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Akazamicin, a cytotoxic aromatic polyketide from marine-derived *Nonomuraea* sp.

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Abstract

In our screening program on marine-derived actinomycetes, *Nonomuraea* sp. AKA32 isolated from deep-sea water collected from a depth of 800 m in Sagami Bay, Japan was found to produce compounds cytotoxic to cancer cells. Activity-guided purification led to the isolation of a new aromatic polyketide, akazamicin (**1**), along with two known compounds, actinofuranone C (**2**) and *N*-formylanthranilic acid (**3**). Structures of these compounds were elucidated through the interpretation of NMR and MS spectroscopic data. Compounds **1**, **2**, and **3** displayed cytotoxicity against murine B16 melanoma cell line with the IC₅₀ value of 1.7, 1.2, and 25 μM, respectively.

Introduction

Actinomycetes are the leading producer of secondary metabolites with high structural diversity ranging from aliphatic to aromatic, linear to polycyclic, and from monomeric to oligomeric compounds [1]. Their metabolites are clinically utilized for a wide range of illness, such as microbial and parasitic infections, cancer, metabolic disorders, and autoimmune diseases. Representative drugs include streptomycin, kanamycin, avermectin, acarbose, and FK506, all of which are produced by *Streptomyces*, the most abundant and common actinomycete genus [2]. During the period from 1950 to 2000, *Streptomyces* was the most productive genus for antibiotics and other bioactive

compounds and is still likely the most prolific source of new secondary metabolites. Meanwhile, the number of new compounds from rare actinomycetes is increasing in recent years, accounting for up to 40% of new bioactive compounds among microorganisms [3–6].

After the intensive and exhaustive screening efforts with actinomycetes of terrestrial origin [7, 8], marine environment is now attracting attention as one of the promising niches for discovering new secondary metabolites [9, 10]. Deep-sea water (DSW) is defined as the sea water present deeper than 200 m and is characterized by several unique features, such as low temperature, rich inorganic nutrient, mineral abundance, and homeostasis [11]. Microorganisms in DSW are expected to have unique metabolic pathways for secondary metabolite production, however, in the past 20 years, only a limited number of studies have been devoted to bioactive compounds from DSW-derived microorganisms [12–20], because of technological barriers associated with isolation strategies and accessibility to DSW samples. In order to validate the uniqueness of bacterial community structure in DSW, we performed the denaturing gel gradient electrophoresis (DGGE) and pyrosequencing analyses for the DSW samples collected around Japan, which revealed that the bacterial community structure is distinctively different between DSW and the surface sea water (SSW), and DSW contains a large number of unknown actinobacteria comparing with SSW [21].

In this study, a total of 131 actinomycete strains were isolated from DSW in Sagami Bay collected by the

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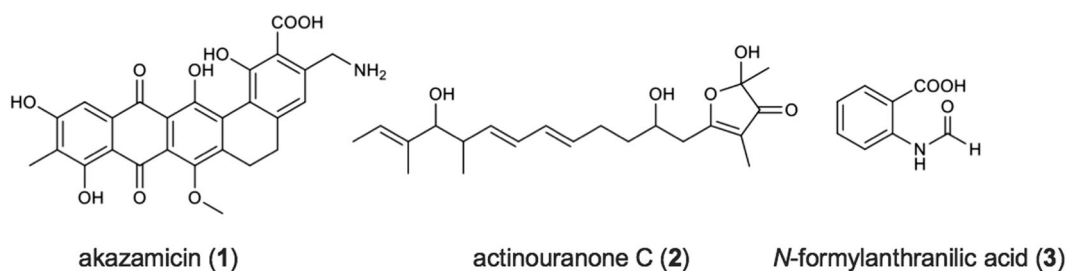


Fig. 1 Structures of akazamicin (1), actinofuranone C (2), and *N*-formylanthranilic acid (3)

Izu-Akazawa DSW pumping station in Shizuoka, Japan. Isolated strains were cultured in liquid medium, and the culture broth was subjected to cytotoxicity test against B16 melanoma cells. Among the several candidate strains which showed cytotoxicity against B16 melanoma cells, *Nonomuraea* sp. AKA32 was chosen for further chemical study because of its low homology of 16S rRNA gene sequence to known actinomycete species. Herein, we describe the isolation, structure determination, and cytotoxic activity of a new aromatic polyketide akazamicin (1) and two known compounds actinofuranone C (2) and *N*-formylanthranilic acid (3) isolated from the culture extract of strain AKA32 (Fig. 1).

