

ORIGINAL ARTICLE

Pyramidamycins A-D and 3-hydroxyquinoline-2-carboxamide; cytotoxic benzamides from *Streptomyces* sp. DGC1

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Four new benzamides, pyramidamycins A-D (2–5) along with the new natural 3-hydroxyquinoline-2-carboxamide (6) were isolated from the crude extract of *Streptomyces* sp. DGC1. Additionally, five other known compounds, namely 2-aminobenzamide (anthranilamide) (1), 4',7-dihydroxyisoflavanone (7), 2'-deoxy-thymidine, 2'-deoxy-uridine and adenosine were also isolated and identified. The structures of the new compounds 2–6 were elucidated by 1D and 2D NMR studies along with HR MS analyses. The isolated compounds 1–6 contained the same amide side chain. The isolated compounds 1–7 were biologically evaluated in comparison with landomycin A against a prostate cancer cell line (PC3) and non-small cell lung cancer cell line (H460) for 48 h and against several bacterial strains. Pyramidamycin C (4) was the most active compound against both PC3 and H460 cell lines ($GI_{50} = 2.473$ and $7.339 \mu\text{M}$, respectively). Benzamides (1–3) demonstrated inhibitory activity against *Kocuria rosea* B-1106 (a diameter halo of 13 ± 2 mm for 1; 10 ± 2 mm for 2 and 3). Compound 6 was slightly active against both *Escherichia coli* DH5 α and *Micrococcus luteus* NRRL B-2618 (diameter halos 8 ± 2 and 9 ± 2 mm, respectively). Taxonomically, the amplified 500-bp 16S rRNA fragment of the *Streptomyces* sp. DGC1 had 99% identity (BLAST search) to the 16S rRNA gene of *Streptomyces atrovirens* strain NRRL B-16357.

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INTRODUCTION

Most currently marketed antibiotics are natural products of microbial origin, and >120 of the most important medicines in use today are obtained from terrestrial microorganisms.^{1,2} Often because of drug-resistance phenomena, 17 million lives every year are lost to infectious diseases,³ leading to the global concern that we may soon be facing a post-antibiotic era with reduced capabilities to combat microbes. As a consequence, a concerted worldwide search for new antibiotics from microbial origin is on-going, with focus on the potential of marine and terrestrial bacteria as source for novel metabolites with interesting biological and pharmaceutical properties.^{4–7} Diverse habitats, for example, tropical forests, deep sea sediments, sites of extreme temperature, salinity or pH, were explored and were successful to yield new microorganisms, which in turn provide the potential for novel metabolic pathways and new bioactive natural compounds.⁸ *Streptomyces* spp. are widespread in nature and continue to have a significant role in the production of bioactive metabolites. *Streptomyces* spp. produce many classes of secondary metabolites with great biofunctional diversity (antibiotics, antifungal, antiviral,

anticancer, immunosuppressants, insecticides, herbicides etc.) and diverse chemical structures, which makes them useful as pharmaceuticals and agricultural agents.^{1,9,10}

During our continued search for bioactive constituents from bacteria, strain DGC1 was isolated from a soil sample collected from the Devil's Golf Course salt pan (Death Valley National Park, CA, USA). Phylogenetic studies of DGC1 strain were conducted as described earlier,¹¹ and the amplified 500-bp 16S rRNA fragment was found to have 99% identity (BLAST) to the 16S rRNA gene of *Streptomyces atrovirens* strain NRRL B-16357. The extract obtained from the small-scale fermentation of *Streptomyces* sp. DGC1 on SG medium^{12,13} exhibited several unusual green fluorescent bands under long UV (365 nm), which stained to yellow with anisaldehyde/sulfuric acid in the pre-screening. A large-scale fermentation of the strain in SG medium afforded a crude extract from which different chromatographic techniques led to the isolation of five new benzamides: pyramidamycins A-D (2–5) and 3-hydroxyquinoline-2-carboxamide (6), whose structures were determined by NMR (1D and 2D) spectroscopy and MS (ESI and HR-ESI) studies (Figure 1).

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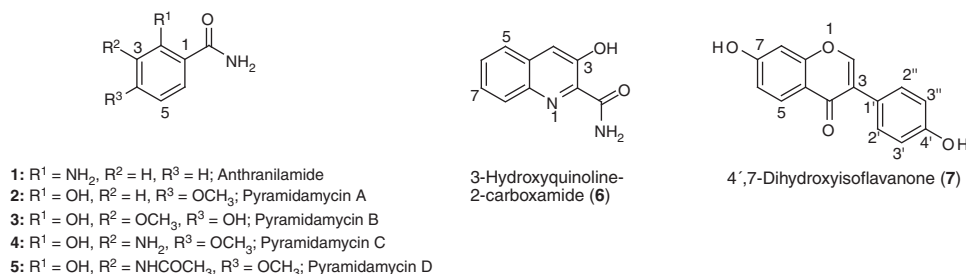


Figure 1 Chemical structures of compounds 1–7.

