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Variable-temperature ^{1}H-NMR Studies on Three C-glycosylflavones Exhibiting Rotational Isomerism

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Abstract: Rotational isomerism has been observed on some C-glycosyl flavonoids with special chemical strutures. It may bring rotamers arising from restricted rotation about one single bond. C-glycosyl flavonoids showed a wide range of biological activities. This emphasizes the significance to understand the conformational stability of C-glycosyl flavonoids in drug development. Three C-glycosyl flavonoids, vaccarin (1), isovitexin-2”-O-arabinoside (2) and spinosin (3), were found to exist as rotamers in the light of their featured behavior in nuclear magnetic resonance spectroscopy (NMR). Some separated signals were observed in their ^{1}H-NMR and ^{13}C NMR spectra. Variable-temperature (VT) ^{1}H-NMR experiments were then conducted to study the observation and to determine their rotational energy barriers and then evaluate their conformational stability. The temperature varied from 298K to 363K and separated signals merged at high temperature. With the help of Eyring equation, the rotational energy barriers of compounds 1-3 were calculated and found much lower than 24 kcal/mol. When the energy barrier is high enough (> 24 kcal/mol) and the time scale for interconversion (halflife > 1000 s) is long enough for isolation of individual rotamers, the isomers are termed atropisomers, which thus will display axial charity. According to this standard, the compounds 1-3 may not exhibit atropisomerism and isolation of rotamers should be difficult. Their relatively low energy barriers indicate they can be developed as rapidly equilibrating mixture.

Keywords: Rotamer, Atropisomer, Rotational Isomerism, C-glycosyl Flavonoids, VT-NMR

1. Introduction

Rotational isomer or rotamer is one of a set of conformers arising from restricted rotation about one single bond, which is due to the presence of a sufficiently large rotational barrier to make the phenomenon observable on the time scale of the experiment. When the energy barrier is high enough (> 24 kcal/mol) and the time scale for interconversion (halflife > 1000 s) is long enough for isolation of individual rotamers, the isomers are termed atropisomers, which thus will display axial charity [1-3].

Rotational isomerism has been observed on a portion of C-glycosyl flavonoids. Nuclear magnetic resonance spectroscopy (NMR) is the technique that has contributed most to the understanding of this phenomenon. The signals in ^{1}H and ^{13}C NMR spectra unexpectedly separate in low temperature but duplication collapses in high temperature, which indicates the existence of rotational isomers [4].

C-glycosyl flavonoids showed a wide range of biological activities and had higher antioxidant and anti-diabetes potential than their corresponding O-glycosyl flavonoids and aglycones [5]. It has become clear that in many cases the majority of activity (toward a desired target) belongs to one rotational isomer, while the other contributes very little [6, 7]. This emphasizes the significance to understand the conformational stability of C-glycosyl flavonoids in drug development. The stability of rotamers is often evaluated by kinetics measurements that include various physical terms such as the rate constant (k), half-life ($t_{1/2}$), and energy barrier ($\Delta G^{\ddagger}$) to rotation [3].

Our previous phytochemical studies on *Vaccaria hispanica* (Miller) Rauschert isolated two C-glycosyl flavonoids, vaccarin (1) and isovitexin-2”-O-arabinoside (2) (Figure 1), both of which exhibited rotational isomerism and existed as rotamers [8]. However, it is still unclear whether the rotational barrier is high enough for atropisomerism to be realized or not.

The conformational stability of these molecules needs to be analyzed. In this paper, we are aimed to determine their rotational energy barriers and then evaluate their conformational stability through variable-temperature (VT) ^{1}H-NMR experiments. In order to research the effect of the C-7 substituents, commercial spinosin (3) was purchased and subjected to the experiments, as well.