Results and discussion

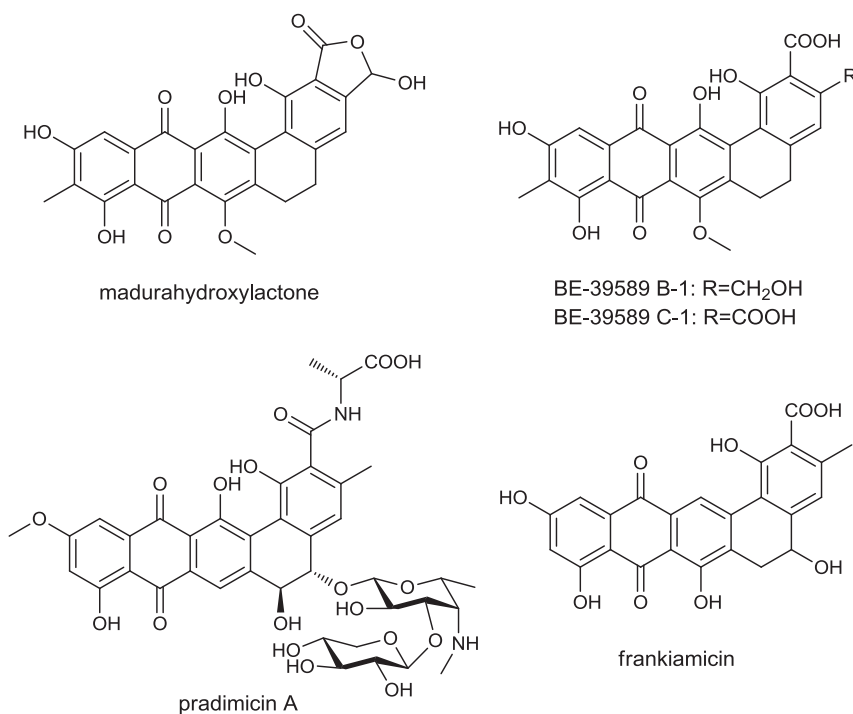
The producing strain AKA32 was isolated from a bag filter used for the filtration of DSW in Sagami Bay pumped up at the Izu-Akazawa DSW pumping station in Shizuoka, Japan. On the basis of 16S rRNA gene sequence analysis, it was identified as a member of the genus *Nonomuraea*. Because of the low similarity to known species and the strong cytotoxicity against murine B16 melanoma cell line shown by the two kinds of culture broth, strain AKA32 was chosen for further chemical investigation. Activity-guided purification from the crude extract of two kinds of media led to the isolation of a new aromatic polyketide akazamicin (1) and a known compound actinofuranone C (2) from the culture broth of A16 and another known compound *N*-formylanthranilic acid (3) from the culture broth of ISP-2. Subsequent biological evaluation elucidated that 1, 2, and 3 inhibited the growth of B16 melanoma, Hep G2, and Caco-2 cell lines. Herein, we describe the isolation, structure determination, and biological activity of these three compounds.

Akazamicin (1) was obtained as a red powder. The IR spectrum indicated the presence of hydroxy (2928 cm^{-1}) and carbonyl (1626 and 1603 cm^{-1}) groups. The UV spectrum [λ_{max} 292 (ϵ 18,500), 319 (ϵ 11,400), 476 (ϵ 10,300) nm in MeOH] was closely similar to that of madurahydroxylactone [22, 23], suggesting that 1 has a

benzo[*a*]naphthacenequinone framework (Fig. 2). The molecular formula was determined as $\text{C}_{26}\text{H}_{21}\text{NO}_9$ on the basis of the HRESITOFMS that was corroborated by NMR data obtained in $\text{DMSO-}d_6$ (Table 1). Combined analysis of ^1H and ^{13}C NMR, DEPT, and HSQC spectral data revealed the presence of two aromatic protons (δ_{H} 6.70 and 7.27), methylene protons (δ_{H} 2.63, 2.79, and 4.15), one methyl (δ_{H} 2.10) and one methoxy (δ_{H} 3.80) group, four exchangeable protons (δ_{H} 8.27, 11.25, 13.66, and 13.74). In addition, the presence of 19 sp^2 carbons that do not bear protons was also confirmed.

