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Phenylnannolones A–C: Biosynthesis of New Secondary Metabolites from the Myxobacterium *Nannocystis exedens*

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Myxobacteria are gliding bacteria that belong to the δ-Proteobacteria and are known for their unique biosynthetic capabilities. Among myxobacteria, Nannocystis spp. *are most closely related to marine myxobacteria and their secondary metabolism has hardly been investigated. Phenylnannolones A (**1**), B (**2**) and C (**3**) were obtained from a culture of* Nannocystis exedens *that was isolated from the intertidal region of Crete. Compound **1** had inhibitory activity toward the ABCB1 gene product P-glycoprotein and reversed daunorubicin resistance in cultured cancer cells. Phenylnannolone A has an unusual structural architecture; it is composed of an ethyl-substituted polyene chain linked to a pyrone moiety on one side and to a phenyl ring on the other. The investigation of the biosynthesis with labelled precursors revealed acetate, butyrate and phenylalanine as building blocks for **1**. The labelling pattern suggested novel biochemical reactions for the biosynthesis of the starter unit.*

Introduction

Bacteria of the genus *Nannocystis*[1] are representatives of the myxobacteria, which belong to the δ-subdivision of the Proteobacteria. These microorganisms are highly organised as expressed in features like social gliding and swarm formation to facilitate feeding by predation on other bacteria. Under unfavourable living conditions, fruiting bodies containing myxospores are formed.[2] The complexity of their social behaviour and life cycle is reflected in their large genomes, some of which (e.g., *Sorangium cellulosum* So ce56[3] and *Myxococcus xanthus* DK1622[4]) have been sequenced. In both species, genes devoted to regulation and secondary metabolism are overrepresented. The latter explains the frequent encounter of fascinating natural products in myxobacteria, which may even play a role in hunting prey.[2] Since the 1970s, more than 100 new basic chemical structures[5,6] have been discovered from myxobacteria, including the epothilones, which are in clinical trials as anticancer agents.[7] The epothilone B derivative ixabepilone (Ixempra™) has recently been approved by the FDA for advanced breast cancer.[8]

In recent years, truly marine myxobacteria belonging to the genera *Engygromyxa*,[9] *Plesiocystis*[10] and *Haliangium*[11] were discovered. Myxobacteria of the family Nannocystaceae, with the main representative *Nannocystis*, show the closest phylogenetic relation to marine myxobacteria.[9,12] Even though the secondary metabolism of *Nannocystis* species has hardly been investigated, their biosynthetic potential is obvious from their production of natural products such as germacran,[13] geosmin,[14] and siderophores of the citrate-hydroxamate type called nannochelins (Figure 1).[15] Additionally, *Nannocystis* spp. belong to the few groups of bacteria that are known to produce true steroids.[16,17]

Our research program is aimed at discovering unique structures from bacterial sources that have not been investigated to date and at deciphering novel biosynthetic pathways. Over the course of our research, our attention was drawn to *Nannocystis exedens* (strain 150), which was isolated from the intertidal region of Crete. The structurally related phenylnannolones A (**1**), B (**2**) and C (**3**) were obtained from a culture of this bacterial strain. Phenylnannolone A has an unprecedented structure, which is composed of an ethyl-substituted polyene chain linked to a pyrone moiety on one side and to a phenyl ring on the other. The investigation of the biosynthesis of **1** with labelled precursors revealed acetate, butyrate and phenylalanine as building blocks. Thus, **1** is a polyketide with a phenylalanine-derived starter unit, which might be formed through a C_6C_2 intermediate. In this respect, the biosynthesis may be similar to that of the myxobacterial compounds ripostatin[18] and phenalamide[19] (Figure 2), which are suggested to be poly-

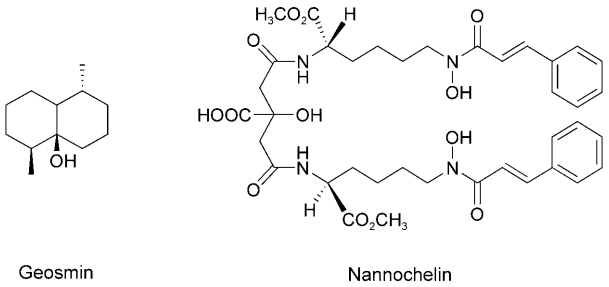

Figure 1. Structures of geosmin and nannochelin, metabolites reported to be produced by *Nannocystis* species.

