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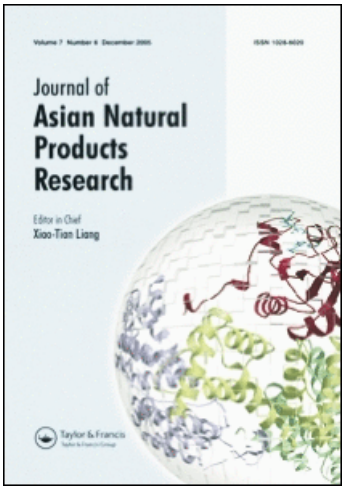

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Chromone glycosides from *Knoxia corymbosa*

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Chromone glycosides from *Knoxia corymbosa*

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Four new chromone glycosides, corymbosins K_1-K_4 (**3–6**), together with two known compounds, noreugenin (**1**) and undulatoside A (**2**), were isolated from the whole plant of *Knoxia corymbosa* (Rubiaceae). The structures of the new compounds were established through extensive NMR or X-ray spectroscopic analysis as 7-*O*-β-D-allopyranosyl-5-hydroxy-2-methylchromone (corymbosin K_1, 3), 7-*O*-β-D-6-acetylglucopyranosyl-5-hydroxy-2-methylchromone (corymbosin K_2, **4**), 7-*O*-[6-*O*-(4-*O*-*trans*-caffeoyl-*β*-D-allopyranosyl)]-*β*-D-glucopyranosyl-5-hydroxy-2-methylchromone (corymbosin K_3, **5**) and 7-*O*-[6-*O*-(4-*O*-*trans*-feruloyl-*β*-D-allopyranosyl)]-*β*-D-glucopyranosyl-5-hydroxy-2- methylchromone (corymbosin K_4, **6**). Compounds 2–5 were subjected to test their immunomodulatory activity *in vitro*.

Keywords: *Knoxia corymbosa*; Rubiaceae; Chromone glycoside; Corymbosin K1, K2, K3, K4; Immunomodulatory activity

1. Introduction

Knoxia corymbosa Willd., belonging to the genus of *Knoxia* (Rubiaceae), is wildly used for the treatment of watery diarrhoea and dropsy in traditional Chinese folk medicine [1]. Sometimes, it mixed as "Hongyadaji" with *Knoxia valerinoides,* of which the characteristic components were anthraquinones [2,3]. Bhattacharya et al. have previously reported some ursolic acid and sterols from the title plant [4]. In order to investigate bioactivities components and to offer chemical evidence for its application in traditional Chinese folk medicine, further chemical investigation is warranted. We have previously reported some flavonol glycosides from *K. corymbosa* [5–8]. Further chemical investigation of the residue of this plant resulted in the isolation of four new chromone glycosides, namely, corymbosin K_1, K_2, K_3 and K_4, and two known related compounds, noreugenin (**1**) [9] and undulatoside A (**2**) [10]. In this paper we describe the isolation and structure elucidation of four new chromone glycosides (**3**-**6**) and their immunomodulatory bioactivity.

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2. Results and discussion

The EtOH extract of the plant was dissolved and suspended in water, and partitioned by EtOAc and n-BuOH in water. The EtOAc extract afforded compounds **1–4** while the n-BuOH extract provided compounds **5** and **6** (figure 1). ^{1}H NMR and ^{13}C NMR spectral data of compounds **1** and **2** were identical with those reported for noreugenin (**1**) [9] and undulatoside A (**2**) [10].