Structure analysis was started with the HMBC from the methyl proton at δ_{H} 2.10 to C-9, C-10, and C-11. The downfield shifted resonances of phenolic protons at δ_{H} 13.66 (9-OH) and 11.15 (11-OH) displayed correlations to C-8a and C-9, and to C-11 and C-12, respectively. In addition, an aromatic proton at δ_{H} 7.27 (H-12) was correlated strongly to C-8a and C-10 and weakly to C-11 and C-12a. All these correlations, together with the highfield shifted resonances of C-8a, C-10, and C-12, established a pentasubstituted benzene ring in which two hydroxyl groups were placed in *meta*-position. Furthermore, a quinone carbonyl carbon C-13 was connected at C-12a based on a long-range correlation from H-12 to C-13. Another quinone carbonyl carbon C-8 was connected to C-8a by a four-bond correlation from H-12 to C-8, which was supported by the sharp singlet resonance of 9-OH that indicated its hydrogen bonding to the adjacent carbonyl oxygen. On the basis of these spectroscopic evidences, a naphthoquinone substructure was established. The remaining part of 1 was constructed from another aromatic proton at δ_{H} 6.70 (H-4). This proton showed HMBC to sp^2 carbons C-2, C-3, C4a, and C-14b and sp^3 carbons C-5 and C-16. In turn, the methylene protons H-16 were correlated to C-2, C-3, and C-4, establishing the connectivity between C-3 and C-16. H-16 protons were also COSY-correlated with an exchangeable proton at δ_{H} 6.70, which was deduced to be an amino group in consideration of molecular formula. The H-5 methylene showed a COSY correlation to H-6 methylene and HMBC to C-4, C-4a, C-6a, and C-14b. In addition, another phenolic proton at δ_{H} 13.74 showed correlations to C-13a, C-14, and C14a, establishing the

Fig. 2 Benzo[*a*]naphthacenequinone antibiotics from actinomycetes



connectivity among these three carbons. HMBC from methoxy protons at δ_{H} 3.80 to C-7 established the methoxy substitution at this carbon. As compound **1** did not bear any more protons that could give HMBC information critical to the complete assignment (Fig. 3), it became necessary for us to compare the ^1H and ^{13}C NMR chemical shifts with known benzo[*a*]naphthacenequinones of which structure was established with full NMR data. We selected BE-39589 B-1 and BE-39589 C-1 for this purpose because these compounds are structurally closest to **1** with the same carbon skeleton, and NMR spectroscopic data are fully reported [23]. Although long-range correlations were not available, careful chemical shift comparison allowed to assign the position of the remaining carbons C-1, C-15, and C-7a (Figure S21).

The only remaining concern, however, was the connectivity between C-7a and C-8 and between C-13 and C-13a, as two possible structures existed (Fig. 4). In order to eliminate one of these two possibilities, chemical shift comparison was made for phenolic OH protons in some structurally related anthraquinones. In the case of five compounds in which one phenolic proton is hydrogen-bonded to one quinone carbonyl, proton signals are observed at from δ_{H} 13.08 to 14.30 (Figure S22, type A). On the other hand, in the case of six compounds in which two phenolic protons are hydrogen-bonded to one quinone carbonyl, proton signals are observed at from δ_{H} 11.90 to 12.62 (Figure S23, type B). Previous literatures rationalizing this chemical shift difference were not

