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NOTE

MBJ-0086 and MBJ-0087, new bicyclic depsipeptides, from *Sphaerisporangium* sp. 33226

Teppei Kawahara[1], Masashi Itoh[2], Miho Izumikawa[1], Junko Hashimoto[1], Noriaki Sakata[2], Toshio Tsuchida[2] and Kazuo Shin-ya[3]

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We have constructed a library of isolated natural products referred to as the 'CB library' for efficient implementation of biological screenings.[1] To expand and variegate this library, we have conducted chemical and biological screenings using the advanced compound-identification system based on accumulated HPLC-MS profiling data combined with strain information designated as 'MBJ's special selection'. During screening, we have identified several new natural bioactive compounds such as the cytotoxic eremophilane derivatives MBJ-0009, MBJ-0010, MBJ-0011, MBJ-0012 and MBJ-0013 from *Nectria* sp. f26111[2] and *Apiognomonia* sp. f24023,[3] the chaetoglobosin derivatives MBJ-0038, MBJ-0039 and MBJ-0040 from *Chaetomium* sp. f24230,[4] a hydroxamate metabolite MBJ-0003 from *Micromonospora* sp. 29867[5] and the aziridine containing-peptide MBJ-0035 from *Streptosporangium* sp. 32552.[6] Further screening was used to isolate new bicyclic depsipeptides designated as MBJ-0086 (**1**) and MBJ-0087 (**2**) from the culture broth of *Sphaerisporangium* sp. 33226 (Figure 1). Here, we describe the fermentation, isolation, structure elucidation and antibacterial activity of **1** and **2**.

Sphaerisporangium sp. 33226 was isolated from a soil sample collected in Kochi Prefecture, Japan. This producing strain was cultivated in 250-ml Erlenmeyer flasks, each containing 25 ml of a seed medium consisting of 2% potato starch (Tobu Tokachi Nosan Kako Agricultural Cooperative Assoc., Hokkaido, Japan), 2% glucose (Junsei Chemical, Tokyo, Japan), 2% soy bean powder (SoyPro, J-Oil Mills, Tokyo, Japan), 0.5% yeast extract powder (Oriental Yeast, Tokyo, Japan), 0.25% NaCl (Junsei Chemical), 0.32% $CaCO_3$ (Wako Pure Chemical Industries, Osaka, Japan), 0.0005% $CuSO_4 \cdot 5H_2O$ (Wako Pure Chemical Industries), 0.0005% $ZnSO_4 \cdot 7H_2O$ (Wako Pure Chemical Industries) and 0.0005% $MnCl_2 \cdot 4H_2O$ (Junsei Chemical) for 3 days at 28 °C on a rotary shaker at 220 r.p.m. (pH 7.4). The seed culture (0.5 ml) was transferred into 500-ml Erlenmeyer flasks containing 50 ml of the same medium and cultivated for 4 days at 28 °C on a rotary shaker at 220 r.p.m. After cultivation, an equal volume of *n*-BuOH was added to the culture broth (2 liters). The *n*-BuOH extract was evaporated *in vacuo* and partitioned between water (300 ml) and EtOAc (300 ml, 3 times).

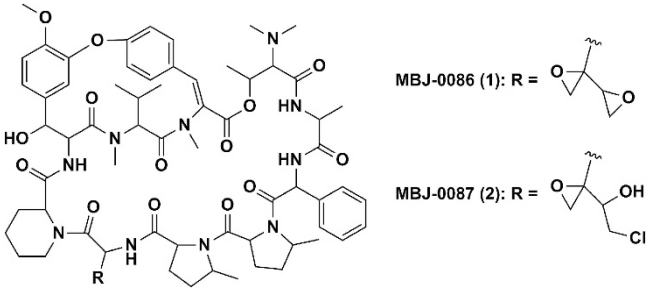

Figure 1 Structures of MBJ-0086 (**1**) and MBJ-0087 (**2**).