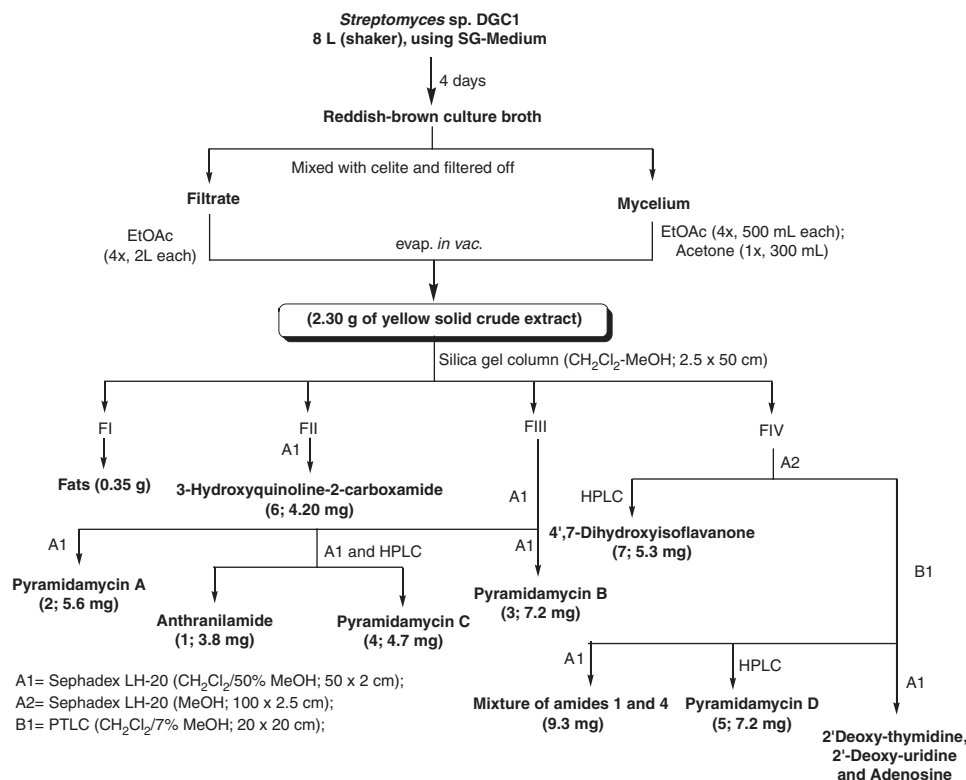


Figure 2 Work-up procedure of extracts from *Streptomyces* sp. DGC1.

Benzamides are of increased interest, because Ning *et al.*¹⁴ have demonstrated recently that the synthetic benzamide chidamide is a potent histone deacetylase inhibitor in T-cell lymphoma cell lines. The new compounds were examined for antimicrobial and cytotoxic activities.

RESULTS AND DISCUSSION

In our search for new bioactive compounds from streptomycetes, *Streptomyces* sp. DGC1 (Supplementary Figure S2) was cultivated on ISP4 agar plates at 28 °C for 3 days. After grown over, small agar pieces (circa 1 cm³) of the strain were used to inoculate 12 2-l Erlenmeyer flasks, each containing 670 ml of SG medium.^{12,13} The cultures were kept on a rotary shaker for 4 days at 28 °C. The reddish brown broth was harvested, mixed with celite, filtered off and extracted with ethyl acetate, and the mycelium was extracted with ethyl acetate followed by acetone. The combined organic extracts from supernatant and cells were concentrated *in vacuo* to afford 2.30 g of yellow-solid crude extract.

A TLC analysis of the strain extract exhibited several UV yellowish-green fluorescent bands at 366 nm, which turned yellow by staining with anisaldehyde/sulfuric-acid spraying reagent. The HPLC-MS analysis of the crude extract displayed several components with UV spectrum (Supporting Information, Supplementary Figures S3 and S4). Work-up and purification of the 2.30-g crude extract using various chromatographic techniques (Figure 2) led to the isolation of five new compounds, including pyramidamycins A-D (2–5) and 3-hydroxyquinoline-2-carboxamide (6), all five possessing an amide group (–CONH₂). In addition, the five known compounds 2-aminobenzamide (anthranilamide, 1, Supplementary Figures S5–S8),^{15,16} 4',7-dihydroxyisoflavanone (daidzein, 7, Supplementary Figures S48–S56),^{17,18} 2'-deoxy-thymidine,¹⁹ 2'-deoxy-uridine²⁰ and adenosine,^{19,21,22} were also isolated and characterized.

Structure elucidation

The physicochemical properties of compounds 1–6 are summarized in Tables 1 and 2. The known compounds were identified from their

Table 1 Physicochemical properties of pyramidamycins A-C (2–4)^a

	Pyramidamycin A (2)	Pyramidamycin B (3)	Pyramidamycin C (4)
Appearance	White solid, UV absorbing and blue fluorescence under long UV (365 nm)	Colorless solid, UV absorbing	White powder, UV absorbing
Anisaldehyde/H ₂ SO ₄ ^b	Pale yellow	Pale yellow	Yellow
R _f	0.32 (CH ₂ Cl ₂ /5% MeOH)	0.22 (CH ₂ Cl ₂ /5% MeOH)	0.57 (CH ₂ Cl ₂ /2% MeOH), 0.30 (CH ₂ Cl ₂)
Molecular formula	C ₈ H ₉ NO ₃	C ₈ H ₉ NO ₄	C ₈ H ₁₀ N ₂ O ₃
(–)-ESI MS: <i>m/z</i>	166 [M-H] [–]	182 [M-H] [–]	181 [M-H] [–]
(+)-ESI MS: <i>m/z</i>	168 [M+H] ⁺ , 190 [M+Na] ⁺	184 [M+H] ⁺ , 206 [M+Na] ⁺	183 [M+H] ⁺ , 205 [M+Na] ⁺
(+)-HR-ESI MS (<i>m/z</i>)			
Found	168.0654 [M+H] ⁺ and 190.0489 [M+Na] ⁺	184.0609 [M+H] ⁺ , 206.0435 [M+Na] ⁺ and 389.0963 [2M+Na] ⁺	183.0774 [M+H] ⁺ , 205.0600 [M+Na] ⁺ and 387.1292 [2M+Na] ⁺
Calculated	168.0655 for C ₈ H ₁₀ NO ₃ and 190.0475 for C ₈ H ₉ NO ₃ Na	184.0604 for C ₈ H ₁₀ NO ₄ , 206.0424 for C ₈ H ₉ NO ₄ Na and 389.0955 for C ₁₆ H ₁₈ N ₂ O ₈ Na	183.0770 for C ₈ H ₁₁ N ₂ O ₃ , 205.0589 for C ₈ H ₁₀ N ₂ O ₃ Na, 387.1275 for C ₁₆ H ₂₀ N ₄ O ₆ Na
UV/VIS (MeOH)			
λ _{max} (log ε)	212 (4.45), 255 (4.17), 295 (3.90) nm	214 (4.20), 260 (3.94), 295 (3.62) nm	235 (4.42), 271 (4.02), 314 (3.63) nm

