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Cytochrome P450 3A4 Inhibitory Constituents of the Wood of *Taxus yunnanensis*

Yasuhiro Tezuka,*[,†] Kouhei Morikawa,[‡] Feng Li,[†] Lidyawati Auw,[†] Suresh Awale,[†] Takahiro Nobukawa,[‡] and Shigetoshi Kadota[†]

Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan, and School of Allied Health Science, Kitasato University, 1-15-1 Kitasato, Sagamihara 228-8555, Japan

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From the aqueous extract of the wood of *Taxus yunnanensis*, which showed cytochrome P450 3A4 (CYP3A4) inhibition, a new isoflavan [(3*S*,4*R*)-4′-hydroxy-6,3′-dimethoxyisoflavan-4-ol (**1**)], a new degraded lignan [2,3-bis(hydroxymethyl)-7-hydroxy-6-methoxy-1-tetralone (**2**)], and a new lignan [(7*R*)-7-hydroxytaxiresinol (**3**)] were isolated, together with nine known lignans. Among the isolates obtained, α-conidendrin (**12**) showed strong CYP3A4 inhibition with an IC_{50} value of 0.2 μM.

Taxus yunnanensis Cheng et L.K. Fu (Taxaceae) is an evergreen tree distributed widely in Yunnan Province of the People's Republic of China and is commonly known as "Yunnan Hongdoushan".[1] The wood of this plant has been used in traditional Chinese medicine by people of different ethnic groups of Yunnan Province for the treatment of kidney problems, diabetic ailments, and various other purposes.[2] *Taxus brevifolia* is well-known as the source of taxane-type diterpenoids such as paclitaxel (Taxol), a potent anticancer drug.[3] Besides taxane-type diterpenoids, several other classes of compounds including biflavonoids, cinnamic acid derivatives, lignans, and steroids were also reported from *Taxus* species,[4] including *T. yunnanensis*.[5,6]

A recent investigation by our group on cytochrome P450 (CYP) inhibitory natural products has shown that certain lignans inhibit CYP3A4.[7] CYPs are the principal enzymes for oxidative metabolism of drugs and other xenobiotics, in addition to endogenous substances in many mammalian tissues. Drug metabolism has a critical role in the termination of drug action and influences the residence time of toxic chemicals in the body. Thus, the effects of oxidation by CYPs can be manifested by poor drug bioavailability and various acute and chronic toxic effects, including birth defects, cancer susceptibility, and adverse drug interactions.[8] In human liver microsomes, CYP3A4 is the most abundant enzyme, and recent investigations have revealed that more than 50% of drugs clinically used are oxidized by CYP3A4, while about 30% of drugs are metabolized by CYP2D6.[9] Inhibition of CYP-mediated metabolism is the major mechanism underlying numerous drug−drug interactions. Moreover, the CYP inhibitors can be used as drug-sparing agents when taken concomitantly with conventional medicines, to decrease the dosage and financial cost of expensive drug regimes.[10] As part of a continuing study on CYP inhibitors from crude drugs, we examined the inhibitory activities on CYP3A4 and CYP2D6 of the H_2O extract of *T. yunnanensis* and found that this extract inhibited CYP3A4 (71.4% inhibition at 100 μg/mL) and CYP2D6 (46.7% inhibition at 100 μg/mL). Therefore, the inhibitory constituents of the H_2O extract of *T. yunnanensis* were examined.

The wood of *T. yunnanensis* was extracted with H_2O, and the resultant extract was separated by a combination of medium-pressure liquid chromatography (MPLC) and preparative TLC techniques to give three new compounds (**1**−**3**), together with nine known lignans. The known lignans were identified by analyzing their spectroscopic data and comparing them with those in the literature, and they included isotaxiresinol[11] (**4**), (7*R*)-7-hydroxylariciresinol[12] (**5**), tanegool[13] (**6**), isolariciresinol[14] (**7**), taxiresinol[11] (**8**), secoisolariciresinol[15] (**9**), lariciresinol[16] (**10**), (7*S*,8*S*)-methoxy-3′,7-epoxy-8,4′-oxyneoligna-4,9,9′-triol[17] (**11**), and α-conidendrin[18] (**12**).