Corymbosin K_1 (**3**) was obtained as colourless needles and gave a molecular ion peak at *m/z* 377.0851 by HRESI-MS, consistent with a molecular formula of $C_{16}H_{18}O_9$. It showed chromone properties (dark purple spot on silica gel chromatogram under UV light) and colour reaction (an orchid colour with 3% $FeCl_3$ on polyamide paper). The UV absorptions at 287, 310 nm, IR absorptions at 1664, 1626, 1590, 1401 cm^{-1} and ^{1}H NMR at δ_H 6.40 and 6.61 (d, $J = 2.2$ Hz), an olefinic proton at δ_H 6.26 (s), a methyl group at δ_H 2.39 (s), and a strongly chelated OH group at δ_H 12.83 (s) of **3** indicated the existence of a 5,7-dioxygenated chromone nucleus [11,12]. Compared with **1**, the remaining ^{13}C NMR data (table 1) and anomeric proton at δ_H 5.20 (d, $J = 7.9$ Hz) showed the sugar moiety of **3** should be a β anomer. The ^{13}C NMR data of the sugar moiety are different to those of **2**, but similar to those of kaempferol-**3**-*O*-β-D-allopyranoside [13]. Acid hydrolysis of 3 gave an aglycone identical with **1** and a sugar moiety identical with allose using authentic samples. HMBC correlation between δ_H 5.20 and δ_C 163.3 indicated that the allose was linked at C-7. Its X-ray crystallographic analysis (figure 2) further confirmed the structure. Thus, the structure of **3** was determined to be 7-*O*-β-D-allopyranosyl-5-hydroxy-2-methylchromone.

Corymbosin K_2 (**4**) was isolated as white amorphous powder and exhibited a molecular ion peak at *m/z* 396.1118 in HRESI-MS, compatible with the molecular formula $C_{18}H_{20}O_{10}$. The IR spectrum of **4** showed hydroxyl absorptions at 3460, 3265 cm^{-1}, a ketone carbonyl at 1668 cm^{-1} and an ester group at 1740 cm^{-1}. The NMR data of **4** were similar to those of **2** except for additional resonances at δ_H 2.03 (3H, s), δ_C 20.9 and 170.5, showing the presence of an acetyl moiety in **4**. Comparing the ^{13}C NMR data of the sugar moiety with **2**, the downfield shift of C-6′ and upfield shift of C-5′ of **4** indicated that the acetyl moiety was linked at C-6′. The ^{13}C NMR data of the sugar moiety were similar to those of

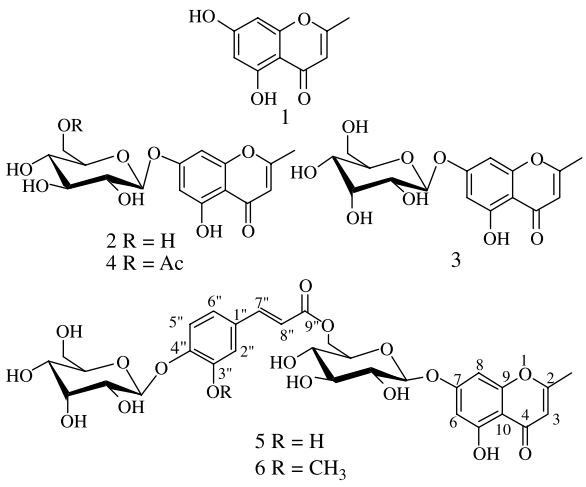

Figure 1. Structures of corymbosins K_1-K_4.

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Table 1. ^{13}C NMR spectral data for compounds **3–6**.

Carbon	***3***[a]	***4***[b]	***5***[a]	***6***[a]
2	168.3	168.7	168.3	168.3
3	108.3	108.7	108.3	108.2
4	182.0	182.4	181.9	181.9
5	161.1	161.5	161.1	161.2
6	99.4	99.9[c]	99.8	99.5
7	163.2	162.9	162.6	162.6
8	94.4	94.9	94.3	94.5
9	157.4	157.7	157.4	157.4
10	105.0	105.5	105.1	105.1
1′	98.4	99.8[c]	99.5	98.1
2′	70.1	73.3	72.9	73.0
3′	71.4	76.5	76.1	76.2
4′	66.9	70.2	69.9	69.9
5′	74.9	74.1	73.9	73.9
6′	60.9	63.7	63.4	63.4
1″			128.5	127.9
2″			114.8	114.9
3″			146.9	148.9
4″			147.7	149.3
5″			120.7	122.5
6″			115.9	115.8
7″			144.6	144.7
8″			115.7	115.8
9″			166.1	166.2
1‴			99.5	99.5
2‴			70.3	71.2
3‴			71.0	71.6
4‴			67.1	67.1
5‴			75.0	74.8
6‴			61.0	61.0
2-Me	20.4	20.3	19.8	19.8
6′-MeCO		20.9		
6′-MeCO		170.5		
3″-OMe				55.8