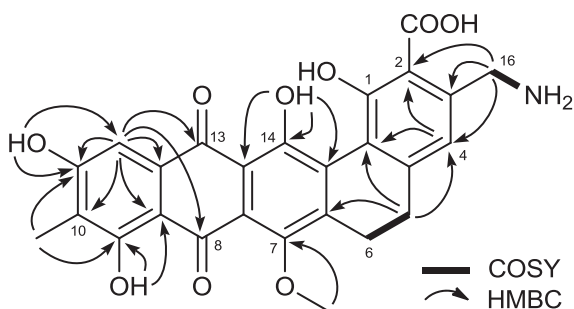
found, but it can be explained by the electron density on the nucleus of the phenolic proton. In type A, one quinone carbonyl oxygen attracts electron only from one phenolic proton, while one quinone carbonyl oxygen in type B must attract electron from two phenolic protons, implying that electron density of the phenolic proton in type A is lower than that in type B. In the case of akazamicin, two phenolic protons are observed at δ_{H} 13.66 and 13.74. These chemical shifts are obviously closer to those for compounds in type A (Figure S24), which led us to propose the structure A (Fig. 4) as a correct structure for **1**.

Known natural products bearing the benzo[*a*]naphthacenequinone core are exclusively produced by actinomycetes. Among this class, only three compounds (madurahydroxylactone, BE-39589 B-1, and BE-39589 C-1) have the carbon skeleton identical with **1** (Fig. 2). Meanwhile, antibiotic R2 and nomurcin are the variants of this group that have a different carbon substituent at C10: antibiotic R2 has an ethyl group and nomurcin has a hydroxymethyl group instead of a methyl group of **1** (Fig. 5). Pradimicins, benanomycin, and frankiamicin A belong to this class, but they have no carbon substitution at C-10. All the known compounds of benzo[*a*]naphthacenequinone have the hydroxyl group at C-9 and C-11, whereas compounds which have the hydroxyl group at C-10 and C-12 do not exist according to SciFinder database search. From the viewpoint of biosynthesis, the presence of hydroxyl groups at C-9 and C-11 seems more probable.

Table 1 NMR data of Compound (**1**) in DMSO-*d*₆

Position	δ_C^a	δ_H mult ^b	HMBC ^c
1	163.3		
2	117.0		
3	136.7		
4	118.8	6.70 s	2, 3, 4a, 5, 14b, 16
4a	143.1		
5	28.7	2.63 t	4, 4a, 6, 6a, 14b
6	22.5	2.79 br. t	
6a	145.1		
7	150.4		
7a	121.7		
8	186.0		
8a	109.8		
9	162.3		
10	117.6		
11	162.1		
12	106.0	7.27 s	8a, 10, 11, 12a, 13
12a	131.6		
13	186.6		
13a	114.6		
14	157.1		
14a	131.9		
14b	120.6		
15	171.8		
16	43.6	4.15 s	2, 3, 4
7-OCH ₃	61.0	3.80 s	7
10-CH ₃	8.2	2.10 s	9, 10, 11
1'		8.27 s	
9-OH		13.66 s	8a, 9
11-OH		11.25 s	
14-OH		13.74 s	13a, 14, 14a

^aRecorded at 125 MHz., ^brecorded at 500 MHz., ^cHMBC are from proton(s) stated to the indicated carbon

**Fig. 3** COSY and key HMBC for akazamicin (**1**)

According to the incorporation experiments of ¹³C-labeled acetates into pradimicin, C-9 and C-11 are derived from the carbonyl carbon of acetate (malonate) (Fig. 6). Based on these considerations, we concluded that our proposed

structure of akazamicin is much more likely to be correct than another possible structure.

The structures of actinofuranone C (**2**) and *N*-formylanthranilic acid (**3**) were determined on the basis of NMR and MS analysis. The NMR spectroscopic data of **2** were totally identical with the reported one [24]. The structure of **3** was confirmed by the NMR spectral comparison with the commercially available **3** purchased from Sigma-Aldrich Co. Ltd.

IC₅₀ values of compounds **1–3** against B16, Hep G2, and Caco-2 cells are summarized in Table 2. Compounds **1** and **2** displayed cytotoxicity at the same level against B16 melanoma cells with IC₅₀ values of 1.7 and 1.2 μM, respectively. Compound **3** was *ca* 10-fold less potent than **1** and **2**. The three compounds were not significantly cytotoxic against Hep G2 and Caco-2 cells with IC₅₀ values ranging from 10 to 200 μM.