Compound **1** was obtained as a white, amorphous solid with a molecular formula of $C_{17}H_{18}O_5$. The ^{1}H NMR spectrum of **1** (Table 1) showed signals due to two 1,2,4-trisubstituted benzene rings [δ_H 6.66 (d, J = 2.0 Hz), 6.67 (dd, J = 8.1, 2.0 Hz), 6.80 (d, J = 8.1 Hz); δ_H 6.62 (d, J = 1.7 Hz), 6.81 (d, J = 8.1 Hz), 6.70 (dd, J = 8.1, 1.7 Hz)], an oxygen-substituted methylene [δ_H 3.72 (dd, J = 10.6, 6.6 Hz), 3.80 (dd, J = 10.6, 6.6 Hz)], an oxygen-substituted methine (δ_H 4.90, d, J = 6.6 Hz), and a methine (δ_H 3.01, q, J = 6.6 Hz) together with the signals of two methoxy groups (δ_H 3.80, 3.77). In turn, the ^{13}C NMR spectrum of **1** showed 17 carbon signals consisting of six sp^2 methines, four oxygen-substituted sp^2 quaternary carbons, two sp^2 quaternary carbons, an oxygen-substituted methylene, an oxygen-substituted methine, a methine, and two methoxy carbons (Table 1). On analysis of the COSY, HMQC, and HMBC spectra, compound **1** was suggested to be an isoflavone with two methoxy groups (Figure 1). The locations of the methoxy groups were determined as C-6 and C-3′ on the basis of the NOE enhancements of H-5 and H-2′ on irradiation of the methoxy protons at δ_H 3.77 and δ_H 3.80, respectively. The *trans* relationship between H-3 (δ_H 3.01) and H-4 (δ_H 4.90) was deduced from the large coupling constant (6.6 Hz), while the absolute stereochemistry as 3*S*,4*R* was determined from the positive Cotton effect at 260 nm and negative Cotton effect at 226 nm.[19] Thus, compound **1** was determined as (3*S*,4*R*)-4′-hydroxy-6,3′-dimethoxyisoflavan-4-ol.

The HREIMS of compound **2** indicated a molecular formula of $C_{13}H_{16}O_5$. The ^{1}H NMR spectrum of **2** (Table 1) showed the signals of two isolated aromatic protons (δ_H 7.34, 6.81), two oxygen-substituted methylenes (δ_H 3.67, 2H; δ_H 4.17, 3.84), a methylene (δ_H 3.03, 2.92), and two methines (δ_H 2.47, 2.41) together with the signal of a methoxy group (δ_H 3.92). The ^{13}C NMR spectrum of this compound showed 13 carbon signals consisting of two sp^2 methines, two oxygen-substituted sp^2 quaternary carbons, two sp^2 quaternary carbons, two oxygen-substituted methylenes, a methylene, and two methines (Table 1) together with a methoxy carbon (δ_C 56.5) and a carbonyl carbon (δ_C 199.5). The analysis of the COSY, HMQC, and HMBC spectra of **2** indicated an α-tetralone derivative having hydroxymethyl substituents at C-2 and C-3 and oxygenated substituents at C-6 and C-7 (Figure 1). The location of the methoxy group was determined to be at C-6 on the basis of the NOE enhancement of H-5 on irradiation of the methoxy protons and the ROESY correlations of H-5 with the methoxy protons and H-4 at δ_H 3.03. In the ROESY spectrum, H-2 and H-3 showed correlations with H-4 at δ_H 2.92 and with H-4 at δ_H 3.03, respectively. Thus, H-2 and H-3 (i.e., two hydroxymethyl groups)

* To whom correspondence should be addressed. Tel: +81 (0)76 434 7627. Fax: +81 (0)76 434 5059. E-mail: tezuka@inm.u-toyama.ac.jp.
† University of Toyama.
‡ Kitasato University.

