Anthraquinones from *Neonauclea calycina* and Their Inhibitory Activity against DNA Topoisomerase II

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In a series of searches for DNA topoisomerase II inhibitors from naturally occurring compounds, a wood extract of *Neonauclea calycina* Merr. (Rubiaceae) showed a moderate effect *in vitro*. Purification of the extract resulted in the isolation of seven known anthraquinones. The structures were characterized as damnacanthal, rubiadin 1-methyl ether, nordamnacanthal, morindone, damnacanthol, lucidin 3-*O*-primeveroside and morindone 6-*O*-primeveroside by spectral analysis, respectively. Damnacanthal and morindone showed an intensive inhibitory effect against topoisomerase II (IC_{50}: 20 μg/ml and 21 μg/ml).

Key words *Neonauclea calycina*; Rubiaceae; anthraquinone; damnacanthal; morindone; topoisomerase II inhibitory activity

Topoisomerases are enzymes which control the topological state of DNA. Type II topoisomerases, which catalyze DNA strand passage through transient double strand breaks in the DNA, can relax supercoiled DNA and resolve knotted or catenated DNA rings.[1] The prime biological importance of these enzymes makes them critical targets for the action of a wide variety of anticancer drugs. Etoposide analogs have been applied clinically, but their severe side effects remain a serious problem. The development of a new class of inhibitors of topoisomerase II is thus awaited. In the development of new drugs, natural products obtained from plants are sometimes useful directly or may serve as starting materials for the development of semisynthetic active agents. Furthermore, naturally occurring compounds can supply suitable leads for the subsequent design of structurally related molecules that are more active or less toxic. In our previous paper, we reported the inhibitory effects of xanthone and benzophenone derivatives against topoisomerases I and II.[2] As part of our continuous search for topoisomerase II inhibitors in phenolic compounds, we report here the inhibitory effect of anthraquinones from *Neonauclea calycina*, which is classified in the tribe of Naucleae (family: Rubiaceae)[3] and has traditionally been used for the treatment of tumors in some areas in Southeast Asia. No phytochemical studies of the plant has been found through available literature.

MATERIALS AND METHODS

General IR spectra were recorded on a JASCO IR-AI spectrophotometer. UV spectra were taken on a Shimadzu UV-2200 spectrophotometer. MS were obtained on a JEOL JMS-D300 operating at 70 eV. NMR spectra were measured at 270 MHz for ^{1}H- and at 67.5 MHz for ^{13}C-NMR on a JEOL JMN GX-270 instrument; chemical shifts are given in δ values (ppm) with tetramethylsilane (TMS) as an internal standard. The following adsorbents were used for purification: analytical TLC, Merck Kieselgel 60 F_{254}; column chromatography; Fuji Davison Silica gel BW-300.

Plant Materials Wood of *N. calycina* was collected in Bali, Indonesia in July, 1992. The voucher specimen is deposited in the Herbarium of Gifu Pharmaceutical University.

Extract and Isolation The dried and ground wood (1.0 kg) of *N. calycina* was successively extracted with CH_2Cl_2 (2 l×12 h×3), acetone (2 l×12 h×3) and 70% MeOH (2 l×12 h×3) under reflux. The CH_2Cl_2 extract (18 g) was recrystallized from MeOH to give a crude mass of anthraquinones. The crude anthraquinones (5 g) were subjected to column chromatography on silica gel (Si CC) (350 g) eluted with $CHCl_3$ to give **1** (2.67 g), **2** (500 mg), **3** (230 mg) and **4** (30 mg). The acetone extract (10 g) was subjected to Si CC eluting with a benzene–acetone system to give **5** (20 mg) from the benzene–acetone (20 : 1) eluent. The 70% MeOH extract (20 g) was also subjected to Si CC eluting with a $CHCl_3$–MeOH system. Compounds **6** (20 mg) and **7** (15 mg) were obtained from the $CHCl_3$–MeOH (7 : 1) and the (5 : 1) eluents, respectively, and were purified by recrystallization from EtOAc–hexane.

Structures of Compounds 1—7 In the electron ionization (EI) MS, **1**—**5** showed $[M]^+$ at *m/z* 282, 268, 268, 270 and 284, respectively. When **1** was methylated with MeI and K_2CO_3 in acetone, 2-formyl-1,3-dimethoxyanthraquinone (**8**) was obtained ($[M]^+$: *m/z* 296). Reduction of **1** with $NaBH_4$ in MeOH gave **5**. The same methylation of **3** as **1** afforded **8**. Compounds **6** and **7** showed $[M-H]^-$ both at *m/z* 563 in the negative ion fast atom bombardment (FAB) MS. Usual acid-hydrolysis (0.1 N H_2SO_4) of **6** and **7** gave 2-carbinol-1,3-dihydroxyanthraquinone (lucidin) and **4** as aglycones, respectively. In addition to the MS data mentioned above, spectral analysis of IR, UV, and ^{1}H- and ^{13}C-NMR, including a 2D-NMR spectrum, revealed that the structures of **1**—**7** were 2-formyl-3-hydroxy-1-methoxyanthraquinone (damnacanthal)[4] (**1**), 3-hydroxy-2-methyl-1-methoxyanthraqinone (rubiadin 1-methyl ether)[5] (**2**), 2-formyl-1,3-dihydroxyanthraquinone (nordamnacanthal)[4] (**3**), 1,5,6-trihydroxy-2-methylanthraquinone (morindone)[6] (**4**), 2-carbinol-3-hydroxy-1-methoxyanthraquinone (damnacantol)[4] (**5**), 2-carbinol-1,3-dihydroxy-