The EtOAc-soluble material (1.26 g) was subjected to silica gel medium-pressure liquid chromatography (Si-MPLC, Purif-Pack SI-30, Shoko Scientific, Yokohama, Japan) eluted with a gradient system of *n*-hexane–EtOAc (0–25% EtOAc) followed by a stepwise solvent system of $CHCl_3$–MeOH (0, 2, 5, 10, 20, 30 and 100% MeOH). Fractions were monitored using the UPLC-DAD-ELS-MS system. The 5% MeOH eluate (553.9 mg) was subjected to Sephadex LH-20 column chromatography ($CHCl_3$/MeOH = 1:1, GE Healthcare BioSciences AB, Uppsala, Sweden). The target fraction (415.1 mg) was then separated by Si-MPLC (Purif-Pack SI-30) using isocratic elution with 4% MeOH in $CHCl_3$ to give fractions A (199.5 mg) and B (129.6 mg). Fraction A was subjected to a second round of chromatography using Si-MPLC (Purif-Pack SI-30, 3% MeOH in $CHCl_3$, isocratically) to obtain crude **2** (59.5 mg). The pure sample of **2** (8.2 mg, retention time: 11.0 min) was obtained by two preparative HPLC runs on an XSelect CSH C_{18} column (5.0 μm, 19 i.d. × 150 mm; Waters, Milford, MA, USA) with 55% aq. MeCN containing 0.1% formic acid (flow rate: 10 ml min^{-1}). Fraction B was re-chromatographed using Si-MPLC (Purif-Pack SI-30, isocratic, 3% MeOH in $CHCl_3$) to give semi-purified **1** (51.5 mg). Final purification of **1** was carried out by reversed-phase HPLC (column: CAPCELL PAK C18 MGII column, 5.0 μm, 20 i.d. × 150 mm, Shiseido, Tokyo, Japan; solvent: 40% aq. MeCN containing 0.1%

[1]Japan Biological Informatics Consortium (JBIC), Tokyo, Japan; [2]Bioresource Laboratories, MicroBiopharm Japan Co., Ltd. (MBJ), Shizuoka, Japan and [3]National Institute of Advanced Industrial Science and Technology (AIST), Tokyo, Japan
Correspondence: Dr K Shin-ya, National Institute of Advanced Industrial Science and Technology (AIST), 2-4-7 Aomi, Koto-ku, Tokyo 135-0064, Japan.
E-mail: k-shinya@aist.go.jp
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Table 1 ^{13}C (150 MHz) and ^{1}H (600 MHz) NMR spectroscopic data for MBJ-0086 (1) and MBJ-0087 (2) in $CDCl_3$

	1		*2*	
Position	δ_C	δ_H, *mult (J in Hz)*	δ_C	δ_H, *mult (J in Hz)*
5MePro-1				
1	171.9		172.0	
2	61.1	4.38, d (7.2)	61.2	4.34, d (7.8)
3	28.8	2.63, dd (7.2, 12.6); 1.77, ovl[a]	29.3	2.47, m; 1.88, ovl[a]
4	31.2	2.16, ovl[a]; 1.47, ovl[a]	31.2	2.15, ovl[a]; 1.56, ovl[a]
5	54.8	4.16, m	55.1	4.17, ovl[a]
6	20.8	1.35, d (6.0)	20.5	1.41, d (6.0)
5MePro-2				
1	170.1		169.8	
2	60.6	4.66, ovl[a]	60.5	4.60, dd (7.8, 7.8)
3	25.9	2.14, ovl[a]; 1.96, m	26.0	2.19, ovl[a]; 1.94, m
4	33.1	2.10, ovl[a], 1.73, ovl[a]	33.0	2.10, ovl[a]; 1.71, ovl[a]
5	54.6	4.27, m	54.8	4.29, m
6	19.71	0.92, d (6.6)	19.5	0.86, d (6.0)
PhGly				
N*H*		7.86, d (7.2)		7.72, d (6.6)
1	167.8[b]		167.9	
2	54.4	5.75, d (7.2)	54.4	5.82, d (7.2)
3	137.4		137.3	
4/8	128.1	7.45, d (7.8)	128.2	7.47, d (7.8)
5/7	128.4	7.31, ovl[a]	128.6	7.35, dd (7.8, 7.8)
8	128.0	7.26, t (7.8)	128.1	7.29, ovl[a]
Ala				
N*H*		7.05, ovl[a]		7.25, ovl[a]
1	170.6		170.4	
2	48.3	4.58, m	48.6[c]	4.54, m
3	19.74	1.38, d (6.6)	20.0	1.51, d (6.6)
Me2Thr				
1	168.6	h	169.0	
2	73.94	2.95, d (6.0)	73.9	2.95, d (6.0)
3	70.7	5.08, dq (6.0, 6.6)	70.6	5.17, ovl[a]
4	13.0	1.44, d (6.6)	13.1	1.45, d (6.0)
5/6	43.7	2.18, s	43.8	2.21, s
Δ-MeTyr				
1	163.2		163.0	
2	129.5		129.8	
3	140.3	8.20, s	140.0	8.10, s
4	129.6		129.5	
5	127.5	7.32, ovl[a]	127.6	7.30, ovla
6	124.9	7.11, dd (2.4, 8.4)	124.8	7.11, dd (1.8, 8.4)
7	156.6		156.6	
8	123.1	7.13, dd (2.4, 8.4)	123.7	7.19, dd (1.8, 8.4)
9	135.2	7.60, dd (1.8, 8.4)	134.7	7.58, d (8.4)
10	36.0	3.32, s	36.1	3.29, s
MeVal				
1	170.2		170.1	
2	56.3	5.22, d (7.2)	56.7	5.12, d (6.6)
3	29.4	1.88, m	29.4	1.88, ovl[a]
4	19.0	0.89, d (7.2)	19.1	0.91, d (6.6)
5	18.8	0.64, d (7.2)	18.7	0.67, d (6.6)
6	33.7	3.33, s	34.0	3.34, s

