* sp. NTK 937\*

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**Caboxamycin, a new benzoxazole antibiotic, was detected by HPLC-diode array screening in extracts of the marine strain *Streptomyces* sp. NTK 937, which was isolated from deep-sea sediment collected in the Canary Basin. The structure of caboxamycin was determined by mass spectrometry, NMR experiments and X-ray analysis. It showed inhibitory activity against Gram-positive bacteria, selected human tumor cell lines and the enzyme phosphodiesterase.**

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**Keywords:** benzoxazole antibiotic; fermentation; isolation; marine *Streptomyces*; structural elucidation

## INTRODUCTION

A set of 600 actinomycetes isolated from marine sediments from various sites in the Atlantic and Pacific Oceans were screened using our HPLC-diode array technology for the production of bioactive secondary metabolites. It is becoming increasingly clear that actinomycetes isolated from marine habitats are a rich source of new natural products, including drug candidates.<sup>1–5</sup> Extracts from the culture filtrate of strain NTK 937 showed a prominent peak in the HPLC-diode array analysis that had an unusual UV-visible spectrum, albeit it was similar to that of nataxazole from our HPLC-UV-Vis database which contains 867 entries, mostly antibiotics.<sup>6</sup> Nataxazole, a new member of the benzoxazole family of antibiotics, was extracted from terrestrial *Streptomyces* sp. Tü 6176.<sup>7</sup> Nataxazole has a structure similar to that of benzoxazoles UK-1<sup>8</sup> and AJI9561,<sup>9</sup> which were derived from *Streptomyces* strains, and were shown to have potent anti-tumor properties. The similarity of the UV-visible spectrum of the metabolite produced by strain NTK 937 to nataxazole underlines its structural relationship to benzoxazoles; the new compound was named caboxamycin (**1**) composed from the strain's collection site, the Canary Basin and the benzoxazole scaffold of its structure, which is shown in Figure 1. Herein, we report on the taxonomy of the producing strain, the fermentation, isolation and structural elucidation of caboxamycin and its biological activities.

## RESULTS

### Taxonomy of the producing strain

The chemical and morphological properties of strain NTK 937 were consistent with its classification in the genus *Streptomyces*.<sup>10</sup> The organism formed an extensively branched substrate mycelium, straight spore chains on the aerial mycelium, contained hexa- and octahydrogenated menaquinones with nine isoprene units as the predominant isoprenologues, and gave whole-cell hydrolysates rich in LL-diaminopimelic acid. Comparison of an almost complete 16S rRNA sequence of strain NTK 937 with corresponding sequences of *Streptomyces* type strains showed that it formed a distinct phyletic line in the *Streptomyces* 16S rRNA gene tree.

### Screening, fermentation and isolation

When grown in the 20-liter fermentor strain NTK 937 reached a maximal biomass of 12 mg dry weight per ml after 5 days of incubation. Production of **1** started at 72 h, reaching a maximal yield of 15 mg l<sup>-1</sup> after incubation for 5 days. The HPLC elution profile of the corresponding culture filtrate is shown in Figure 2. **1** was isolated from the culture filtrate by separation on an Amberlite XAD-16 column; the extracts were concentrated to an aqueous residue and extracted with ethylacetate and concentrated *in vacuo* to dryness. The

