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NOTE

Arcyriaflavin E, a new cytotoxic indolocarbazole alkaloid isolated by combined-culture of mycolic acid-containing bacteria and *Streptomyces cinnamoneus* NBRC 13823

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Among all actinomycetes, *Streptomyces* species are prolific sources of secondary metabolites, leading to the isolation of thousands of antibiotics and other bioactive compounds.[1] Genomic analyses of several *Streptomyces* strains revealed that each contains around 30 biosynthesis gene clusters for secondary metabolite production.[2–4] However, many of these genes remain cryptic under standard laboratory conditions, and these 'silent genes' are expected to be untapped sources for novel bioactive compounds.

Recent research has illustrated that some mycolic acid-containing bacteria, such as *Tsukamurella*, *Rhodococcus*, *Corynebacterium*, *Gordonia* and *Nocardia* species, interact with a broad range of *Streptomyces* strains and induce the production of cryptic secondary metabolites by co-culture.[5,6] This 'combined-culture' method promises to lead to the discovery novel cryptic bioactive compounds produced by *Streptomyces*.

In this report we describe the isolation of arcyriaflavin E (**2**), a new cytotoxic indolocarbazole alkaloid, along with two known related alkaloids, BE-13793C (**1**) and arcyriaflavin A (**3**), from *Streptomyces cinnamoneus* NBRC 13823 (provided by the National Institute of Technology and Evaluation Biological Resource Center (Japan)). The production of these indolocarbazole alkaloids was induced by culturing the strain together with *Tsukamurella pulmonis*, a mycolic acid-containing bacterium.[5,7]

HPLC-UV ANALYSIS OF THE METABOLIC PROFILE OF *S. CINNAMONEUS NBRC* 13823

S. cinnamoneus NBRC 13823 and *T. pulmonis* were separately inoculated into a 500-ml baffled Erlenmeyer flask (*S. cinnamoneus* NBRC 13823) and a 500-ml Erlenmeyer flask (*T. pulmonis*), each containing 100 ml of V-22 medium, composed of 1.0% starch, 0.5% glucose, 0.5% Bacto Tryptone (Difco), 0.3% NZ case (Wako), 0.2% Yeast Extract (Difco), 0.1% K_2HPO_4, 0.05% $MgSO_4{\cdot}7\,H_2O$ and 0.3% $CaCO_3$ (pH = 7.0). *S. cinnamoneus* NBRC 13823 was cultured at 30 °C for 3 days on a rotary shaker at 220 r.p.m., and *T. pulmonis* was cultivated in the same manner for 2 days.

A 3-ml portion of the *S. cinnamoneus* NBRC 13823 culture and 1 ml of the *T. pulmonis* culture were simultaneously added to a 500-ml baffled Erlenmeyer flask containing 100 ml of A-3 M medium, consisting of 2.0% starch, 2.0% glycerol, 0.5% glucose, 1.5% Pharma media (Archer Daniels Midland Co.), 1.0% HP-20 (Nihon Rensui) and 0.3% yeast extract (pH = 7.0). In addition, 3 ml of the *S. cinnamoneus* NBRC 13823 culture and 1 ml of the *T. pulmonis* culture were individually inoculated in 500-ml baffled Erlenmeyer flasks containing 100 ml of A-3M medium, as control cultures. They were all cultured at 30 °C for 5.5 days on a rotary shaker at 160 r.p.m.