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Chart 1

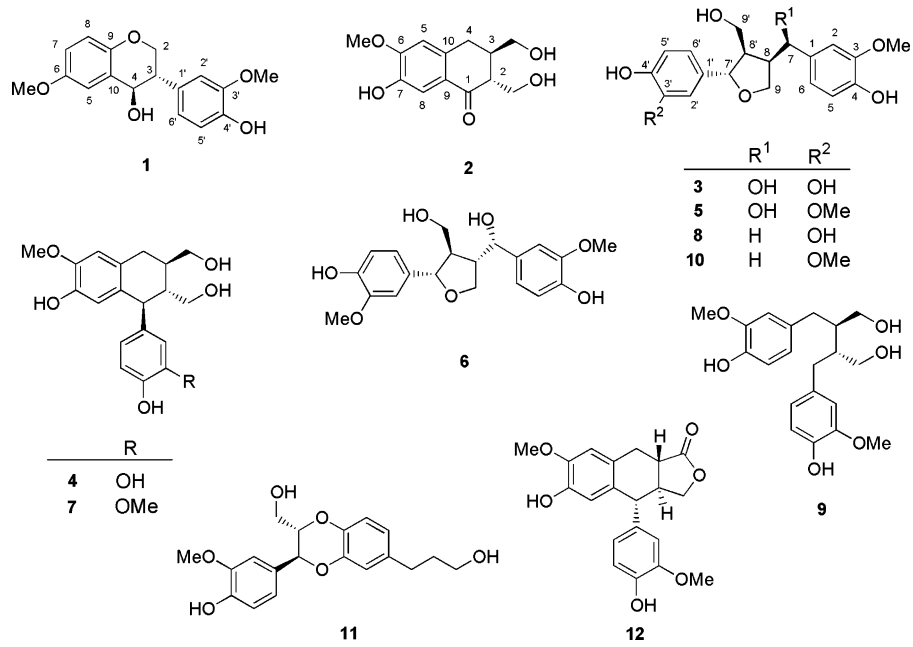

Table 1. NMR Spectroscopic Data (400 MHz) for Compounds **1**−**3**

	1[a]		**2**[b]		**3**[b]	
position	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.
1				199.5, C		136.2, C
2	3.80 dd (10.6, 6.6) 3.72 dd (10.6, 6.6)	63.8, CH_2	2.47 m	52.4, CH	6.86 br s	111.6, CH
3	3.01 q (6.6)	55.1, CH	2.41 m	39.8, CH		149.0, C
4	4.90 d (6.6)	75.4, CH	3.03 dd (16.6, 4.4) 2.92 dd (16.6, 9.0)	31.5, CH_2		147.1, C
5	6.66 d (2.0)	109.8, CH	6.81 s	111.7, CH	6.73 d	116.0, CH
6		146.8, C		154.6, C	6.73 d	120.8, CH
7	6.67 dd (8.1, 2.0)	119.6, CH		146.5, C	4.44 d (8.8)	76.9, CH
8	6.80 d (8.1)	114.2, CH	7.34 s	113.4, CH	2.51 m	50.8, CH
9		145.2, C		138.3, C	4.23 dd (9.0, 4.4) 3.92 dd (9.0, 7.3)	71.7, CH_2
10		134.2, C		126.7, C		
1′		130.5, C				134.9, C
2′	6.62 d (1.7)	127.7, CH			6.82 d (2.0)	114.7, CH
3′		146.9, C				146.4, C
4′		144.9, C				145.9, C
5′	6.81 d (8.1)	114.7, CH			6.70 d (8.1)	116.1, CH
6′	6.70 dd (8.1, 1.7)	121.7, CH			6.67 dd (8.1, 2.0)	119.1, CH
7′					4.57 d (7.2)	84.9, CH
8′					1.84 m	53.5, CH
9′					3.25 dd (11.4, 4.4) 3.14 dd (11.4, 5.4)	62.2, CH_2
OMe-3					3.82 s	56.4, CH_3
OMe-6	3.77 s	55.9, CH_3	3.92 s	56.5, CH_3		
OMe-3′	3.80 s	55.9, CH_3				
CH_2OH-2			4.17 dd (10.4, 4.2) 3.84 dd (10.4, 4.9)	60.0, CH_2		
CH_2OH-3			3.67 d (5.2)	64.7, CH_2		

[a] Measured in $CDCl_3$ + 3 drops of CH_3OH-d_4. [b] Measured in CH_3OH-d_4.

should have a *trans* relationship. From these data, compound **2** was determined as 2,3-bis(hydroxymethyl)-7-hydroxy-6-methoxy-1-tetralone. Compound **2** may be derived from isotaxiresinol (**4**), isolariciresinol (**7**), or their congeners, and as such, **2** has the configuration 2*R*,3*R*.

