

## Complete NMR Assignments of the Antibacterial Biflavonoid GB1 from *Garcinia kola*

Quan-Bin HAN,<sup>a</sup> Song-Fong LEE,<sup>b</sup> Chun-Feng QIAO,<sup>a</sup> Zhen-Dan HE,<sup>a</sup> Jing-Zheng SONG,<sup>a</sup> Han-Dong SUN,<sup>c</sup> and Hong-Xi XU<sup>\*a</sup>

<sup>a</sup> Chinese Medicine Laboratory, Hong Kong Jockey Club Institute of Chinese Medicine, Hong Kong, P. R. China;

<sup>b</sup> Department of Applied Oral Sciences, Faculty of Dentistry, Dalhousie University, Halifax, Nova Scotia, Canada B3H 3J5; and <sup>c</sup> State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, Yunnan, P. R. China.

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**From the antibacterial fraction of the roots of *Garcinia kola*, 3'',4',4''',5,5'',7,7''-heptahydroxy-3,8''-biflavanone (GB1) was isolated as the major constituent, whose interesting conformations were studied on the basis of its 1D and 2D NMR spectra obtained at different temperatures and in different solvents. GB1 showed antibacterial activities against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *enterococci* (VRE) with MIC of 32 and 128 µg/ml, respectively.**

**Key words** *Garcinia kola*; biflavonoid; GB1; antibacterial

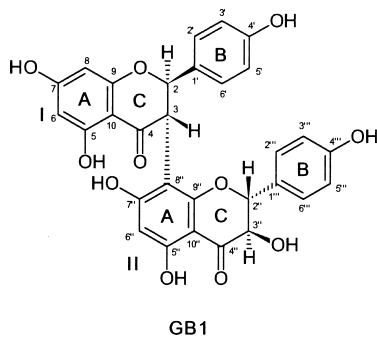
The nut of *Garcinia kola* HECKEL, native to Nigeria and Ghana, is chewed together with the kola nut by the local people before each meal to promote digestion, and is therefore called the false kola nut. It is believed to improve the flavor of anything eaten after it and even to render putrid water palatable. The beneficial effects of chewing this nut are generally thought to include the mechanical cleansing effect and anti-microbial substances in the seeds. During the initial screening of 10 common Nigerian chewing sticks for antibacterial activity, we noted the methanol extract of *Garcinia kola* displayed good activity.<sup>1)</sup> Previous studies indicated that water-soluble polyphenolic compounds may be responsible for the anti-bacterial activity of *G. kola*.<sup>2,3)</sup> However, we reported that the active components could be fractionated into the ether fraction, suggesting that a different class of compounds may be responsible for the activity.<sup>1)</sup> Many phytochemical studies have revealed that biflavonoids are the major constituents of *G. kola*, and GB1 is one of the major biflavonoids.<sup>4–12)</sup> In this study, a biflavonoid was isolated as the major component of the active fraction, which showed inhibitory effects against methicillin-resistant *Staph. aureus* (MRSA), and vancomycin-resistant *enterococci* (VRE).

However, it is difficult to identify the chemical structure of this biflavonoid due to the lack of sufficient reference data. This compound exhibits two sets of signals in its nuclear magnetic resonance (NMR) spectra at room temperature, which commonly leads to the conclusion of mixed compounds. As a single compound, it presented one spot or one

peak in various TLC and HPLC tests. The biflavonoids always present substantial spectral complexity at the dimeric level due to hindered rotation between the flavanone and the flavanonol moieties around the C-3/C-8'' axis.<sup>4–12)</sup> The confusing signals due to its rotameric behavior (atropisomerism) collapse with increasing rotation around 3/8'' bond at higher temperature like 100 °C to a single set of signals.<sup>13)</sup> This phenomenon indicated a characteristic of this kind of 3,8''-linked biflavonoids.<sup>14)</sup> We compared its NMR data obtained at different temperatures including –50 °C, –30 °C, 21 °C, 70 °C, and 90 °C, and in different solvents such as acetone, pyridine, and dimethyl sulfoxide (DMSO). The results support its complicated conformations due to hindered rotation. From these analyses, we have identified this biflavonoid to be GB1 on the basis of its NMR data which was assigned ambiguously by two dimensional (2D) NMR spectra obtained at 90 °C in DMSO-*d*<sub>6</sub>. This is the first report of its complete assignment of its NMR data. In this paper, we wish to report the NMR analysis of its interesting rotameric behavior, the complete data assignment, and the related bioassays.

