Prenylated Flavanones Isolated from Flowers of *Azadirachta indica* (the Neem Tree) as Antimutagenic Constituents against Heterocyclic Amines

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Four prenylated flavanones were isolated from the methanol extract of the flowers of *Azadirachta indica* (the neem tree) as potent antimutagens against Trp-P-1 (3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole) in the *Salmonella typhimurium* TA98 assay by activity-guided fractionation. Spectroscopic properties revealed that those compounds were 5,7,4′-trihydroxy-8-prenylflavanone (**1**), 5,4′-dihydroxy-7-methoxy-8-prenylflavanone (**2**), 5,7,4′-trihydroxy-3′,8-diprenylflavanone (**3**), and 5,7,4′-trihydroxy-3′,5′-diprenylflavanone (**4**). All isolated compounds were found for the first time in this plant. The antimutagenic IC_{50} values of compounds **1**−**4** were 2.7 ± 0.1, 3.7 ± 0.1, 11.1 ± 0.1, and 18.6 ± 0.1 μM in the preincubation mixture, respectively. These compounds also similarly inhibited the mutagenicity of Trp-P-2 (3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole) and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine). All of the compounds **1**−**4** strongly inhibited ethoxyresorufin O-dealkylation activity of cytochrome P450 1A isoforms, which catalyze N-hydroxylation of heterocyclic amines. However, compounds **1**−**4** did not show significant inhibition against the direct-acting mutagen NaN_3. Thus, the antimutagenic effect of compounds **1**−**4** would be mainly based on the inhibition of the enzymatic activation of heterocyclic amines.

KEYWORDS: Antimutagen; neem tree (*Azadirachta indica*); Ames test; Trp-P-1; prenylated flavanones

INTRODUCTION

The neem tree, *Azadirachta indica* A. Juss (Meliaceae), is indigenous to India, and now it is cultivated widely in tropical areas of the world (*1*−*3*). Various parts of the neem tree have been used for food, medicine, and insecticides since ancient times (*1*−*3*). Many bioactive constituents have been isolated and identified from various parts of neem tree (*3*). Azadirachtin and other limonoids occurring in the seeds of neem tree show potent insecticidal effects against a wide variety of insect pests and low toxicity to humans (*3*). The flower of the neem tree (locally called "dok sadao" in Thai) is commonly consumed as a vegetable in Thailand, and it is also known to have some medicinal effects (*4*). There are some reports suggesting health uses of the neem tree (*5*−*9*). Okpanyi and Ezeukwu (*5*) reported that the methanol extract of the leaves of the neem tree exerts anti-inflammatory and antipyretic effects. In previous studies, we have reported an antimutagenic effect and active constituents of some edible Thai plants (*6*−*9*). The methanol extract of the flowers of the neem tree and other plant species, *Micromelum minutum*, *Oroxylum indicum*, *Cuscuta chinensis*, *Litsea petiolata*, and *Boesenbergia pandurata*, exhibited remarkable antimutagenic effects against Trp-P-1 (3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole), Trp-P-2 (3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole), and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine). The extract of the flowers of the neem tree inhibited 90% of the mutagenic activity of Trp-P-1 at a dose of 0.1 mg of dry plant material equivalent (*9*). Kusamuran et al. (*10*, *11*) also reported the extract of the flowers of the neem tree showed both antimutagenic activity in bacteria and anticarcinogenic activity in rats. However, the antimutagenic principles of the flowers of the neem tree have not yet been elucidated. We report here the identification and properties of the antimutagenic constituents of the methanol extract of the flowers of the neem tree.

MATERIALS AND METHODS

Chemicals. The flowers of the neem tree were purchased in a market in Khon Kaen, Thailand, in 2002. Mutagens (Trp-P-1, Trp-P-2, and

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PhIP) were purchased from Wako Pure Chemicals (Osaka, Japan). The S9 fraction from the liver of drug-treated Sprague−Dawley rats (male) for the Ames test was a product of Kikkoman (Noda, Japan). Acetone-d_6 and $CDCl_3$ were of NMR grade (Wako). Resorufin, 7-ethoxyresorufin, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). Unless otherwise stated, all other reagents were of analytical grade.

Determination of Antimutagenic Activities. The antimutagenic effect against Trp-P-1 in *Salmonella typhimurium* TA98 was assayed according to the Ames preincubation method (*12*, *13*) with some minor modifications as previously described (*7*). The activity was calculated using the formula defined by Kanazawa et al. (*14*).