^aSee also Supplementary Figures S4, S9–S11, S23 and S28–S30 (for comparison).^bColoration with anisaldehyde/sulfuric-acid spraying reagent and heating.**Table 2** Physicochemical properties of pyramidamycin D (5) and 3-hydroxyquinoline-2-carboxamide (6)^a

	Pyramidamycin D (5)	3-Hydroxyquinoline-2-carboxamide (6)
Appearance	White powder, UV absorbing	Pale-yellow solid, UV absorbing, green fluorescence under long UV (365 nm)
Anisaldehyde/sulfuric acid ^b	Pale yellow	—
R _f	0.31 (CH ₂ Cl ₂ /7% MeOH)	0.30 (CH ₂ Cl ₂)
Molecular formula	C ₁₀ H ₁₂ N ₂ O ₄	C ₁₀ H ₈ N ₂ O ₂
(–)-ESI MS: <i>m/z</i>	223 [M-H] [–]	187 [M-H] [–]
(+)-ESI MS: <i>m/z</i>	225 [M+H] ⁺	189 [M+H] ⁺
(+)-HR-ESI MS (<i>m/z</i>)		
Found	225.0875 [M+H] ⁺ , 247.0704 [M+Na] ⁺ and 263.0438 [M+K] ⁺	189.0653 [M+H] ⁺ and 211.0473 [M+Na] ⁺
Calculated	225.0870 for C ₁₀ H ₁₃ N ₂ O ₄ , 247.0689 for C ₁₀ H ₁₂ N ₂ O ₄ Na and 263.0429 for C ₁₀ H ₁₂ N ₂ O ₄ K for C ₁₀ H ₈ N ₂ O ₂ Na	189.0658 for C ₁₀ H ₉ N ₂ O ₂ and 211.0478
UV/VIS (MeOH): λ _{max} (log ε)	235 (4.13), 271 (3.84), 297 (3.63) nm	218 (4.18), 231 (4.24), 296 (4.59), 358 (3.58) nm

^aSee also Supplementary Figures S4, S35 and S40–S42 (for comparison).^bColoration with anisaldehyde/sulfuric-acid spraying reagent and heating.

NMR and mass data, by comparison with literature data. Structures **1** and **7** were determined by 1D and 2D NMR studies (Supplementary Figures S5–S8, and S48–S53), and by comparison with literature data.

Compound **2** was obtained as a white solid. It is UV absorbing, exhibits a blue fluorescence under long UV (365 nm) and gave a pale-yellow color discoloration on spraying with anisaldehyde/sulfuric acid. The molecular formula of **2** was determined by HR-ESI MS as C₈H₉NO₃ (Table 1, Supplementary Figures S9–S22). The proton NMR spectrum of **2** in DMSO-*d*₆ (Table 3) displayed one chelated broad signal for a hydroxyl group at δ 13.40 along with two broad singlets at δ 8.21 and 7.68, typical for an amide group (–CONH₂), which converted to a broad signal (as known from anthranilamide **1**) of 2H at δ 5.75 when measured in CDCl₃ solvent. In addition, the ¹H NMR spectrum displayed ortho-coupled protons at δ 7.76 (d, J = 9.0 Hz) and 6.43 (dd, J = 9.0, 2.5 Hz), a meta-coupled proton at δ 6.39 (d, J = 2.5 Hz) as well as a methoxy singlet at δ 3.75 (s), representing a

trisubstituted benzene. The ¹³C NMR/HSQC spectra (Table 4) confirmed compound **2** to be 2-hydroxy-4-methoxybenzamide and showed the OH group at C-2 (δ 163.6) chelated with the amide carbonyl (δ 172.4) and the methoxy group (δ 55.5) located at C-4 (δ 164.0). The HMBC correlations (Figure 3) of compound **2** finalized the structure, showing ³J correlations from the doublet proton H-6 (δ 7.76) to the amide carbonyl (δ 172.4), C-2 (δ 163.6) and C-4 (δ 164.0). The methoxy group (δ 3.75) could be determined as being attached at C-4 due to its significant HMBC correlation with C-4 (δ 164.0). Furthermore, NOESY correlations between this methoxy group and H-3 as well as H-5 were observed, all of which confirmed structure **2** as 2-hydroxy-4-methoxybenzamide, (Figure 3, Tables 3 and 4). A database search (chemical abstracts) confirmed the novelty of structure **2**, which was subsequently named pyramidamycin A.