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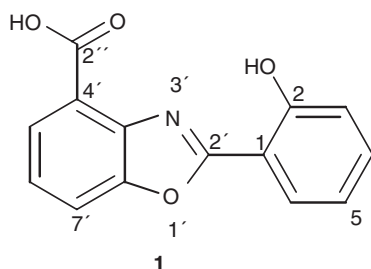
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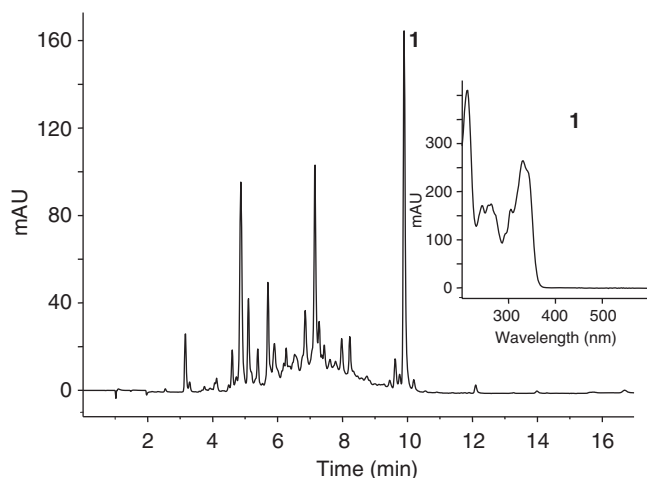
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**Figure 1** Structure of caboxamycin (**1**).



**Figure 2** HPLC analysis of a culture filtrate from *Streptomyces* sp. NTK 937 at a fermentation time of 120 h, monitored at 310 nm, and UV-visible spectrum of caboxamycin (**1**), retention time 9.9 min.

crude product was purified by chromatography on a diol-modified silica gel column followed by chromatography on a Sephadex LH-20 and Toyopearl HW-40S column. **1** was obtained as a white, yellow-fluorescent powder after concentrated to dryness.

### Structural elucidation

The mass spectrum derived from the HPLC-ESI-MS chromatograms revealed the molecular mass for **1** [(M+H)<sup>+</sup>=256.0]. The exact molecular mass was determined by high-resolution ESI-FT-ICR-MS as 256.06010 Da [(M+H)<sup>+</sup>] (**1**), this corresponds to the molecular formulae C<sub>14</sub>H<sub>9</sub>NO<sub>4</sub> (**1**) [(M+H)<sup>+</sup><sub>theor</sub>=256.06043; Δm=1.29 p.p.m.]. The ESI-FTICR-MS measurement of **1** showed an additional molecular mass of 564.02409 Da [(M-3H+Fe+H)<sup>+</sup>]. This corresponds well with the molecular formula C<sub>28</sub>H<sub>16</sub>N<sub>2</sub>O<sub>8</sub>Fe [(2M-3H+Fe+H)<sup>+</sup><sub>theor</sub>=564.02506; Δm=1.72 p.p.m.] and suggests that the compound is able to complex metal ions. The physico-chemical properties of **1** are summarized in Table 1.

The <sup>1</sup>H-NMR-spectrum of **1** showed seven signals in the aromatic region between 8.2 and 7 p.p.m. and one broad signal at 11.8 p.p.m. <sup>13</sup>C-NMR and DEPT spectra revealed the presence of seven aromatic CH groups and seven quaternary carbons between 170 and 110 p.p.m. The correlation of <sup>1</sup>H-NMR-signals to the corresponding C-atoms was determined in a Heteronuclear Multiple Quantum Coherence NMR experiment. Seven signals were found in accordance with the DEPT spectrum. Compared to the molecular formula, C<sub>14</sub>H<sub>9</sub>N<sub>1</sub>O<sub>4</sub>, two protons should be bound to heteroatoms (OH or NH), one of which giving a broad signal at 11.8 p.p.m. in the <sup>1</sup>H-NMR-spectrum.