Instrumental Analysis. The UV absorption spectra of the compounds in methanol were recorded on a UV-1600 spectrophotometer (Shimadzu, Kyoto, Japan). The mass spectra were recorded on a JMS-HX/HX-110A spectrometer (JEOL, Tokyo, Japan) by direct electron ionization (EI). The molecular formula was verified by high-resolution mass measurement using an ApexII70e electrospray ionization Fourier transform ion cyclotron resonance mass spectrometer (ESI-FTICRMS) (Bruker Daltonics, Billerica, MA) within an error of 4×10^{-4} u in mass assignment on the sample. The ^{1}H NMR and ^{13}C NMR spectra were recorded on a DRX 600 or Avance 800 spectrometer (Bruker Biospin, Karlsruhe, Germany) at 298 K. HSQC and HMBC spectra were acquired using the standard Bruker software.

Isolation of Antimutagenic Compounds. The lyophilized and ground materials (141 g) were extracted with methanol (1.0 L) three times at room temperature for 12 h. The extract was evaporated at 35 °C, and the syrup obtained was partitioned twice with diethyl ether (500 mL) and water (200 mL). The major part of the activity was recovered in the diethyl ether fraction. The diethyl ether fraction (11.9 g) was evaporated, dissolved in methanol, and then mixed with 100 g of Wakogel LP-40 C18 to remove highly lipophilic substances, which sometimes interfere with the chromatographic separation in column chromatography. The filtrate that displayed a high activity was collected. The resin was washed with 250 mL of methanol using a Büchner funnel to increase the yield of the active compounds. The obtained fraction (3.1 g) was dissolved in 500 mL of 10% methanol and subjected to chromatography on a 10 cm × 5.0 cm i.d. column of Wakogel LP-40 C18 equilibrated with 10% methanol. The column was eluted by 10, 20, 40, 60, 80, 90, and 100% methanol. The major active substances were eluted out at 80% (fraction A, 75 mg) and 90% (fraction B, 83 mg). Fractions A and B were concentrated to ∼10 mL and subjected to a semipreparative HPLC on a PX-8020 system equipped with a photodiode array detector (Tosoh, Tokyo, Japan) with a 100 × 10 mm i.d. TSK gel super-ODS column (Tosoh) maintained at 40 °C. The mobile phase system was a linear gradient of water and acetonitrile, operating at a flow rate of 3 mL/min. Wavelengths used for monitoring were 240 and 255 nm. Fraction A was chromatographed using a gradient elution of 40−50% acetonitrile, 0−20 min, by which compounds **1** and **2** were obtained. Fraction B was chromatographed using a gradient elution of 50−65% acetonitrile, 0−20 min, by which compounds **2**−**4** were obtained. Compounds **1**−**4** were further purified by repeating semipreparative HPLC. Finally, the active fractions **1**−**4** were purified to homogeneity (>99% by HPLC) and identified as antimutagens. The physicochemical propeties of compounds **1**−**4** were as listed below. Assignments of chemical shifts of ^{1}H and ^{13}C NMR were confirmed by 2D NMR analyses, HSQC, and HMBC.

*Compound **1**:* ESI-FTICRMS, *m*/*z* ([M + H]$^+$) 341.1390 (341.1384 calcd for $C_{20}H_{21}O_5^+$); UV, λ_{max}, 294 nm (methanol, log ϵ = 4.25) and 338 nm (3.74); ^{1}H NMR (acetone-d_6, 800.03 MHz) δ 1.606 (br s, H-4″), 1.610 (d, J = 0.9 Hz, H-5″), 2.76 (dd, J = 17.0, 3.0 Hz, H-3a), 3.14 (dd, J = 17.0, 12.7 Hz, H-3b), 3.22 (br d, J = 7.4 Hz, H-1″), 5.19 (ddqq, J = 7.4, 7.4, 0.9, 0.7 Hz, H-2″), 5.45 (dd, J = 12.0, 3.0 Hz, H-2), 6.04 (s, H-6), 6.91 (d, J = 8.6 Hz, H-3′ and H-5′), 7.41 (d, J = 8.6 Hz, H-2′ and H-6′); ^{13}C NMR (acetone-d_6, 201.19 MHz) δ 17.9 (C-4″), 22.3 (C-1″), 25.9 (C-5″), 43.5 (C-3), 79.7 (C-2), 96.4 (C-6), 103.3 (C-4a), 108.3 (C-8), 116.1 (C-3′ and C-5′), 123.7 (C-2″), 128.9 (C-2′ and C-6′), 131.1 (C-1′), 131.2 (C-3″), 158.6 (C-4′), 161.1 (C-8a), 163.0 (C-5), 165.1 (C-7), 197.5 (C-4).

