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# Isolation of 2 new naphthablin analogs, JBIR-79 and JBIR-80, from *Streptomyces* sp. RI24

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Ischemic disorders of the central nervous system are one of the main causes of death. In brain ischemia there is an extensive release of L-glutamate, a major neurotransmitter in the central nervous system, which subsequently induces neuronal cell death. It has also been reported that free radical scavengers effectively ameliorate brain ischemia injury by blocking glutamate toxicity.[1,2] Free radical scavengers such as phenazostatins, benzostatins, aestivophoenins, and carbazomadurins that were isolated from actinomycete have been reported to show protective activity against L-glutamate toxicity in neuronal hybridoma N18-RE-105 cells.[3–5] In the course of our screening for inhibitors of glutamate toxicity in N18-RE-105 cells to prevent the brain ischemia injury, we isolated two new naphthablin[6] analogs, designated as JBIR-79 (**1**) and JBIR-80 (**2**), from the culture of *Streptomyces* sp. RI24 (Figure 1a). This article describes the fermentation, isolation and elucidation of the structure of these two analogs, as well as their protective activities against L-glutamate toxicity.

*Streptomyces* sp. RI24 was isolated from a soil sample collected at Shuri, Okinawa Prefecture, Japan by the sodium dodecyl sulfate-yeast extract method.[7] The strain was cultivated in 50-ml test tubes, each of which contained 15 ml of a seed medium consisting of starch (Kosokagaku, Tokyo, Japan) 1.0%, polypeptone (Nihon Pharmaceutical, Tokyo, Japan) 1.0%, molasses (Dai-Nippon Meiji Sugar, Tokyo, Japan) 1.0% and meat extract (Extract Ehlrich; Wako Pure Chemical Industry, Osaka, Japan) 1.0%, pH 7.2 (before sterilization). The test tubes were shaken in a reciprocal shaker (355 r.p.m.) at 27 °C for 2 days. Aliquots (2.5 ml) of the seeding culture were transferred to 500-ml baffled Erlenmeyer flasks containing 100 ml of a production medium that contained starch (Kosokagaku) 2.5%, soybean meal (Nisshin Oillio, Tokyo, Japan) 1.5%, dry yeast (Mitsubishi Tanabe Pharma, Osaka, Japan) 0.2%, $CaCO_3$ (Kozaki Pharmaceutical, Tokyo, Japan) 0.4% and Diaion HP-20 resin (Mitsubishi Chemical, Tokyo, Japan) 1.0%, pH 7.4 (before sterilization), and then cultured in a rotary shaker (180 r.p.m.) at 27 °C for 5 days.

The mycelial cake was separated from the fermentation broth (2 liters) by centrifugation, followed by extraction with acetone (400 ml). After being concentrated *in vacuo*, the residual aqueous concentrate was extracted with EtOAc (100 ml, ×3). The organic layer was dried over $Na_2SO_4$ and then allowed to evaporate under reduced pressure until thoroughly dry. The dried residue (770 mg) was reconstituted in $CHCl_3$ (1 ml) subjected to normal-phase medium-pressure liquid chromatography (Purif-Pack SI-60 column packing with 30-g silica gel; Shoko Scientific, Tokyo, Japan) using a $CHCl_3$–MeOH stepwise solvent system (0, 2 and 5% MeOH). The 2% MeOH eluate (38 mg) was further purified by preparative reversed-phase HPLC using an L-column 2 ODS column (20 i.d. × 150 mm; Chemical Evaluation and Research Institute, Tokyo, Japan) with a 2996 photodiode array detector (Waters, Milford, MA, USA) and a 3100 mass detector (Waters), using 70% aqueous MeOH containing 0.1% formic acid (flow rate, 10 ml $min^{-1}$) as a developing solvent to yield **1** (11.6 mg, retention time (Rt) 27.3 min). Likewise, $CH_3Cl$ eluate (17 mg) from silica gel chromatography was purified by preparative reversed-phase HPLC that was developed using 75% aqueous MeOH containing 0.1% formic acid to yield **2** (1.1 mg, Rt 33.6 min).