Compound **3** was obtained as a colorless solid, with a molecular weight of 183 Da corresponding to a molecular formula of C₈H₉NO₄,

Table 3 ^1H NMR (500 MHz) data of the benzamides 1–5 in $\text{DMSO-}d_6$, δ in p.p.m. relative to TMS, multiplicities (JHz^{-1})

Position	Anthranilamide (1) ^a δ_{H} (JHz^{-1})	Pyramidamycin A (2) ^{a,b} δ_{H} (JHz^{-1})	Pyramidamycin B (3) ^a δ_{H} (JHz^{-1})	Pyramidamycin C (4) ^a δ_{H} (JHz^{-1})	Pyramidamycin D (5) ^a δ_{H} (JHz^{-1})
1- <u>CONH₂</u>	7.70 (1H, br s) 7.03 (1H, br s)	8.21 (1H, br s) 7.68 (1H, br s)	8.29 (1H, br s) 7.63 (1H, br s)	8.16 (1H, br s) 7.59 (1H, br s)	8.30 (1H, br s) 7.78 (1H, br s)
2-OH	—	13.40 (1H, br s)	13.49 (1H, br s)	13.0–11.0 (1H, br s)	13.52 (1H, br s)
2-NH ₂	6.54 (2H, br s)	—	—	—	—
3	6.66 (1H, dd, 8.5, 1.0)	6.39 (1H, d, 2.5)	—	—	—
3-NH	—	—	—	—	8.87 (1H, br s)
3-NH ₂	—	—	—	13.0–11.0 (2H, br s)	—
3-OCH ₃	—	—	3.68 (3H, s)	—	—
3-NHCOCH ₃	—	—	—	—	1.96 (3H, s)
4	7.12 (1H, td, 7.0, 1.5)	—	—	—	—
4-OH	—	—	9.86 (1H, br s)	—	—
4-OCH ₃	—	3.75 (3H, s)	—	3.79 (3H, s)	3.80 (3H, s)
5	6.47 (1H, td, 8.0, 1.0)	6.43 (1H, dd, 9.0, 2.5)	6.33 (1H, d, 8.5)	6.47 (d, 9.0)	6.58 (d, 8.5)
6	7.51 (1H, dd, 8.0, 1.5)	7.76 (1H, d, 9.0)	7.43 (1H, d, 9.0)	7.16 (d, 9.0)	7.77 (d, 9.0)

Abbreviation: TMS, tetramethylsilane.

^aSee also Supplementary Figures S5–S8, S12–S22, S24–S27, S31–S34, S36–S39 and S43–S47 for comparison.^bFor ^1H NMR data in CDCl_3 , see the experimental part.

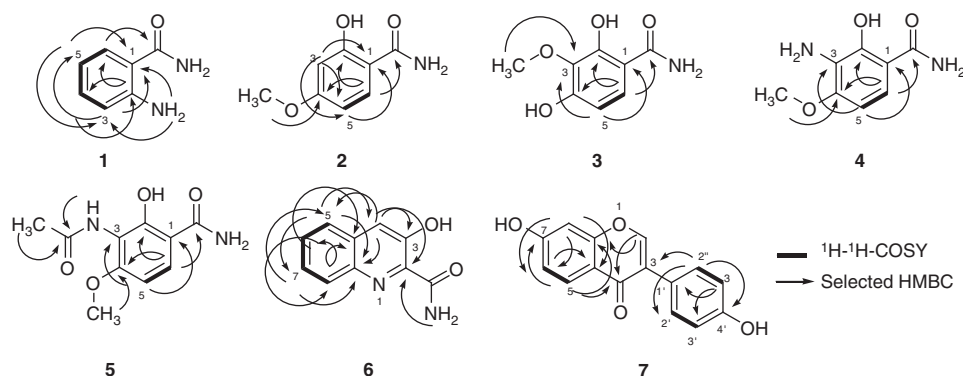
Underlined entries refer to the protons (or carbons) of the listed position.

Table 4 ^{13}C NMR (125 MHz) data of benzamides 1–5 in $\text{DMSO-}d_6$, (δ_{C} , mult.)

Position	Anthranilamide (1) ^a δ_{C} , mult.	Pyramidamycin A (2) ^{a,b} δ_{C} , mult.	Pyramidamycin B (3) ^a δ_{C} , mult.	Pyramidamycin C (4) ^a δ_{C} , mult.	Pyramidamycin D (5) ^a δ_{C} , mult.
1	113.7 s	107.3 s	106.9 s	107.5 s	107.8 s
1-CO	171.3 s	172.4 s	172.9 s	173.4 s	172.5 s
2	150.2 s	163.6 s	156.7 s	149.6 s	158.8 s
3	116.4 d	101.2 d	135.1 s	124.9 s	113.9 s
3-NHCO	—	—	—	—	168.3 s
3-NHCOCH ₃	—	—	—	—	22.7 q
3-OCH ₃	—	—	59.8	—	—
4	131.9 d	164.0 s	154.9 s	149.7 s	159.4 s
4-OCH ₃	—	55.5 q	—	55.9 q	55.9 q
5	114.5 d	106.1 d	107.0 d	102.3 d	102.0 d
6	128.5 d	129.5 d	123.5 d	123.5 d	127.1 d

^aSee also Supplementary Figures S6, S13, S25, S32 and S37 for comparison.^bFor ^{13}C NMR data in CDCl_3 , see the experimental part.

Underlined entries refer to the protons (or carbons) of the listed position.

**Figure 3** ^1H - ^1H -COSY (bold lines) and selected HMBC (\rightarrow) correlations in compounds 1–7.

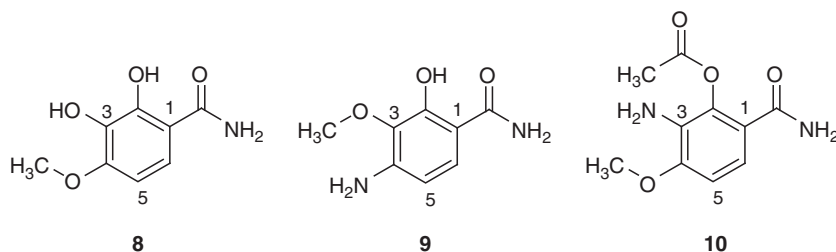


Figure 4 Chemical structures of compounds 8–10.

as deduced by HR-ESI MS (Table 1, Supplementary Figures S23–S27). The proton NMR spectrum (Table 3) and the ^{13}C NMR/HSQC spectra (Table 4) of **3** showed that it contains the same benzamide core as compound **2** with $\Delta m/z = 16$ amu higher than **2** corresponding to an additional oxygen atom. An additional broad signal at δ 9.86 in the proton NMR spectrum and the absence of the meta-coupled aromatic proton (at C-3 of compound **2**) suggested that the extra OH group (δ 9.86) might be located at C-3 (like in the hypothetical structure **8**, Figure 4). However, based on the full 2D NMR studies, the methoxy group (δ 3.68) showed an HMBC correlation with C-3 (δ 135.1), confirming its linkage at C-3, rendering the OH group at position 4 (structure **3**). All of the remaining HMBC correlations (Figure 3) and NMR data (Tables 3 and 4) are in full agreement with structure **3**. Compound **3** is a new structural analogue of **2**, 2,4-dihydroxy-3-methoxybenzamide, and was named pyramidamycin B.