### Results and Discussion

Compound **1**, obtained as a faint brown powder, showed the [M+H]<sup>+</sup> ion at *m/z* 559 in its electrospray ionization mass spectroscopy (ESI-MS) corresponding to the molecular formula C<sub>30</sub>H<sub>22</sub>O<sub>11</sub>. It was identified as 3'',4',4''',5,5'',7,7''-heptahydroxy-3,8''-biflavanone (GB1) by <sup>1</sup>H-, <sup>13</sup>C-, and 2D NMR data recorded at 90 °C in DMSO-*d*<sub>6</sub>. The <sup>1</sup>H-NMR spectra recorded at 90 °C showed four signals for five exchangeable OH protons, two at low field with δ=12.08 and 11.62, two in the range from δ=9.20 to 8.80 and one upfield at δ=5.94 (Table 1). The signals for two other OH protons recorded at 21 °C in the range from δ=11.20 to 10.50 disappeared at 90 °C. Based on the <sup>1</sup>H–<sup>1</sup>H correlation spectroscopy (COSY), and the heteronuclear multiple bond connectivity (HMBC) spectra and the coupling constants, the four doublets for eight aromatic protons at δ=7.16, 7.07, 6.75, and 6.69 for one proton each with *J*=8.4 Hz were assigned to H-2''/6''', H-2'/6'', H-3''/5''', and H-3'/5'', respectively. The doublet for two protons at δ=5.91 (*J*=2.0 Hz)



\* To whom correspondence should be addressed. e-mail: xuhongxi@hkjicm.org

Table 1. <sup>1</sup>H- and <sup>13</sup>C-NMR Data of **1** at 21 °C and 90 °C in DMSO-*d*<sub>6</sub>, δ in ppm, *J* in Hz

	δ at 21 °C		δ at 90 °C	
	δ <sub>H</sub>	δ <sub>C</sub>	δ <sub>H</sub>	δ <sub>C</sub>
2	5.63, d, 11.2	81.8	5.54, d, 11.2	81.2
	5.30, d, 11.2	81.2		
3	4.64, d, 11.2	47.4	4.53, d, 11.2	47.3
	4.41, d, 11.2			
4		196.8		195.9
		196.4		
5 OH	12.28, s	163.7	12.08, s	163.3
	12.20, s	163.9		
6	5.95, s	96.2	5.91, d, 2.0	95.8
	5.89, <sup>a</sup> s			
7 OH	11.20—10.50 <sup>c</sup>	166.5	—	165.9
		166.4		
8	5.89, <sup>a</sup> s	95.9	5.91, d, 2.0	95.5
	5.85, <sup>a</sup> s	95.4		
9		162.8		162.4
		162.7		
10		101.2		101.1
1'		128.2		127.9
		127.6		
2'/6'	7.09, d, 7.6	129.1	7.07, d, 8.4	128.1
		128.0		
3'/5'	6.74, d, 7.6	114.8	6.69, d, 8.4	114.5
	6.64, d, 7.6	115.0		
4' OH	9.61, s	157.6 <sup>b</sup>	8.80—9.20 <sup>b</sup>	157.2
	9.51, s	157.4		
2''	5.13, d, 11.2	82.6	5.01, d, 11.2	82.4
	4.96, d, 11.2			
3''	4.21, d, 11.2	72.4	4.43, d, 11.2	71.7
	3.99, d, 11.2	72.0		
3'' OH	5.95, s		5.94, s	
4''		197.5		196.6
5'' OH	11.84, s	161.8	11.62, s	161.7
	11.72, s	162.3		
6''	5.80, s	95.1	5.83, s	94.6
	5.76, s			
7'' OH	11.20—10.50 <sup>c</sup>	165.1	—	164.4
		164.6		
8''		101.4		101.2
9''		159.4		159.5
		160.1		
10''		100.3		99.8
		99.8		
1'''		128.8		127.2
		127.8		
2'''/6'''	7.16, d, 7.6	129.0	7.16, d, 8.4	128.3
		128.4		
3'''/5'''	6.83, d, 7.6	114.8	6.75, d, 8.4	114.5
	6.64, d, 7.6	115.0		
4''' OH	9.59, s	157.8 <sup>b</sup>	8.80—9.20 <sup>d</sup>	157.2
	9.47, s			

*a, b*) Signals exchangeable, *c, d*) signals overlapped, — signals not exhibited.

was accordingly located at H-6, and H-8. The proton H-6'' exhibited a singlet at δ=5.83. In the same way, the remaining four doublets at δ=5.54, 5.01, 4.53, and 4.43 for one proton each with *J*=11.2 Hz were indicated to be H-2, H-2'', H-3, H-3'', respectively.