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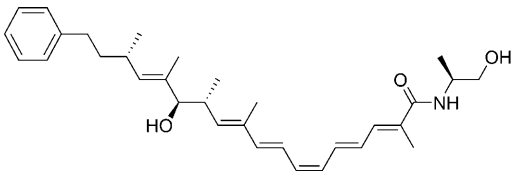
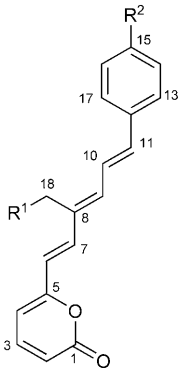


Figure 2. Structure of phenalamide A1, a myxobacterial multidrug resistance reversal agent.

ketides with phenylacetate as their starter unit. The labelling pattern for phenylnannolone A, however, suggests further unprecedented biochemical reactions for the biosynthesis of the aromatic starter unit.

Results

The *Nannocystis exedens* strain 150 was isolated from the intertidal region of a sandy beach in Crete and taxonomically characterised by 16S rDNA analysis. After the bacteria were cultured in a liquid medium in the presence of an adsorber resin (Amberlite XAD-16), extraction of the resin with acetone provided an extract that was fractionated by liquid-liquid partitioning and normal phase vacuum liquid chromatography (VLC). Subsequent HPLC separations yielded the structurally closely related compounds phenylnannolone A (**1**), B (**2**) and C (**3**) as yellow solids (Figure 3).



1 (phenylnannolone A): $R^1 = CH_3$, $R^2 = H$
2 (phenylnannolone B): $R^1 = H$, $R^2 = H$
3 (phenylnannolone C): $R^1 = CH_3$, $R^2 = OH$

Figure 3. Structures of phenylnannolones A, B and C (**1**–**3**).

The molecular formula of **1** ($C_{19}H_{18}O_2$) was determined by mass spectral analysis (HREIMS; measured 278.1304 g mol^{-1}, calcd. 278.1307 g mol^{-1}), and implied eleven degrees of unsaturation. UV maxima at 239, 304 and 394 nm indicated an extended aromatic chromophore. The IR spectrum showed characteristic absorption bands for the presence of a lactone carbonyl group (1710 cm^{-1}), a monosubstituted phenyl ring (750 and 692 cm^{-1}) and double bonds conjugated with a phenyl ring (1625 cm^{-1}).[20]

The ^{13}C NMR spectra (^{1}H decoupled and DEPT) included two strong signals at 127.7 (C-13/17) and 129.5 (C-14/16) ppm, a less intense signal at 128.9 (C-15) ppm and a resonance for a quaternary carbon at 138.3 (C-12) ppm; this proved the presence of a monosubstituted phenyl ring. Additionally, nine sp^2-hybridized carbon atoms with chemical shifts characteristic of an olefinic partial structure (from 105.7 (C-4)–144.9 (C-3) ppm) were discernible, one of which (C-8) was a quaternary carbon. Two sp^2-hybridized carbons attached to an oxygen were obvious from ^{13}C NMR resonances at 161.5 (C-1) and 161.0 (C-5) ppm. The molecule also contained two sp^3-hybridized carbons with high-field resonance signals [20.4 (C-18) and 14.6 (C-19)] ppm.

A ^{1}H-^{13}C HSQC spectrum correlated all proton resonances to the ^{13}C NMR resonances of directly bonded carbon atoms. Subsequently, a ^{1}H-^{1}H COSY spectrum indicated five isolated spin systems, one of which included the protons of the phenyl ring (H-13–H-17). A second spin system formed an ethyl residue (CH_2-18, $J = 7.7$ Hz and CH_3-19, $J = 7.7$ Hz). A third spin system comprised H-9–H-11 with H-10 coupling with both H-9 ($J = 11.7$ Hz) and H-11 ($J = 15.4$ Hz), thus clarifying the C-9 to C-11 part of the structure of **1**. A disubstituted double bond ($\Delta^{6,7}$) within **1** was obvious from the ^{1}H-^{1}H coupling of H-6 ($J = 16.1$ Hz) and H-7 ($J = 16.1$ Hz), whereas the diene $\Delta^{2,3}$ and $\Delta^{4,5}$ was apparent from the ^{1}H-^{1}H spin system (including H-2 ($J = 9.2$ Hz), H-3 ($J = 9.2$ Hz, 6.6 Hz) and H-4 ($J = 6.6$ Hz)). In this way, five partial structures were delineated. These structures had to be connected by the four quaternary carbons C-12, C-8, C-5 and C-1. Carbon C-12 was part of the phenyl ring and located adjacent to C-11, as proven by HMBC correlations from H-13/17 to C-11. ^{1}H-^{13}C HMBC correlations from H_2-18 as well as from H-7 and H-9 to C-8 showed that the corresponding carbons were linked by C-8. We established the connection of C-4 and C-6 by C-5 from the HMBC correlations of H-6 to C-4 and C-5. Carbons C-1 and C-5 are linked by an oxygen bridge which forms a 2-pyrone ring. These conclusions are consistent with the eleven degrees of unsaturation (Figure 4).