[a]Measured on 125 MHz in DMSO-d_6, TMS as internal standard.
[b]Measured on 75 MHz.
[c]Data interchangeable.

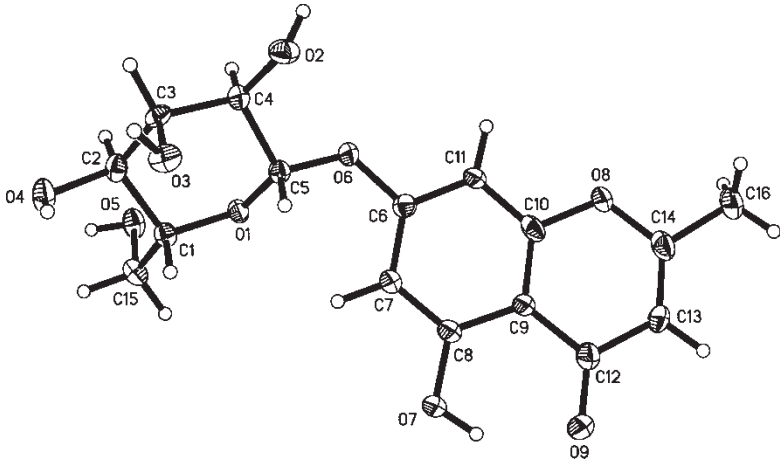

Figure 2. The perspective structure of corybosin K_1.

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kaempferol-3-*O*-β-D-6″-acetylglucopyranoside [14] and 6″-acetylphloridzoside [15]. An acetyl moiety at C-6′ in **4** was further supported by the correlations between the CH_2 group at δ_H 4.33 and 4.05 of the glucose residue and carbonyl carbon at δ_C 170.5 in the HMBC spectrum. Thus, the structure of compound **4** was deduced to be 7-*O*-β-D-6-acetylglucopyranosyl-5-hydroxy-2-methylchromone.

Corymbosin K_3 (**5**) was isolated as white amorphous powder. The molecular formula of $C_{31}H_{34}O_{17}$ was determined by HRFAB-MS. The UV absorptions at 290 and 321 nm indicated the presence of substituted aromatic rings and α,β-unsaturated ketone. The ^{1}H NMR and ^{13}CNMR data (table 1) showed a chromone aglycone [δ_H 6.19 (s), 6.44 (d, $J = 1.8$ Hz), 6.65 (d, $J = 1.8$ Hz), 2.22 (s)], a caffeoyl residue [δ_H 7.11 (d, $J = 1.2$ Hz), 7.08 (d, $J = 8.4$ Hz), 7.03 (dd, $J = 8.4$ and 1.2 Hz)], two *trans* olefinic protons [δ_H 7.48 (d, $J = 15.9$ Hz), 6.41 (d, $J = 15.9$ Hz)] and two anomeric protons [δ_H 5.14 (d, $J = 7.3$ Hz), 5.06 (d, $J = 7.9$ Hz)]. The β configuration of each sugar moiety was confirmed by the coupling constant of the anomeric proton. On acid hydrolysis of **5** [16], both glucose and allose were detected in comparison with the authentic samples on TLC. On mild alkaline hydrolysis of **5** [17], one hydrolysis product showed the same spectral data as those of **2**. Thus the glucose was linked at C-7 and the allose was linked to the caffeoyl residue. HMBC correlations of δ_H 5.14 with δ_C 162.6 and δ_H 5.06 with δ_C 147.7 further confirmed that the glucose was linked to C-7 and the allose to C-4″. Based on the above evidence, the structure of compound **5** was determined as 7-*O*-[6-*O*-(4-*O*-*trans*-caffeoyl-β-D-allopyranosyl)]-β-D-glucopyranosyl-5-hydroxy-2-methylchromone.

