A New Pyranonaphtoquinone Derivative, 4-Oxo-rhinacanthin A, from Roots of Indonesian Rhinacanthus nasutus

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A new pyranonaphtoquinone derivative, named 4-oxo-rhinacanthin A (1), was isolated from the roots of the Indonesian Rhinacanthus nasutus together with two known congeners, rhinacanthin A (2) and 3,4-dihydro-3,3-dimethyl-2H-naphtho[2,3-b]pyran-5,10-dione (3). The structure of 1 was elucidated based on its spectroscopic data. The absolute configuration of 1 was assigned by comparing its experimental Electronic Circular Dichroism (ECD) spectrum with the calculated ECD spectrum. Compounds 2 and 3 inhibited the growth of Staphylococcus aureus with inhibition zones of 16 and 20 mm at 25 µg/disc, respectively. Compound 3 also exhibited inhibitory activity against Mycobacterium smegmatis (20 mm at 25 µg/disc).

Key words 4-oxo-rhinacanthin A; pyranonaphthoquinone; Rhinacanthus nasutus; Mycobacterium smegmatis

Rhinacanthus nasutus, a small shrub in the family Acanthaceae, is widely distributed throughout Southeast Asia and has been used as a traditional medicine for the treatment of skin diseases, diabetes, hypertension, and pneumonia. Several naphthoquinone and lignan derivatives have been reported from R. nasutus. In the course of our investigation on useful bioactive substances from terrestrial and marine organisms, the EtOH extract from the roots of R. nasutus, collected at Manado, Indonesia in 2014, was found to exhibit antibacterial activity against Staphylococcus aureus (inhibition zone of 8 mm at 50 µg/disc). Bioassay-guided separation of the extract led to the isolation of a new pyranonaphthoquinone derivative, 4-oxo-rhinacanthin A (1), together with two known homologues (2 and 3) (Fig. 1). Compound 3 inhibited the growth of Mycobacterium smegmatis as well as S. aureus. M. smegmatis has been used most widely for the studies on anti-mycobacterial substances because of its fast-growing and non-pathogenic properties. We herein describe the isolation, structural elucidation, and biological activities of compounds 1–3.

Results and Discussion

Isolation The EtOH extract from the roots of R. nasutus exhibited antibacterial activity against S. aureus with an inhibition zone of 8 mm at 50 µg/disc and was separated into seven fractions using an octadeylsilane (ODS) column. The bioactive fractions were further purified by preparative HPLC (ODS) fractions using an octadecylsilane (ODS) column. The bioactive fractions were further purified by preparative HPLC (ODS) columns. The bioactive fractions were further purified by preparative HPLC (ODS) columns. The bioactive fractions were further purified by preparative HPLC (ODS) columns.

Compounds 2 and 3 were identified as rhinacanthin A and 3,4-dihydro-3,3-dimethyl-2H-naphtho[2,3-b]pyran-5,10-dione, respectively, by comparing their spectroscopic data with the reported values.

Structure Elucidation The molecular formula of compound 1 was C_{15}H_{12}O_{5} based on high resolution (HR)-FAB-MS (m/z 273.0754 [M+H]^+), Δ = -0.9 ppm) and NMR data (Table 1). The ^{1}H- and ^{13}C-NMR spectra of 1 (in CDCl₃) indicated 11 proton and 15 carbon signals, which were classified into two methyls, one sp² oxygenated methine, one sp³ oxygenated quaternary carbon, four sp² methines, three sp² quaternary carbons, one sp² oxygenated quaternary carbon, and three carbonyl carbons in an analysis of the ^{1}H-detected heteronuclear multiple quantum coherence (HMOC) spectrum of 1 (Table 1).

The ^{1}H- and ^{13}C-NMR data for 1 resembled those for 2, suggesting that compound 1 also possessed the same naphthoquinone skeleton as 2. This structure of 1 was confirmed by analyses of two dimensional (2D)-NMR data (Table 1, Fig. 2). A marked difference in the NMR spectra of 1 and 2 was the presence of an additional carbonyl carbon signal (δ 192.0) in 1 instead of the sp³ methylene signal at C-4 in 2. Thus, the planar structure of 1 was elucidated as shown in Fig. 2 and named 4-oxo-rhinacanthin A.