Closely related to pyramidamycin B (**3**) compound **4** was obtained as a white powder from the same fraction FIII, exhibiting a molecular formula of $\text{C}_8\text{H}_{10}\text{N}_2\text{O}_3$ (HR-ESI MS), which is 1 amu smaller than **3**, indicating that one of the OH groups was replaced by an NH_2 group (Table 1). The ^1H and ^{13}C NMR data of **4** were similar to those of **3** (Tables 3 and 4), giving two alternative possible structures (**4** and **9**, see Figure 4 for alternative structures **8–10**) depending on the positions of the methoxy and the amino groups. In the HMBC spectra, a ^3J correlation was observed from the methoxy group (δ 3.79) to C-4 (δ 149.7), confirming its linkage to C-4 as in compound **2**, and not to C-3 (δ 124.9) as in **3**. All the remaining HMBC correlations (Figure 3) and NMR data (Tables 3 and 4) are in full agreement with structure **4**. Therefore, structure **4** was determined as 3-amino-2-hydroxy-4-methoxybenzamide, and consequently named pyramidamycin C (for spectra, see Supplementary Figures S28–S34).

Structurally related to pyramidamycin C (**4**), compound **5** was obtained as an orange solid, with a molecular formula of $\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}_4$ (HR-ESI MS); that is by 42 amu (typical for an acetyl group) higher than **4** (for physicochemical properties, see Tables 1 and 2). The comparison of the NMR data of compound **5** with those of pyramidamycin C (**4**) confirmed that **5** contains an additional acetyl group ($-\text{COCH}_3$), and the chemical shift of its carbonyl (δ 168.3) suggested an amide or ester connectivity, leaving the two alternative structures **5** and **10**. The proton NMR spectra of **5** showed a chelated broad signal at δ 13.52, indicating a free hydroxyl group at C-2 and a broad signal of an NH group at δ 8.87, thus excluding the isomeric structure **10**. Compound **5** was further subjected to 2D NMR (HSQC and HMBC) experiments, and ^2J correlations were observed from the NH (δ 8.87) and CH_3 (δ 1.96) to the carbonyl at δ 168.3, confirming the acetamide moiety. The observed $^3\text{J}_{\text{C-H}}$ HMBC coupling from the methoxy group (δ 3.80) to C-4 (δ 159.4) also confirmed its linkage to C-4. Also the remaining HMBC couplings (Figure 3) and the other NMR data (Tables 3 and 4) are in full agreement with structure **5**. Thus, compound **5** was identified as 3-acetamido-2-hydroxy-4-

Table 5 ^1H (500 MHz) and ^{13}C NMR (125 MHz) data of 3-hydroxyquinoline-2-carboxamide (**6**) in $\text{DMSO}-d_6$, δ in p.p.m. relative to TMS

Position	3-hydroxyquinoline-2-carboxamide (6) ^a	
	δ_{C} , mult.	δ_{H} (JHz–1)
2	135.8 s	—
2-CO	171.8 s	—
2-CONH ₂	—	8.78 (1H, br s), 8.23 (1H, br s)
3	153.9 s	—
3-OH	—	12.32 (1H, br s)
4	120.5 d	7.75 (1H, s)
4a	132.2 s	—
5	127.2 d	7.82 (1H, br d, 8.0)
6	129.6 d	7.57 (1H, br t, 6.5)
7	128.3 d	7.59 (1H, br t, 7.0)
8	129.9 d	7.99 (1H, br d, 7.5)
8a	141.6 s	—

Abbreviation: TMS, tetramethylsilane.

^aSee also Supplementary Figures S43–S47 for comparison.

Underlined entries refer to the delta value of the carbon atom.

methoxybenzamide, and named pyramidamycin D (for spectra, see Supplementary Figures S35–S39).

Compound **6** was isolated from fraction FII as a pale-yellow solid. It shows green fluorescence under long UV (365 nm), and has a molecular weight of m/z 188, corresponding to the molecular formula $\text{C}_{10}\text{H}_8\text{N}_2\text{O}_2$ determined by HR-ESI MS. The ^1H NMR spectrum revealed signals for a disubstituted benzene ring, a chelated OH group (δ 12.32), one singlet aromatic proton (δ 7.75) along with the typical broad signals of the amide protons ($-\text{CONH}_2$) as in the above discussed compounds (**1–5**, Figure 3). The ^{13}C NMR/HMBC spectra revealed ten carbons, five sp^2 methine (δ 129.9, 129.6, 128.3, 127.2 and 120.5, Table 5) and four quaternary sp^2 carbon atoms (δ 171.8, 153.9, 141.6, 135.8 and 132.2), of which the first one is the carbonyl amide. In the HMBC spectrum (Figure 3), the disubstituted benzene ring was confirmed and the chemical shift of one of its quaternary carbons at δ 141.6 should be linked to a heterocyclic nitrogen atom, such as in indole or quinoline moieties. The remaining singlet methine proton was in peri-position to H-5 (δ 7.82) based on the observed HMBC correlations between H-4 (δ 7.75) and C-5 (δ 127.2) and from H-5 (δ 7.82) to C-3 (δ 120.5). In addition, $^3\text{J}_{\text{C-H}}$ HMBC correlations (Figure 3) were observed from the amide protons ($-\text{CONH}_2$) and the singlet methine H-4 (δ 7.75) to C-2 (δ 135.8), along with the $^2\text{J}_{\text{C-H}}$ HMBC coupling of H-4 (δ 7.75) to C-3 (δ 153.9), confirming the structure of **6** as 3-hydroxyquinoline-2-carboxamide. Based on literature search, compound **6** is a new