Table 1 (Continued)

	1		*2*	
Position	δ_C	δ_H, *mult (J in Hz)*	δ_C	δ_H, *mult (J in Hz)*
mDOPS				
N*H*		7.03, ovl[a]		6.95, br s
1	168.9		169.1	
2	59.5	4.68, ovl[a]	59.6	4.72, d (4.2)
3	73.86	4.95, br s	74.1	4.94, d (10.2)
4	131.9		131.7	
5	112.0	5.32, br s	112.2	5.28, br s
6	147.1		149.4	
7	149.3		147.2	
8	111.3	6.85, d (8.4)	111.2	6.86, d (7.8)
9	118.7	7.06, ovl[a]	118.6	7.04, d (7.8)
10	56.0	3.92, s	56.0	3.92, s
O*H*		6.25, br s		6.29, d (10.2)
Pip				
1	171.1		171.0	
2	54.3	5.12, m	54.3	5.18, ovl[a]
3	25.5	2.22, ovl[a]; 1.44, ovl[a]	25.6	2.27, ovl[a]; 1.44, ovl[a]
4	20.2	1.59, ovl[a]; 1.20, m	20.2	1.61, ovl[a]; 1.21, ovl[a]
5	24.6	1.70, ovl[a]; 1.12, m	25.0	1.59, ovl[a]; 1.19, ovl[a]
6	43.2	3.37, br d (14.4); 1.62, ovl[a]	43.8	3.66, m; 1.75, ovl[a]
*Bx (**1**)/Ceo (**2**)*				
N*H*		9.59, d (5.4)		8.70, br s
1	167.8[b]		168.0	
2	51.9	4.77, d (5.4)	53.2	4.88, d (6.0)
3	57.6		58.7	
4	49.5	3.08, d (4.2); 2.75, d (4.2)	48.6[c]	3.26, d (3.0); 3.02, br s
5	51.4	3.48, dd (2.0, 4.0)	69.0	4.20, ovl[a]
6	45.5	2.85, dd (4.0, 4.0); 2.83, dd (4.0, 4.0)	46.4	3.84, dd (2.4, 12.0); 3.60, dd (6.6, 12.0)

NMR spectra were taken on a Varian NMR System 600 NB CL in $CDCl_3$ with the residual solvent peak as an internal standard (δ_C 77.0, δ_H 7.25 p.p.m.).
[a]Overlapped with other signals.
[b]Interchangeable.
[c]Interchangeable.

formic acid; flow rate: 10 ml min^{-1}; retention time: 5.5 min) to yield **1** (21.6 mg).