The absolute configuration of 1 was investigated by comparing its experimental Electronic Circular Dichroism (ECD) spectrum with the calculated ECD spectrum, since the ^{1}H-NMR spectrum of 1 gave a few signals around the C-3 position to apply the modified Mosher’s method. The experimental ECD spectrum of 1 (solid line) matched the ECD spectrum calculated for (3S)-1 (dashed line) (Fig. 3). Consequently, the absolute structure of 1 was assigned as shown in Fig. 1.

Biological Activity The antibacterial activities of compounds 1–3 against S. aureus IAM12544T were evaluated using the paper disc method (Table 2). Compounds 2 and 3 displayed inhibition zones of 16 and 20 mm at 25 µg/disc, respectively, whereas compound 1 did not exhibit any activity (Table 2). Compound 3 also exhibited antibacterial activity against M. smegmatis NBRC 3207 with an inhibition zone of 20 mm at 25 µg/disc (Table 2). Due to structural similarities between 1–3, the oxidation patterns of the pyran ring will affect their antibacterial activities.

Since R. nasutus is used as a folk medicine for several diseases, further investigations on its other bioactivities and the isolation of bioactive components are now underway.
Experimental

General Experimental Procedures Specific rotations were assessed with a JASCO P-2300 digital polarimeter (JASCO, Ltd., Tokyo, Japan). UV spectra were measured on a U-3310 UV-Vis spectrophotometer (Hitachi, Ltd., Tokyo, Japan) and IR spectra on a PerkinElmer, Inc. Spectrum One Fourier transform infrared spectrometer (PerkinElmer, Inc., Waltham, MA, U.S.A.). ECD spectra were measured with a J-720 spectrometer (JASCO). NMR spectra were recorded on a JEOL JNM-AL-400 NMR spectrometer (400 MHz for $^1$H and 100 MHz for $^{13}$C) in CDCl$_3$ ($\delta^H_{H} 7.24$, $\delta^C_C 77.0$). FAB-MS and HR-FAB-MS were performed using a JMS-MS 700 mass spectrometer (JEOL, Tokyo, Japan). Preparative HPLC was performed with a Hitachi L-6200 system (Hitachi, Ltd.).

Materials Middlebook 7H9 broth, polysorbate 80, and Middlebook OADC were purchased from BD (Franklin Lakes, NJ, U.S.A.). All other chemicals including organic solvents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Isolation of Compounds 1–3 The roots of R. nasutus were collected in Manado, Indonesia, in 2014. A voucher specimen was deposited at the Faculty of Fisheries and Marine Science, Sam Ratulangi University as W14-02-20.

The plant (1.0 kg) was extracted with EtOH. The extract was evaporated, and the residue was suspended in H$_2$O and extracted with EtOAc. The EtOAc extract (1.5 g) was separated by an ODS column (100 g) with CH$_3$OH–H$_2$O (stepwise elution) into seven fractions (Frs. 1–7). Active Fr. 5 (85.0 mg, eluted with 70% CH$_3$OH) was further separated into five fractions (Frs. 5-1–5-5) using preparetive HPLC [column, PEGASIL ODS (Senshu Sci. Co., Ltd., Tokyo, Japan), i.d. 10 × 250 mm; solvent, 70% CH$_3$OH in H$_2$O containing 0.05% trifluoroacetic acid (TFA); flow rate, 2.0 mL/min; detection, UV 210 nm], and compounds 1 (0.7 mg) and 2 (3.3 mg) were isolated from fr. 5-1 (8.7 mg) by repeated HPLC (column, PEGASIL ODS, i.d. 10 × 250 mm; solvent, 60% CH$_3$OH in H$_2$O containing 0.05% TFA; flow rate, 2.0 mL/min; detection, UV 210 nm). Compound 3 (1.2 mg) was obtained from active Fr. 3 (33.8 mg, eluted with 30% CH$_3$OH) by HPLC purification (column, PEGASIL ODS, i.d. 10 × 250 mm; solvent, 60% CH$_3$OH in H$_2$O containing 0.05% TFA; flow rate, 2.0 mL/min; detection, UV 210 nm).