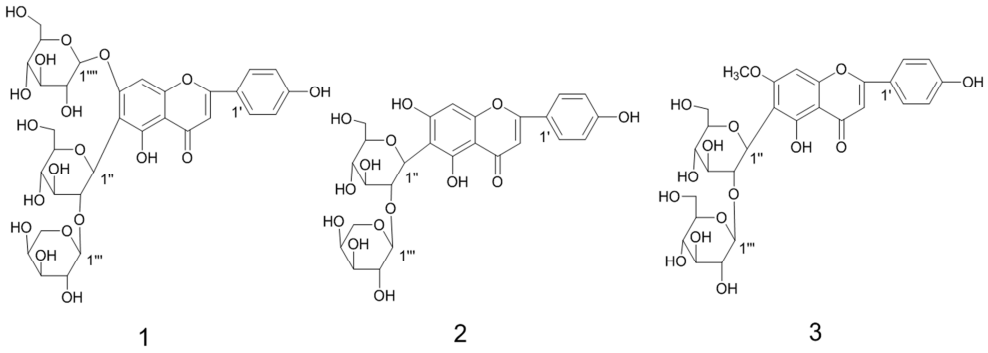

Figure 1. *Structures of compounds 1-3.*

2. Results and Discussion

C-glycosyl flavonoids 1-3 were subjected to ^{1}H-NMR and ^{13}C-NMR experiments at room temperature. Duplicated signals of a part of nucleus appeared in both spectra. Variable-temperature (VT) ^{1}H-NMR experiments were then conducted from 298 K to 363 K. It turned out that the separated signals coalesced at higher temperature (Figure 2). This feature suggested compounds 1-3 existed as rotational isomers at room temperature, which should be caused by the restricted rotation around the C (sp^3)–C (sp^2) glucosyl-flavone linkage according to [4].

With the 5-OH signals as markers, the ^{1}H-NMR spectrum of vaccarin (1) in DMSO-d_6 at 298 K showed that the relative proportion of the major and minor rotamers was 1.336: 1. The two rotamers of vaccarin (1) were independently detected because they were in relatively slow interconverting. At higher temperature, the signals for the two rotamers moved even closer but were still detected. At last, the two signals coalesced to a single peak at δ 13.55 ppm at 353 K, namely at coalescence temperature, T_c (Figure 2). Actually, the other separated signals also coalesced, some of which coalesced at lower temperatures, because their smaller chemical shift difference.

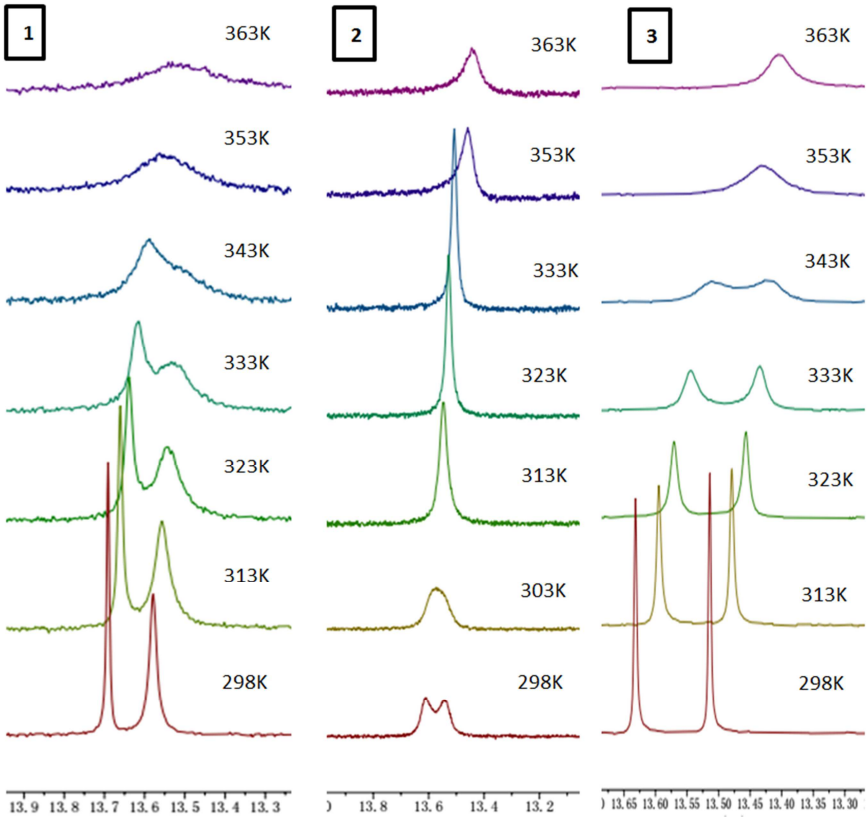

Figure 2. *The 5-OH signal markers in the ^{1}H NMR spectra of compounds 1-3 at different temperatures.*