Compounds **1** and **2** were obtained as red and orange amorphous solids, respectively ($[\alpha]_D^5$ −172.2 for **1** and −148.2 for **2**, *c* 0.1, in MeOH). Using HR-electrospray ionization-MS (LCT Premier XE Mass Spectrometer; Waters), the molecular formulae of **1** and **2** were determined to be $C_{27}H_{32}O_8$ (found: 485.2183 $[M+H]^+$, calcd: 485.2175) for **1**, and $C_{25}H_{28}O_6$ (found: 425.1974 $[M+H]^+$, calcd: 425.1964) for **2**. The presence of hydroxyl and carbonyl groups was deduced from their IR spectra ($\nu_{max}$ (KBr) 3450, 1735, 1241 $cm^{-1}$ for **1**, and 3432, 1700, 1222 $cm^{-1}$ for **2**). The UV absorptions of **1** and **2** were also identical: ($\lambda_{max}$ (ε) 220 (22 480), 270 (15 230), 310 (11 510), 420 (4340) nm for **1**, and 220 (21 860), 270 (14 860), 310 (11 240), 420 (3910) nm for **2**, in MeOH). These UV spectra showed the typical

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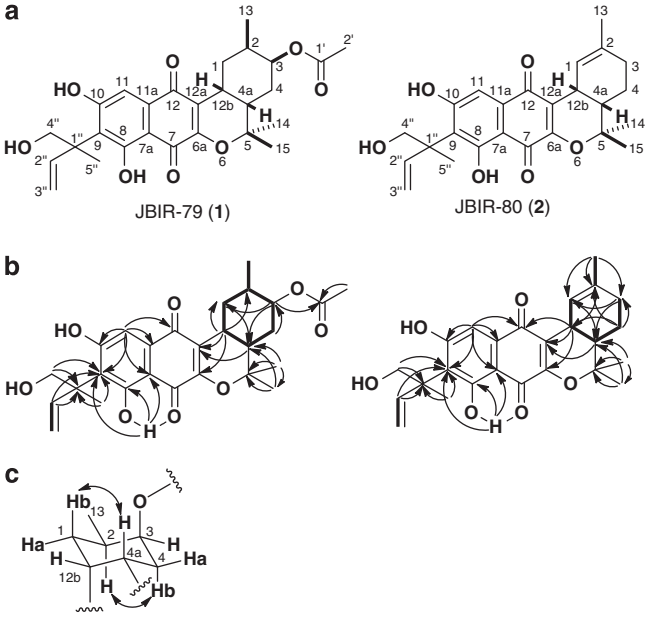


**Figure 1** (**a**) Structures of JBIR-79 (**1**) and JBIR-80 (**2**). (**b**) Correlations in DQF-COSY (bold lines) and CT-HMBC (arrows) spectra of **1** and **2**. (**c**) ROE correlations (double arrows) of the cyclohexane ring moiety of **1**.

absorption of a naphthoquinone with phenolic hydroxyl residue as a chromophore,[8–10] like that of naphthablin.[6]

The structures of **1** and **2** were elucidated by a series of double-quantum-filtered (DQF)-COSY and constant time heteronuclear multiple bond correlation (CT-HMBC) spectra.[11] The direct connectivity of protons and carbons was established by heteronuclear single quantum coherence spectra, and the tabulated $^{13}C$ and $^{1}H$ NMR spectral data for **1** and **2** are listed in Table 1. In the HMBC spectrum of **1**, strong $^{1}H$-$^{13}C$ *meta*-couplings from an aromatic proton 11-H ($\delta_H$ 7.05) to aromatic quaternary carbons C-7a ($\delta_C$ 108.0) and C-9 ($\delta_C$ 121.9) were observed. A phenolic hydroxyl proton 8-OH ($\delta_H$ 13.21) that was hydrogen-bonded with a quinone carbonyl at the *peri* position was $^{1}H$-$^{13}C$ long-range coupled to C-7a, C-8 ($\delta_C$ 164.2) and C-9. The aromatic proton 11-H was also long-range coupled to aromatic quaternary carbons C-10 ($\delta_C$ 163.3) and C-11a ($\delta_C$ 132.4). These correlations and the values of their $^{13}C$ chemical shift established the assignment of carbons in a 1,3-dihydroxy-2,5,6-trisubstituted benzene moiety. In addition, the aromatic proton 11-H was long-range coupled to a quinone carbonyl carbon C-12 ($\delta_C$ 183.5) at the *peri* position. By taking into consideration these relationships and the UV spectral data (*vide supra*), the presence of a naphthoquinone moiety of **1** was determined. $^{1}H$-$^{13}C$ long-range couplings from an olefinic proton 2″-H ($\delta_H$ 6.05), which was $^{1}H$–$^{1}H$ coupled to exomethylene protons 3″-H ($\delta_H$ 5.17, 5.01), from hydroxymethylene protons 4″-H ($\delta_H$ 3.91, 3.73) and from a methyl proton 5″-H ($\delta_H$ 1.49) to a quaternary carbon C-1″ ($\delta_C$ 46.8), constructed a 2-methylbut-3-en-1-ol moiety. The $^{1}H$-$^{13}C$ long-range couplings of the olefinic proton 2″-H, the hydroxymethylene protons 4″-H and the methyl proton 5″-H to the aromatic quaternary carbon C-9 elucidated that the 2-methylbut-3-en-1-ol moiety was substituted at the C-9 position. Thus, a naphthoquinone substructure of **1** was established, as shown in Figure 1b (left).