Benzo[*a*]naphthacenequinone antibiotic is a unique, pentangular type II polyketide of actinomycete origin, including madurahydroxyalacone from *Nonomuraea* [22, 24], pradimicin from *Actinomadura* [25], and frankiamicin from *Frankia* [26]. Compound **1** is structurally closest to BE-39589 B1 and BE-39589 C-1 from *Actinomadura* [27]. To the best of our knowledge, cytotoxicity has not been reported with this class of antibiotics. Compound **2** and its congeners actinofuranones A and B are the secondary metabolites of *Amycolatopsis* [24] and *Streptomyces* [28], respectively. Furanone-type polyketides have been isolated from various organisms such as mollusks, fungi, and actinomyetes, however, there is no report on the identification of this class from *Nonomuraea*. Compound **3** is known as a degraded compound of indole. There is no report on cytotoxicity of compounds **2** and **3** against normal and tumor cells.

In summary, this is the first report on the secondary metabolites of actinomycetes collected from DSW in the Pacific Ocean. Further chemical investigation along this line is currently in progress in our laboratory, being directed to prove the biosynthetic potential of DSW-derived actinomycetes and the scientific value of DSW as an alternative source for new bioactive compounds.

Experimental section

General experimental procedures

NMR experiments were performed on a Bruker AVANCE 500 spectrometer (Bruker Biospin K. K., Yokohama, Japan). UV spectra were recorded on Shimadzu SPD-10A spectrophotometer. High-resolution ESITOFMS were recorded on a Bruker micrOTOF focus (Bruker Daltonics K. K., Yokohama, Japan). IR spectra were recorded on a Shimadzu FT-IR-300 spectrophotometer.

