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Effective assignment of positional isomers in dimeric shikonin and its analogs by ^{1}H NMR spectroscopy

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ABSTRACT

An approach for distinguishing two types of positional isomers of dimeric shikonin and its analogs was explored with $^{4}J_{C,H}$ long-range correlation by prolonging the acquisition time at $^{2,3}J_{C,H}$ values of 2.0 and 8.0 Hz. Furthermore, the ^{1}H (proton) nuclear magnetic resonance (NMR) pattern of phenolic hydroxyl protons was developed as a "diagnosis signal" to ascertain the relative location of each side chain in DMSO–d_6 at sample concentrations of 0.022–0.034 mol/L. The chemical shift differences of 0.6 ppm between OH-5′ and OH-1 and between OH-8′ and OH-4 are assigned to Type A and Type B, respectively. All reported ambiguous structures were corrected by this pattern. Additionally, the steric structures of isolated compounds were elucidated by quantum chemical calculations of electronic circular dichroism (ECD) spectra.

Hydroxyl naphthoquinones, such as alkannin, shikonin and their derivatives, naturally occur in Boraginaceae, are the characteristic and high-content constituents that are generally regarded as an index for quality control of the traditional Chinese medicine "Zicao" [1–3]. In the last decade, their dimers were isolated from *Arnebia euchroma* [4,5], *Lithospermum erythrorhizon* [6] and *Onosma paniculatum* [7]. Some of them exhibited synergistic antibacterial, neuraminidase inhibitory and antitumor activities. Structurally, their common skeleton feature is the existence of a dibenzo[*b*,*h*]fluorene unit bearing two alkyl chains. The presence of 18 continuous quaternary carbons in the dibenzo[*b*,*h*]fluorene unit makes their structural determination difficult by $^{2}J_{C,H}$ and $^{3}J_{C,H}$ correlations in heteronuclear multiple bond correlation (HMBC) experiments, especially, the substitutive position of the isohexenyl side chain.

The first two dimers, shikometabolins A and B (Fig. 1), were reported by Meselhy *et al.* in 1994 and were acquired through biotransformation of shikonin by human intestinal bacteria [8–11]. Based on sufficient sample amounts, multiple nuclear magnetic resonance (NMR) technologies, such as two-dimensional (2D) incredible natural abundance double quantum transfer experiment (INADEQUATE) [12] and heteronuclear Overhauser effect spectroscopy (HOESY) [13] experiment, were used to ascertain the conjunctive ordering of carbons and the position of side chains in the linkage area: in shikometabolin A, two side chains were determined to behave in the opposite orientation, while in shikometabolin B, two side chains behaved the same. We defined them as Type A and Type B, respectively. Incredibly, since 1994, the isolated hydroxyl naphthoquinone dimers from natural products [4–7] have all been determined to be Type B through the comparison of NMR data and without the help of 2D INADEQUATE and HOESY experiments. To our knowledge, the 2D INADEQUATE experiment is time-consuming and requires large sample amounts because of its naturally low sensitivity. Therefore, it is not suitable for trace amounts of natural products. On the other hand, NMR comparison, widely used in the structure determination for structurally related compounds, is responsible for numerous structural misassignments [14–16]. These problems inspired us to investigate thoroughly for a simple and effective method to elucidate the structures of these dimers.

As a continuation of our efforts to explore hydroxyl naphthoquinones from the roots of *A. euchroma*, 17 dimeric hydroxyl naphthoquinones (Fig. 1, **1a**–**13**) were isolated by various column chromatography methods. Due to their extremely similar NMR data of the dibenzo[*b*,*h*]fluorene unit, the most difficult problem encountered in their structure determination was how to distinguish whether they belong to Type A or Type B without using INADEQUATE and HOESY experiments due to the limited sample amount. A detailed and comprehensive literature survey disclosed a method by diminishing the *J* value ($^{2,3}J_{C,H}$) to 4.0 Hz