Compound **3** was obtained as a yellow, amorphous solid, having a molecular formula of $C_{19}H_{22}O_7$. The ^{1}H NMR spectrum of **3** (Table 1) showed the signals due to two sets of 1,2,4-trisubstituted benzene rings [δ_H 6.86 (br s), 6.73 (2H, br s); δ_H 6.82 (d, J = 2.0 Hz), 6.70 (d, J = 8.1 Hz), 6.67 (dd, J = 8.1, 2.0 Hz)], two oxygen-substituted methylenes (δ_H 4.23, 3.92; δ_H 3.25, 3.14), two oxygen-substituted methines (δ_H 4.44, 4.57), and two methines (δ_H 2.51, 1.84) together with the signal of a methoxy group (δ_H 3.82). In the ^{13}C NMR spectrum, **3** showed 19 carbon signals consisting of six sp^2 methines, four oxygen-substituted sp^2 quaternary carbons, two sp^2 quaternary carbons, two oxygen-substituted methylenes, two oxygen-substituted methines, two methines, and a methoxy carbon (Table 1). These spectroscopic data were similar to those of (7*R*)-7-hydroxylariciresinol[12] (**5**) isolated from the same extract, except for a lack of one of two methoxy groups in **5**. The location of the methoxy group was determined to be at C-3 on the basis of the NOE enhancement of H-2 on irradiation of the methoxy protons (Figure 1). The absolute configuration was determined to be the same as that of **5** on the basis of the ROESY correlations between

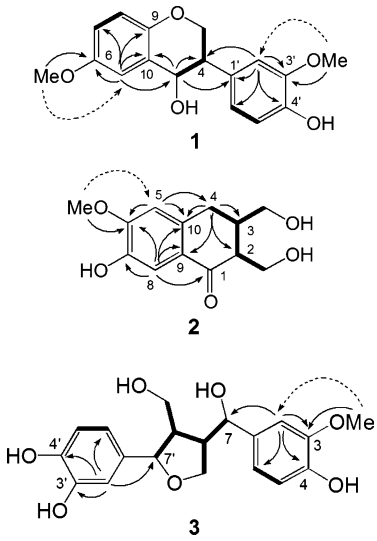

Figure 1. COSY (bold line) and HMBC (arrows: $^1H \rightarrow {}^{13}C$) correlations and NOEs (dashed arrows) observed in difference NOE spectra for **1**−**3**.

Table 2. CYP Inhibitory Activities (IC_{50}, μM) of Compounds **1**−**12**[a]

compound	CYP3A4	CYP2D6
1	99.5	>100
5	66.5	>100
7	>100	97.7
8	85.6	68.1
9	47.3	>100
10	25.6	>100
11	54.2	>100
12	0.2	17.7
ketokonazol[b]	0.0078	
quinidine[b]		0.01

[a] Compounds **2**−**4** and **6** were inactive (IC_{50} > 100 μM) for both CYP3A4 and CYP2D6. [b] Positive controls.

H-7′/H-9′, H-8/H-8′, and H-8/H-7 and on comparison of the specific rotation value and CD spectrum ($[\alpha]^{22}_D$ −34.6; $[\theta]_{300}$ +836, $[\theta]_{280}$ −1001, $[\theta]_{251}$ +417, $[\theta]_{233}$ −2422, $[\theta]_{217}$ −4518) with those of **5** ($[\alpha]^{22}_D$ −30.6; $[\theta]_{301}$ +557, $[\theta]_{283}$ −423, $[\theta]_{256}$ +397, $[\theta]_{236}$ −2271, $[\theta]_{216}$ −3634). Thus, compound **3** was determined as the 3′-*O*-demethylated derivative of **5** [(7*R*)-7-hydroxytaxiresinol].

All compounds isolated were tested for their inhibitory activities on CYP3A4 and CYP2D6. Among the 12 compounds tested, compounds **1**, **5**, and **8**−**12** showed CYP3A4 inhibition, while compounds **7**, **8**, and **12** inhibited CYP2D6. For both CYP3A4 and CYP2D6, α-conidendrin (**12**) showed the most potent inhibition, with IC_{50} values of 0.2 and 17.7 μM, respectively (Table 2).