Fig. 4 Two possible structures for **1** deduced from NMR analysis

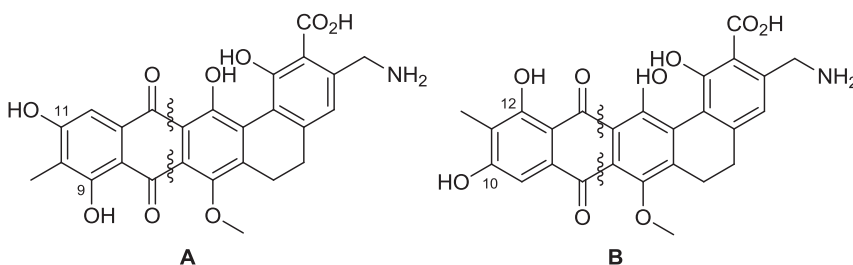


Fig. 5 Benzo[*a*]naphthacenequinones bearing carbon substitution at C-10

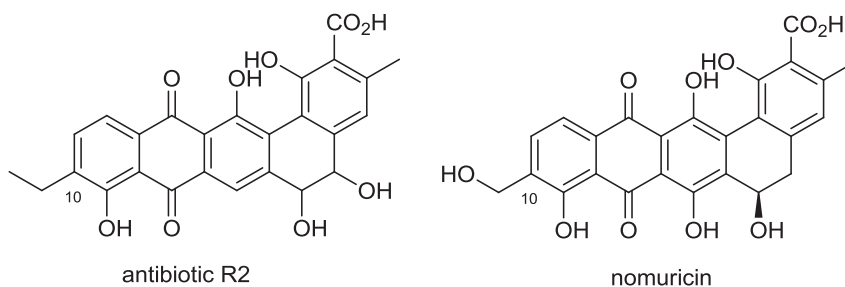


Fig. 6 Biosynthesis of pradimicin aglycon

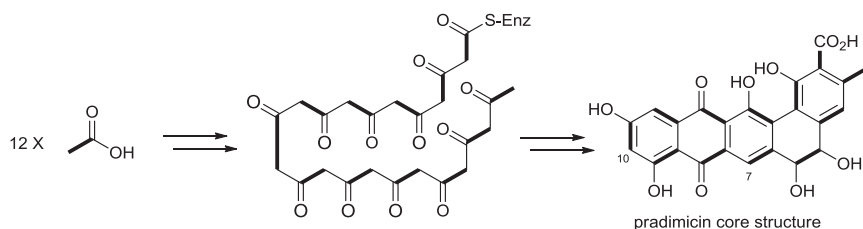


Table 2 Cytotoxicity of compounds **1–3** against tumor cells

Cell lines	IC ₅₀ (μM)		
	1	2	3
B16	1.7	1.2	25
Hep G2	75	38	84
Caco-2	185	13	81

DSW sampling and filtration

Deep-sea water (DSW) was collected at the DSW pumping station of DHC Co. Ltd. (<http://www.dhc.co.jp>; coordination 34°52'40" N, 139°06'09" E) in Izu-Akazawa, Shizuoka Prefecture, Japan. The sampling point (−800 m in depth) is the deepest among the all DSW facilities in Japan (Deep Ocean Water Applications Society: <http://www.dowas.net/facilities/index.html> [In Japanese]). The DSW pumped up is passed through a bag filter (18 cm in diameter × 80 cm in length, Figure S20) in order to eliminate debris and small particles in DSW. After 30,000 tons of DSW was filtered, the bag filter was removed from the line and the bottom part was cut into small pieces (3 cm × 3 cm), which was suspended in sterilized DSW under axenic condition. A portion of the liquid was spread onto the ISP-2 and HV-agar medium and the plate was incubated at 4 °C, 27 °C, and

38 °C for 31 days. Strain AKA32 was obtained from the plate incubated at 27 °C.

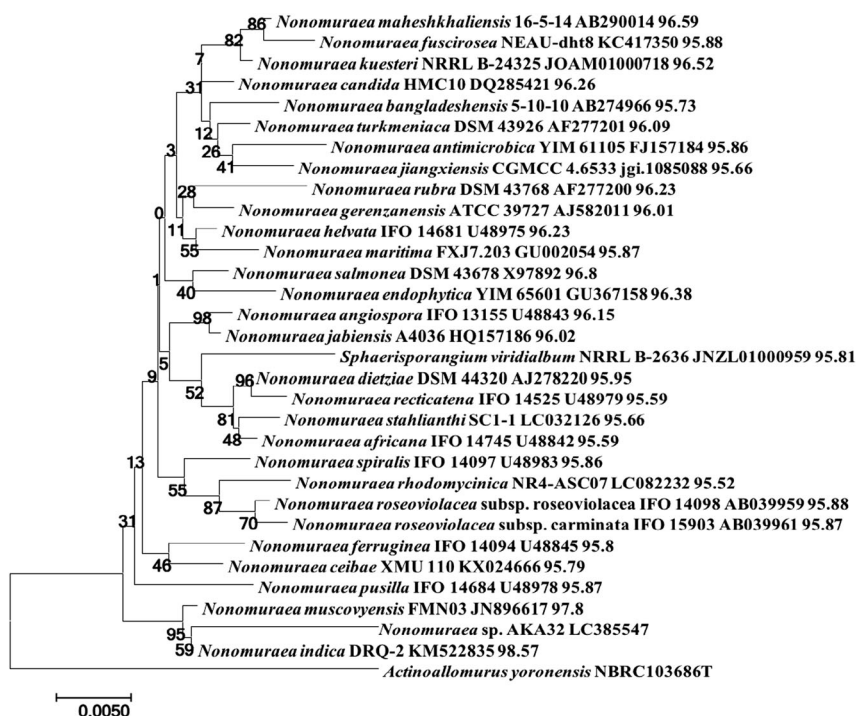
Microorganism

Nonomuraea sp. AKA32 was isolated from a DSW collected at the Izu-Akazawa DSW pumping station in Shizuoka, Japan. The isolated strain was identified *Nonomuraea* on the basis of 98.5% similarity (1435 nucleotides; DDBJ accession number: LC385547) to *Nonomuraea indica* DRQ-2^T (DDBJ accession number: KM522835). The phylogenetic tree generated by a neighbor-joining method [29] based on 16S rRNA gene sequence clearly revealed the evolutionary relationship of strain AKA32 within a group of known *Nonomuraea* species (Fig. 7).