The free energy of activation for the interconversion between the two unequally populated rotamers of vaccarin (1) can be calculated using Eyring's equations (1 and 2) as modified by Shanan-Atidi and Bar-Eli [9, 10]:

$$\Delta G^{\ddagger}_{B} = 4.57T_c \{10.62 + \log [X/2\pi (1 - \Delta P)] + \log (T_c/ \Delta\nu)\} \quad (1)$$

$$\Delta G^{\ddagger}_{B} = 4.57T_c \{10.62 + \log [X/2\pi (1 + \Delta P)] + \log (T_c/ \Delta\nu)\} \quad (2)$$

where $X = 2\pi\tau\Delta\nu$ and $\Delta P = P_A - P_B$

P_A and P_B mean the respective population of the conformers A and B ($P_A > P_B$, $P_A + P_B = 1$). τ is the mean lifetime. T_c means the coalescence temperature and $\Delta\nu$ is the chemical shift difference between conformers A and B. X is obtained using equation (3):

$$P_A - P_B = \Delta P = [(X^2 - 2)/3]^{3/2}*1/X \quad (3)$$

From the ^{1}H-NMR spectrum of vaccarin (**1**) at 298 K, the frequency difference, $\Delta\nu$, between the 5-OH signals was 44.7 Hz. The coalescence temperature, T_c, was 353 K.

$$\Delta P = (1.336 - 1)/2.336 = [(X^2 - 2)/3]^{3/2}*1/X$$

$$X = 1.793$$

$$\Delta G^{\ddagger}_{A} = 4.57*353* \{10.62 + \log [1.793/2\pi (1 - 0.1438)] + \log (353/44.7)\} = 17.8 \text{ kcal/mol}$$

$$\Delta G^{\ddagger}_{B} = 4.57*353* \{10.62 + \log [1.793/2\pi (1 + 0.1438)] + \log (353/44.7)\} = 17.6 \text{ kcal/mol}$$

The ^{1}H-NMR spectrum of isovitexin-2"-O-arabinoside (2) in DMSO-d_6 at 298 K showed that the relative proportion of the major and minor rotamers was 1.018: 1, using the 5-OH signals as markers. The frequency difference, $\Delta\nu$, between the 5-OH signals was 27.96 Hz. The coalescence temperature, T_c, was 303 K.

$$\Delta P = (1.018 - 1)/2.018 = [(X^2 - 2)/3]^{3/2}*1/X$$

$$X = 1.472$$

$$\Delta G^{\ddagger}_{A} = 4.57*303* \{10.62 + \log [1.472/2\pi (1 - 0.009)] + \log (303/27.96)\} = 15.3 \text{ kcal/mol}$$

$$\Delta G^{\ddagger}_{B} = 4.57*303* \{10.62 + \log [1.472/2\pi (1 + 0.009)] + \log (303/27.96)\} = 15.3 \text{ kcal/mol}$$

Since the relative proportion of the two rotamers was approximately 1:1 at 298 K, the free energy of activation for rotation, $\Delta G^{\ddagger}$, was also calculated using Eyring equation for equally populated rotamers. The rate of rotation, k_c, at the temperature of coalescence, T_c, 303 K, can be calculated using the following equation [10]:

$$k_c = \pi(\Delta\nu)/\sqrt{2}$$

$$k_c = \pi*27.96/\sqrt{2} = 62.1 \text{ s}^{-1}$$

The free energy of activation for rotation, $\Delta G^{\ddagger}$, at 303 K was calculated using the Eyring equation:

$$k = (K\, k_B T\, e^{-\Delta G^{\ddagger}/RT})/h$$

where k is the rate constant at the temperature T, k_B is Boltzmann's constant, h is Planck's constant, K is the transmission coefficient, T is the temperature in K, R is the universal gas constant and $\Delta G^{\ddagger}$ is the free energy of activation.

Assuming the transmission coefficient, K, to be unity, converting natural log (ln) to log10, and substituting k_c and T_c into the Eyring equation, this equation becomes:

$$\Delta G^{\ddagger} = 4.57\, T_c\, (10.32 + \log_{10} T_c - \log_{10} k_c) \text{ in units of cal/mol}$$

$$= 4.57*303*(10.32 + \log_{10} 303 - \log_{10} 62.1)$$

$$= 15.2 \text{ kcal/mol}$$

The two methods gave similar results.