**Table 1** Physico-chemical properties of caboxamycin (**1**)

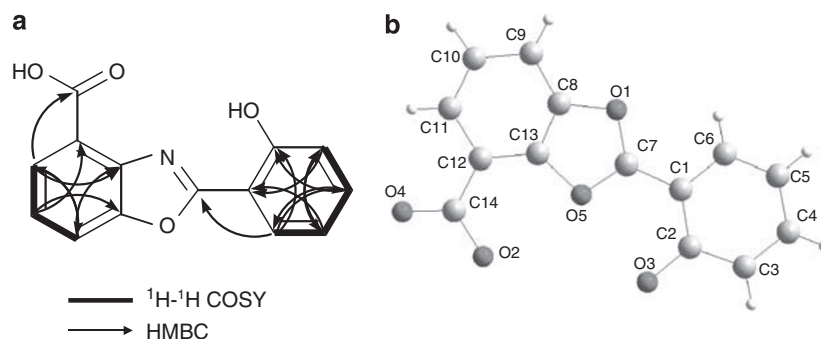
| <b>1</b>                                  |  |
|---|--|
| Appearance                                | White powder   |
| Molecular weight                          | 255  |
| Molecular formula                         | C <sub>14</sub> H <sub>9</sub> N <sub>1</sub> O <sub>4</sub>   |
| ESI-FT-ICR MS (m z <sup>-1</sup> )        |  |
| Found                                     | 256.06010 (M+H) <sup>+</sup>   |
| Calcd                                     | 256.06043 (M+H) <sup>+</sup>   |
| UV λ <sub>max</sub> <sup>MeOH</sup> [nm]  |  |
| (ε [cm <sup>2</sup> mmol <sup>-1</sup> ]) | 212 (20), 243 (8.16), 257 (sh, 8.16), 263 (8.37), 273 (sh, 7.35), 292 (sh, 5.51), 305 (sh, 7.76), 331 (11.43), 342 (sh, 10.82)                         |
| IR ν <sub>max</sub> (cm <sup>-1</sup> )   | 3466, 3088, 3046, 3006, 2956, 2920, 2850, 2676, 2570, 1690, 1633, 1591, 1546, 1486, 1433, 1296, 1264, 1247, 1239, 1183, 1155, 1065, 875, 752, 740, 724 |

The structure was fully elucidated using COSY and HMBC spectra. The <sup>1</sup>H-<sup>1</sup>H-COSY experiment revealed two spin systems, one of which showed correlations from H-4 to H-6 and the other one from H-10 to H-13 (Figure 3). The structure was fully elucidated using the HMBC spectrum; structure consists of two constituents, a salicylic acid and a hydroxy-anthranilic acid connected by a ring closure between the carboxylic acid group of the salicylic acid and the hydroxyl- and amino-groups of the hydroxy-anthranilic acid (Figure 1). The correlations from H-5' to C-2'', C-3'a and C-7', from H-6' to C-4' and C-7'a and from H-7' to C-3'a and C-5', shown in Figure 3, together with the chemical shifts of C-3'a, 138.7 p.p.m. and C-7'a, 149.5 p.p.m., prove the presence of a hydroxy-anthranilic acid, especially for the positions of the nitrogen-, the oxygen-atoms and of the carboxylic acid group at C-3'a. The presence of a salicylic acid moiety was proven by the connectivities from H-3 to C-1 and C-5, from H-4 to C-2 and C-6, from H-5 to C-1 and C-3 and from H-6 to C-2', C-2 and C-4 (Figure 3). Comparing the NMR data obtained for **1**, summarized in Table 2, to the NMR data of known metabolites (Table 2) with a similar structure; for example, AJ19561<sup>11</sup> (Figure 4), verifies the structure of **1**. Moreover, parallel to NMR studies, crystallization experiments were performed with a single crystal obtained for compound **1**. Subsequent X-ray structural determination fully confirmed the structure of compound **1** (Figure 3).

### Biological activity

Caboxamycin (**1**) showed antibiotic activity against the Gram-positive bacteria *Bacillus subtilis* (IC<sub>50</sub>=8 μM), *Staphylococcus lentus* (IC<sub>50</sub>=20 μM) and the yeast *Candida glabrata* (IC<sub>50</sub>=117 μM). Also the biofilm formation of *Staphylococcus xylosus* was weakly inhibited. A significant activity was present against the phytopathogenic bacteria *Xanthomonas campestris* (IC<sub>50</sub>=43 μM) and *Ralstonia solanacearum* (IC<sub>50</sub>=176 μM) and against the opportunistic pathogen *Staphylococcus epidermidis* (IC<sub>50</sub>=43 μM). The corresponding data for chloramphenicol (positive control prokaryotes) were: *Bacillus subtilis* (IC<sub>50</sub>=9 μM), *Staphylococcus lentus* (IC<sub>50</sub>=14 μM), *Xanthomonas campestris* (IC<sub>50</sub>=6 μM), *Ralstonia solanacearum* (IC<sub>50</sub>=75 μM), *Staphylococcus epidermidis* (IC<sub>50</sub>=7 μM) and for cycloheximide (positive control eukaryotes): *Candida glabrata* (IC<sub>50</sub>=4 μM).