Fermentation

Nonomuraea sp. AKA32 preserved as a frozen glycerol stock was inoculated onto ISP 2 agar. A pure colony of this strain was inoculated into 500-ml K-1 flasks, each containing 100 ml of seed medium consisting of soluble starch 1%, glucose 0.5%, NZ-case (Humco Sheffield Chemical Co.) 0.3%, yeast extract (Difco Laboratories) 0.2%, Tryptone (Difco Laboratories) 0.5%, K₂HPO₄ 0.1%, MgSO₄ · 7H₂O 0.005%, and CaCO₃ 0.3% (pH 7.0). The flasks were shaken

Fig. 7 Neighbor-joining phylogenetic tree [29] based on almost-complete 16S rRNA gene sequences showing the position of strain AKA32 in the genus *Nonomuraea*. Numbers at the nodes indicate the levels of bootstrap support are shown. GenBank accession numbers are given in parentheses. Bar, 0.005 substitutions per site



at 30 °C for 6 days on a rotary shaker (200 rpm). Three-milliliter aliquots of the seed culture were transferred into 500-ml K-1 flasks each containing 100 ml of A-16 production medium consisting of glucose 2%, Pharmamedia (Trader's Protein) 1%, CaCO₃ 0.5%, Diaion HP-20 resin (Mitsubishi Chemical Corporation, 50 g l⁻¹) for compounds **1** and **2**, and ISP-2 production medium consisting of glucose 1%, malt extract (Difco Laboratories) 0.4% and yeast extract (Difco Laboratories) 0.4% for compound **3**. The flasks were shaken at 30 °C for 7 days on a rotary shaker (200 rpm).

Extraction and isolation of compounds **1** and **2**

After the fermentation of production medium for compounds **1** and **2**, the whole-culture broth was extracted with 1-butanol (100 ml per flask) on a shaker for 1 h. The mixture was centrifuged at 8000 rpm for 15 min, and the organic layer was separated from the aqueous layer containing the mycelium. Evaporation of the solvent in vacuo provided 10.0 g of crude extract from 21 of culture, which was subjected to silica gel column chromatography with a step gradient of CHCl₃/MeOH (1:0, 20:1, 10:1, 4:1, 2:1, 1:1, 1:2, and 0:1 v/v). Concentration of fraction 7 (CHCl₃/MeOH = 1:2) provided 1.8 g of red oily residue, which was further purified by preparative C-18 HPLC (10 mm × 250 mm COSMOSIL 5C₁₈-AR-II Packed, Nacalai Tesque) using a gradient of MeCN/0.1% HCO₂H (MeCN concentration: 50–80% for 0–30 min) at 4 ml min⁻¹ afforded compound **1** (10.0 mg) as a red powder with a retention time of 18.5 min.

Also, concentration of fraction 4 (CHCl₃/MeOH = 4:1) provided 1.0 g of red powder, which was repeatedly purified by reversed-phase ODS column chromatography with a gradient of MeCN/0.1% HCO₂H (2:8, 3:7, 4:6, 5:5, 6:4, and 7:3 v/v). Concentration of fraction 4 (MeCN/0.1% HCO₂H = 5:5) gave 61 mg of brown solid, which was purified by preparative C-18 HPLC (10 mm × 250 mm COSMOSIL 5C₁₈-AR-II Packed, Nacalai Tesque) using a gradient of MeCN/0.1% HCO₂H (MeCN concentration: 50% for 0–5 min; 50–60% for 5–10 min; 60% for 10–20 min; 60–80% for 20–30 min) at 4 ml min⁻¹, yielding compound **2** (8.0 mg) as a colorless, white powder with a retention time of 13.5 min.