Compound **1** was a colorless amorphous powder; $[\alpha]^{26}_D$ +107 (MeOH; *c* 0.05). The IR spectrum of **1** showed absorption bands for hydroxy (3400 cm^{-1}) and amide (1640 cm^{-1}) functionalities. The UV spectrum showed absorption maxima at 286 (logε 4.0) and 303 (logε 4.0) nm in MeOH. The molecular formula of **1** was found to be $C_{67}H_{86}N_{10}O_{16}$ by high-resolution-ESI-MS (*m/z* 1287.6339 $[M+H]^+$, calcd for $C_{67}H_{87}N_{10}O_{16}$ 1287.6302). The ^{1}H and ^{13}C NMR spectra of **1** suggested the peptidic nature of this molecule, including 4 amide resonances, signals for 9 α-methine protons, 4 *N*-methyl groups and 10 amide carbonyl carbons. The direct connectivity between protons and carbons was determined based on the heteronuclear single quantum coherence spectrum; the tabulated ^{13}C and ^{1}H NMR spectroscopic data for **1** are shown in Table 1.

Detailed analyses of DQF-COSY and constant-time HMBC[7] spectra revealed the presence of Ala, *N*-MeVal (MeVal), two 5-MePro (5MePro-1 and 5MePro-2), *N*,*N*-diMeThr (Me2Thr), dehydro-*N*-MeTyr (Δ-MeTyr), β-hydroxy-4-methoxyTyr (4-methoxydroxidopa,

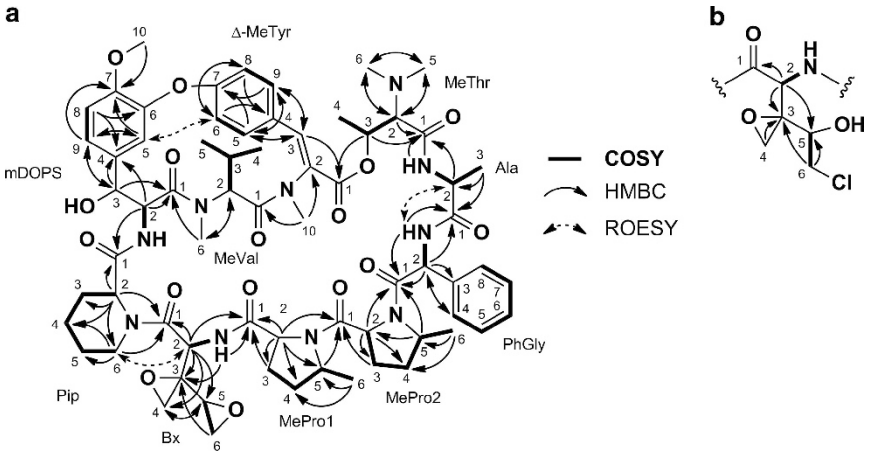

Figure 2 (**a**) Structure determination of **1**. (**b**) Structure determination of Ceo moiety of **2**.

mDOPS), 2-phenylglycine (PhGly) and a pipecolic acid (Pip) residues, as shown in Figure 2a.

The structure of the residual amino acid moiety with a 2,2′-bioxirane moiety (abbreviated as Bx), which is considered to be derived from Ile, was determined based on the COSY correlations from an amide proton NH-Bx (δ_H 9.59) to a doublet α-methine proton H-Bx-2 (δ_H 4.77) and from an oxymethine proton H-Bx-5 (δ_H 3.48) to oxymethylene protons H_2-Bx-6 (δ_H 2.85 and 2.83), and HMBCs from the H-Bx-2 to an amide carbonyl carbon C-Bx-1 (δ_C 167.8), an oxygenated quaternary carbon C-Bx-3 (δ_C 57.6), an oxymethylene carbon C-Bx-4 (δ_C 49.5) and an oxymethine carbon C-Bx-5 (δ_C 51.4). High-field shifted ^{13}C NMR chemical values of C-Bx-3, C-Bx-4, C-Bx-5 and C-Bx-6 (δ_C 45.5), and the large $^1J_{CH}$ values of C-Bx-4 (174 Hz) and C-Bx-6 (180 Hz) also supported this bis-epoxide substructure.