The ^{1}H-NMR spectrum of spinosin (3) in DMSO-d_6 at 298 K showed that the relative proportion of the major and minor rotamers was 1.091: 1, using the 5-OH signals as markers. The frequency difference, $\Delta\nu$, between the 5-OH signals was 47.14 Hz. The coalescence temperature, T_c, was 353 K.

$$\Delta P = (1.091 - 1)/2.091 = [(X^2 - 2)/3]^{3/2}*1/X$$

$$X = 1.583$$

$$\Delta G^{\ddagger}_{A} = 4.57*353* \{10.62 + \log [1.583/2\pi (1 - 0.0435)] + \log (353/47.14)\} = 17.6 \text{ kcal/mol}$$

$$\Delta G^{\ddagger}_{B} = 4.57*353* \{10.62 + \log [1.583/2\pi (1 + 0.0435)] + \log (353/47.14)\} = 17.5 \text{ kcal/mol}$$

The VT ^{1}H-NMR studies of C-glycosyl flavonoids 1-3 confirmed the hypothesis that the doubling of signals in the ^{1}H- and ^{13}C-NMR spectra at 298 K was owing to the presence of two rotamers separated by a relatively high energy barrier (Table 1). C-glycosyl flavonoids 1 with the largest C-7 substituent had the highest energy barriers. This result indicated that the C-7 substituents had an effect to the rotational isomerism of 6-C-glycosyl flavonoids. The bulker C-7 substituents gave rise to the higher rotational energy barriers.

Table 1. *Energy barriers of C-glycosyl flavonoids 1-3 (kcal/mol).*

	Compound 1	Compound 2	Compound 3
$\Delta G^{\ddagger}_{A}$	17.8	15.3	17.6
$\Delta G^{\ddagger}_{B}$	17.6	15.3	17.5

However, they may not exhibit atropisomerism and the isolation of rotamers should be difficult because their energy barriers are much lower than 24 kcal/mol. Based on the amount of rotational barriers, suspected atropisomeric compounds were classified into three categories and accompanying drug development strategies were suggested by LaPlante [1]. Class 1 compounds have rotation barriers around the chiral axis of <20 kcal/mol and possess fast axial rotation rates ($t_{1/2}$ less than minutes) with no axial chirality. According to [1], Class 1 rotamers should be developed as purified, single compounds (rapidly equilibrating mixture). Obviously, 6-C-glycosyl flavonoids 1-3 belong to the Class 1 category and can be developed as rapidly equilibrating mixture. We can also presume that other common natural 6-C-glycosyl flavonoids should belong to the Class 1 category, judging from their similar structures and other reported data of energy barriers [11-15]. Therefore, common 6-C-glycosyl flavonoids usually can be developed as rapidly equilibrating mixture.

3. Experimental

3.1. General Experimental Procedure

^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker Avance III HD 500 MHz instrument. VT-^{1}H NMR was recorded with a Bruker Avance III 400 MHz instrument (Bruker, Breman, Germany).

3.2. Materials

Compounds 1-2 were isolated from *Vaccaria hispanica* seeds and have been identified to vaccarin (1) and isovitexin-2”-O-arabinoside (2) in our previous report [8]. Spinosin (3) 20 mg was purchased from Chengdu Must Bio-Technology Co., Ltd, China with certificate of analysis (ID: MUST-18051904).

3.2.1. Compound 1 (Vaccarin)

Yellow crystals (EtOH), ^{1}H NMR (500 MHz, DMSO-d_6, 298 K) δ: 13.69 and 13.58 (1H, s, 5-OH), 6.85 and 6.82 (1H, s, 3-H), 6.89 and 6.89 (1H, s, 8-H), 7.94 and 7.93 (2H, d, J = 8.8 Hz, 2’,6’-H), 6.89 (2H, d, J = 8.8 Hz), 4.88 and 4.73 (1H, d, J = 9.6 Hz, 1”-H), 4.30 and 4.09 (1H, d, J = 6.0, 1”’-H), 5.02 and 5.01 (1H, d, J = 7.6 Hz, 1”“-H).