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-140 digital polarimeter. CD measurements were carried out on a JASCO J-805 spectropolarimeter. The ^1H, ^{13}C, and 2D NMR spectra were taken on a JEOL JNM-LA400 spectrometer with tetramethylsilane (TMS) as internal standard. HREIMS measurements were performed on a JEOL JMS-700T spectrometer at 70 eV. MPLC was performed with a Büchi MPLC double gradient pump system. Analytical and preparative TLC were conducted on precoated Merck Kieselgel 60F254 plates (Merck, 0.25 or 0.50 mm thickness).

Plant Material. The wood of *Taxus yunnanensis* was collected from cultivated plants in Yunnang Province on October, 2000, and kindly provided by Kotosugi Co., Ltd. (Sagamihara, Japan). A voucher specimen (TMPW 21495) is preserved at the Museum of Materia Medica, Analytical Research Center for Ethnomedicines, Institute of Natural Medicine, University of Toyama.

Extraction and Isolation. The air-dried wood of *T. yunnanensis* was crushed into a powder form. The powder (600 g) was extracted with H_2O (4 L, reflux, 30 min, × 3), and the insoluble portion was separated by filtration. The filtrate was lyophilized to give a H_2O extract (48.7 g). The H_2O extract was subjected to MPLC on ODS using a gradient system of (A) H_2O and (B) CH_3CN−acetone (1:1) to give eight fractions: fraction 1, H_2O eluate, 381 mg; fraction 2, 1−5% B/A eluate, 200 mg; fraction 3, 6−10% B/A eluate, 1.5 g; fraction 4, 11−20% B/A eluate, 204 mg; fraction 5, 21−30% B/A eluate, 275 mg; fraction 6, 31−40% B/A eluate, 1.2 g; fraction 7, 41−60% B/A eluate, 340 mg; fraction 8, 61−100% B/A eluate, 210 mg. Fraction 2 (200 mg) was separated by normal-phase preparative TLC with MeOH−$CHCl_3$ (2:8), followed by normal-phase preparative TLC with acetone−C_6H_6 (1:2), to give compounds **1** (7.1 mg), **2** (3.5 mg), and **3** (3.2 mg). Fraction 3 (1.5 mg) was dissolved in MeOH and left overnight to give isotaxiresinol[11] (**4**, 1.0 g). Fraction 4 (204 mg) was subjected to normal-phase preparative TLC with 15% MeOH−$CHCl_3$, followed by reversed-phase preparative TLC with 15% 2-propanol−H_2O, to give **4** (51.0 mg), (7*R*)-7-hydroxylariciresinol[12] (**5**, 17.1 mg), and tanegool[13] (**6**, 7.5 mg). Fraction 5 (275 mg) was purified by normal-phase preparative TLC with 40% acetone−C_6H_6, followed by reversed-phase preparative TLC with H_2O−CH_3CN−2-propanol (6:1:1), to give isolariciresinol[14] (**7**, 21.1 mg), taxiresinol[11] (**8**, 25.4 mg), secoisolariciresinol[15] (**9**, 55.1 mg), and lariciresinol[16] (**10**, 11.2 mg). Fraction 6 (1.2 g) yielded **9** (930 mg). Fraction 7 (340 mg) was subjected to normal-phase preparative TLC with 10% MeOH−CH_2Cl_2, followed by reversed-phase preparative TLC with H_2O−CH_3CN (3:1), to give (7*S*,8*S*)-methoxy-3′,7-epoxy-8,4′-oxyneoligna-4,9,9′-triol[17] (**11**, 5.6 mg) and α-conidendrin[18] (**12**, 8.5 mg).

Compound 1: white, amorphous solid; $[\alpha]^{22}_D$ −38.2 (*c* 0.1, MeOH); CD (*c* 3.3 × 10^{-4} M, EtOH) $[\theta]_{260}$ +426, $[\theta]_{244}$ −843, $[\theta]_{226}$ −1776; ^1H and ^{13}C NMR, see Table 1; HREIMS *m*/*z* 302.1166 (calcd for $C_{17}H_{18}O_5$ $[M]^+$, 302.1154).