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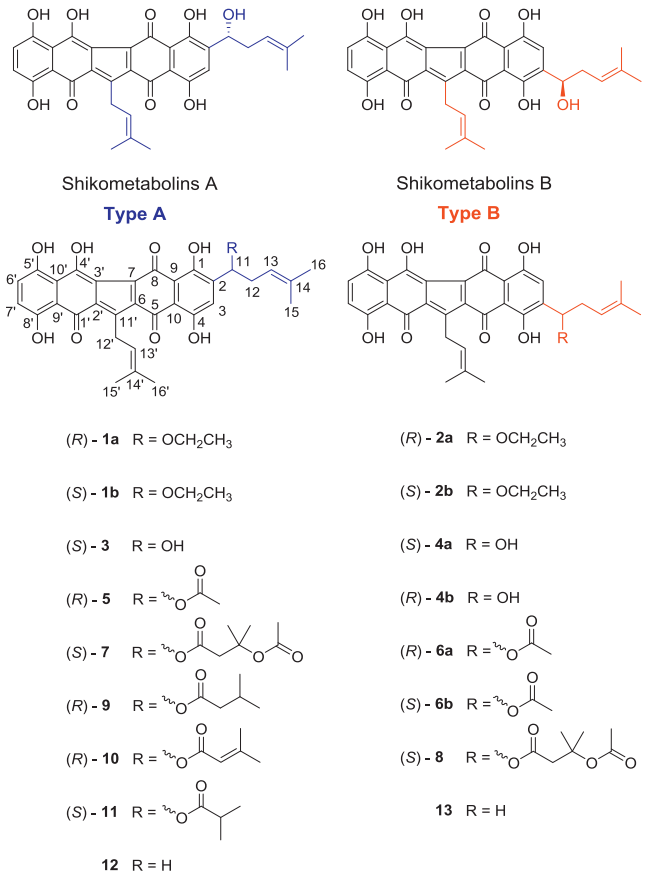

Fig. 1. Shikometabolins A and B together with **1a**–**13** isolated from *A. euchroma*.

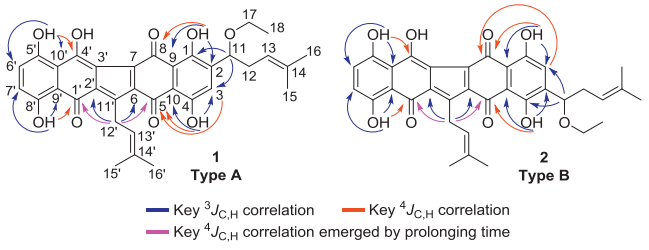

Fig. 2. The key $^{3}J_{C,H}$ and $^{4}J_{C,H}$ correlations in **1** and **2**.

for a long-range correlation to bring out the oxygen-interval cross-peaks [17,18]. In addition, in our previous experiments directed toward polyacetylene glycosides [19], which featured two or more conjugated acetylenic and olefinic bonds, four-bond or even longer-range couplings in a large conjugate system were detected and strengthened as the acquisition time increased. These phenomena inspired us to determine the effect of some parameters, such as the $^{2,3}J_{C,H}$ and acquisition time, on the four-bond ^{1}H-^{13}C correlations in the HMBC experiment.