*Compound **2**:* ESI-FTICRMS, *m*/*z* ([M + H]$^+$) 355.1542 (355.1540 calcd for $C_{21}H_{23}O_5^+$); UV, λ_{max}, 291 nm (methanol, log ϵ = 4.11) and 338 nm (3.74); ^{1}H NMR ($CDCl_3$, 600.13 MHz) δ 1.61 (d, J = 0.9 Hz, H-4″), 1.65 (d, J = 1.1 Hz, H-5″), 2.81 (dd, J = 17.1, 3.0 Hz, H-3a), 3.05 (dd, J = 17.1, 12.7 Hz, H-3b), 3.21 (br d, J = 7.4 Hz, H-1″), 3.85 (s, 7-O-Me), 5.13 (ddqq, J = 7.4, 7.4, 1.1, 0.9 Hz, H-2″), 5.34 (dd, J = 12.7, 3.0 Hz, H-2), 6.09 (s, H-6), 6.87 (d, J = 8.6 Hz, H-3′ and H-5′), 7.33 (d, J = 8.6 Hz, H-2′ and H-6′); ^{13}C NMR ($CDCl_3$, 150.90 MHz) δ 17.7 (C-4″), 21.6 (C-1″), 25.8 (C-5″), 43.3 (C-3), 55.9 (7-O-Me), 78.4 (C-2), 92.4 (C-6), 103.0 (C-4a), 109.0 (C-8), 115.5 (C-3′ and C-5′), 122.4 (C-2″), 127.7 (C-2′ and C-6′), 131.2 (C-1′), 131.4 (C-3″), 155.8 (C-4′), 158.8 (C-8a), 162.6 (C-5), 165.8 (C-7), 196.5 (C-4).

*Compound **3**:* ESI-FTICRMS, *m*/*z* ([M + H]$^+$) 409.2007 (409.2010 calcd for $C_{25}H_{29}O_5^+$); UV, λ_{max}, 292 nm (methanol, log ϵ = 4.08) and 332 nm (3.53); ^{1}H NMR ($CDCl_3$, 600.13 MHz) δ 1.72 (m, H-4″ and H-5″), 1.79 (br s, H-4‴ and H-5‴), 2.80 (dd, J = 17.1, 3.0 Hz, H-3a), 3.05 (dd, J = 17.1, 13.0 Hz, H-3b), 3.31 (d, J = 7.2 Hz, H-1″), 3.39 (d, J = 7.1 Hz, H-1‴), 5.21 (ddqq, J = 7.2, 7.2, 1.4, 0.8 Hz, H-2″), 5.326 (dd, J = 13.0, 3.0 Hz, H-2), 5.328 (ddqq, J = 7.1, 7.1, 1.4, 0.8 Hz, H-2‴), 6.02 (s, H-6), 6.84 (d, J = 8.1 Hz, H-5′), 7.15−7.20 (m, H-2′ and H-6′); ^{13}C NMR ($CDCl_3$, 150.90 MHz) δ 17.8 (C-4″), 17.9 (C-4‴), 21.8 (C-1″), 25.8 (C-5″ and C-5‴), 29.8 (C-1‴), 43.2 (C-3), 78.9 (C-2), 96.8 (C-6), 103.2 (C-4a), 106.1 (C-8), 115.9 (C-5′), 121.3 (C-2″), 121.6 (C-2‴), 125.5 (C-6′), 127.2 (C-1′), 128.0 (C-2′), 130.8 (C-3′), 134.9 (C-3″), 135.4 (C-3‴), 154.7 (C-4′), 159.8 (C-8a), 162.3 (C-5), 163.7 (C-7), 196.6 (C-4).