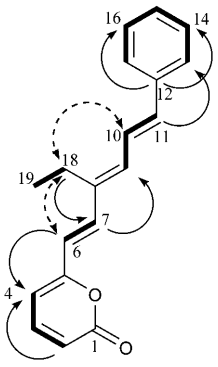


Figure 4. Selected ^{1}H-^{13}C HMBC (plain) and NOE (dashed) correlations, ^{1}H-^{1}H COSY fragments are highlighted in bold.

We deduced the configuration of $\Delta^{2,3}$ as *Z* from a ^{1}H-^{1}H coupling constant of 9.2 Hz. The double bonds within the 2-pyrone ring, $\Delta^{2,3}$ and $\Delta^{4,5}$, had to be in the *Z*-conformation to allow for lactonisation. The $\Delta^{6,7}$ and $\Delta^{10,11}$ however, had to be in the *E*-conformation, according to their ^{1}H-^{1}H coupling constants of 16.1 and 15.4 Hz, respectively. In order to clarify the configuration of $\Delta^{8,9}$, we carried out selective 1D-NOE experiments. Irradiation at the resonance frequency of H_2-18 led to a strong enhancement of the H-10 and H-6 signals, but not of the H-9 signal. In contrast, H-9 had a strong NOE with H-7. Therefore, H-9 and the ethyl residue C-18/19 had to be located on opposite sides of the $\Delta^{8,9}$ double bond, which dictated an *E*-configuration.

The only difference between **1** and **2** was the replacement of the ethyl group of the former by a methyl group in the

latter. Accordingly, LC-MS data showed a mass difference of 14 indicating that **2** contained one CH_2 group fewer than **1**, and we established the molecular formula of **2** as $C_{18}H_{16}O_2$ by HREIMS analysis (measured 264.1142, calcd. 264.1150). As expected, the NMR and IR data of **2** were very similar to those of **1**; however, the ^{1}H NMR spectrum of **2** did not show the ethyl group signals but instead a singlet resonance accounting for three protons ($\delta_H = 1.27$, $\delta_C = 12.6$ ppm, CH_3-18). Thus, we established the structure of phenylnannolone B (**2**).

In phenylnannolone C (**3**), the phenyl residue was substituted with a hydroxyl group in the *para*-position. Accordingly, the molecule was more polar than **1** and **2**, and we observed a mass difference of 16 between **1** and **3**. We confirmed a molecular formula of $C_{19}H_{18}O_3$ by HREIMS measurements (found 294.1256, calcd 294.1256). The only difference in the ^{1}H NMR spectra of **1** and **3** concerned the aromatic resonances. In the ^{1}H NMR spectrum of **3**, the complex multiplet was reduced to two distinct doublets, confirming a *para*-substituted phenyl ring.

The structural features of phenylnannolone A (**1**) and its derivatives **2** and **3** led to the assumption that the compounds were polyketides with a phenylalanine- and tyrosine-derived starter unit, presumably cinnamoyl- and coumaryl-CoA, respectively. We supposed that the biosynthetic origin of the ethyl group was ethylmalonyl-CoA. In order to gain insight into the details of the biosynthetic pathways for **1**–**3**, we carried out feeding experiments with ^{13}C-labelled precursors.

Feeding experiments with 2-^{13}C-acetate led to an enrichment of ^{13}C in positions 2, 4, 6 and 9. The labelling of carbons C-2, C-4 and C-6 agreed well with the proposed biosynthesis, but we did not expect the labelling of C-9. However, we confirmed acetate incorporation at C-9 by labelling studies with 1,2-$^{13}C_2$-acetate. The latter experiment yielded enhanced ^{13}C NMR signals for C-1–C-6, with C-1 and C-2, C-3 and C-4 and C-5 and C-6 showing intra-acetate couplings, which proved the incorporation of intact acetate units. The same experiment also showed C-9 to be enriched. However, the ^{13}C NMR signal was only a singlet, indicating that this acetate unit had to be cleaved during the biosynthetic process (Figure 5).

We did not observe an enrichment for any of the aromatic carbons upon feeding ^{13}C-labelled acetate, thus excluding a polyketide-derived aromatic moiety. The formation of the phenylic part of **1**–**3** through the shikimate pathway with a phenylalanine-derived starter unit thus appeared most probable. We confirmed the incorporation of L-phenylalanine by feeding 3-^{13}C -L-phenylalanine, leading to an enhancement of the ^{13}C NMR signal for C-11. Feeding with U-$^{13}C_9$-^{15}N-L-phenylalanine proved that C-10 also originated from phenylalanine.