The sequence from a methine proton 12b-H ($\delta_H$ 3.15) to a methine proton 4a-H ($\delta_H$ 1.85), which were $^{1}H$ spin-coupled to each other, through methylene protons 1-H ($\delta_H$ 2.72, 1.68), a methine proton 2-H ($\delta_H$ 1.38), an oxymethine proton 3-H ($\delta_H$ 4.94) and methylene protons 4-H ($\delta_H$ 1.97, 1.28) observed in DQF-COSY spectrum of **1**, revealed a six-membered substructure, as shown in Figure 1b. The spin coupling between a methyl proton 13-H ($\delta_H$ 0.76) and the methine proton 2-H determined that the substitution position of the methyl residue is at the C-2 position ($\delta_C$ 30.2). $^{1}H$-$^{13}C$ long-range couplings from a singlet methyl proton 2′-H ($\delta_H$ 2.05) and the oxymethine proton 3-H to an ester carbonyl carbon C-1′ ($\delta_C$ 171.0) determined an acetyl functional group and its substituted position. Two singlet methyl protons 14-H ($\delta_H$ 1.42) and 15-H ($\delta_H$ 1.26) were $^{1}H$-$^{13}C$ long-range coupled to each other, and are commonly coupled to the methine carbon C-4a ($\delta_c$ 36.1) and a quaternary oxycarbon C-5 ($\delta_c$ 80.8), which indicated that an *i*-propyl moiety was connected with C-4a. Thus, a terpenoid substructure was established, as shown in Figure 1b. $^{1}H$-$^{13}C$ long-range couplings from 12b-H to an oxygenated $sp^2$ carbon C-6a ($\delta_c$ 155.8) and an aromatic quaternary carbon C-12a ($\delta_c$ 121.0), together with the molecular formula of **1**, indicated that the naphthoquinone and terpenoid moieties were connected through an ether bond between C-5 and C-6a or between C-5 and C-12a. Generally, hydrogen-bonded quinone carbonyl carbon is observed at low fields as compared with those of non-hydrogen-bonded carbonyl carbon, as is observed in juglone compounds.[12] Because the two quinone carbonyl carbons C-7 and C-12 showed almost identical $^{13}C$ chemical shift values ($\delta_C$ 182.1 and 183.5, respectively), the atoms that induce an upfield shift against C-7 have to be substituted at the position of C-6a. Thus, it was proven that the oxygen atom connects to C-6a, and the linkage of the naphthoquinone and terpenoid moieties was established, as shown in Figure 1b. The NMR spectral data of other naphterpin-type molecules[6,8,9] also supported the presence of the linkage of a six-membered ether ring system with the naphthoquinone moiety. Thus, the planar structure of **1** was determined to be a naphthablin derivative in which the isobutyl group was replaced by the acetyl residue, as shown in Figure 1a.

The relative configuration of the cyclohexane ring moiety was established from the $^{3}J_{H,H}$ coupling constants and a ROESY experiment. Large coupling constants between 1-Hb ($\delta_H$ 1.68)/2-H ($^{3}J$=13.4 Hz) and 4a-H/4-Hb ($\delta_H$ 1.28) ($^{3}J$=12.6 Hz) indicated that these protons were in a diaxial orientation. Since the spin-coupling constants between 1-Ha ($\delta_H$ 2.72)/12b-H, 12b-H/4a-H, 4a-H/4-Ha ($\delta_H$ 1.97) and 4-Ha/3-H were relatively small ($^{3}J$=3.2, 4.8, 5.4 and 3.2 Hz, respectively), it was deduced that 1-Ha, 12b-H, 4-Ha and 3-H were in equatorial positions. The ROEs between 1-Hb/4a-H and 2-H/4-Hb confirmed that this cyclohexane ring assumes a chair conformation (Figure 1c). Thus, the relative configuration of the cyclohexane moiety was established, as shown in Figure 1a.