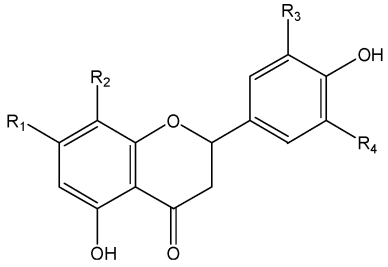
*Compound **4**:* ESI-FTICRMS, *m*/*z* ([M + H]$^+$) 409.2011 (409.2010 calcd for $C_{25}H_{29}O_5^+$); UV, λ_{max}, 288 nm (methanol, log ϵ = 4.02) and 323 nm (3.61); ^{1}H NMR ($CDCl_3$, 600.13 MHz) δ 1.767 (d, J = 0.7 Hz, H-4″ and H-4‴), 1.773 (d, J = 1.2 Hz, H-5″ and H-5‴), 2.74 (dd, J = 17.2, 2.9 Hz, H-3a), 3.11 (dd, J = 17.2, 13.3 Hz, H-3b), 3.36 (br d, J = 7.1 Hz, H-1″ and H-1‴), 5.28 (dd, J = 13.3, 2.9 Hz, H-2), 5.31 (ddqq, J = 7.1, 7.1, 1.2, 0.7 Hz, H-2″ and H-2‴), 5.97 (d, J = 2.2 Hz, H-8), 5.99 (d, J = 2.2 Hz, H-6), 7.05 (s, H-2′ and H-6′), 12.1 (s, 5-OH); ^{13}C NMR ($CDCl_3$, 150.90 MHz) δ 17.9 (C-4″ and C-4‴), 25.8 (C-5″ and C-5‴), 29.7 (C-1″ and C-1‴), 43.1 (C-3), 79.4 (C-2), 95.6 (C-8), 96.6 (C-6), 102.9 (C-4a), 121.6 (C-2″ and C-2‴), 126.1 (C-2′ and 6′), 127.6 (C-3′ and C-5′), 129.8 (C-1′), 134.8 (C-3″ and C-3‴), 153.3 (C-4′), 163.4 (C-8a), 164.3 (C-5), 165.5 (C-7), 196.3 (C-4).

Ethoxyresorufin *O*-Deethylase (EROD) Assay. The EROD assay was performed according to the method of Burke et al. (*15*). The reaction mixture, containing S9 fraction (2.5 nmol of cytochrome P450), 5 μM 7-ethoxyresorufin (10 μL of a 1 mM solution in DMSO), and 1 mM NADPH was incubated with or without compounds **1**−**4** (25 μg, dissolved in DMSO) in a total of 2 mL of 0.1 M potassium phosphate buffer (pH 7.4) at 37 °C. The reaction rate was determined by the increasing fluorescence of the reaction mixture recorded using a Shimadzu RF-5300 PC fluorospectrometer with excitation and emission wavelengths at 530 and 585 nm, respectively. Resorufin was used as standard for quantification.

RESULTS AND DISCUSSION

Identification of Antimutagenic Constituents of the Flowers of the Neem Tree. Activity-guided fractionation of the methanol extract of freeze-dried flowers of the neem tree by reversed phase column chromatography led to the isolation of four antimutagenic compounds (**1**−**4**). Final yields of compounds **1**−**4** from 141 g of the plant material were 15.8 mg (0.011%), 11.2 mg (0.008%), 30.9 mg (0.022%), and 27.7 mg (0.020%), respectively.

^{1}H NMR [δ 2.74−2.81 (Ha-3), δ 3.05−3.14 (Hb-3), and δ 5.28−5.45 (H-2)], ^{13}C NMR [δ 43.14−43.45 (C-3) and δ 78.43−79.74 (C-2)], and UV (λ_{max} around 290 nm and 323−338 nm) spectroscopic data revealed compounds **1**−**4** to be flavanone derivatives. Sets of ^{1}H NMR signals [1.60−1.78 (two methyl groups), 3.21−3.36 (CH_2), and 5.19−5.32 (CH, coupled with two methyl groups and CH_2)] indicated the presence of prenyl groups in the structures of compounds **1**−**4**. ^{1}H NMR spectra and molecular formulas of compounds **1**−**4** corresponding to ESI-FTICRMS data suggested that compounds



	R_1	R_2	R_3	R_4
1	OH	CH_2-CH=C(CH_3)$_2$	H	H
2	OCH_3	CH_2-CH=C(CH_3)$_2$	H	H
3	OH	CH_2-CH=C(CH_3)$_2$	CH_2-CH=C(CH_3)$_2$	H
4	OH	H	CH_2-CH=C(CH_3)$_2$	CH_2-CH=C(CH_3)$_2$

Figure 1. Antimutagenic compounds (**1**–**4**) isolated from *A. indica*.