The cell pellets were collected after centrifugation of the fermentation broths, and were subjected to lyophilization. The freeze-dried cells were extracted with 50 ml of a CH_3OH-$CHCl_3$ mixture (50:50, v/v). The extracts were then subjected to an HPLC-UV analysis, performed on a 4.6 × 250 mm Cosmosil $5C_{18}$-MS-II column (Nacalai Tesque, Kyoto, Japan), in a CH_3CN (solvent A)/H_2O-containing 1% acetic acid (solvent B) gradient system (solvent A: 95–80% (15 min) − 0% (45–50 min)), using a JASCO PU2080 pump to control the flow rate at 1.0 ml min^{-1}. All eluates were monitored by UV absorption at 315 nm (JASCO, Tokyo, Japan, MD-2010 Plus Multiwavelength Detector). As a result of the HPLC analysis, one significantly enhanced peak (compound **1**) and two newly induced peaks (compounds **2** and **3**) were observed when *S. cinnamoneus* NBRC 13823 was cultured with *T. pulmonis* (Figure 1).

EXTRACTION AND ISOLATION OF COMPOUNDS 1–3

To elucidate the structures of **1**, **2** and **3**, the cells from 1.5 l of the combined culture were extracted with 800 ml of a mixture of CH_3OH-$CHCl_3$ (50:50, v/v), according to the same method described above. The extract was concentrated under reduced pressure to yield an oily liquid (0.73 g), and was separated by silica gel column

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chromatography using Silica Gel 60 N (63–210 μm, Kanto Chemical Co., Tokyo, Japan) by stepwise elution with $CHCl_3$-CH_3OH mixtures (CH_3OH:$CHCl_3$ = 0:100, 1:99, 2:98, 5:95, 10:90, 20:80, 50:50 and 100:0, v/v). The eluates containing compounds **1** (50:50 and 100:0, 150 mg), **2** (25:75, 150 mg) and **3** (2:98 and 5:95, 160 mg) were further purified by semi-preparative reverse-phase HPLC with a 10 × 250 mm Cosmosil 5C_{18}-MS-II column (Nacalai Tesque), using an aqueous CH_3CN isocratic mode containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 3.0 ml min^{-1}, to yield **1** (5.73 mg, 35% aqueous CH_3CN), **2** (3.23 mg, 53% aqueous CH_3CN) and **3** (1.21 mg, 56% aqueous CH_3CN).

STRUCTURE ELUCIDATION OF COMPOUNDS 1–3

The structures of **1** and **3** were determined to be BE-13793C and arcyriaflavin A, respectively (Figure 2a), by comparing their molecular formulae, UV spectra and a series of NMR spectra with literature data.[8,9]