Extraction and isolation of compound **3**

After the fermentation of production medium for compound **3**, the whole-culture broth was extracted with 1-butanol (100 ml per flask) on a shaker for 1 h. The mixture was centrifuged at 8000 rpm for 15 min, and the organic layer was separated from the aqueous layer containing the mycelium. Evaporation of the solvent in vacuo afforded 10.0 g of crude extract from 301 of culture, which was subjected to reversed-phase ODS column chromatography with a gradient of MeCN/0.1% HCO₂H (5:95, 10:90, 20:80, 30:70, 40:60, 50:50, and 60: 40 v/v). Concentration of fraction 4 (MeCN/0.1% HCO₂H = 30:70) provided 180 mg of brown solid that was further purified by preparative C-18 HPLC (10 mm × 250 mm COSMOSIL 5C₁₈-AR-II Packed, Nacalai Tesque) using a gradient of MeCN/0.1% HCO₂H (MeCN

concentration: 15–80% for 0–30 min) at 4 ml min⁻¹, yielding compound **3** (10.0 mg) as a colorless, amorphous solid with a retention time of 8.2 min.

Akazamicin (**1**)

Red powder; UV (MeOH) λ_{\max} (ϵ) 292 (18,500), 319 (11,400), 476 (10,300); IR (ATR) ν_{\max} 2928, 1626, 1603 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; HRESI-TOFMS [M-H]⁻ 490.1144 (calcd for C₂₆H₂₀NO₉ 490.1144).

Actinofuranone C (**2**)

Colorless amorphous solid; ¹H and ¹³C NMR data, see Table S1; ESI-MS [M + Na]⁺ 387.25 (calcd for C₂₁H₃₂O₅Na 387.22), [M-H]⁻ 363.25 (calcd for C₂₁H₃₁O₅ 363.22).

N-formylanthranilic acid (**3**)

White powder; ¹H and ¹³C NMR data, see Table S2; ESI-MS [M + Na]⁺ 188.03 (calcd for C₈H₇NO₃Na 188.03).

Cell culture

B16 murine melanoma and Hep G2 human liver cancer cells were maintained in DMEM medium containing 3.7 g l⁻¹ sodium bicarbonate supplemented with 1% (w/v) non-essential amino acids, 10% (v/v) FBS, and 1% (v/v) penicillin (10,000 units ml⁻¹)-streptomycin (10 mg l⁻¹), while Caco-2 human colon carcinoma cells were maintained in high glucose DMEM medium containing 1 g l⁻¹ glucose, 3.7 g l⁻¹ sodium bicarbonate supplemented with 1% (w/v) non-essential amino acids, 10% (v/v) FBS, and 1% (v/v) penicillin (10,000 units ml⁻¹)-streptomycin (10 µg ml⁻¹) solution in a 5% CO₂ incubator under 37 °C. Each cell lines were seeded into the culture flasks (75 cm², IWAKI, Japan) and incubated at 37 °C under 5% CO₂ atmosphere until ~80% confluence, then harvested by Trypsin-EDTA solution (10×, Sigma Diagnostics, Inc., St Louis, MO, USA) and seeded to a new flask until use.

Cytotoxicity test

B16 cells were seeded at 1.5 × 10⁵ cells/well and Hep G2 and Caco-2 cells were seeded at 2.0 × 10⁵ cells/well into 96-well microplate (IWAKI, Japan), respectively, and incubated at 37 °C under 5% CO₂ atmosphere. After 24 h for incubation of each cell line, medium was discarded and replaced with fresh DMEM and high glucose DMEM containing 5% FBS and the samples were prepared in DMSO at a final concentration of 2–200 µM and each test was performed in quadruplicate. The control group was cultured only in DMEM and high glucose DMEM, with 5%

FBS and 0.9% DMSO in the culture medium. After additional 48 h of incubation, cell viability was measured by MTT assay (Sigma Diagnostics, Inc., St Louis, MO, USA): MTT was dissolved in 1X sterile PBS at 5 mg ml⁻¹ concentration. Ten-microliter of MTT solution was added into each well of cell culture plate and incubated at 37 °C for 2 h. After incubation, medium was removed, 100 µl of 0.04 M of hydrogen chloride-isopropyl alcohol was added to each well to solubilize the crystals, and the plates were read at 570–655 nm using a microplate reader. Relative cell viability was calculated from the absorbance at 570–655 nm of treated cells/that of untreated cells.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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