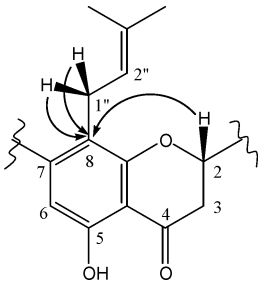


Figure 2. HMBC analyses to determine the position of the prenyl group on the A ring of compounds **1**–**3**.

1 and **2** possessed one prenyl group and that compounds **3** and **4** had two prenyl groups in a molecule **(Figure 1**).

^{1}H NMR and ^{13}C NMR spectra of compound **1** were closely similar to those of both 5,7,4′-trihydroxy-6-prenylflavanone and 5,7,4′-trihydroxy-8-prenylflavanone (sophoraflavanone B) **(Figure 1)** previously isolated from hops (*16*). As Mizobuchi has reported (*17*), several classical methods such as the Gibbs test (*18*), UV shift (*19*), acid cyclization (*20*), and chemical shifts on 1D-NMR (*21*) are not fully reliable in determining the position of substitution by the prenyl group at C-6 or C-8. In this study, we confirmed the position of substitution by the prenyl group on the flavanone skeleton by ^{1}H−^{13}C HMBC analysis. The HMBC correlation between H-2 (δ 5.45) and a quaternary carbon (δ 108.3) was attributed to C-8, and another HMBC correlation between H-1″ and the C-8 was also observed **(Figure 2**). Thus, compound **1** was identified as sophoraflavanone B **(Figure 1)**. Antimicrobial activity and inhibitory activity against cyclooxygenase 2 of this compound were reported previously (*16*, *22*).

Compound **2** was identified as 5,4′-trihydroxy-7-methoxy-8-prenylflavanone (munduleaflavanone B) **(Figure 1),** which was previously isolated from *Mundulea suberosa* (*23*) because both compounds showed identical molecular formulas and UV, ^{1}H NMR, and ^{13}C NMR spectra (*23*). The positions of the methoxy and prenyl groups were revealed by HMBC analysis **(Figure 2)**. Biological activity of munduleaflavanone B has not previously been reported.

Compound **3** was identified as 5,7,4′-trihydroxy-8,3′-diprenylflavanone **(Figure 1)**, isolated from *Euchresta japonica* and reported as euchrestaflavanone A (*21*, *24*−*26*). The positions of two prenyl groups were revealed by HMBC analyses as previously reported (*25*). An antifungal activity of euchrestaflavanone A toward *Cladosporium herbarum* has been reported (*26*).

Table 1. Antimutagenic Activity of Isolated Compounds **1**–**4** against Trp-P-1, Trp-P-2, and PhIP

	% inhibition of mutagenesis[a]		
compound[b] (0.03 μmol/plate)	Trp-P-1 (50 ng/plate)	Trp-P-2 (20 ng/plate)	PhIP (250 ng/plate)
1	92	94	99
2	89	81	94
3	92	90	99
4	79	74	89

[a] Means of four independent experiments. [b] Compounds were dissolved in DMSO (50 μL).

Compound **4** was identified as 5,7,4′-trihydroxy-3′,5′-diprenylflavanone (abyssinone V) **(Figure 1)**, previously isolated from *Erythrina sigmoidea* and *Erythrina burttii*, by comparing spectroscopic data (*27*, *28*). HSQC and HMBC correlations in the 2D-NMR analyses of compound **4** agreed well with the attributed structure of abyssinone V (*27*, *28*). Biological activity of abyssinone V has not previously been reported.

The presence of compounds **1**−**4** in *A. indica* is reported here for the first time. A prenylated flavanone (nimbaflavone, 8,3′-di-isoprenyl-5,7-dihydroxy-4′-methoxyflavanone) has previously been isolated from the leaves of the neem tree grown in India (*29*). Because nimbaflavone has a structure very similar to that of compounds **1**−**4**, particularly compound **3**, it might exhibit antimutagenic activity. However, nimbaflavone was not found in activity-guided fractionation of the present study. It is well recognized that the chemical composition of the same plant varies depending on many factors including the culture variety and growing conditions (*30*).