Compound **6** was obtained as white amorphous powder and exhibited $[M - H]^-$ ion at *m/z* 691.1850 in HRESI-MS, corresponding to the molecular formula of $C_{32}H_{36}O_{17}$. Its ^{1}H NMR and ^{13}C NMR data (table 1) were similar to those of **5**, except for the presence of the extra methoxyl group at δ_H 3.78 (s) and δ_C 55.8. The HMBC correlation between δ_H 3.78 and δ_C 149.3 showed a feruloyl residue in **6**. On acid and alkaline hydrolysis of **6**, glucose, allose and undulatoside A were detected by comparison with authentic samples in TLC. Its HMBC correlations of δ_H 5.20 with δ_C 162.6 and δ_H 5.14 with δ_C 149.3 established that the glucose was linked to the chromone aglycone on C-7 and the allose linked at the feruloyl residue on C-4″. The structure of **6** was established as 7-*O*-[6-*O*-(4-*O*-*trans*-feruloyl-β-D-allopyranosyl)]-β-D-glucopyranosyl-5-hydroxy-2-methylchromone.

Compounds **2–5** were subjected for the evaluation of their immunomodulatory activity *in vitro*. It was found that **4** significantly inhibited ($P < 0.01$) the proliferation of murine B lymphocytes *in vitro* at a concentration of 1×10^{-5} M (table 2).

3. Experimental

3.1 General experimental procedures

The melting points were determined on an XT-4 melting point apparatus and are uncorrected. The $[\alpha]_D$ values were obtained on a Jasco-20C digital polarimeter. UV spectra were taken with UV 2501 spectrometer. IR spectra were recorded with a Bio-Rad Win-IR spectrometer. MS spectra were measured with a VG Autospec-3000 spectrometer, and ESI-MS were measured on a API QSTAR Pulsar I system. NMR experiments were conducted with Bruker AV-500 MHz and AV-300 MHz instruments. X-Ray structural analysis was recorded on Smart Apex CCD.

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Table 2. Effect of compounds **2–5** on murine lymphocyte proliferation induced by concanavalin A (ConA) (5 mg/ml) or lipopolysaccharide (LPS) (10 mg/ml).

Compound	*Concentration*	*[^{3}H]TdR incorporation × 10^{-3} (cpm)*	
		ConA-induced T cell proliferation	*LPS-induced B cell proliferation*
Negative control		2.826 ± 0.028	0.92 ± 0.109
Positive control (ConA or LPS)		44.711 ± 0.251	37.825 ± 1.833
2	1×10^{-7}	44.411 ± 0.891	25.828 ± 2.859
	1×10^{-6}	43.737 ± 3.413	25.469 ± 3.281
	1×10^{-5}	41.255 ± 1.815	24.077 ± 1.799
4	1×10^{-7}	40.220 ± 1.043	24.703 ± 1.891**
	1×10^{-6}	34.356 ± 0.863	23.829 ± 2.323
	1×10^{-5}	30.281 ± 1.256	30.209 ± 3.911
Negative control		1.633 ± 0.090	2.854 ± 0.096
Positive control (ConA or LPS)		31.422 ± 1.788	38.194 ± 1.686
3	1×10^{-7}	34.550 ± 1.898	38.348 ± 0.767
	1×10^{-6}	36.114 ± 3.700	38.377 ± 0.972
	1×10^{-7}	29.839 ± 3.321	34.837 ± 4.566
5	1×10^{-7}	34.318 ± 1.179	36.823 ± 5.599
	1×10^{-6}	42.142 ± 5.625	35.780 ± 6.434
	1×10^{-5}	6.670 ± 2.254	5.748 ± 8.600

Results are represented as mean ± S.D. based on three independent experiments ($n = 3$).