Taking **2** as an example, a series of HMBC spectra for **2** at different $^{2,3}J_{C,H}$ values (1.0, 2.0, 4.0, 8.0 and 12.0 Hz) combined with its time-divisional intercepts (every 30 min for 180 min) were recorded at a sample concentration of 0.022 mol/L (Figs. S5–S25 in Supporting information). When the acquisition time was 30 min, two- or three-bond ^{1}H-^{13}C correlations (blue in Fig. 2) and some four-bond ^{1}H-^{13}C correlations (red in Fig. 2) were observed at $^{2,3}J_{C,H}$ values of 1.0, 2.0, 4.0, 8.0 and 12.0 Hz. Surprisingly, when the acquisition time proceeded to 150 min at the setting $^{2,3}J_{C,H}$ value of 8.0 Hz, two key four-bond ^{1}H-^{13}C correlations from H_2-12′ (δ_H 4.15) to C-1′ (δ_C 186.8) and C-5 (δ_C 187.1) appeared, as shown in Fig. 2 (bright pink) (Fig. S19). Coupling with the correlation of H-2 (δ_H 7.04) to C-8 (δ_C 183.0) and other conventional HMBC signals, the relative position of two alkyl chains in **2** was doubtlessly attributed to the same side, as depicted in Fig. 2 (Type B). Similarly, for **1**, the key four-bond ^{1}H-^{13}C correlations from H_2-12′ (δ_H 4.16) to C-1′ (δ_C 186.9) and C-5 (δ_C 186.6) were also observed at the $^{2,3}J_{C,H}$ value of 8.0 Hz in the extended acquisition time (Figs. S36 and S37 in Supporting information). Together with the cross-peak of H-3 (δ_H 7.03) to C-5 and other conventional signals in the HMBC spectrum, the two side chains of **1** were classified on the opposite side, as depicted in Fig. 2 (Type A). Additionally, the HMBC correlations of H_2-12′ with two "silent" quaternary carbons (C-3′ and C-7) were observed at a $^{2,3}J_{C,H}$ value of 2.0 Hz in **2** (Fig. S12). Therefore, 18 continuous quaternary carbons in the dibenzo[*b*,*h*]fluorene unit were accurately assigned. Detailed assignments of the ^{1}H and ^{13}C NMR spectra for **1** and **2** are presented (Tables S7–S10 in Supporting information).

Further careful analysis of the ^{1}H NMR data of **1** and **2** in DMSO–d_6 revealed an interesting phenomenon. The four phenolic hydroxyl proton signals of **1** appeared at δ_H 14.00 (OH-5′), 14.60 (OH-1), 13.72 (OH-8′) and 13.72 (OH-4), while the corresponding signals of **2** appeared at δ_H 13.97 (OH-5′), 13.99 (OH-1), 13.73 (OH-8′) and 14.29 (OH-4). Obviously, when there was no isohexenyl chain adjacent, two active protons were displayed at approximately δ_H 14.0, such as OH-5′ and OH-1, in **2**, while they were nearly at δ_H 13.7, such as OH-8′ and OH-4, in **1**. Notably, the existence of an isohexenyl chain was found to increase the chemical shift of the adjacent phenolic hydroxyl proton by approximately 0.6 ppm. For instance, the chemical shift of OH-1 changed from approximately δ_H 14.0 to δ_H 14.6 compared with OH-5′ in **1**; the chemical shift of OH-4 changed from approximately δ_H 13.7 to δ_H 14.3 compared with OH-8′ in **2** (Fig. 3). The ^{1}H NMR patterns and the chemical shift differences (0.6 ppm) of phenolic hydroxyl protons can be recognized as a "diagnosis signal" for the series of dimeric hydroxyl naphthoquinones to ascertain the relative location of side chains. Subsequently, other dimers (**3**–**13**) were studied carefully, and their ^{1}H NMR data follow the above pattern: **3**, **5**, **7**, **9**, **10**, **11** and **12** belong to Type A; **4**, **6**, **8** and **13** belong to Type B (Fig. 1, Table 1).

Notably, in DMSO–d_6, the ^{1}H NMR signals of OH-5′ and OH-1 of shikometabolins A and B in Meselhy's data [8] were evidently at a higher field than the corresponding data of **3** and **4**, while the chemical shifts of OH-8′ and OH-4 were almost identical (Table 1). After clarifying that purification processes (involving acids or bases) and the content of D_2O in DMSO–d_6 do not have a significant effect on the chemical shifts of phenolic hydroxyl protons (Figs. S26–S28 in Supporting information), the sample concentration, a factor affecting intramolecular hydrogen bonding, was