Antimutagenic Properties of Compounds 1—4. The antimutagenic IC_{50} values, which are concentrations in 1 mL of the preincubation mixture for 50% inhibition against mutagenesis in the presence of 50 ng of Trp-P-1, of compounds **1**−**4** were 2.7 ± 0.1, 3.7 ± 0.1, 11.1 ± 0.1, and 18.6 ± 0.1 μM, respectively. The antimutagenic IC_{50} values for all of them were quite low, ranging from 10^{-5} to 10^{-6} M, comparable to that of other flavonoid antimutagens (*31*, *32*). Therefore, these compounds would be the major components responsible for the antimutagenicity of the extract from flowers of the neem tree. Compounds **1**−**4** showed strong inhibitory effect against the other cooked food mutagens, Trp-P-2 and PhIP, at a dose of 0.03 μmol/plate **(Table 1**). Antimutagenic compounds previously isolated from some edible Thai plants (*6*−*9*) also showed strong inhibition against these mutagens at similar concentrations.

Inhibitory Effects of Compounds 1—4 on Ethoxyresorufin *O*-Deethylase. Antimutagens are classified into two groups, desmutagens and bioantimutagens, on the basis of the mode of action (*33*). Desmutagens neutralize mutagens before or during attack of DNA, and bioantimutagens activate DNA repair processes. If the compounds were bioantimutagens, the inhibitory effect would be detected when direct-acting mutagens are used in the Ames test. In the previous study, we found the crude methanol extract of the flowers of the neem tree showed a weak inhibition toward enzymatically activated Trp-P-1 (*9*). Kusamuran et al. (*10*) also reported that the methanol extract of neem leaves exhibited a weak antimutagenicity against direct-acting mutagens AF-2 and NaN_3 at a dose of 5 mg/plate. However, the isolated compounds **1**−**4** showed little or no inhibition toward the mutagenicity of NaN_3 at a dose of 30 μM. Hence, compounds **1**−**4** could not be bioantimutagens but desmutagens, and constituents responsible for the bioantimutagenic activity

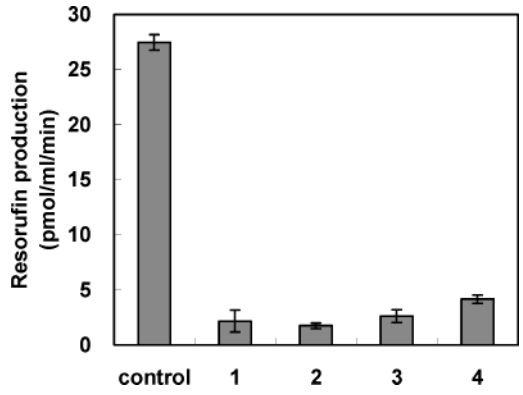


Figure 3. Inhibitory effect of compounds **1**–**4** toward the ethoxyresorufin *O*-deethylase (EROD) activity of the S9 fraction. Data are the average of three independent experiments.

in the crude extract must be some other compounds. In the Ames test, heterocyclic amines such as Trp-P-1 are first activated by cytochrome P450 (P450) 1A isoforms in the S9 fraction of the rat liver homogenate (*34*, *35*). These *N*-hydroxyl derivatives are then converted to *N*-acetoxy, or *N*-sulfonyloxy esters, that are regarded as the direct-acting mutagen (*35*). To confirm that compounds **1**−**4** are the desmutagens, we determined whether compounds **1**−**4** inhibit P450 1A isoforms. In this study we selected the EROD assay, which is a sensitive and specific method to determine P450 1A isoforms (*15*). As shown in **Figure 3**, compounds **1**−**4** markedly inhibited the EROD activity of S9. Therefore, the antimutagenic effects of compounds **1**−**4** are mostly due to the inhibition of N-hydroxylation catalyzed by P450 1A isoforms in the S9 fraction of rat liver homogenate.

The present study provides information on the antimutagenic constituents of the flowers of the neem tree, which is widely consumed in Thailand and the surrounding area. Moreover, the flowers of the neem tree can be used as a functional food to reduce cancer risks after confirming the stability, particularly during cooking, and bioavailability of its antimutagenic constituents. Some in vivo studies (*10*, *11*), which revealed anticarcinogenic effects of the flowers of the neem tree in rats, support its potential use as a functional food. Studies on the bioavailability and retainability of compounds **1**−**4** in rats are in progress.

LITERATURE CITED

(1) Koul, O.; Isman, M. B.; Ketkar, C. M. Properties and uses of neem, *Azadirachta indica*. *Can. J. Bot.* **1990**, *68*, 1−11.

(2) van der Nat, J. M.; van der Sluis, W. G.; de Silva, K. T.; Labadie, R. P. Ethnopharmacognostical survey of *Azadirachta indica* A. Juss (Meliaceae). *J. Ethnopharmacol.* **1991**, *35*, 1−24.