**$P < 0.01$ compared with control group.

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3.2 Plant material

The plant *Knoxia corymbosa* was collected in the Honghe district of Yunnan Province in June 2001. It was authenticated by Professor Yao-Hua Wang and checked by Professor Wei-Ming Zhu, Department of Biology, Yunnan University. A voucher specimen (No.L-KC-WYB-1) has been deposited at the School of Pharmacy, Yunnan University.

3.3 Extraction and isolation

The whole plant powder (8 kg) was extracted with 95% EtOH at room temperature for three times (3 days each). After concentration of the extract *in vacuo*, the residue was suspended in water and partitioned successively with petroleum ether (60–90°C), EtOAc and n-BuOH. The EtOAc residue (63 g) was separated repeatedly by chromatography on a silica gel column, eluted with $CHCl_3$/MeOH (from 80:1 to 1:1) to afford **1** (81 mg), **2** (4.5 g), **3** (1.3 g) and **4** (300 mg). The n-BuOH extract (317 g) was subjected to silica gel column chromatography using gradient elution with $CHCl_3$/MeOH (from 40:1 to 1:2) to offer a subfraction. The fraction ($CHCl_3$/MeOH = 8:1) was further chromatographed on silica gel column eluting with $CHCl_3$/MeOH/H_2O (from 16:1:0.1 to 1:1:0.1) and further purified with Pharmadex LH-20 eluted with MeOH repeatedly. Compounds **5** (79 mg) and **6** (36 mg) were then obtained.

3.3.1 Corymbosin K_1 (3). Colourless needles, mp 196–198°C; $[\alpha]_D^{27}$ − 57.8 (*c* 0.29, MeOH); UV (DMSO) λ_{max} nm 287, 310; IR (KBr) ν_{max} 3488, 3383, 3184, 1664, 1626, 1590, 1401 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 6.26 (s, H-3), 6.40 (d, $J = 2.2$ Hz, H-6), 6.61 (d, $J = 2.2$ Hz, H-8), 2.39 (s, CH_3-2), 12.83 (s, O*H*-5), 5.20 (d, $J = 7.9$ Hz, H-1′), 3.44 (m, H-2′), 3.93 (d, $J = 3.0$ Hz, H-3′), 3.34 (m, H-4′), 3.75 (m, H-5′), 3.69 (m, H-6′); ^{13}C NMR

(DMSO-d_6, 125 MHz): see table 1; EI-MS *m/z* 354 ([M]$^+$, 15), 192 ([M-glucosyl]$^+$, 100); HRESI-MS [M + Na]$^+$*m/z* 377.0851 (calcd for $C_{16}H_{18}O_9Na$, 377.0848).

3.3.2 Corymbosin K_2 (4). White amorphous powder; mp 162–164°C; $[\alpha]_D^{26}$ − 68.7 (*c* 0.18, MeOH); UV (DMSO) λ_{max} nm 286, 311; IR (KBr) ν_{max} 3460, 3265, 1740, 1668, 1622, 1586, 1401, 1079 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 6.26 (s, H-3), 6.42 (d, J = 2.1 Hz, H-6), 6.65 (d, J = 2.1 Hz, H-8), 2.37 (s, C*H*$_3$-2), 12.81 (s, O*H*-5), 2.03 (s, C*H*$_3$CO-6′), 5.07 (d, J = 7.3 Hz, H-1′), 3.16–3.35 (m, H-2′, 3′, 4′), 3.72 (m, H-5′), 4.33 and 4.05 (m, H-6′); ^{13}C NMR (DMSO-d_6, 75 MHz): see table 1; EI-MS *m/z* 396 ([M]$^+$, 33), 192 ([M-glucosyl-Ac]$^+$, 100); HRESI-MS *m/z* 397.1118 [M + H]$^+$ (calcd for $C_{18}H_{21}O_{10}$, 397.1134).