In the <sup>13</sup>C-NMR spectrum, ten indicative lowfield signals out of twenty-six were assigned to two carbonyl carbons, six hydroxylated carbons, and two aromatic carbons bearing oxygen-bridge to the pyran rings of the benzopyran moieties. Similarly, the signals at δ=82.4, 81.2, 71.7, and 47.3 were assigned to C-2'', C-2, C-3'', and C-3, respectively. Although

its absolute configuration was recently revised,<sup>14</sup>) the complete <sup>1</sup>H- and <sup>13</sup>C-NMR data assignments have not yet been reported.<sup>4–12</sup>) This has been achieved on the basis of the <sup>1</sup>H-detected heteronuclear multiple quantum coherence (HMQC), HMBC, and the rotating frame Overhauser enhancement spectroscopy (ROESY) spectra. The NMR spectra obtained at 21 °C which seemed like two confusing sets of biflavonoids, were also assigned for the first time by 2D NMR analyses and comparison with those of GB2 recently available.<sup>15</sup>) In the ROESY at 21 °C, the key OH-5 of both conformations showed nuclear Overhauser enhancement (NOEs) with H-2''', and 6'''. This observation suggested that its conformation at 21 °C was not a simple mixture of two extremely characteristic conformations. It should be a dynamic system. The ROESY spectrum recorded a general NOE presence occurring at 21 °C during the whole measuring time.

The NMR spectra were also measured at 70 °C. It was found that the spectra recorded at 70 °C were same as those at 90 °C. The single set of signals exhibited at 90 °C would change back to those confusing data when this compound was measured again at room temperature. This suggested that its NMR spectra were associated closely with the temperature at which it was measured. Along with the rise of temperature, the hindered rotation would be faster and faster. When the temperature was beyond 70 °C, the rotation speed was too high to present any relatively stable conformation, and the spectral complexity at the dimeric level therefore disappeared. For the same reason, all of the NOEs disappeared at 90 °C except those between H-2, 3 with H-2', 6', and H-2'', 3'' with H-2''', 6'''.<sup>15</sup>) The single set of signals appearing accordingly should be the general exhibition of the conformations produced by the hindered rotation, different from any of those two sets of signals revealed at 21 °C.

Furthermore, it could be deduced that the hindered rotation would be slower and slower along with the fall of temperature. At a certain low temperature, the rotation speed was low enough to produce a relatively stable conformation, which could also lead to a single set of signals corresponding to one of those two conformations at 21 °C. This compound was further measured at -50 °C, and -30 °C to observe the change of its spectra at low temperature. As a result, the two sets of signals in 1:1 ratio observed at 21 °C changed to those in 1:0.3 at -30 °C, and even to an almost single set of signals at -50 °C (Fig. 1).

The above results supported the close correlation of its NMR behavior with temperature. Additionally, the effects of solvents were also investigated. At 21 °C, the <sup>13</sup>C-NMR spectra were measured in DMSO-*d*<sub>6</sub>, and acetone-*d*<sub>6</sub>, respectively. The spectral complexity at the dimeric level was observed in both measurements. At 90 °C in Pyridine-*d*<sub>6</sub>, the <sup>13</sup>C-NMR spectra were also a single set of signals. These observations suggested that the solvents had no significant effect on its rotameric behavior.

As the major constituent of the antibacterial fraction,<sup>1</sup>) GB1 was tested for its inhibitory effects against MRSA, and VRE, especially. This compound displayed antibacterial effects with minimum inhibitory concentration (MIC) of 32, and 128 μg/ml, respectively.