(3) Govindachari, T. R. Chemical and biological investigations on *Azadirachta indica* (the neem tree). *Curr. Sci.* **1992**, *63*, 117−121.

(4) Rochanakij, S.; Thebtaranonth, Y.; Yenjai, C.; Yuthavong, Y. Nimbolide, a constituent of *Azadirachta indica*, inhibits *Plasmodium falciparum* in culture. *Southeast Asian J. Trop. Med. Public Health* **1985**, *16*, 66−72.

(5) Okpanyi, S. N.; Ezeukwu, G. C. Anti-inflammatory and antipyretic activities of *Azadirachta indica*. *Planta Med.* **1981**, *41*, 34−39.

(6) Trakoontivakorn, G.; Nakahara, K.; Shinmoto, H.; Tsushida, T. Identification of Antimutagenic Substances (Ames Test) from *Boesenbergia pandurata* Schl. (fingerroot) and *Languas galanga* (galanga). *JIRCAS J.* **1999**, *7*, 105−116.

(7) Trakoontivakorn, G.; Nakahara, K.; Shinmoto, H.; Takenaka, M.; Onishi-Kameyama, M.; Ono, H.; Yoshida, M.; Nagata, T.; Tsushida, T. Structural analysis of a novel antimutagenic compound, 4-hydroxypanduratin A, and the antimutagenic activity of flavonoids in a Thai spice, fingerroot (*Boesenbergia pandurata* Schult.) against mutagenic heterocyclic amines. *J. Agric. Food Chem.* **2001**, *49*, 3046−3050.

(8) Nakahara, K.; Onishi-Kameyama, M.; Ono, H.; Yoshida, M.; Trakoontivakorn, G. Antimutagenic activity against Trp-P-1 of the edible Thai plant, *Oroxylum indicum* vent. *Biosci., Biotechnol., Biochem.* **2001**, *65*, 2358−2360.

(9) Nakahara, K.; Trakoontivakorn, G.; Alzoreky, N. S.; Ono, H.; Onishi-Kameyama, M.; Yoshida, M. Antimutagenicity of some edible Thai plants, and a bioactive carbazole alkaloid, mahanine, isolated from *Micromelum minutum*. *J. Agric. Food Chem.* **2002**, *50*, 4796−4802.

(10) Kusamran, W. R.; Tepsuwan, A.; Kupradinun, P. Antimutagenic and anticarcinogenic potentials of some Thai vegetables. *Mutat. Res.: Fundam. Mol. Mech. Mutagen.* **1998**, *402*, 247−258.

(11) Kusamran, W. R.; Ratanavila, A.; Tepsuwan, A. Effects of neem flowers, Thai and Chinese bitter gourd fruits and sweet basil leaves on hepatic monooxygenases and glutathione *S*-transferase activities, and *in vitro* metabolic activation of chemical carcinogens in rats. *Food Chem. Toxicol.* **1998**, *36*, 475−484.

(12) Ames, B. N.; McCann, J.; Yamasaki, E. Method for detecting carcinogens and mutagens with *Salmonella*/mammalian microsomes mutagenicity test. *Mutat. Res.: Sect. Environ. Mutagen. Relat. Subjects* **1975**, *31*, 347−364.

(13) Maron, D.; Katzenellenbogen, J.; Ames, B. N. Compatibility of organic solvents with the *Salmonella*/microsome test. *Mutat. Res.: Genet. Toxicol. Testing* **1981**, *88*, 343−350.

(14) Kanazawa, K.; Kawasaki, H.; Samejima, K.; Ashida, H.; Danno, G. Specific desmutagens (antimutagens) in oregano against a dietary carcinogen, are galangin and quercetin. *J. Agric. Food Chem.* **1995**, *43*, 404−409.

(15) Burke, M. D.; Thompson, S.; Elcombe, C. R.; Halpert, J.; Haaparanta, T.; Mayer, R. T. Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: a series of substrates to distinguish between different induced cytochromes P-450. *Biochem. Pharmacol.* **1985**, *34*, 3337−3345.

(16) Mizobuchi, S.; Sato, Y. A new flavanone with antifungal activity isolated from hops. *Agric. Biol. Chem.* **1984**, *48*, 2771−2775.

(17) Mizobuchi, S. Study on the structure of 5,7,4′-trihydroxyflavanones. *Agric. Biol. Chem.* **1985**, *49*, 2195−2196.

(18) Gibbs, H. D. Phenol Tests. III. The indophenol test. *J. Biol. Chem.* **1927**, *72*, 649−664.