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3.3.3 Corymbosin K_3 (5). White amorphous powder; mp 162°C; $[\alpha]_D^{24}$ − 77.4 (*c* 0.17, DMSO); UV (DMSO) λ_{max} nm 290, 321; IR (KBr) ν_{max} 3415, 1711,1662, 1626, 1586, 1401, 1081 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 6.19 (s, H-3), 6.44 (d, J = 1.8 Hz, H-6), 6.65 (d, J = 1.8 Hz, H-8), 2.22 (s, C*H*$_3$-2), 12.82 (s, O*H*-5), 5.14 (d, J = 7.3 Hz, H-1′), 4.15 (dd, J = 11.0 and 4.4 Hz, one proton of H-6′), 4.49 (d, J = 11.0, one proton of H-6′), 3.82 (m, H-5′), 5.06 (d, J = 7.9 Hz, H-1‴), 3.72 (m, H-3‴), 3.98 (brs, H-5‴), 3.30–3.50 (m, other proton signals of sugar moieties overlapped with water protons), 7.11 (d, J = 1.2 Hz, H-2″), 7.08 (d, J = 8.4 Hz, H-6″), 7.03 (dd, J = 8.4 and 1.2 Hz, H-5″), 7.48 (d, J = 15.9 Hz, H-7″), 6.41 (d, J = 15.9 Hz, H-8″); ^{13}C NMR (DMSO-d_6, 125 MHz): see table 1; FAB-MS *m/z* 677 [M – H]$^-$ (25), 191 [M – H-caffeoyl-allosyl-glucosyl]$^-$ (100); HRFAB-MS *m/z* 677.1345 [M – H]$^-$ (calcd for $C_{31}H_{33}O_{17}$, 677.1353).

3.3.4 Corymbosin K_4 (6). White amorphous powder; mp 196°C; $[\alpha]_D^{19}$ − 76.7 (*c* 0.18, Pyridine); UV (MeOH) λ_{max} nm 289, 312; IR (KBr) ν_{max} 3435, 1699,1660, 1625, 1557, 1507, 1418, 1079 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) δ 6.19 (s, H-3), 6.45 (d, J = 2.0 Hz, H-6), 6.63 (d, J = 2.0 Hz, H-8), 2.23 (s, C*H*$_3$-2), 12.84 (s, O*H*-5), 3.78 (s, C*H*$_3$O-3″), 5.20 (d, J = 7.8 Hz, H-1′), 4.17 (dd, J = 11.5 and 4.4 Hz, one proton of H-6′), 4.48 (d, J = 11.5, one proton of H-6′), 3.80 (m, H-5′), 5.14 (d, J = 7.2 Hz, H-1‴), 3.92 (m, H-3‴), 3.68 (m, H-5‴), 3.20–3.50 (m, the other proton signals of sugar moieties overlapped with water protons), 7.11 (d, J = 1.2 Hz, H-2″), 7.08 (d, J = 8.4 Hz, H-6″), 7.03 (dd, J = 8.4 and 1.2 Hz, H-5″), 7.54 (d, J = 15.9 Hz, H-7″), 6.56 (d, J = 15.9 Hz, H-8″); ^{13}C NMR (DMSO-d_6, 125 MHz): see table 1; ESI-MS *m/z* 691 [M – H]$^-$, 529 [M – H-allosyl]$^-$, 337 [M – H-feruloyl-allosyl]$^-$; HRESI-MS *m/z* 691.1850 [M – H]$^-$ (calcd for $C_{32}H_{35}O_{17}$, 691.1874).