Table 1. $^{13}$C- (100 MHz) and $^1$H- (400 MHz) NMR Data for 1 (CDCl$_3$)

| C# | $\delta^C_C$ | $\delta^H_{H}$, Mult. (J in Hz) | HMBC |
|----|-------------|-------------------------------|------|
| 2  | 88.3        | 8.20, dd (8.0, 4.0)           |      |
| 3  | 75.2        | 4.36, s                       | 2, 11, 12 |
| 4  | 192.0       |                               |      |
| 4a | 111.8       |                               |      |
| 5  | 179.7       |                               |      |
| 5a | 131.8       |                               |      |
| 6  | 126.7       | 7.84, ddd (8.0 8.0, 4.0)      | 6, 5a, 8 |
| 7  | 135.7       | 7.66, ddd (8.0 8.0, 4.0)      | 7, 9, 9a |
| 8  | 127.1       | 8.12, dd (8.0, 4.0)           | 8, 9a, 10 |
| 9a | 130.9       |                               |      |
| 10 | 179.5       |                               |      |
| 10a| 162.4       |                               |      |
| 11 | 17.3        | 1.34, s                       | 2, 3, 11 |
| 12 | 26.4        | 1.82, s                       | 2, 3, 12 |

Fig. 2. $^1$H–$^1$H COSY and HMBC Correlations of 1

Fig. 3. Experimental ECD Spectrum of 1 (Solid Line) and Calculated ECD Spectrum of (3S)-1 (Dashed Line)

Table 2. Antibacterial Activities of 1–3 (25 µg/disc) against Staphylococcus aureus and Mycobacterium smegmatis

| Compound | S. aureus | M. smegmatis |
|----------|-----------|-------------|
| 1        | —         | —           |
| 2        | 16        | —           |
| 3        | 20        | 20          |
| Kanamycin (10 µg/disc) | 20 | — |
| Streptomycin (2 µg/disc) | — | 25 |

a) An inhibition zone was not detected.
273 [M+H]^+. HR-FAB-MS m/z 273.0754 ([M+H]^+, Calcd for C_{15}H_{13}O_{5}, 273.0763). 1H- and 13C-NMR (CDCl_3), see Table 1.

**Calculation of ECD Spectra** The most stable conformer of (3S)-isomer of I was predicted using Spartan '14 (Wavefunction, Inc., Irvine, CA, U.S.A.) by a preliminary conformational analysis with the MMFF94 force field followed by geometrical optimization using the density functional theory (DFT) with the B3LYP functional and 6-31G(d,p) basis set. The ECD spectrum in CH_3CN was calculated for the predicted most stable conformer using Gaussian 09 (Gaussian, Inc., Wallingford, CT, U.S.A.) by time-dependent DFT (TDDFT) with the B3LYP functional and 6-311++G(d,p) basis set. The relative energies of the other two conformers found were >5 kcal mol^{-1} with respect to the most stable one; therefore, we only used the most stable conformer in the ECD spectrum calculation. The solvent effect was introduced by the polarizable continuum model (PCM). Thirty-five low-lying excited states were calculated corresponding to the wavelength region down to approximately 184 nm, and the calculated spectrum was displayed using GaussView 5.0.9 (Semichem, Inc., Shawnee Mission, KS, U.S.A.) with the peak half-width at half height being 0.333 eV. The calculated spectrum was shifted by −20 nm to match the experimental spectrum.

**Antimicrobial Assay** Growth inhibitory activities were examined using the paper disk method against *S. aureus* IAM 12544T as the test microorganism. Kanamycin (10 µg/disc) was used as a positive control.

**Antimycobacterial Assay** The antibacterial assay was performed using *M. smegmatis* NBRC 3207 with the paper disc method. Strain NBRC 3207 was obtained from the Biological Resource Center (NBRC), NITE (Chiba, Japan) and maintained in 20% glycerol at −80°C. The test microorganism was cultured in Middlebrook 7H9 broth containing 0.05% polysorbate 80, 0.5% glycerol, and 10% Middlebrook OADC at 37°C for 2 d and adjusted to 1.0×10^6 colony forming unit (CFU)/mL. The inoculum was spread on the above medium containing 1.5% agar in a square plate. Each sample in CH_3OH was adsorbed to a sterile filter disc (6mm, Advantec), and, after the evaporation of CH_3OH, the disc was placed on an agar plate and incubated at 37°C for two days. Streptomycin sulfate (5 µg/disc) was used as a positive control.

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**Conflict of Interest** The authors declare no conflict of interest.

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