The ethyl group in **1** could either be derived from acetate or by the incorporation of a butyrate unit through ethylmalonyl-CoA. We excluded the former possibility since feeding experiments with 1,2-$^{13}C_2$-acetate and 2-^{13}C-acetate did not result in the labelling of the appropriate carbons (C-7, C-8, C-18 and C-19). We produced evidence for the incorporation of an ethylmalonyl-CoA building block by feeding with 1-^{13}C-butyrate, which led to the enhancement of the ^{13}C NMR signal for C-7. Interestingly, the signals of C-1, C-3 and C-5 were also slightly enhanced, which can be explained by the degradation of 1-^{13}C-butyrate to 1-^{13}C-acetate.

The feeding studies thus defined the biosynthetic origin of each carbon within **1**. According to our experimental data, the biosynthesis of **1** begins with the complex and unprecedented formation of an aromatic starter unit, possibly through a phenylalanine-derived C_6C_2 intermediate, which most probably is extended with malonyl-CoA and tailored by decarboxylation to obtain a C_6C_3 unit. Subsequently, further extension of this postulated cinnamic acid unit with ethylmalonyl-CoA and three intact acetate units would give rise to the basic carbon skeleton for the phenylnannolones (Figure 6).

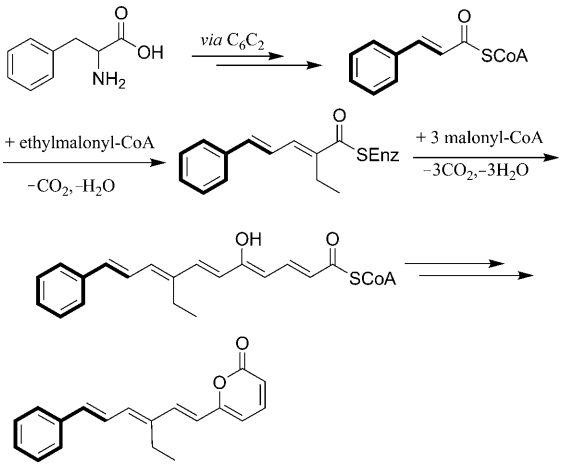


Figure 6. Proposed biosynthesis of phenylnannolone A (**1**), the phenylalanine-derived carbons are highlighted in bold.

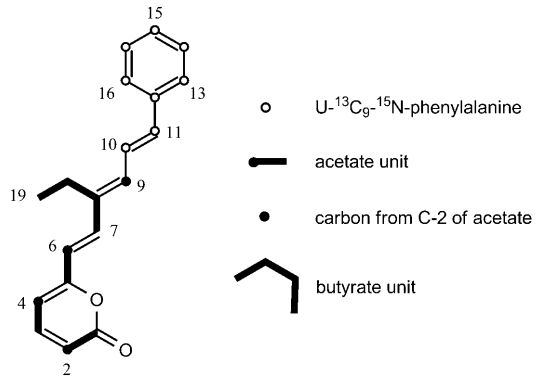


Figure 5. Results of the feeding experiments with labelled precursors.

Compound **1** inhibited the ABCB1 gene product P-glycoprotein (P-gp). P-gp is an ABC transporter with broad substrate acceptance, including cytostatics like etoposide, mitoxantrone and daunorubicin.[21] We indirectly determined the ability of **1** to inhibit P-gp by measuring the increase in daunorubicin cytotoxicity in the presence of phenylnannolone A in the (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. First we determined the toxicity of phenylnannolone A alone in the P-gp-overexpressing cell line A2780adr and in the wild-type cell line as a control. Phenylnannolone A only

showed cytotoxicity at very high concentrations, that is, above 31.6 μM (see the Supporting Information). For daunorubicin, we determined an IC_{50} value of 13.3 μM towards the A2780adr cell line and a value of 91.8 nM for the wild-type cells line (Figure 7). Thus, A2780adr was approximately 145 times more

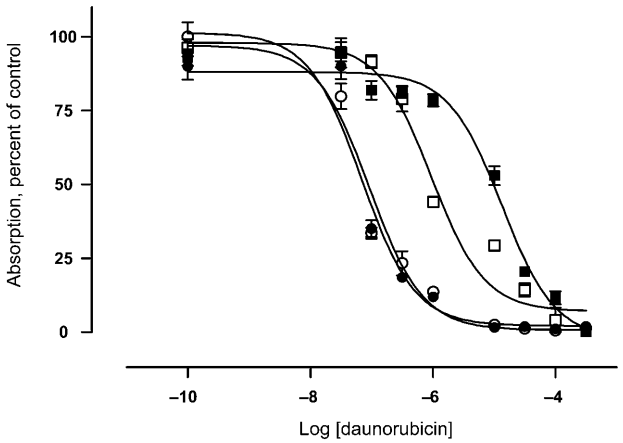


Figure 7. Concentration-effect curve of the cytostatic drug daunorubicin in the resistant A2780adr (squares) and sensitive A2780 (circles) cell lines in the presence (open symbols) or absence (closed symbols) of phenylnannolone A (**1**). The presented data are averages ±SE of one typical experiment with three replicates from a series of three independent experiments.