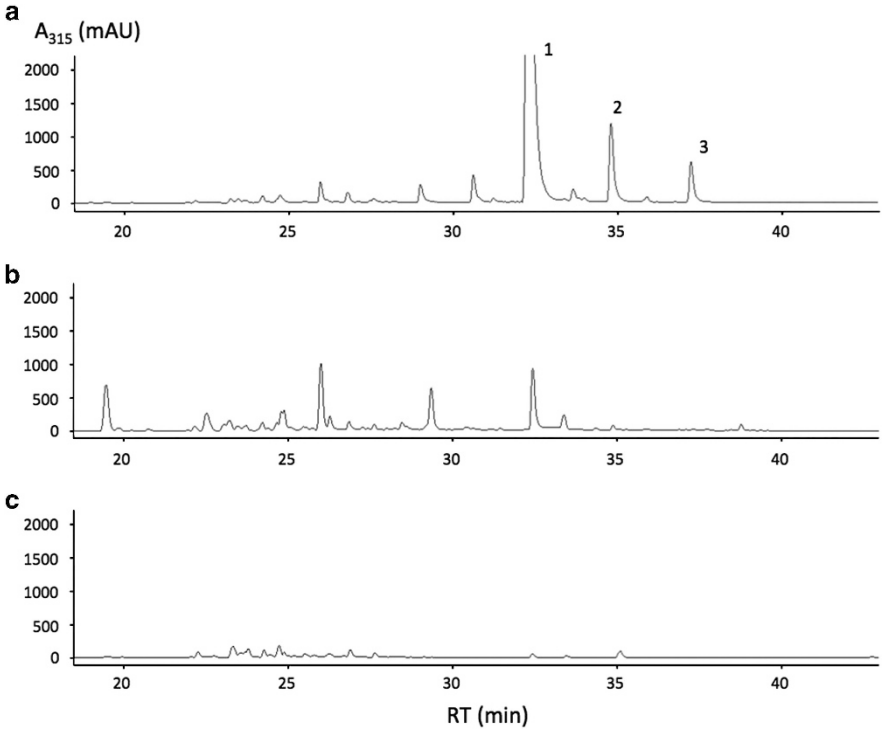

Figure 1 HPLC profiles of the extracts of *Streptomyces cinnamoneus* NBRC 13823 cultivated with *T. pulmonis* (**a**), NBRC 13823 pure culture (**b**) and *T. pulmonis* pure culture (**c**), monitored by UV absorption at 315 nm.

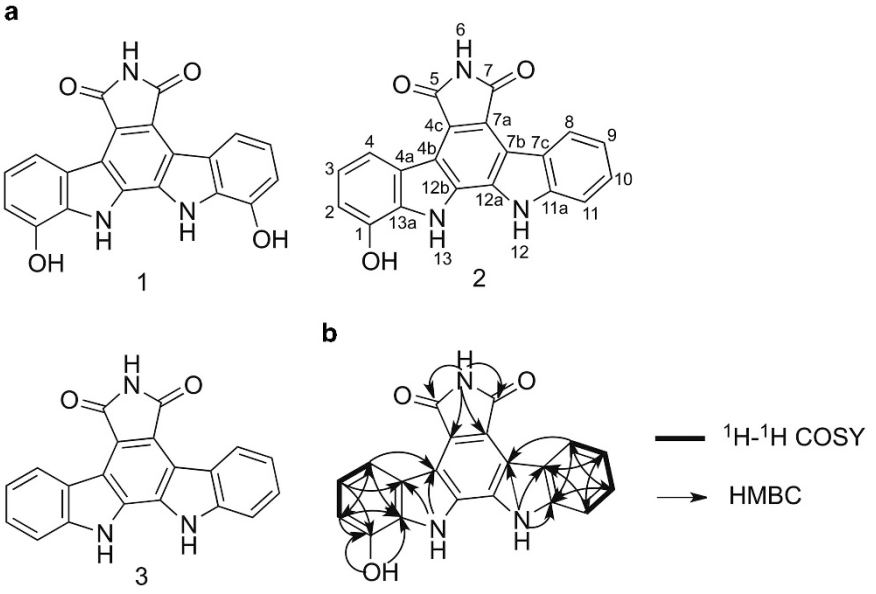

Figure 2 Chemical structures of **1**–**3** (**a**) and ^{1}H–^{1}H COSY and HMBC correlations for **2** (**b**).

Table 1 NMR spectroscopic data of arcyriaflavin E (2)

	2			
Position	δ_C		δ_H J *(Hz)*	*HMBC*
1	143.4	qC	—	—
2	111.1	CH	6.99 1H, d, 8	C1, C4, C13a
3	120.8	CH	7.14 1H, t, 8	C1, C4a
4	115.3	CH	8.43 1H, d, 8	C2, C4b
4a	123.0	qC	—	—
4b	115.9	qC	—	—
4c	119.7[a]	qC	—	—
5	171.3[a]	qC	—	—
6	—	—	10.95 1H, s	C4c, C5, C7, C7a
7	171.4[a]	qC	—	—
7a	120.0[a]	qC	—	—
7b	115.1	qC	—	—
7c	121.6	qC	—	—
8	124.3	CH	8.98 1H, d, 8	C7b, C10, C11a
9	120.2	CH	7.35 1H, t, 8	C7c, C11
10	126.7	CH	7.55 1H, t, 8	C8, C11a
11	112.0	CH	7.82 1H, d, 8	C7c, C9
11a	139.9	qC	—	—
12	—	—	11.50 1H, s	C7b, C7c, C11a, C12a/12b
12a	128.7[a]	qC	—	—
12b	129.2[a]	qC	—	—
13	—	—	11.74 1H, s	C4a, C4b, C12a/12b
13a	130.3	qC	—	—
1-OH	—	—	10.27 1H, s	C1, C13a

[a]May be interchanged.