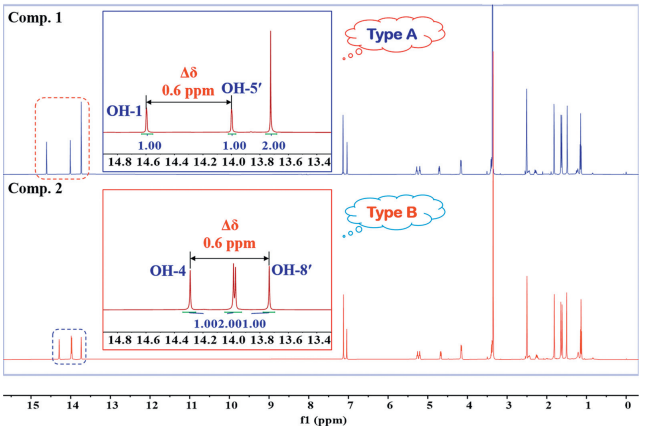

Fig. 3. The characteristic "diagnosis signal" in ^{1}H NMR spectra for **1** and **2**.

Table 1
The ^{1}H NMR chemical shifts of phenolic hydroxyl protons from **1** to **13**[a], and shiko. A and B[b] in DMSO–d_6 (δ in ppm).

Type A compound	Position of phenolic hydroxyl				Type B compound	Position of phenolic hydroxyl			
	5′	1	8′	4		5′	1	8′	4
1	14.00	14.60	13.72	13.72	**2**	13.97	13.99	13.73	14.29
3	13.99	14.60	13.73	13.74	**4**	13.97	13.98	13.74	14.32
5	14.00	14.67	13.71	13.70	**6**	13.97	13.97	13.71	14.33
7	14.00	14.69	13.69	13.69	**8**	13.99	13.99	13.71	14.34
9	14.02	14.69	13.70	13.69					
10	13.99	14.66	13.71	13.69					
11	13.97	14.64	13.70	13.69					
12	14.04	14.61	13.74	13.76	**13**	14.07	14.07	13.74	14.28
Shiko. A	13.54	14.21	13.77	13.79	Shiko. B	13.76	13.76	13.80	14.34

[a] 500 MHz.
[b] Namely shikometabolin reported by Meselhy (400 MHz).

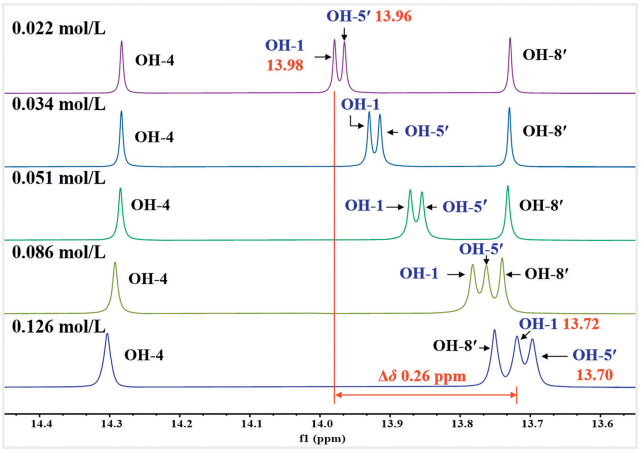

Fig. 4. Effect of concentration factors on ^{1}H NMR patterns of phenolic hydroxyl protons for **2** in DMSO–d_6.

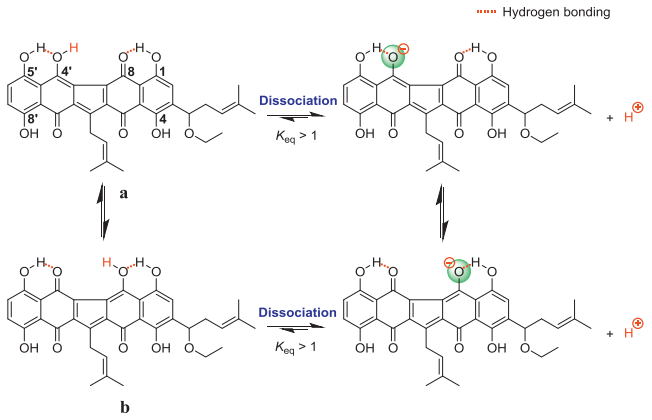

Fig. 5. Possible dissociation equilibrium for **2** affects the strength of intramolecular hydrogen bonds in DMSO–d_6.