resistant to daunorubicin than A2780. In combination with daunorubicin, phenylnannolone A decreased the resistance of the A2780adr cells, resulting in an IC_{50} of 0.946 μM, while the IC_{50} in the wild-type cells remained nearly unaffected (IC_{50} = 68.6 nM). The resistance factor was reduced 10.3-fold from 145 to 14.1, closely corresponding to the maximal effect obtained with third generation modulators such as tariquidar.[22] Phenylnannolone A may thus be of importance for the development of multi-drug resistance reversal agents. The activity of phenylnannolone A correlates to that of phenalamide, which has been taken as a model for the synthesis of new multi-drug resistance reversal agents.[23] A drawback of the phenalamides and their derivatives is their considerable cytotoxic activity, whereas phenylnannolone A showed no cytotoxic effects which is an important advantage of the structural type reported here.

We also tested phenylnannolone A for antimicrobial properties towards six different organisms where it did not show any activity. It was slightly active against influenza A (data not shown) but not towards coxsackievirus B3 and Herpes simplex virus type 1. Additionally, we observed no modulation in the activity of trypsin, acetylcholinesterase, the adenosine A_1 receptor or the phosphatases Cdc25A and MPTPb.

Discussion

Phenylnannolones A, B and C (**1**–**3**) are polyketides with amino acid-derived starter units. During the current study, we also detected phenylnannolones in a different *Nannocystis* species (data not shown), showing that phenylnannolone production may be a characteristic feature of this little-investigated genus. The co-occurrence of **1**, **2** and **3** may be due to a lack of substrate specificity of the biosynthetic enzymes. Thus, using tyrosine instead of phenylalanine for the formation of the starter unit could result in **3**, and incorporation of methylmalonyl-CoA instead of ethylmalonyl-CoA would explain the presence of **2**. In this respect, it is of interest that during the biosynthesis of monensin A and B in *Streptomyces cinnamonensis*, the incorporation of either ethylmalonyl- or methylmalonyl-CoA is dependent on the availability of these substrates.[24,25]

Phenylnannolones A, B and C (**1**–**3**) are new structural types of myxobacterial secondary metabolites. The presence of a polyunsaturated carbon chain substituted with an ethyl group is extremely rare in natural products, one example being a pheromone from a *Carpophilus* beetle.[26] Even though certain structural features such as monosubstituted phenyl rings and polyunsaturated carbon chains are known from other myxobacterial natural products, the overall composition makes these compounds (**1**–**3**) the first members of a new structural class.

Among the various polyketide-derived polyenes isolated from myxobacterial cultures are dawenol,[27] DKxanthenes,[6] myxalamides[28] and phenalamides (also named stipiamides).[19] Of these the phenalamides are most closely related to the phenylnannolones (Figure 2). Even though only preliminary labelling studies were performed for phenalamide, the aromatic and polyene part of this structure was suggested to be formed from a phenylalanine-derived C_6C_2 starter unit, which is extended with methylmalonyl-CoA and malonyl-CoA.[19]

We determined the biosynthetic origin of each carbon within phenylnannolone A by using appropriately labelled precursors. We propose that the starter unit for **1** is cinnamoyl-CoA (C_6C_3). Even though myxobacteria have been reported to possess phenylalanine ammonium lyases (PALs),[29] the direct transformation of L-phenylalanine to cinnamoyl-CoA does not take place in the case of phenylnannolone A, because C-9 originated from the methyl carbon (C-2) of acetate. A benzoate starter unit derived from phenylalanine has been described for soraphen, a myxobacterial metabolite. The starter unit is subsequently elongated with malonate[30] and results in a cinnamic acid analogous structure (3-hydroxy-3-phenylpropanoate). However, due to the labelling pattern observed for **1**, a corresponding scenario is not possible, since in cases like soraphen, the carbon corresponding to the C-9 of phenylnannolone A derives from the C-1 of acetate.