The cytotoxic effect of caboxamycin (**1**) was tested in different tumor cell lines. It showed a moderate growth inhibitory activity (IC<sub>50</sub>: 28.6–29.4 μM) towards gastric adenocarcinoma (AGS), hepato-



**Figure 3** Selected 2D NMR correlations (a) and X-ray structure (b) of caboxamycin (1).

**Table 2**  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectral data of caboxamycin (1) and AJI9561 (3)  $^{11}$  in  $\text{DMSO}-d_6$

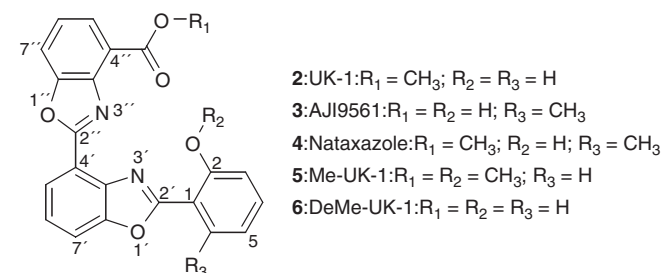
| No.   | 1 in $\text{DMSO}-d_6$                               |                                       | 3 in $\text{DMSO}-d_6$                               |                                       |
|-------|--|---------------------------------------|--|---------------------------------------|
|       | $\delta$ ( $^1\text{H}$ ) [p.p.m.]<br><i>J</i> in Hz | $\delta$ ( $^{13}\text{C}$ ) [p.p.m.] | $\delta$ ( $^1\text{H}$ ) [p.p.m.]<br><i>J</i> in Hz | $\delta$ ( $^{13}\text{C}$ ) [p.p.m.] |
| 1     |  | 109.7                                 | —  | 120.5                                 |
| 2     |  | 158.4                                 | —  | 150.4                                 |
| 3     | 7.12 d (8.13)  | 117.3                                 | 7.06 d (8.0)   | 121.1                                 |
| 4     | 7.53 m   | 134.4                                 | 7.36 t (8.0)   | 131.8                                 |
| 5     | 7.07 t (7.23)  | 119.9                                 | 6.85 d (8.0)   | 128.7                                 |
| 6     | 8.00 d (7.61)  | 127.4                                 |  | 140.8                                 |
| 2'    |  | 163.6                                 |  | 161.5                                 |
| 3'a   |  | 138.7                                 |  | 139.9                                 |
| 4'    |  | 121.8                                 |  | 118.7                                 |
| 5'    | 7.97 d (7.74)  | 127.2                                 | 8.37 d (7.6)   | 125.9                                 |
| 6'    | 7.54 m   | 125.3                                 | 7.59 t (7.6)   | 125.3                                 |
| 7'    | 8.07 d (8.00)  | 115.3                                 | 7.88 d (7.6)   | 113.9                                 |
| 7'a   |  | 149.5                                 |  | 151.2                                 |
| 2''   |  | 165.6                                 |  | 162.8                                 |
| 3'' a |  |                                       |  | 141.4                                 |
| 4''   |  |                                       |  | 122.3                                 |
| 5''   |  |                                       | 8.21 d (8.0)   | 127.3                                 |
| 6''   |  |                                       | 7.58 d (8.0)   | 124.8                                 |
| 7''   |  |                                       | 7.94 d (8.0)   | 115.3                                 |
| 7'' a |  |                                       |  | 151.7                                 |
| Me    |  |                                       | 2.86 s   | 21.3                                  |
| OH    |  |                                       | 12.91 brs  |                                       |
| COOH  |  |                                       | 11.7 br  |                                       |

cellular carcinoma (Hep G2) and breast carcinoma cells (MCF7). UK-1 (2) was more potent than 1 (Table 3).