tested in focus. Using **2** as a model compound, its ^{1}H NMR spectra at different concentrations from 0.022 mol/L to 0.126 mol/L in DMSO–d_6 were recorded (Fig. 4). Amazingly, the peaks of OH-5′ and OH-1 gradually shifted to higher fields as the concentration increased and were in general agreement with the reported values at a sample concentration of 0.126 mol/L, whereas OH-8′ and OH-4 only slightly displaced to low fields. Generally, when the solution is diluted, the antimagnetic shift of the proton signal with hydrogen-bond interactions occurs due to the severing or weakening of intermolecular hydrogen bonds. However, the anomalous low-field shifts of OH-5′ and OH-1 can be explained by the fact that the phenolic hydroxyl groups at C-4′ or C-8 are more easily dissociated in dilute solution (Fig. 5), resulting in stronger hydrogen bonding to OH-5′ or OH-1. Similarly, the same phenomenon was observed in the concentration factor experiments for **1** (Fig. S29 in Supporting information). Therefore, as a "diagnosis signal" for this type of compound, the recommended sample concentration ranges from 0.022 mol/L to 0.034 mol/L.

Additionally, to elucidate whether the "diagnosis signal" applies to other deuterated solvents used in previous literature [4–7], the NMR spectra of **1**–**8** in acetone-d_6 and **1**, **2** in pyridine-d_5 were recorded. Their ^{1}H NMR data were cautiously assigned (Tables S11–S13 in Supporting information). Although the chemical shifts of the corresponding phenolic hydroxyl protons in acetone-d_6 and pyridine-d_5 have certain differences, especially at OH-5′ and OH-1, the expected $\Delta\delta$ pattern (0.6 ppm) still exists.

Based on the above NMR rules, all sixteen reported dimers [4–7], except for shikometabolins A and B [8], were carefully verified. The NMR data of nine "plausible" compounds were summarized (Table S14 in Supporting information), including three cases of inaccurate assignments and six cases of inaccurate side chain positions. The structural accuracy of the remaining seven compounds reported in the patent (Type B) cannot be judged due to the absence of NMR data.

Finally, in consideration of the chirality of C-11 of these dimers, their chiral resolutions were executed by high performance liquid chromatography (HPLC) experiments with normal-phase chiral columns (Figs. S1–S4 in Supporting information), and their absolute configurations were determined by experimental and calculated electronic circular dichroism (ECD) spectra [20,21]. Compounds **2**, **4**, **6** and **8** (Type B) gave (*R*)–**2a**, (*S*)–**2b**, (*S*)–**4a**, (*R*)–**4b**, (*R*)–**6a**, (*S*)–**6b** and (*S*)–**8**, while A-type compounds gave (*R*)–**1a**, (*S*)–**1b**, (*S*)–**3**, (*R*)–**5**, (*S*)–**7**, (*R*)–**9**, (*R*)–**10**, and (*S*)–**11** (Figs. S30–S32 in Supporting information).

In conclusion, the structures of dimeric shikonin and its analogs were more conveniently determined through four-bond ^{1}H-^{13}C correlations in HMBC spectra at $^{2,3}J_{C,H}$ values of 2.0 and 8.0 Hz. Furthermore, the characteristic $\Delta\delta$ of phenolic hydroxyl protons in ^{1}H NMR spectra caused by the adjacent side chain was summarized. Notably, this ^{1}H NMR pattern varies with sample concentration, so the sample concentration needs to be limited when this rule is applied. This is a simple and effective method to elucidate the structures of these dimers. It provides a valuable reference scheme for the structural identification of organic molecules in which several continuous quaternary carbons exist.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2023.108863.

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