All carbons of the C-10–C-17 phenyl-ethyl moiety (C_6C_2) in **1** originated from phenylalanine. Therefore, our data suggest a C_6C_2 intermediate (phenylacetic acid) in the formation of the starter unit. This intermediate was postulated as the starter unit for the biosynthesis of ripostatin, phenalamide and some cyanobacterial metabolites (e.g. microcystin and nodularin[31,32,33]) but was not verified, since feeding experiments with phenylacetic acid proved unsuccessful in every case. For cyanobacterial toxins of the microcystin family, the adenylation-peptidyl carrier protein didomain (A-PCP), postulated to be responsible for the activation and loading of the starter unit, was heterologously expressed, and the direct loading of a phenyl-

acetate starter unit was proven unlikely. Instead, a modification of a phenylpropanoid precursor was suggested. This involves the loss of the carbonyl carbon of the C_6C_3 unit through novel biosynthetic chemistry.[34] In the case of phenylnannolone A, a C_6C_2 intermediate would have to be elongated with acetate and subsequently subjected to further transformations, including a decarboxylative step. In this way, the C-2 of acetate would finally be located at position C-9 of phenylnannolone A, which is in accordance with the observed labelling pattern. Even though the details of the individual biochemical steps for the biosynthesis of the starter unit cannot be deduced from our experimental data, they provide clear evidence that these reactions are unprecedented and represent unique biochemical reactions. The investigation of the biosynthetic gene cluster for phenylnannolone will clarify the picture.

The incorporation of ethylmalonyl-CoA for the formation of an ethyl group is quite a common biosynthetic scenario with examples known for many microorganisms like *Streptomyces cinnamonensis* as well as for insects such as *Carpophilus spp*.[26] However, the incorporation of ethylmalonyl-CoA in **1** was somewhat surprising, as the carbons in question were reported as acetate/succinate-derived for ethyl group-containing myxobacterial metabolites such as tuscorone and tuscolide.[35]

Experimental Section

General experimental procedures: UV and IR spectra were obtained by employing Perkin–Elmer Lambda 40 and Perkin–Elmer Spectrum BX instruments. NMR spectra were recorded at 300 K on a Bruker Avance 300 DPX or Bruker Avance 500 DRX spectrometer with $(CD_3)_2CO$ as the solvent and internal standard. Spectra were referenced to residual solvent signals with resonances at $\delta_{H/C}$ = 2.04/29.8 ppm.

EI-MS were recorded with a Finnigan MAT 95 spectrometer. LC-ESI-MS was performed with an Agilent 1100 system with an API 2000 Triple Quadrupole LC/MS/MS (Applied Biosystems/MDS Sciex, Foster City, Canada) and a Diode Array Detector (DAD).

Analytical TLC: TLC was performed on aluminium sheets of silica gel Si 60 F_{254} (Merck) with a solvent system of dichloromethane/methanol (9:1). Detection was performed by observing the quenching of fluorescence at 254 nm and 366 nm and by staining with a vanillin sulphuric acid reagent followed by heating to 110 °C (blue/green spots).

HPLC was carried out with either an HP system equipped with a 1050 pump, a 1050 autosampler and a 1050 multiple-wavelength detector (isolation of **1** and **2**) or a system consisting of a Waters associated chromatography pump, a Rheodyne 7725i injection system (Milfoard, USA), a Knauer differential refractometer (Berlin, Germany), and a Linseis L200E recorder (Selb, Germany; isolation of **3**).

Isolation and taxonomy of the bacterial strain: The myxobacterial strain (no. 150 of our strain collection) was isolated from a sandy soil sample collected on the island of Crete. Small amounts of the sample were applied onto water cycloheximide (WCX) *E. coli* agar[36] plates, on which the strain was isolated. It was transferred to yeast (VY/2)[36] agar plates until an axenic culture was obtained. The morphology of the swarm and the microscopic appearance of the vegetative cells led to the assumption that strain 150 belonged to the genus *Nannocystis*. The result of the 16S rDNA sequencing analysis supported this assumption. Stock cultures of the strain were kept at −80 °C.