Caboxamycin (1) is a weak inhibitor of bovine brain PDE ( $\text{IC}_{50}=148\ \mu\text{M}$ ) and only slightly inhibited human PDE-4B2 ( $\text{IC}_{50}=1.46\ \text{mM}$ ).

## DISCUSSION

The benzoxazole core structure of caboxamycin (1) is part of the natural products UK-1 (2) from *Streptomyces* sp. 517-02,<sup>8</sup> AJI9561 (3) from *Streptomyces* sp. AJI9561<sup>9</sup> and nataxazole (4) from *Streptomyces* sp. Tü 6176.<sup>7</sup> 2~4 showed potent cytotoxic activities against various tumor cell lines whereas growth inhibition was not observed against Gram-positive or Gram-negative bacteria, or against yeasts and fungi. Furthermore, two derivatives of UK-1, Me-UK-1 (5) and DeMe-UK-1



**Figure 4** Structures of benzoxazole metabolites from *Streptomyces* strains.

**Table 3** Growth inhibitory activity ( $\mu\text{g ml}^{-1}$ ) of caboxamycin (1) and UK-1 (2) against selected human tumor cell lines

| Cell line | 1                |         | 2                |     |
|-----------|------------------|---------|------------------|-----|
|           | $\text{GI}_{50}$ | TGI     | $\text{GI}_{50}$ | TGI |
| AGS       | 7.5              | $>10^a$ | 0.8              | 1.9 |
| Hep G2    | 7.4              | $>10^b$ | 0.085            | 2.4 |
| MCF7      | 7.3              | $>10^c$ | 0.65             | 3.5 |

Abbreviations:  $\text{GI}_{50}$ , 50% growth inhibition; TGI, 100% growth inhibition.

<sup>a</sup>72% inhibition at  $10\ \mu\text{g ml}^{-1}$ .

<sup>b</sup>70% inhibition at  $10\ \mu\text{g ml}^{-1}$ .

<sup>c</sup>80% inhibition at  $10\ \mu\text{g ml}^{-1}$ .

(6), were prepared by methylation with methyl iodide and alkaline hydrolysis, respectively;<sup>12</sup> their structures are shown in Figure 4. Interestingly, 6 exhibited anti-bacterial activity<sup>13</sup> and 5 anti-fungal activity.<sup>14</sup>

In the last decade efforts have been made to understand the selective cytotoxic activity of UK-1 (2) towards cancer cells versus bacteria and fungi, and for this purpose various synthetic derivatives have been prepared (Figure 5) and their biological activities evaluated and compared.<sup>15–17</sup> Reynolds *et al.*<sup>18</sup> investigated the metal ion coordination by 2. It was shown that 2 bound divalent metal ions and DNA in a divalent metal ion-dependent manner. It also bound to double-stranded DNA 10 times more tightly in the presence of  $\text{Mg}^{2+}$  and inhibited human topoisomerase II.<sup>16</sup>

It was demonstrated that the synthetic benzoxazole derivative 7, which is the methyl ester of 1, retained the selectivity of the natural product against cancer cells and therefore represents a minimum structural pharmacophore.<sup>14</sup> The synthetic derivative 8, which constitutes the methyl ether of 1 and 9, which represents the decarboxylated 1, did not exhibit any cytotoxic or anti-bacterial activities.<sup>15</sup> It

