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First finding of Daidzein 7-*O*-phosphate and Genistein 7-*O*-phosphate that are hydrolyzed by sulfatase

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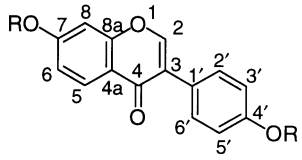
Abstract—Attempted structural assignment of two water soluble isoflavone analogs of Daidzein and Genistein, was initially assumed to be the corresponding sulfates on the basis of the facts that these analogs were hydrolyzed by sulfatase; however, they were eventually determined through NMR technology including $^{31}P-^{13}C$ couplings to be the corresponding phosphates both at the 7-*O*-position; thus, Daidzein 7-*O*-phosphate [4H-1-benzopyran-4-one,7-hydroxy-3-(4-hydroxyphenyl)-, 7-phosphate] and Genistein 7-*O*-phosphate [4H-1-benzopyran-4-one, 5,7 –dihydroxy-3-(4-hydroxyphenyl)-, 7-phosphate].

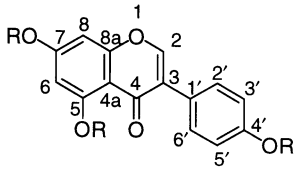
1. Introduction

Isoflavones widely occur in plants as a group of yellow plant pigments, sometimes designated as isoflavonoids. There are 8 congeners of this class of isoflavonoids; thus, isoflavone, Daidzein, Genistein (isolated by Perkin and Newbury[1]), prunetin biochanin A, orobol, santal and pratensein.[2] Some of the better known isoflavones are Daidzein (4′,7-dihydroxyisoflavone) occurring as the 7-*O*-glucoside Daidzein in soybean flour; and Genistein from soybeans and red clover.[3] Some of the isoflavonoids occurring in clover species have estrogenic effects, and other biological activities such as antiarteriosclerotic and antifungal activities.[4] Genistein shows weak activity against Gram-positive bacteria.[5] Isoflavones are reported to show good effects as above and to be superior to a glycoside in those effects. However, attempted application of an isoflavone glycoside seems unsuccessful for use of these isoflavones for food processing due to bitter taste and/or insolubility; thus, it is important for the food processing industry to find the way to reduce its defect. For this study, we found other water-soluble isoflavones with greater solubility by means of microbial transformation.

A bacterium strain, *Bacillus subtilis* NCI-21011, was found to transform isoflavone precursors, Daidzein (**1**) and Genistein (**3**), into water soluble analogs **2** and **4**, respectively. This paper deals with the determination of the linking position and species of water soluble function of compounds **2** and **4**.



1 Daidzein R= H
2 Daidzein derivative R= H, SO_3H or PO_3H_2



3 Genistein R= H
4 Genistein derivative R= H, SO_3H or PO_3H_2

2. Results and discussion

The water soluble isoflavone analogs were obtained from a fermentation broth of *B. subtilis* NCI-21011 (see details in Section 3). Two unknown isoflavone analogs **2** and **4** were, respectively, treated with sulfatase Type VIII (supplied from Sigma) in 50 mM citrate buffer (pH 5.2) at 37°C for 30 min. After treatment with 70% EtOH and centrifugation, the hydrolysates (supernatant) were analyzed on an ODS column (Shiseido, Capcell Pak UG120; 4.6×250 mm^2) at 1.0 mL/mL with gradient elution (15–32% acetonitrile containing 0.1% acetic acid for 60 min). The isoflavone analog **2** was eluted at 18.6 min and hydrolysate Daidzein (**1**) was done at 38.4 min. Similarly, the analog **4** was eluted

Keywords: Daidzein; Genistein; sulfate; phosphate; ^{31}P NMR.
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Table 1. NMR assignment of Daidzein (**1**) and compound **2** (right) based on HMBC correlation