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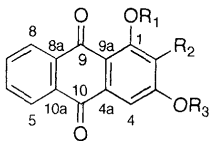


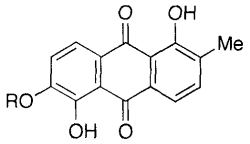
1: R_1=Me, R_2=CHO, R_3=H (damnacanthal)

2: R_1=R_2=Me, R_3=H (rubiadin 1-methyl ether)

3: R_1=R_3=H, R_2=CHO (nordamnacanthal)

5: R_1=Me, R_2=CH_2OH, R_3=H (damnacanthol)

6: R_1=H, R_2=CH_2OH, R_3=prim (lucidin 3-*O*-*β*-primeveroside)



4: R=H (morindone)

7: R=prim (morindone 6-*O*-*β*-primeveroside)

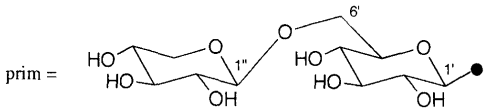


Fig. 1. Structures of Anthraquinones Isolated from *N. calycina*

Table 1. Inhibitory Effects of Compounds **1**—**4** and Etoposide on Topoisomerase II

1	2	3	4	Etoposide
IC_{50} (μg/ml)				
20	300	>500	21	70

anthraquinone 3-*O*-*β*-primeveroside (lucidin 3-*O*-*β*-primeveroside[7] (**6**) and 1,5,6-trihydroxy-2-methylanthraquinone 6-*O*-*β*-primeveroside (morindone 6-*O*-*β*-primeveroside)[7] (**7**), respectively.

Topoisomerase II Assay Purified topoisomerase II (0.75 units/ml) was purchased from TopoGen, Inc. (U.S.A.) and kinetoplast DNA was purified from *Crithidia fasciculata* by cesium chloride step gradient centrifugation.[8] Topoisomerase II activity was assessed by a decatenation reaction of kinetoplast DNA.[9] The assay was performed in a reaction mixture (20 μl) containing 50 mM Tris–HCl (pH 7.9), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA (pH 8.0), 0.5 mM ATP, 30 μg/ml bovine serum albumin, and 0.25 μg/ml of kinetoplast DNA, as described previously.[10] The reaction was started by adding 1 μl of topoisomerase II and 1 μl of the anthraquinones dissolved in Me_2SO at the desired concentrations. After incubation at 30 °C for 30 min, the reaction mixture was treated with 1% SDS and 100 μg/ml proteinase K prior to the analysis of DNA products by 0.8% agarose gel electrophoresis. Gels were stained with 0.5 μg/ml ethidium bromide and photographed under UV light. Monomer minicircles released from the kinetoplast DNA were quantified by densitometry.

RESULTS AND DISCUSSION

The structure of **1** was characterized as damnacanthal[4] by analysis of spectral data and chemical transformation. In the same way, the structures of **2**—**5** were determined to be rubiadin 1-methyl ether[5] (**2**), nordamnacanthal[4] (**3**), morindone[6] (**4**) and damnacanthol[4] (**5**), respectively. The structures of **6** and **7** were characterized as lucidin 3-*O*-*β*-primeveroside[7] and morindone 6-*O*-*β*-primeveroside[7] by spectral analysis and hydrolysis. Only anthraquinones **1**—**4** were subjected to the present screening test. In the first screening, the compounds were added at concentrations of 10, 100 and 500 μg/ml to the topoisomerase II assay system. Although no inhibition was observed in **2** and **3** at 100 μg/ml, **1** and **4** showed a significant inhibition at the concentration. With varying concentrations in a lower range (1, 10, 25, 50, and 100 μg/ml), the IC_{50} values of **1**—**4** were determined and the results are shown in Table 1. The inhibitory activities of **1** and **4** were stronger than that of the positive control of etoposide, but the activity was less than that of subelliptenone F (1,1-dimethylallyl-1,4,5,6-tetrahydroxyxanthone) (IC_{50}<1 μg/ml).[2]

To reduce the toxicity and the side effects of anticancer agents, the development of a new class of compounds is anticipated. In the present study we were able to demonstrate an inhibitory effect of some anthraquinones against topoisomerase II.

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