^{13}C NMR (125 MHz, DMSO-d_6, 298 K) δ: 164.5 and 164.4 (C2), 103.5 and 103.6 (C3), 182.8 and 182.5 (C4), 161.8 and 161.9 (C5), 110.6 and 110.3 (C6), 163.1 and 162.2 (C7), 94.1 and 94.4 (C8), 157.0 and 156.8 (C9), 105.6 and 105.7 (C10), 121.3 and 121.4 (C1’), 129.0 and 129.1 (C2’, C6’), 116.5 (C3’, C5’), 160.1 and 161.6 (C4’), 71.8 and 71.3 (C1”), 81.7 and 81.5 (C2”),78.6 and 78.6 (C3”), 70.2 and 71.2 (C4”), 80.7 (C5”), 61.4 and 62.1 (C6”), 105.3 and 105.4 (C1”’), 67.4 and 67.5 (C2”’), 72.7 and 72.8 (C3”’), 72.0 and 71.8 (C4”’), 64.8 and 65.0 (C5”’), 101.3 and 101.3 (C1”“), 73.8 and 74.3 (C2”“) 77.6 and 77.8 (C3”“), 69.9 and 70.0 (C4”“), 76.4 and 76.8 (C5”“), 61.1 (C6”“).

3.2.2. Compound 2 (Isovitexin-2”-O-arabinoside)

Yellow crystals (MeOH), ^{1}H NMR (500 MHz, DMSO-d_6, 298 K) δ: 13.61 and 13.54 (1H, s, 5-OH), 6.64 (1H, s, 3-H), 6.31 (1H, s, 8-H), 7.86 (2H, d, J = 8.6 Hz, 2’,6’-H), 6.88 (2H, d, J = 8.6 Hz), 4.64 (1H, brs, 1”-H), 4.26 (1H, d, J = 5.5 Hz, 1”’-H).

^{13}C NMR (125 MHz, DMSO-d_6, 298 K) δ: 163.9 (C2), 103.2 (C3), 182.5 and 182.3 (C4), 162.3 and 161.0 (C5), 108.6 (C6), 164.4 and 163.4 (C7), 94.2 and 93.5 (C8), 156.8 (C9), 103.9 and 103.7 (C10), 121.6 (C1’), 128.9 (C2’, C6’), 116.5 (C3’, C5’), 161.6 (C4’), 71.9 and 71.4 (C1”), 80.7 (C2”), 79.1 (C3”), 70.9 and 70.7 (C4”), 82.1 (C5”), 62.0 and 61.8 (C6”), 105.7 and 105.4 (C1”’), 67.4 (C2”’), 72.8 (C3”’), 71.9 (C4”’), 65.0 (C5”’).

3.2.3 Compound **3** (spinosin)

Yellow powder, ^{1}H NMR (500 MHz, DMSO-d_6, 298 K) δ: 13.63 and 13.51 (1H, s, 5-OH), 6.81 and 6.78 (1H, s, 3-H), 6.87 and 6.85 (1H, s, 8-H), 7.98 (2H, d, J = 8.3 Hz, 2’,6’-H), 6.84 (2H, d, J = 8.3 Hz), 4.69 and 4.67 (1H, d, J = 9.7 Hz, 1”-H), 4.18 and 4.16 (1H, d, J = 8.6, 1”’-H), 3.90 (3H, s).

^{13}C NMR (125 MHz, DMSO-d_6, 298 K) δ: 164.2 (C2), 104.6 and 103.5 (C3), 182.8 and 182.5 (C4), 161.7 and 161.0 (C5), 109.1 (C6), 165.5 and 164.3 (C7), 91.2 and 90.7 (C8), 157.6 and 157.4 (C9), 104.9 and 103.4 (C10), 121.5 (C1’), 129.0 (C2’, C6’), 116.4 (C3’, C5’), 160.2 (C4’), 71.1 and 70.9 (C1”), 81.2 (C2”), 77.1 (C3”), 81.5 and 81.8 (C4”), 71.5 (C5”), 61.9 (C6”), 105.8 and 105.7 (C1”’), 75.1 (C2”’), 76.8 (C3”’), 69.9 and 69.9 (C4”’), 75.0 (C5”’), 60.5 (C6”’), 57.0 and 56.5 (7-OCH_3).

Conflict of Interest

The authors declare that they have no competing interests.

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