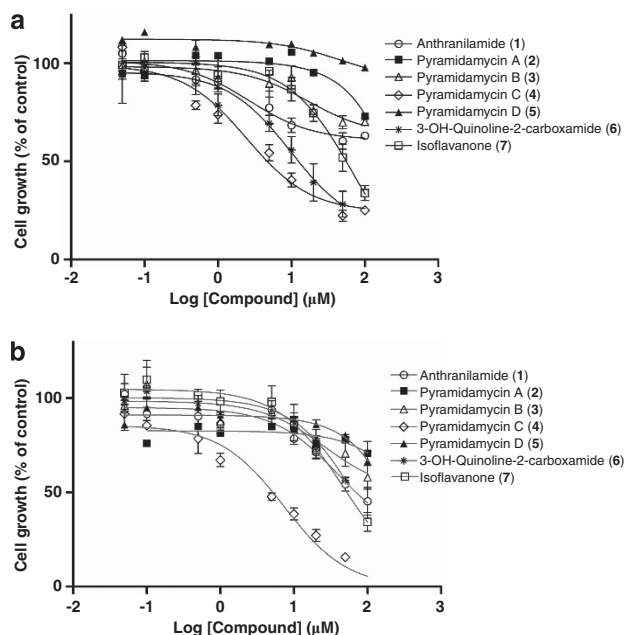


Figure 5 Dose–response curve of anthranilamide, pyramidamycins A–D, 3-hydroxy-Quinoline-2-carboxamide and isoflavanone in PC3 (a) and H460 (b) cell lines at 48 h.

natural product. The compound was previously mentioned by Kaneko and Yameda²³ as one of the intermediates of their synthesis of 3-hydroxyquinoline derivatives, however, no NMR and MS data were reported (for spectra, see Supplementary Figures S40–S47).

Biological activity

Cytotoxicity assays. The cytotoxic activity of anthranilamide (1), pyramidamycins A–D (2–5), 3-hydroxy-quinoline-2-carboxamide (6) and isoflavanone 7 was determined in comparison with the known strong cytotoxic angucyclin landomycin A, using PC3 (Prostate cancer) and H460 (non-small cell lung cancer) cell lines (Figures 5a and b, Table 6). Cell-viability assays showed that pyramidamycin C (4) was the most active compound against both PC3 and H460 cell lines (GI_{50} = 2.473 and 7.339 μ M, respectively). It exhibited cytostatic activity at all tested concentrations, but was less active than landomycin A (GI_{50} = 0.5505 and 4.109 μ M, respectively). Pyramidamycin C (4) has a free amino group ($-NH_2$) connected at C-3, which may be responsible for its cytostatic activity. Acetylation of this amino group, as found in the congener pyramidamycin D (5), led to the complete loss of cytostatic activity. All other compounds showed low or no activity, except 3-hydroxy-quinoline-2-carboxamide (6), which revealed some moderate cytostatic activity in the PC3 cell line (Figure 5a, Table 6).

Antibacterial activity. The antibacterial activity of compounds 1–7 were also determined against the Gram-negative bacterium *Escherichia coli* DH5 α (Invitrogen, Grand Island, NY, USA) and the Gram-positive bacteria *Micrococcus luteus* NRRL B-2618 and *Kocuria rosea* B-1106 (Table 7). *K. rosea* B-1106 was included in the testing as a second representative Gram-positive bacterium of the family *Micrococcaceae*. Of these compounds, benzamides 1–3 were the most active against the Gram-positive bacterium *K. rosea* B-1106, with 1 producing a diameter halo of 13 \pm 2 mm. Compounds 5 and 6 were slightly active against Gram-negative *E. coli* DH5 α (5 = 10 \pm 2 mm, 6 = 8 \pm 2 mm). Furthermore, compound 6 demonstrated the widest

Table 6 Cytotoxic activity of pyramidamycin C (4), 3-hydroxy-quinoline-2-carboxamide (6) and 4',7-dihydroxyisoflavanone (7) in comparison with landomycin A (GI_{50} values, μ M)

Compound	PC3 cells, 48 h		H460 cells, 48 h	
	GI_{50} (μ M)	95% confidence intervals	GI_{50} (μ M)	95% confidence intervals
Pyramidamycin C (4)	2.473	1.349–4.533	7.339	4.456–12.09
3-OH-quinoline-2-carboxamide (6)	9.812	2.823–34.11	—	—
4',7-Dihydroxyisoflavanone (7)	69.93	29.23–167.3	46.54	16.77–129.2
Landomycin A	0.5505	0.4982–0.6081	4.109	2.548–6.626

—, no measurable GI_{50} .

Table 7 Diameter halo measurements (mm) of compounds 1–7 tested against Gram-positive (*K. rosea* NRRL B-1106 and *M. luteus* NRRL B-2618) and Gram-negative bacteria (*E. coli* DH5 α) at 100 μ g per disc

Compound	<i>E. coli</i> DH5 α	<i>K. rosea</i> NRRL B-1106	<i>M. luteus</i> NRRL B-2618
	Anthranilamide (1)	—	13 \pm 2
Pyramidamycin A (2)	—	10 \pm 2	10 \pm 2
Pyramidamycin B (3)	—	10 \pm 2	—
Pyramidamycin C (4)	—	—	—
Pyramidamycin D (5)	10 \pm 2	—	—
3-Hydroxyquinoline-2-carboxamide (6)	8 \pm 2	—	9 \pm 2
4',7-Dihydroxyisoflavanone (7)	—	—	—

—, no measurable halo.

range of activities of those compounds tested, inhibiting also the Gram-positive *M. luteus* NRRL B-2618 (9 \pm 2 mm).