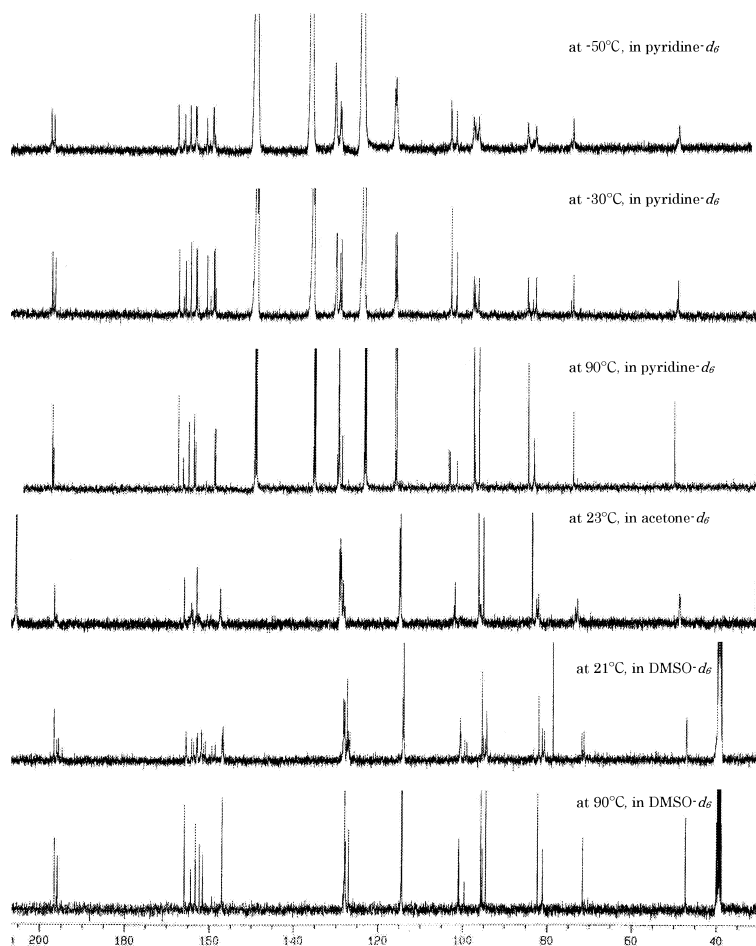


Fig. 1. The  $^{13}\text{C}$ -NMR Spectra Recorded at Different Temperatures and in Different Solvents

### Experimental

**General Procedures** ESI-MS was recorded on a VG Auto Spec-3000 spectrometer. 1D- and 2D-NMR spectra were run on a Bruker AM-400 and a DRX-500 instrument with TMS as internal standard, respectively.

**Plant Material** The dried roots of *G. kola* were obtained at a local market in Lagos, Nigeria and authenticated by Dr. A. O. Adeoye, Department of Pharmacognosy, School of Pharmacy, College of Medicine, the University of Lagos, Lagos, Nigeria. A specimen (No LUH230) was deposited at the Lagos University Herbarium, Lagos, Nigeria.

**Extraction and Isolation** *G. kola* (30 g) was chopped into pieces and refluxed with methanol (150 ml) for  $2 \times 2$  h. The methanol extract was concentrated and successively extracted with hexane ( $3 \times 150$  ml), chloroform ( $3 \times 150$  ml), ether ( $5 \times 150$  ml), and *n*-butanol ( $3 \times 150$  ml). The ether extract containing the active anti-bacterial principal was subjected to silica gel column chromatography eluting with chloroform containing increasing concentration of methanol (5–20%, v/v). GB1 (100 mg) was isolated from the chloroform/methanol 9 : 1 fraction by preparative thin layer chromatography on silica gel using the solvent system of chloroform/methanol (9 : 1, v/v).

**Antibacterial Assays** The agar-diffusion method was used as an antibacterial screening test. Dried samples were dissolved in DMSO and 25  $\mu\text{l}$  aliquots were applied to wells on Mueller Hinton agar plates (BBL, Becton Dickinson and Co., Cockeysville, MD, U.S.A.), which have been inoculated with tested bacteria according to the standard protocol described by the National Committee of Clinical Laboratory Standards (1993, Document M7-A3, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically). The plates were incubated at 35 °C and the diameter of the inhibition zone was measured after 24 h. DMSO without test compound served as controls and no inhibition was observed.

The minimum inhibitory concentration (MIC) was determined by the macrobroth dilution assay (National Committee of Clinical Laboratory Standards, 1993). Briefly, stock solutions (5.12 mg/ml) of compounds in DMSO were two-fold diluted in 0.5 ml of Mueller Hinton broth. To these broths, 0.5 ml of bacterial suspensions (106 superscript 6 cfu/ml) was added. The

tubes were incubated at 35 °C for 24 h. MIC was determined as the lowest concentration of the compound completely inhibited the growth of the test organism.

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