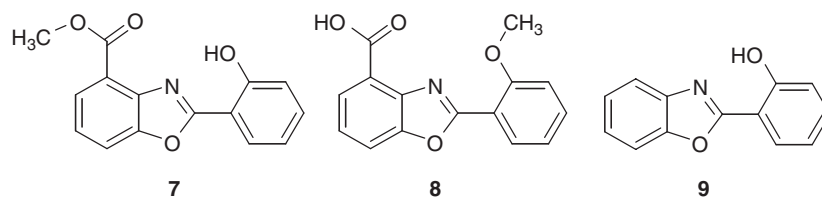


Figure 5 Structures of synthetic benzoxazole derivatives.<sup>14</sup>

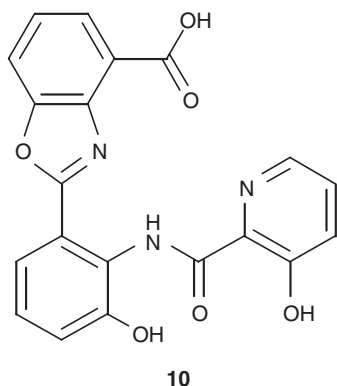


Figure 6 Structure of antibiotic A33853.

was shown that an appropriate substituent in position 4' of the benzoxazole core is important for an efficient  $Mg^{2+}$ -binding ability. The described cytotoxic activity of the described natural product **1** and the observation of an iron-intermediate with ESI-FT-ICR-MS are in accordance with these results. UK-1 displays cytotoxic activity against certain solid tumor-derived cell lines.<sup>15</sup> Caboxamycin is less potent than UK-1. Therefore the interaction between **2** and the  $Mg^{2+}$  ion may be stronger than that of **1**. Synthetic derived **1** has been described as an intermediate in the synthesis of benzoxazole carboxamides, but its biological activity has not been evaluated.<sup>19</sup> The streptomycete metabolite **1** and the evaluation of its biological activity add an element to the investigations of synthetic derivatives of the natural product UK-1 (**2**).

A further benzoxazole natural product related to UK-1 family is antibiotic A-33853 (**10**) which is produced by a *Streptomyces* strain;<sup>20</sup> its structure is shown in Figure 6. Although benzoxazoles **2–6** are composed of two molecules, hydroxy-anthranilic acid and one salicylic acid, which form two ring closures resulting in the bis(benzoxazole) structure, **10** consists of two molecules, hydroxy-anthranilic acid and one 3-hydroxy-picolinic acid, which build one benzoxazole and one amide bond. Interestingly, **10** showed anti-bacterial activity against penicillin-resistant *Staphylococcus* and *Streptococcus* strains, and anti-viral and anti-trichomonal activities *in vitro*.<sup>20</sup> Unfortunately, data are not available on the cytotoxic activity and the metal-binding ability of **10**, hence it is not possible to consider the contributions of the various structural elements to the biological activity.

Among biological activities of caboxamycin (**1**) the inhibition of phosphodiesterases are notable. Phosphodiesterases are essential regulators of cyclic nucleotide signalling with diverse physiological functions. PDE-4-selective inhibitors have therapeutic potential for treating major diseases, such as asthmatic inflammation and chronic obstructive pulmonary disease<sup>21,22</sup> and several PDE-4 inhibitors have reached the clinical trial of phases II and III status.<sup>22</sup> The PDE-4B enzyme is widely expressed in human inflammatory cells, negatively regulates a wide range of pro-inflammatory and immune cells<sup>21,23</sup> and appears to be involved in the production of the tumor necrosis factor

TNF- $\alpha$  and other cytokines promoting the inflammatory response.<sup>22</sup> Therefore, by inhibiting the PDE-4B enzyme, inflammatory response is blocked or strongly reduced.

The results pointed out, that caboxamycin has a 10-fold stronger inhibitory effect to the mixture of the three bovine PDE isoforms ( $IC_{50}=148\ \mu M$ ) than on the human PDE-4B2 ( $IC_{50}=1.46\ mM$ ). This might be based on significantly stronger inhibition of PDE-2 and PDE-10 by caboxamycin than PDE-4. Despite this weak inhibition, the effect is notable because the effect against other phosphodiesterases possibly may be much stronger than the measured effects against the selection of enzymes tested. In addition, structural modifications of the caboxamycin molecule may yield molecules demonstrating much stronger effects. Structural modification according to the binding properties to human PDE-4B2 already has successfully been carried out with other PDE-4 inhibitors.<sup>22</sup>