3.4 Acid hydrolysis

A solution of each compound (5 mg of each) in H_2O (5 ml) and 2 mol/l aqueous HCl (5 ml) was refluxed in a water bath for 3 h, then the reaction mixture was evaporated to dryness. The residue was dissolved in MeOH and detected sugar (EtOAc/MeOH/H_2O/HOAc = 12:3:3:4) and noreugenin (petroleum ether/acetone = 3:1) by comparison with authentic samples on TLC.

3.5 Mild alkaline hydrolysis

Compounds **5** and **6** (10 mg of each) were hydrolysed with 1% aqueous KOH at room temperature. After 1 h, the mixture was neutralised with dilute HCl and extracted with EtOAc. The EtOAc layer gave undulatoside A, which was identified by TLC (authentic sample, $CHCl_3$/MeOH = 5:1).

3.6 X-ray crystallographic data of corymbosin K_1

A colourless crystal from MeOH of **3** with dimension of 0.206 × 0.100 × 0.053 mm was selected for X-ray structure analysis. The crystallographic data were collected on a CCD diffractometer using graphite monochromated MoKα radiation. The structure was solved by the direct methods and expanded using Fourier transformation techniques, and refined by a full-matrix least-square calculation on F^2 with the aid of the program SHELXL97. The compound crystallised in the space group $P2_1$, $a = 9.3978$ (17)Å, $b = 7.4895$ (14)Å, $c = 11.596$ (2)Å, monoclinic, $\beta = 104.095$ (4)°, $V = 791.6$ (3)Å^3, $Z = 2$, $D_{calc} = 1.486$ g/cm^3, $\lambda = 0.71073$ Å, μ(MoKα) = 0.123 mm^{-1}, F (000) = 372, and $T = 293$ (2) K. A total of 4921 reflections were collected in the range $1.81 \leq \theta \leq 28.26°$ of which 3442 unique reflections with $I > 2\sigma(I)$ were used for the analysis. The structure was solved using direct methods and refined by full-matrix least squares on F^2 values. Non-hydrogen atoms were refined anisotropically. Hydrogen atoms were fixed at calculated positions and refined using a riding mode. The final indices were $R = 0.0436$, $R_w = 0.0588$ with goodness-of-fit = 0.641. Scattering factors were taken from the *International Tables for X-ray Crystallography*. Crystallographic data for **1** have been deposited with the Cambridge Crystallographic Data Centre (deposition number CCDC 223380). Copies of the data can be obtained, free of charge, on application to the director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-1223-330633; e-mail: . E-mail: deposit@ccdc.cam.ac.uk).

3.7 Bioassays

Lymphocyte proliferation test of **2–5**: The prepared spleen cells of mice (4×10^6) were seeded into each well of a 96-well microplate and various concentrations of compounds **2–5** and 5 mg/ml of concanavalin A (Con A, from *Canavalia ensiformis* Type III, Sigma) or lipopolysaccharide (LPS, from *Escherichia coli*, Sigma) were added alone or in combination. The plates were cultured at 37°C with 5% CO_2 in a humidified atmosphere for 48 h. For the last 6 h, each well was pulsed with 0.25 μCi/well [^{3}H]TdR (thymidine, [methyl-^{3}H]), ICN Pharmaceuticals Inc., Irvine, CA). The cells were harvested and the radioactivity incorporated was counted by a liquid scintillation counter. All counts/min values shown were the mean of triplicate samples ± SD. Statistical analysis was carried out by Student's *t*-test. ConA or LPS was used as positive control [18,19]. An MTT assay was performed to evaluate the cytotoxicity of the compounds. Splenocytes were cultured in a 96-well plate at 4×10^5 cells/180 μl/well in a humidified CO_2 incubator at 37°C for 48 h in the presence or absence of various concentrations of tested compounds. 18 μl of 5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was added to each well for the final 5 h. Then 90 μl of lysis buffer (10% SDS, 50% DMF, pH 7.2) was added to each well for 6–7 h and the OD_{570} values were read by a microplate reader (Bio-Rad, Model 550).

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