Isolation procedure: Cultivation was performed in Erlenmeyer flasks (15×5 L), each containing 1 L of a peptone medium (MD1 medium, supplemented with 0.2% glucose and 0.2% starch) with Amberlite XAD-16 (2%, Fluka, Germany). MD1 medium consists of Casiton (3 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.7 g L^{-1}) and $MgSO_4 \cdot 7H_2O$ (2 g L^{-1}). The flasks were inoculated with a preculture (same medium, 200 mL) and shaken on a rotary shaker (140 rpm) at 30 °C for seven days. At the end of the cultivation, the bacterial cells and adsorber resin were separated from the culture broth by centrifugation and extracted with acetone (15×200 mL). After removal of the solvent, the residue (5.79 g) was suspended in 60% aqueous methanol (200 mL) and extracted ten times with dichloromethane (100 mL). The dichloromethane layers were combined and dried (2.19 g). The separation of this extract by column chromatography over silica gel (Merck, 63–200 μM, ≈30 g) by consecutively employing dichloromethane, ethyl acetate, acetone and methanol as eluents gave seven fractions between 20 mL and 120 mL. ^{1}H NMR spectroscopic analysis indicated two of the dichloromethane fractions to be very similar and of further interest. They were combined and after evaporation of the solvent, this fraction (240 mg) was subjected to semipreparative RP-HPLC: column: Eurospher-100 C-8, Knauer, 5 μm, 250×8 mm; eluent: methanol/water (75:25), flow rate: 2.5 mL min^{-1}. The retention times of **1** and **2** were 15 min and 13 min, respectively. The separation yielded 42 mg of **1** and 6 mg of **2**.

One of the ethyl acetate fractions seemed to contain a similar compound to **1** and **2** by TLC (same colour and colour reaction upon staining) but in a very low concentration. The fraction was combined with the corresponding fraction from another cultivation (approximately 235 mg) and separated by Vacuum liquid chromatography (VLC) (RP-18, from methanol/water 60:40 to methanol leading to eight fractions with a volume of 50–100 mL each). The HPLC separation of fraction 5 (14 mg) (column: Eurospher-100 Si, Knauer 250×8 mm; solvent system: petroleum ether (PE)/acetone, 80:20; flowrate of 2.5 mL min^{-1}, retention time: 22 min) led to the isolation of **3** (1 mg).

Biosynthetic studies: For the feeding experiments, strain 150 was cultivated in the same liquid medium as described above. In each feeding experiment, the preculture was grown for seven days in 3×200 mL medium which was the inoculum for the main culture (3×1 L), to which 2% Amberlite XAD-16 was added. After 48 h, the labelled compounds were added in the form of sterile filtered solutions. The yields of labelled phenylnannolone A were between 0 mg and 4 mg.

Labelled reagents: 1 g of 1,2-$^{13}C_2$-sodium acetate, 1 g of 2-^{13}C-sodium acetate, 1 g of 1-^{13}C-sodium butyrate, 500 mg of 3-^{13}C-L-phenylalanine and 250 mg of U-$^{13}C_9$-^{15}N-L-phenylalanine. The labelled compounds were obtained from Cambridge Isotope Laboratories or Aldrich. The isolation of labelled phenylnannolone A was carried out according to the isolation procedure described above.

The feeding experiment with 2-^{13}C-sodium acetate led to the following carbon enrichment: C-2 (8.1%), C-4 (6.9%), C-6 (6.7%), C-9 (4.0%, for details see the Supporting Information).

In the other feeding experiments, phenylnannolone A was obtained in yields so low that the signals of the natural abundance carbons could not be detected in the ^{13}C NMR experiments. The enrichment rate could not be calculated. Additionally, in the cases

of 1-^{13}C-butyrate, 3-^{13}C-phenylalanine and U-$^{13}C_9$-^{15}N-L-phenylalanine, the yield was extremely low. Thus the isolated labelled phenylnannolone A was not measured, but a VLC fraction containing the compound was measured (Supporting Information).

In the feeding experiments with 1,2-$^{13}C_2$-phenylacetic acid and carboxy-^{13}C-benzoic acid, no incorporation was observed.

MTT assay: A2780 cells and their P-gp-overexpressing counterparts, A2780adr, were cultured in RPMI-1640 medium (Sigma Chemical, Taufkirchen, Germany) with 10% fatal calf serum and 50 μg mL^{-1} of penicillin G and streptomycin (Sigma Chemical). For MTT survival testing, cells were harvested with trypsin, centrifuged at 1200 rpm at 4 °C and resuspended in medium. Cells were counted, diluted and seeded into 96-well plates at a density of approximately 12500 cells/well either in 90 μL (for the measurement of the resistance factor) or in 80 μL (for testing the effect of **1** on P-gp-mediated daunorubicin resistance). Afterwards, plates were incubated for 6 h at 37 °C and 5% CO_2, and cell attachment was checked by microscopy.

For the measurement of the resistance factor, 10 μL of increasing daunorubicin concentrations were added to each well. To determine the reversal effect of **1**, 10 μL of various increasing daunorubicin concentrations were added plus 10 μL of **1** (at a fixed, final concentration of 31.6 μM), resulting in a volume of 100 μL per well.