## METHODS

### Producing organism and taxonomy

Strain NTK 937 was isolated from an Atlantic Ocean deep-sea sediment core collected in 2001 at the southern edge of the Saharan debris flow near the Canary Islands (27°02'392N, 18°29'022W) at a depth of 3814 m. Isolations were made from the turbidite fraction of the core (0–38 cm from surface) which was deposited approximately 1,000 years ago, but derived from older (approximately 200 000 years) previously deposited sediment from further upslope.<sup>11</sup>

Strain NTK 937 was examined using a combination of genotypic and phenotypic procedures known to produce data of value in the delineation of *Streptomyces* species.<sup>24,25</sup>

### Fermentation and isolation

Batch fermentations of strain NTK 937 were carried out in a 20-liter fermentor equipped with a turbine impeller system (b20; B.Braun, Melsungen, Germany) in a complex medium consisting of mannitol 20 g and soybean meal 20 g in 1 l tap water; pH was adjusted to 7.5 prior to sterilization. The fermentor was inoculated with 5% by volume of a shake flask culture grown in a seed medium at 27 °C in 500 ml-Erlenmeyer flasks with a single baffle for 72 h on a rotary shaker at 120 r.p.m. The fermentation was carried out for 5 days with an aeration rate of 0.5-volume air/volume/minute and agitation at 250 r.p.m.

Hyphlo Super-cel (2%) was added to the fermentation broth, which was separated by multiple sheet filtration into culture filtrate and mycelium. The culture filtrate was applied to an Amberlite XAD-16 column (resin volume 800 ml) and the resin washed with  $H_2O$  and  $H_2O$ -MeOH (8:2); caboxamycin (**1**) was eluted with 100% MeOH and concentrated *in vacuo* to an aqueous residue. The concentrate was extracted three times with ethylacetate, and the organic extracts combined and concentrated *in vacuo* to dryness. The crude product was dissolved in MeOH and applied to a diol-modified silica gel column (40×2.5 cm; LiChroprep Diol, Merck, Darmstadt, Germany). **1** was separated using a linear gradient of cyclohexane-DCM-MeOH and was eluted with DCM-10% MeOH at a flow rate of 420 ml  $h^{-1}$ . Further purification was achieved by chromatography on Sephadex LH-20 using MeOH-DCM (2:1) and Toyopearl HW-40S with MeOH (each column 90×2.5 cm) at a flow rate of 30 ml  $h^{-1}$ .

## HPLC-DAD analyses

The chromatographic system consisted of a HP 1090M liquid chromatograph equipped with a diode-array detector and a HP Kayak XM 600 ChemStation (Agilent Technologies, Waldbronn, Germany). Multiple wavelength monitoring was performed at 210, 230, 260, 280, 310, 360, 435 and 500 nm, and UV-visible spectra were measured from 200 to 600 nm. A 10-ml aliquot of the fermentation broth was centrifuged, and the supernatant adjusted to pH 4 and extracted with the same volume of EtOAc. After centrifugation, the organic layer was concentrated to dryness *in vacuo* and resuspended in 1 ml MeOH. Here, 10 µl aliquots of the samples were injected onto an HPLC column (125×4.6 mm) fitted with a guard-column (20×4.6 mm) filled with 5-µm Nucleosil-100 C-18 (Maisch, Ammerbuch, Germany). The samples were analyzed by linear gradient elution using 0.1% ortho-phosphoric acid as solvent A and MeCN as solvent B at a flow rate of 2 ml min<sup>-1</sup>. The gradient was from 0 to 100% for solvent B in 15 min with a 2-min hold at 100% for solvent B.

## Structural elucidation

LC-MS experiments were performed on an Applied Biosystems QTrap 2000 (Applied Biosystems, Darmstadt, Germany) coupled to an Agilent 1100 HPLC system (Agilent). High-resolution ESI-FT-ICR mass spectra were recorded on an APEX II FTICR mass spectrometer (4.7 T, Bruker-Daltonics, Bremen, Germany) and NMR experiments on a DRX 500 NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a BBI probe head with z gradients.