The structure of **2** was almost identical to that of **1**, except for the lack of an acetoxyl group, which was replaced by methylene protons 3-H ($\delta_H$ 1.99, 1.26) in **2**. The sequence from an olefinic proton 1-H ($\delta_H$ 6.05) to the methylene protons 3-H through a methine proton 12b-H ($\delta_H$ 3,47), a methine proton 4a-H ($\delta_H$ 1.74) and methylene protons 4-H ($\delta_H$ 1.93, 1.24) observed in the DQF-COSY spectrum confirmed the structure of **2** as shown in Figure 1a. The key $^{1}H$-$^{1}H$ and $^{1}H$-$^{13}C$ correlations are shown in Figure 1b (right).

To evaluate the free radical scavenging activities of **1** and **2**, we tested their protective activity against L-glutamate toxicity in N18-RE-105 cells,[3–5] which can be used to assess free radical scavenging activity. Compounds **1** and **2** showed moderate protective activity against L-glutamate toxicity in cells with $EC_{50}$ values of 42 and 83 μM, respectively.

**Table 1** $^{13}C$ (150 MHz) and $^{1}H$ (600 MHz) NMR data for 1 and 2

| | 1 | | 2 | |
|---|---|---|---|---|
| *No.* | $\delta_C$ | $\delta_H$ *(multiplicity, J in Hz)* | $\delta_C$ | $\delta_H$ *(multiplicity, J in Hz)* |
| 1 | 28.4 | 2.72 (ddd, 13.8, 3.2, 3.2) | 120.0 | 6.05 (d, 3.5) |
| | | 1.68 (ddd, 13.8, 13.4, 5.4) | | |
| 2 | 30.2 | 1.38 (m) | 135.9 | |
| 3 | 72.3 | 4.94 (ddd, 4.3, 3.4, 3.2) | 29.7 | 1.99 (ddd, 13.7, 5.8, 3.4) |
| | | | | 1.26 (ddd, 13.7, 11.2, 4.1) |
| 4 | 27.8 | 1.97 (ddd, 15.0, 5.4, 3.2) | 20.3 | 1.93 (dddd, 15.2, 5.0, 4.1, 3.4) |
| | | 1.28 (ddd, 15.0, 12.6, 4.3) | | 1.24 (dddd, 15.2, 12.2, 11.2, 5.8) |
| 4a | 36.1 | 1.85 (ddd, 12.6, 5.4, 4.8) | 39.7 | 1.74 (ddd, 12.2, 5.0, 4.1) |
| 5 | 80.8 | | 80.5 | |
| 6a | 155.8 | | 153.1 | |
| 7 | 182.1 | | 183.2 | |
| 7a | 108.0 | | 108.2 | |
| 8 | 164.2 | | 163.9 | |
| 8-OH | | 13.21 (s) | | 13.38 (s) |
| 9 | 121.9 | | 121.1 | |
| 10 | 163.3 | | 163.3 | |
| 11 | 112.3 | 7.05 (s) | 112.4 | 7.12 (s) |
| 11a | 132.4 | | 132.5 | |
| 12 | 183.5 | | 183.3 | |
| 12a | 121.0 | | 123.8 | |
| 12b | 30.1 | 3.15 (ddd, 5.4, 4.8, 3.2) | 31.1 | 3.47 (dd, 4.1, 3.5) |
| 13 | 17.6 | 0.76 (d, 7.2) | 23.6 | 1.65 (s) |
| 14 | 25.7 | 1.42 (s) | 25.7 | 1.55 (s) |
| 15 | 25.0 | 1.26 (s) | 24.8 | 1.31 (s) |
| 1′ | 171.0 | | | |
| 2′ | 21.2 | 2.05 (s) | | |
| 1″ | 46.8 | | 47.2 | |
| 2″ | 143.7 | 6.05 (dd, 17.6, 10.8) | 143.7 | 6.11 (dd, 17.6, 10.6) |
| 3″ | 114.7 | 5.17 (d, 10.8) | 116.0 | 5.32 (d, 10.6) |
| | | 5.01 (d, 17.6) | | 5.08 (d, 17.6) |
| 4″ | 69.0 | 3.91 (d, 12.0) | 68.7 | 4.09 (d, 11.7) |
| | | 3.73 (d, 12.0) | | 3.63 (d, 11.7) |
| 5″ | 21.9 | 1.49 (s) | 22.0 | 1.49 (s) |

NMR spectra were obtained using a Varian NMR system 600 NB CL (Palo Alto, CA, USA) in $CDCl_3$, and the solvent peak was used as an internal standard ($\delta_H$ 7.25 and $\delta_C$ 77.0 p.p.m.).

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