The amino acid sequence of **1** was determined based on ^1H–^{13}C long-range couplings from an α-proton H-5MePro-1-2 (δ_H 4.38) to an amide carbonyl carbon C-5MePro-2-1 (δ_C 170.1), from an α-proton H-5MePro-2-2 (δ_H 4.66) to an amide carbonyl carbon C-PhGly-1 (δ_C 167.8), from an α-proton H-PhGly-2 (δ_H 5.75) to an amide carbonyl carbon C-Ala-1 (δ_C 170.6), from an α-proton H-Ala-2 (δ_H 4.58) to an amide carbonyl carbon C-Me2Thr-1 (δ_C 168.6), from a β-proton H-Me2Thr-3 (δ_H 5.08) to an amide carbonyl carbon of Δ-MeTyr (δ_C 163.2), from an *N*-methyl proton of Δ-MeTyr (δ_H 3.32) to an amide carbonyl carbon C-MeVal-1 (δ_C 170.2), from an *N*-methyl proton of MeVal (δ_H 3.33) to an amide carbonyl carbon C-mDOPS-1 (δ_C 168.9), from an α-proton H-mDOPS-2 (δ_H 4.68) to an amide carbonyl carbon C-Pip-1 (δ_C 171.1) and from an α-proton H-Pip-2 (δ_H 5.12) to an amide carbonyl carbon C-Bx-1. The presence of an ether bond between C-Δ-MeTyr-7 and C-mDOPS-6 was determined based on the rotating frame NOE between the H-Δ-MeTyr-6 (δ_H 7.11) and H-mDOPS-5 (δ_H 5.32), and index of hydrogen deficiency deduced from the molecular formula. Furthermore, the ^1H and ^{13}C NMR chemical shifts of mDOPS moiety in **1** were well matched with those of RA-IV, a cyclic hexapeptide obtained from Rubiae Radix.[8] Hence, the structure determination of **1** was accomplished as shown in Figure 1. The stereochemistry of the trisubstituted double bond was determined as *Z* by means of a *J*-resolved HMBC-2 spectrum.[9] The ester smaller ^1H–^{13}C long-range coupling value of 4.2 Hz between carbonyl carbon signal of Δ-MeTyr and H-Δ-MeTyr-3 indicated that these two nuclei were *cis* to each other, thus concluding the *Z* geometry for the double bond.[10,11]

MBJ-0087 (**2**) was a colorless amorphous powder with the following properties: $[\alpha]^{26}_D$ +113 (MeOH; *c* 0.1); UV λ_{max} (logε) in MeOH: 286 (4.0) and 303 (4.0) nm; high-resolution-ESI-MS: *m/z* 1321.5912 $[M–H]^-$, calcd for $C_{67}H_{86}{}^{35}ClN_{10}O_{16}$ *m/z* 1321.5912; and IR absorption (ν_{max}) 3400 and 1640 cm^{-1}. Analyses of 1D and 2D NMR spectra of **2** revealed that the partial structure of **2** was the same as that of **1** with the exception of the 2,2′-bioxirane moiety, as described below.

The presence of a 2-amino-2-(2-(2-chloro-1-hydroxyethyl)oxiran-2-yl)acetic acid (Ceo) moiety was determined based on COSY correlations from an amide proton NH-Ceo (δ_H 8.70) to an α-methine proton H-Ceo-2 (δ_H 4.88), from an oxymethine proton H-Ceo-5 (δ_H 4.20) to chlorinated methylene protons H_2-Ceo-6 (δ_H 3.84, 3.60), and ^1H–^{13}C long-range couplings from the H-Coe-2 to an amide carbonyl carbon C-Coe-1 (δ_C 168.0), an oxygenated quaternary carbon C-Coe-3 (δ_C 58.7) and an oxymethine carbon C-Coe-5 (δ_C 69.0), from oxymethylene protons H_2-Ceo-4 (δ_H 3.26, 3.02) to the C-Coe-3 and from the H_2-Ceo-6 to the C-Coe-3 and C-Coe-5, and low-field shifted chemical shifts of C-Ceo-3 and C-Ceo-4 (δ_C 48.6; Figure 2b). HMBCs from the H-Ceo-2 to an amide carbonyl carbon C-5MePro-1-1 (δ_C 172.0) and from an α-methine proton H-Pip-2 (δ_H 5.18) to C-Ceo-1 revealed the gross structure of **2** as shown in Figure 1.