EXPERIMENTAL PROCEDURE

General experimental procedures

UV spectra were recorded on a Shimadzu UV-1800 (Model TCC-240A) UV spectrometer (Shimadzu, Kyoto, Japan). NMR spectra were measured on a Varian VnmrJ 500 (¹H, 500 MHz; ¹³C, 125.7 MHz) spectrometer; the δ values were referenced to the solvent signals (82.5 and 40.6 p.p.m., respectively, for DMSO-*d*₆ and 87.21 and 77.06 p.p.m., respectively, for CDCl₃). ESI mass spectra were recorded on a Finnigan LCQ ion trap mass spectrometer (Thermo Finnigan, Waltham, MA, USA). HR-ESI mass spectra were recorded on an Agilent LC/MSD TOF (resolution: 10 000; 3 p.p.m. mass accuracy; inlet systems: Agilent Technologies 1200 Series LC pumps) mass spectrometer (Agilent Technologies, Palo Alto, CA, USA). Samples were introduced by means of a syringe pump. Prep C₁₈ 7- μ m column (7.8 \times 300 mm²) on a binary LC system (solvent A: H₂O/0.2%/formic acid; solvent B: acetonitrile; flow rate: 2.0 ml min⁻¹; 0–15 min, 0–75% A (linear gradient); 15–20 min, 0% A and 100% B; 20–22 min, 0–75% A (linear gradient); 22–27 min, 75% A). HPLC-MS analyses were carried out using a Symmetry Anal C₁₈ 5- μ m column (4.6 \times 250 mm²) on a binary LC system. Flash chromatography was carried out on silica gel MN 60 (140–270 mesh ASTM). *R_f* values were measured on Polygram SIL G/UV₂₅₄ (Macherey–Nagel & Co., Düren, Germany). Size-exclusion chromatography was performed on Sephadex LH-20 (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Taxonomy

A soil sample containing DGC1 was collected from the Devil's Golf Course salt pan (Death Valley National Park). Approximately 1 g of soil sample was

suspended in 25 ml sterile H₂O and was subsequently heated to 80 °C water for 30 min. After heating, the sample was briefly vortexed and the large sediment was allowed to settle before plating 100 µl serial dilutions of the supernatant (10⁻¹, 10⁻², 10⁻⁴ and 10⁻⁸) onto oatmeal-agar plates supplemented with nalidixic acid (1 mg) and cycloheximide (10 mg). After 5 days of incubation at 30 °C, several bacterial colonies were observed. After a total incubation time of 7 days, over 50 individual colonies were streaked on oatmeal, M2, ISP2²⁴ and ISP4²⁴ agar plates. Colonies with general morphological similarities to streptomycetes were taken and streaked onto agar plates, in which they were found to have optimal growth (oatmeal, M2, ISP2 or ISP4). Finally, strains were grown in tryptic soy broth liquid media and stored as glycerol stocks for further study.

Phylogenetic studies of DGC1 were conducted as previously described.¹¹ The amplified 500-bp 16S rRNA fragment was found to have 99% identity (BLAST search) to the 16S rRNA gene of *S. atrovirens* strain NRRL B-16357. The sequenced 16S rRNA gene fragment from DGC1 has been deposited in the NCBI nucleotide database with an accession number JN836739.

Cell-viability assay

Prostate cancer cell line PC3 and non-small cell lung cancer cell line H460 were used to determine the cytotoxicity of amides 1–7 in comparison with landomycin A. Experiments were performed in four replicates and conducted as previously described.²⁵

Antibacterial activity test

The Gram-negative bacterium *E. coli* DH5α (Invitrogen) and the Gram-positive bacteria *M. luteus* NRRL B-2618 and *K. rosea* B-1106 were maintained in lysogeny broth liquid media and Mueller–Hinton agar (beef infusion 2 g l⁻¹, casein peptone 17.5 g l⁻¹, starch 1.5 g l⁻¹, Difco granulated agar 17 g l⁻¹, 1 l of double distilled H₂O, autoclaved). A sterile loopful of each organism was inoculated into a 7-ml culture of lysogeny broth and incubated in a 37 °C orbital shaker at 200 r.p.m. for 10 h. Each test organism was streaked on a sterile Mueller–Hinton agar plate with a sterile cotton swab. Compounds 1–7 were dissolved in methanol and were aliquoted in 100 µg amounts per each 6-mm sterile filter disc and were allowed to dry in a laminar flow hood. The discs were placed on the plates, which were then incubated for 24 h at 37 °C.^{26,27} The resulting diameter halos were measured (Table 7).

Culture material, fermentation and isolation

SG medium. Glucose (20 g, Sigma-Aldrich, Milwaukee, WI, USA), yeast extract (5 g, Acros Organics), soytone (10 g, Becton, Dickinson & Co., Franklin Lakes, NJ, USA), CoCl₂ × 6 H₂O (1 mg, Acros Organics, Geel, Belgium) and calcium carbonate (2 g, Sigma-Aldrich) were dissolved in 1 l of demineralized water. The suspension (pH 7.2) was sterilized by autoclaving for 33 min at 121 °C.

Oatmeal-agar medium. Oatmeal (60 g) and agar (12.5 g, Becton, Dickinson & Co) were dissolved in 1 l of demineralized water. The pH of the medium was adjusted to 6.0 with 1 M NaOH and 1 M HCl before sterilization.