Compound 2: brown, amorphous solid; $[\alpha]^{22}_D$ −46.6 (*c* 0.2, MeOH); CD (*c* 4.0 × 10^{-4} M, EtOH) $[\theta]_{330}$ −32346, $[\theta]_{244}$ +20302, $[\theta]_{229}$ −15204; ^1H and ^{13}C NMR, see Table 1; HREIMS *m*/*z* 252.1046 (calcd for $C_{13}H_{16}O_5$ $[M]^+$, 252.0998).

Compound 3: yellow, amorphous solid; $[\alpha]^{22}_D$ −34.6 (*c* 0.4, MeOH); CD (*c* 2.8 × 10^{-4} M, EtOH) $[\theta]_{300}$ +836, $[\theta]_{280}$ −1001, $[\theta]_{251}$ +417, $[\theta]_{233}$ −2422, $[\theta]_{217}$ −4518; ^1H and ^{13}C NMR, see Table 1; HREIMS *m*/*z* 362.1364 (calcd for $C_{19}H_{22}O_7$ $[M]^+$, 362.1365).

CYP Inhibition Assay. CYP3A4 and CYP2D6 inhibitory activities were determined using fluorometric measurement[20] of the metabolites 7-hydroxy-4-trifluoromethylcoumarin (7-HFC) and 3-[2-(diethylamino)ethyl]-7-hydroxy-4-methylcoumarin hydrochloride (AHMC), formed by *O*-dealkylation reactions of 7-benzyloxy-4-trifluoromethylcoumarin (7-BFC) and 3-[2-*N*,*N*-diethyl-*N*-methylammonium]ethyl]-7-methoxy-4-methylcoumarin (AMMC) as substrates, respectively, in 96-well black microtiter plates (Corning Inc., Corning, NY). Briefly, one row (12 wells) was used for each test sample: wells 1−8 were for the serial dilution of test samples, wells 9 and 10 were for a control, and wells 11 and 12 were for a blank. For the serial dilution of test samples, 148 and 100 μL of cofactor/serial dilution (C/SD) buffer [50 mM pottasium phosphate buffer, pH 7.4 containing 2.6 mM $NADP^+$ (Oriental Yeast Co., Ltd., Tokyo, Japan), 6.6 mM glucose-6-phosphate (Oriental Yeast Co., Ltd.), 0.8 U/mL glucose-6-phosphate dehydrogenase (Oriental Yeast Co., Ltd.), and 6.6 mM $MgCl_2$] were dispensed into well 1 and wells 2−8, respectively. Into well 1, 2 μL of MeOH solution of test sample was added, and 50 μL of the inhibitor solution from well 1 was then transferred into well 2. Similarly serially 1:3 diluted solutions were prepared in wells 1−8 (50 μL of well 8 was discarded). Wells 9 and 10 (control wells) contained 100 μL of C/SD buffer and 2 μL of MeOH, and wells 11 and 12 were used as blanks for background fluorescence (enzyme and substrate were added after the reaction was terminated). After a prewarm for 10 min at 37 °C, the reaction was initiated by the addition of 100 μL of prewarmed enzyme/substrate mixture in 0.35 M potassium phosphate buffer (pH7.4) containing 2 μM cDNA-expressed CYP3A4 and CYP2D6 in insect cell microsomes (GENTEST Corporation, Woburn, MA), 15 mg/mL insect cell control protein (GENTEST Corporation), and 50 mM 7-BFC or 10 mM AMMC. After incubation for 30 min at 37 °C, the reaction was stopped by addition of 80 μL of a 4:1 acetonitrile−0.5 M tris(hydroxymethyl)aminomethane (Tris-base) solution, and the fluorescence was measured using a Perkin-Elmer HTS 7000 bioassay reader with excitation and emission wavelengths of 405 and 535 nm for CYP3A4 and 390 and 465 nm for CYP2D6. The assays were performed in triplicate for all test compounds. Ketoconazole and quinidine were used as positive

controls for CYP3A4 and CYP2D6, respectively. Data were exported and analyzed using an Excel spreadsheet. The IC_{50} values were calculated by linear interpolation.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of the new compounds **1**−**3**. This information is available free of charge via the Internet at http://pubs.acs.org.

Note Added after ASAP Publication: This paper was published on the Web on Dec 7, 2010, with an error in the title. The corrected version was reposted on Dec 22, 2010.

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