The new compound **2** was isolated as an orange powder, and its UV spectrum (λ_{max}: (CH_3CN): 239, 287, 311 and 411 nm) is similar to those of **1** and **3**. The molecular formula of **2** was determined to be $C_{20}H_{11}N_3O_3$ (*m/z* found: 340.0743 $[M\text{-}H]^-$, calculated: 340.0722) by an HR-Time-of-flight mass spectrometry (TOFMS) analysis (AccuTOF LC-plus JMS-T 100LP (JEOL)), and corresponds to the mono-deoxygenated product of **1** or the mono-oxygenated product of **3**.

The structure of **2** was deduced by 1D and 2D NMR analyses. The ^1H NMR spectrum revealed the presence of 11 protons, consisting of 7 aromatic methine protons at δ 6.99 (d, $J=8$ Hz), 7.14 (t, $J=8$ Hz), 7.35 (t, $J=8$ Hz), 7.55 (t, $J=8$ Hz), 7.82 (d, $J=8$ Hz), 8.43 (d, $J=8$ Hz) and 8.98 (d, $J=8$Hz), and 4 protons attached to the oxygen or nitrogen atoms at δ 10.27 (s), 10.95 (s), 11.50 (s) and 11.74 (s). The ^{13}C NMR and HMQC spectra revealed that **2** contains 20 carbons, consisting of 2 carbonyl carbons at δ 171.26 and 171.35, 11 aromatic quaternary carbons at δ 115.1, 115.9, 119.7, 120.0, 121.6, 123.0, 128.7, 129.2, 130.3, 139.9 and 143.4, and 7 aromatic methine carbons at δ 111.1, 112.0, 115.3, 120.2, 120.8, 124.3 and 126.7 (Table 1). These signals were assigned primarily on the basis of comparisons of the chemical shifts to those of **1** and **3**. The presence of an isolated proton spin system of three aromatic protons at δ 6.99, 7.14 and 8.43, along with two exchangeable protons at δ 10.27 and 11.74, was reminiscent of the hydroxyindole found in **1**. On the other hand, the other spin system of the four remaining aromatic protons at δ 7.35, 7.55, 7.82 and 8.98, with one exchangeable proton at δ 11.50, was diagnostic for the indole moiety of **3**. As for the remaining exchangeable proton at δ 10.95, the ^1H NMR spectra of **1** and **3** also showed corresponding signals (Supplementary Figures S1 and S8), assigned as maleimide protons. Finally, the HMBC correlations also support the presence of two indole rings (left hydroxyindole: correlations from H2, H3, H4, H13 and C1-OH protons; right indole: correlations from H8, H9, H10, H11 and H12, Figure 2b) and a maleimide moiety (correlation from H6, Figure 2b).

Thus, the structure of **2**, named arcyriaflavin E, was elucidated as shown in Figure 2a as the C1-deoxy form of **1** (or the C1-hydroxy form of **3**).

CYTOTOXICITY ASSAY OF COMPOUNDS 1–3

The cytotoxic activities of compounds **1–3** against P388 murine leukemia cells were tested, using the methyl-thiazole tetrazolium (MTT) assay.[10] As a result, **1** and **2** showed cytotoxic activities with IC_{50}s of 33 and 39 μM, respectively, while **3** did not show any cytotoxicity up to a concentration of 100 μM.

In conclusion, we isolated a novel indolocarbazole, arcyriaflavin E, by the combined culture of *T. pulmonis* and *S. cinnamoneus* NBRC 13823. Our results illustrate that the combined culture with mycolic acid-containing bacteria is an efficient and convenient method for searching for novel natural compounds produced by *Streptomyces* strains.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)