After 72 h of incubation, 20 μL of MTT solution (5 mg mL^{-1}) were added to each well. Cells were incubated for an additional hour and then lysed by the injection of 150 μL of propan-2-ol/10 M HCl (50 mL:165 μL). Afterwards, the plates were kept at 4 °C for approximately 1–2 h to complete lysis, and finally, the absorption was measured at 595 (test wavelength) and 690 (reference wavelength) nm with a BMG Fluostar (BMG Labtechnologies, Offenburg, Germany) instrument. The absorption at the test wavelength was subtracted from the absorption at the reference wavelength.

Compound **1:** yellow solid; UV/Vis (MeOH): λ_{max} (log ε): 239 (3.59), 304 (3.90), 394 (3.84) nm; IR ν_{max} 3028, 2967, 2360, 1710, 1625, 1519, 1359, 1220, 1098, 966, 750, 692, 632 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 1; EIMS: *m/z* (%) = 278 (100), 250 (4), 249 (20), 221 (4), 204 (4), 193 (6), 183 (44), 169 (14), 141 (6), 128 (5), 115 (12), 105 (3), 95 (44), 91 (16), 77 (4); HR-EIMS: *m/z* 278.1304 (calcd for $C_{19}H_{18}O_2$: 278.1307).

Compound **2**: yellow solid; UV/Vis (MeOH): λ_{max} (log ε): 252 (3.74), 310 (3.83), 382 (3.84) nm; IR ν_{max} 2925, 2361, 1705, 1627, 1527, 1360, 1221, 1092, 968, 751, 699, 640, 610 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 1; EIMS: *m/z* (%) = 264 (100), 249 (5), 169 (47), 95 (28); HR-EIMS: *m/z* 264.1142 (calcd for $C_{18}H_{16}O_2$: 264.1150).

Compound **3**: yellow solid; UV/Vis (MeOH): λ_{max} (log ε): 260 (3.94), 309 (4.01), 409 (3.87) nm; IR ν_{max} 3384, 2926, 2855, 2358, 1704, 1517, 1361, 1227, 1169, 1104, 969, 636, 616 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 1; EIMS: *m/z* (%) = 294 (100), 265 (15), 199 (50), 95 (40); HR-EIMS: *m/z* 294.1256 (calcd for $C_{19}H_{18}O_3$: 294.1256).

Table 1. NMR spectroscopic data (300 MHz, $(CD_3)_2CO$) for phenylnannolones A (**1**), B (**2**) and C (**3**).

Phenylnannolone A (**1**)			Phenylnannolone B (**2**)		Phenylnannolone C (**3**)	
Position	δ_C, mult.	δ_H [*J* in Hz][a]	δ_C	δ_H [*J* in H][a]	δ_C	δ_H ([*J* in H][a]
1	161.5, qC		161.5		161.7[b]	
2	114.1, CH	6.12, d (9.2)	114.2	6.12, d (9.1)	113.7	6.12, d (9.5)
3	144.9, CH	7.48, dd (6.6, 9.2)	145.0	7.48, dd (6.7, 9.1)	145.0	7.48, dd (6.7, 9.5)
4	105.7, CH	6.35, d (6.6)	105.7	6.34, d (6.7)	105.4	6.32, d (6.7)
5	161.0, qC		160.9		161.3[b]	
6	119.0, CH	6.44, d (16.1)	119.5	6.39, d (15.7)	118.2	6.40, d (15.7)
7	138.1, CH	7.07, d (16.1)	137.8	7.16, d (15.7)	138.4	7.07, d (15.7)
8	142.3, qC		139.4		140.7	
9	137.1, CH	6.62, d (11.7)	135.8	6.69, d (11.7)	137.8	6.58, d (11.3)
10	125.7, CH	7.34, dd (11.7, 15.4)	126.0	7.34, dd (11.7, 15.4)	122.9	7.16, dd (11.3, 15.4)
11	136.7, CH	6.84, d (15.4)	136.5	6.83, d (15.4)	137.0	6.77, d (15.4)
12	138.3, qC		138.3		130.0[b]	
13/17	127.7, CH	7.57, d (7.7)	127.6	7.58, d (7.7)	129.3	7.44, d (8.5)
14/16	129.5, CH	7.35, t (7.7)	129.5	7.35, t (7.7)	116.5	6.83, d (8.5)
15	128.9, CH	7.25, t (7.7)	128.8	7.25, t (7.7)	158.8, qC	
18	20.4, CH_2	2.61, q (7.7)	12.6, CH_3	1.27, s	23.2	2.58, q (7.3)
19	14.6, CH_3	1.12, t (7.7)			14.3	1.10, t (7.3)

[a] The assignment was based on extensive 1D and 2D NMR measurements (^{1}H-^{1}H COSY, HSQC and ^{1}H-^{13}C HMBC); [b] The ^{13}C chemical shift was deduced from the ^{1}H-^{13}C HMBC spectrum.

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