## Biological activity

Anti-microbial assays were performed as described earlier.<sup>26</sup> The following strains were used: *Staphylococcus epidermidis* (DSM 20044), *Pseudomonas syringae* pvar. *aptata* (DSM 50252), *Pseudomonas fluorescens* (NCIMB 10586), *Xanthomonas campestris* (DSM 2405), *Ralstonia solanacearum* (DSM 9544) and *Candida glabrata* (DSM 6425).

Biofilm formation with *Staphylococcus xylosum* (DSM 20267) was analyzed by a crystal violet staining assay<sup>27</sup> in 96-well microtiter plates with the following modifications: cells were cultivated at 37 °C instead of 30 °C; the brain heart medium (BHI) was replaced by the trypticase soy yeast extract medium (30 g l<sup>-1</sup> Bacto tryptic soy broth and 3 g l<sup>-1</sup> yeast extract), the cell density at 600 nm of the initial bacterial suspension was 0.01 and EtOH was used instead of glacial acetic acid for solubilization of the incorporated dye.

The inhibitory action of caboxamycin (**1**) on the growth of tumor cells was tested according to NCI guidelines<sup>28</sup> with the human tumor cell lines AGS (gastric adenocarcinoma), MCF7 (breast adenocarcinoma) and Hep G2 (hepatocellular carcinoma). Aliquots of cell suspensions (5×10<sup>3</sup> cells) were placed in 96-well microtiter plates in 0.2 ml culture medium (RPMI-1640 with 10% fetal calf serum) and incubated in a humidified atmosphere of 5% CO<sub>2</sub> in air. After 24 h caboxamycin (**1**) (0.1~10 µg ml<sup>-1</sup>) and UK-1 (**2**) (0.1~10 µg ml<sup>-1</sup>) as positive control were added to the cells and the cells cultivated for additional 48 h. The cell count was surveyed by protein determination with sulforhodamine B. From the resulting concentration–activity curves, the GI<sub>50</sub> (concentration at which half of the cells were inhibited in their growth) and TGI (concentration at which a total inhibition of cell growth was observed) values were obtained.

Analysis of the effect of **1** on human recombinant cAMP-specific phosphodiesterase (PDE-4B2) was carried out with final concentrations of the substance of 488 µM (125 µg ml<sup>-1</sup>) in 50 mM Tris-HCl buffer (pH 7.5) containing 8.3 mM MgCl<sub>2</sub>, 1.7 mM EGTA and 10 mU recombinant human cAMP-specific PDE 4B2 (Merck Bioscience 524736-10U, Darmstadt, Germany) in a volume of 20 µl per well. The reaction was started with 20 µl of 40 µM cAMP (Sigma A9501, Taufkirchen, Germany) dissolved in 50 mM Tris-HCl (pH 7.5) containing 8.3 mM MgCl<sub>2</sub> and 1.7 mM EGTA. After an incubation period of 30 min at 30 °C the reaction was stopped and the AMP concentration was quantified with the PDELight HTS cAMP Phosphodiesterase Assay Kit (Lonza, LT07-600, Wuppertal, Germany) according to the instructions of the supplier. The luminescence was measured using the microtiter plate reader Infinite M200 (Tecan, Crailsheim, Germany) with 0.1 s integration time. For inhibition of the PDE, 100 µM of Rolipram (4-[3-(Cyclopentylloxy)-4-methoxyphenyl]-2-pyrrolidinone) (Merck Bioscience Cat.No. 557330) were used as a positive control. The effect of **1** on PDE activity from bovine brain (a mixture of three isoforms PDE-2, PDE-4

and PDE-10) was tested accordingly. 10 mM Tris-HCl buffer (pH 7.4) was used containing 1 mU (1 µl) of 3,5-cyclic-nucleotide-specific PDE from bovine brain (Sigma P9529)<sup>29</sup> in a total volume of 20 µl, and the reaction was started with 20 µl of 40 µM cAMP (Sigma A9501) solution in 10 mM Tris-HCl pH 7.4.

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