Antimicrobial activity against *Bacillus subtilis* was tested. First, 20 µl of a log-phase *B. subtilis* culture (2×10^6 CFU ml^{-1}) was dispensed into each well in a 384-well plate and compounds were added at various concentrations. Dimethyl sulfoxide (1%) was used as a vehicle control. After incubation for 18 h at 37 °C, the OD_{620} was measured using a 2103 Envision Multilabel Reader (PerkinElmer, Waltham, MA, USA). Compounds **1** and **2** were active against *B. subtilis* with IC_{50} values of 1.1 and 24 µM, respectively. In contrast, neither compound showed cytotoxicity against human ovarian adenocarcinoma SKOV-3 cells ($IC_{50} > 50$ µM) or antimicrobial activity against *Micrococcus luteus* ($IC_{50} > 50$ µM).

The structures of **1** and **2** are extremely unique and include bicyclic depsipeptides, diphenyl ether and rare amino acid residues such as 5-MePro, Δ-MeTyr, mDOPS and Bx (**1**) or Ceo (**2**). Compounds **1** and **2** are the first natural peptide compounds possessing Bx (**1**) or a Ceo (**2**) units, respectively. Furthermore, there have been no reports describing peptide compounds possessing a diphenyl ether moiety except for dityromycin[12] isolated from *Streptomyces* sp. and the bicyclic hexapeptides isolated from Rubiaceae.[13,14]

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1 Kawahara, T., Nagai, A., Takagi, M. & Shin-ya, K. JBIR-137 and JBIR-138, new secondary metabolites from *Aspergillus* sp. fA75. *J. Antibiot.* **65,** 535–538 (2012).
2 Kawahara, T. *et al.* Cytotoxic sesquiterpenoids MBJ-0009 and MBJ-0010 from a saprobic fungus *Nectria* sp. f26111. *J. Antibiot.* **66,** 567–569 (2013).
3 Kawahara, T. *et al.* Three eremophilane derivatives, MBJ-0011, MBJ-0012 and MBJ-0013, from an endophytic fungus *Apiognomonia* sp. f24023. *J. Antibiot.* **66,** 299–302 (2013).
4 Kawahara, T. *et al.* New chaetoglobosin derivatives, MBJ-0038, MBJ-0039 and MBJ-0040, isolated from the fungus *Chaetomium* sp. f24230. *J. Antibiot.* **66,** 727–730 (2013).
5 Kawahara, T. *et al.* New hydroxamate metabolite, MBJ-0003, from *Micromonospora* sp. 29867. *J. Antibiot.* **67,** 261–263 (2014).
6 Kawahara, T. *et al.* Novel aziridine-containing peptides MBJ-0034 and MBJ-0035 from *Streptosporangium* sp. 32552. *J. Antibiot.* **67,** 577–580 (2014).
7 Furihata, K. & Seto, H. Constant time HMBC (CT-HMBC), a new HMBC technique useful for improving separation of cross peaks. *Tetrahedron Lett.* **39,** 7337–7340 (1998).
8 Itokawa, H. *et al.* Studies on antitumor cyclic hexapeptides RA obtained from Rubiae Radix, Rubiaceae. VI. Minor antitumor constituents. *Chem. Pharm. Bull.* **34,** 3762–3768 (1986).
9 Furihata, K. & Seto, H. *J*-Resolved HMBC, a new NMR technique for measuring heteronuclear long-range coupling constants. *Tetrahedron Lett.* **40,** 6271–6275 (1999).
10 Palermo, J. A. *et al.* Celenamide E, a tripeptide alkaloid from the Patagonian sponge *Cliona chilensis*. *J. Nat. Prod.* **61,** 488–490 (1998).
11 Shigematsu, N. *et al.* Structure of WS9326A, a novel tachykinin antagonist from a *Streptomyces*. *J. Org. Chem.* **58,** 170–175 (1993).
12 Teshima, T. *et al.* The structure of an antibiotic, dityromycin. *Tetrahedron Lett.* **29,** 1963–1966 (1988).
13 Jolad, S. D. *et al.* Bouvardin and deoxybouvardin, antitumor cyclic hexapeptides from *Bouvardia ternifolia* (Rubiaceae). *J. Am. Chem. Soc*. **99,** 8040–8044 (1977).
14 Singh, R., Geetanjali & Chauhan, S. M. 9,10-Anthraquinones and other biologically active compounds from the genus *Rubia*. *Chem. Biodivers.* **1,** 1241–1264 (2004).

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