M2-agar medium. Glucose (4.0 g, Sigma-Aldrich), yeast extract (4.0 g, Acros Organics), malt extract (10.0 g, MP Biomedicals, LLC, Santa Ana, CA, USA) and agar (15.0 g, Becton, Dickinson & Co) were dissolved in 1 l of demineralized water.

Fermentation, extraction and isolation. *Streptomyces* sp. DGC1 was cultivated on ISP4 agar plates at 28 °C for 3 days. Pieces of well-grown agar subculture of the strain were used to inoculate 12 2-l flasks, each containing 670 ml of SG medium, which was grown at 28 °C and harvested after 4 days. The obtained reddish brown culture broth was mixed with celite and filtered off. The water phase was extracted with EtOAc (4 × 2 l) and the biomass was extracted with EtOAc (4 × 500 ml), then with acetone (1 × 300 ml). The organic extracts were evaporated *in vacuo* at 38 °C, and combined after evaporation based on the TLC profile affording 2.30 g of yellow-solid crude extract.

Separation of the obtained crude extract on silica gel column (column 2.5 × 50 cm², 120 g), using a stepwise MeOH/CH₂Cl₂ gradient (0.41

0% MeOH → fraction FI, then 0.21 3% MeOH and 0.21 5% MeOH combined → fraction FII, then 0.21 10% MeOH and 0.21 20% MeOH combined → fraction FIII, then 0.31 50% MeOH and 0.21 100% MeOH combined → fraction FIV), yielded four fractions, FI (350 mg, yellow oil), FII (40.8 mg, yellow solid), FIII (700 mg, yellow solid) and FIV (400 mg, yellow solid). Fraction FI was identified as fats based on TLC, HPLC-MS and anisaldehyde/sulfuric-acid spraying reagent. Fraction FII was further purified using Sephadex LH-20 (2 × 50 cm², 50% MeOH/CH₂Cl₂) to give 3-hydroxyquinoline-2-carboxamide (6; pale-yellow solid, 4.2 mg). Purification of fraction FIII was carried out by Sephadex LH-20 (2 × 50 cm², 50% MeOH/CH₂Cl₂) followed by HPLC to yield anthranilamide (1; pale-yellow solid, 3.8 mg), pyramidamycin A (2; white solid, 5.6 mg), B (3; white solid, 7.2 mg) and C (4; white powder, 4.7 mg). Finally, fractionation and purification of fraction FIV using Sephadex LH-20, PTLC and HPLC afforded pyramidamycin D (5, 7.2 mg), 4',7-dihydroxyisoflavanone (7; yellow solid, 5.3 mg), 2'-deoxythymidine (white solid, 3.8 mg), 2'-deoxy-uridine (white solid, 5.1 mg) and adenosine (white solid, 10.3 mg) in pure forms (Figure 2).

2-Aminobenzamide; anthranilamide (1). Pale-yellow solid; UV absorbing (254 nm), blue fluorescence under long UV (365 nm); R_f 0.59 (7% MeOH/CH₂Cl₂), 0.31 (CH₂Cl₂); yellow coloration with anisaldehyde/sulfuric-acid spraying reagent; UV (MeOH) λ_{max} (log ε) 214 (4.14), 256 sh (3.66), 330 (3.53) nm; ¹H NMR (DMSO-*d*₆, 500 MHz) and ¹³C NMR (DMSO-*d*₆, 125 MHz), see Tables 3 and 4; (–)-APCI MS *m/z* 135 [M-H][–]; (–)-ESI MS *m/z* 135 [M-H][–]; (+)-ESI MS *m/z* 137 [M+H]⁺.

Pyramidamycin A (2). Physicochemical properties, see Table 1; ¹H NMR (DMSO-*d*₆, 500 MHz) and ¹³C NMR (DMSO-*d*₆, 125 MHz), see Tables 3 and 4; ¹H NMR (CDCl₃, 500 MHz) δ 12.48 (1H, br s, 2-OH), 7.25 (1H, d, 9.0, H-6), 6.45 (1H, d, 2.5, H-3), 6.40 (1H, dd, 9.5, 2.5, H-5), 5.75 (2H, br s, NH₂), 3.80 (3H, s, 4-OCH₃); ¹³C NMR (CDCl₃, 125 MHz), δ 172.5 (1-CO), 165.2 (Cq-4), 164.6 (Cq-2), 127.9 (CH-6), 107.5 (CH-5), 101.7 (CH-3), 55.7 (4-OCH₃).

4',7-Dihydroxyisoflavanone; daidzein (7). Yellow solid; R_f 0.43 (7% MeOH/CH₂Cl₂); UV absorbing (254 nm); ¹H NMR (DMSO-*d*₆, 500 MHz) δ 10.84 (1H, br s, 4'-OH), 9.54 (1H, br s, 7-OH), 8.28 (1H, s, H-2), 7.96 (1H, d, 8.5, H-5), 6.93 (1H, dd, 8.5, 2.5, H-6), 6.86 (1H, d, 2.0, H-8), 7.38 (2H, dd, 6.5, 2.5, 2'-H, 2''-H), 6.80 (2H, dd, 7.0, 2.0, 3'-H, 3''-H) p.p.m.; ¹³C NMR (DMSO-*d*₆, 125 MHz), δ 152.8 (CH-2), 123.5 (Cq-3), 174.7 (CO-4), 116.6 (Cq-4a), 127.3 (CH-5), 113.9 (CH-6), 162.6 (Cq-7), 102.1 (CH-8), 157.4 (Cq-8a), 122.5 (Cq-1'), 130.1 (CH-2'/CH-2''), 115.0 (CH-3'/CH-3''), 157.2 (Cq-4'); (–)-APCI MS *m/z* 253 [M-H][–].

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