(19) Sherif, E. A.; Gupta, R. K.; Krishnamurti, M. Anomalous $AlCl_3$ induced U.V. shift of C-alkylated polyphenols. *Tetrahedron Lett.* **1980**, *21*, 641−642.

(20) Jain, A. C.; Gupta, R. K.; Sarpal, P. D. Synthesis of (±)-lupinifolin, di-*O*-methyl xanthohumol and isoxanthohumol and related compounds. *Tetrahedron* **1978**, *34*, 3563.

(21) Shirataki, Y.; Yokoe, I.; Endo, M.; Komatsu, M. Determination of C-6 and C-8 substituted flavanone using ^{13}C-^{1}H long-range coupling and the revised structures of some flavanones. *Chem. Pharm. Bull.* **1985**, *33*, 444−447.

(22) Jang, D. S.; Cuendet, M.; Hawthorne, M. E.; Kardono, L. B.; Kawanishi, K.; Fong, H. H.; Mehta, R. G.; Pezzuto, J. M.; Kinghorn, A. D. Prenylated flavonoids of the leaves of *Macaranga conifera* with inhibitory activity against cyclooxygenase−2. *Phytochemistry* **2002**, *61*, 867−872.

(23) Rao, E. V.; Sridhar, P.; Prasad, R. Two plenylated flavanones from *Mundulea suberosa*. *Phytochemistry* **1997**, *46*, 1271−1274.

(24) Wang, M.; Li, J.; Liu, W. Two flavanones from the root bark of *Lespedeza daidii*. *Phytochemistry* **1987**, *26*, 1218−1219.

(25) Schuetz, B. A.; Wright, A. D.; Rali, T.; Sticher, O. Prenylated flavanones from leaves of *Macaranga pleiostemona*. *Phytochemistry* **1995**, *40*, 1273−1277.

(26) Tahara, S.; Katagiri, Y.; Ingham, J. L.; Mizutani, J. Prenylated flavanones in the roots of yellow lupin. *Phytochemistry* **1994**, *36*, 1261−1271.

(27) Promsattha, R.; Tempesta, M. S.; Fomum, Z. T.; Mbafor, J. T. (−)-Sigmoidin E: A new prenylated flavonoid from *Erythrina sigmoidea*. *J. Nat. Prod.* **1988**, *51*, 611−613.
(28) Yenesew, A.; Midiwo, J. O.; Miessner, M.; Heydenreich, M.; Peter, M. G. Two prenylated flavanones from stem bark of *Erythrina burtii*. *Phytochemistry* **1998**, *48*, 1439−1443.
(29) Garg, H. S.; Bhakuni, D. S. An isoprenylated flavanone from leaves of *Azadirachta indica*. *Phytochemistry* **1984**, *23*, 2115−2118.
(30) Islam, M. S.; Yoshimoto, M.; Yahara, S.; Okuno, S.; Ishiguro, K.; Yamakawa, O. Identification and characterization of foliar polyphenolic composition in sweet potato (*Ipomoea batatas* L.) genotypes. *J. Agric. Food Chem.* **2002**, *50*, 3718−3722.
(31) Edenharder, R.; von Petersdorff, I.; Rauscher, R. Antimutagenic effects of flavonoids, chalcones and structurally related compounds on the activity of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and other heterocyclic amine mutagens from cooked food. *Mutat. Res.: Fundam. Mol. Mech. Mutagen.* **1993**, *287*, 261−274.
(32) Edenharder, R.; Rauscher, R.; Platt, K. L. The inhibition by flavonoids of 2-amino-3-methylimidazo[4,5-*f*]quinoline metabolic activation to a mutagen: A structure−activity relationship study. *Mutat. Res.: Fundam. Mol. Mech. Mutagen.* **1997**, *379*, 21−32.
(33) Kada, T.; Inoue, T.; Ohta, T.; Shirasu, Y. Antimutagens and their modes of action. *Basic Life Sci.* **1986**, *39*, 181−196.
(34) Guengerich, F. P. Comparisons of catalytic selectivity of cytochrome P450 subfamily enzymes from different species. *Chem.−Biol. Interact.* **1997**, *106*, 161−182.
(35) Kato, R.; Yamazoe, Y. Metabolic activation and covalent binding to nucleic acids of carcinogenic heterocyclic amines from cooked foods and amino acid pyrolysates. *Jpn. J. Cancer Res.* **1987**, *47*, 297−311.

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