Daidzein (**1**)							Compound **2**						
Carbon	Proton						Carbon	Proton					
	2 (8.27)	5 (7.95)	6 (6.92)	8 (6.84)	2′ (7.37)	3′ (6.79)		2 (8.31)	5 (7.97)	6 (7.22)	8 (7.40)	2′ (7.37)	3′ (6.80)
2 (152.9)							2 (153.3)						
3 (123.6)	α				β		3 (123.7)	α				β	
4 (174.8)	β	β		γ			4 (175.1)	β	β		γ		
4a (116.8)	γ		β	β			4a (118.3)			β	β		
5 (127.4)							5 (126.4)						
6 (115.2)				β			6 (118.7)				β		
7 (162.7)		β	α	α			7 (159.2)		β	α	α		
8 (102.2)		γ	β				8 (107.2)			β			
8a (157.6)	β	β	γ	α			8a (156.9)	β	β		α		
1′ (122.7)	β					β	1′ (123.7)	β					β
2′ (130.2)	γ				β	α	2′ (130.2)					β	
3′ (115.1)					α	β	3′ (115.1)						β
4′ (157.3)					β	α	4′ (157.4)					β	α

The marks α, β, γ represent the long range coupling between 2, 3 and 4 bonds in the HMBC.

Table 2. NMR assignment of Genistein (**3**) and compound **4** (right) based on HMBC correlation

Genistein **3**						Compound **4**					
Carbon	Proton					Carbon	Proton				
	2 (8.31)	6(6.21)	8 (6.37)	2′ (7.36)	3′ (6.81)		2 (8.34)	6 (6.59)	8 (6.87)	2′ (7.36)	3′ (6.80)
2 (154.1)						2 (154.4)					
3 (122.4)	α			β		3 (122.4)	α			β	
4 (180.4)	β	γ	γ			4 (180.6)	β				
4a (104.6)	γ	β	β			4a (105.7)		β	β		
5 (162.1)		α				5 (161.1)		α			
6 (99.10)			β			6 (103.0)			β		
7 (164.4)		α	α			7 (161.3)		α	α		
8 (93.79)		β				8 (98.06)		β			
8a (157.7)	β		α			8a (156.9)	β		α		
1′ (121.4)	β				β	1′ (121.3)	β				β
2′ (130.2)	γ			β	α	2′ (130.2)				β	
3′ (115.2)				α	β	3′ (115.2)					β
4′ (157.6)				β	α	4′ (157.6)				β	α

The marks α, β, γ represent the long range coupling between 2, 3 and 4 bonds in the HMBC.

at 30.3 min and Genistein (**3**) was done at 57.2 min under the same conditions on HPLC. Almost complete hydrolyses of **2** and **4** (98 and 89%, respectively) were observed by the sulfates treatment as above. These results strongly suggested the structures **2** and **4** to be a sulfate ester of **1** and **3**, respectively. Enzyme hydrolysis has usually been employed as the evidence of sulfate structure.[6,7] Mass spectrometry of isoflavonoids were reported,[7a,8] but in this case one of the fragment ions *m/z* 79 might suggest the presence of a sulfate or phosphate ester. The high resolution

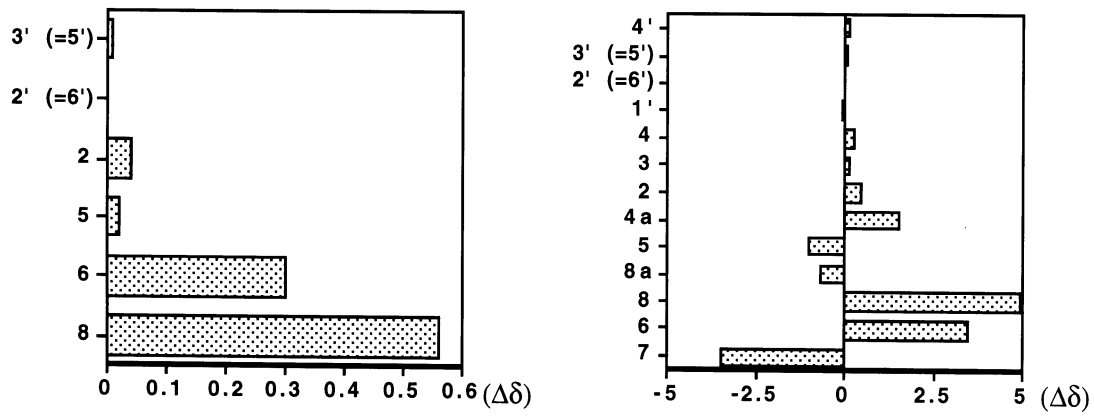

Figure 1. Chemical shift difference of ^{1}H NMR (left) and ^{13}C NMR (right) between Daidzein (**1**) and compound **2**.

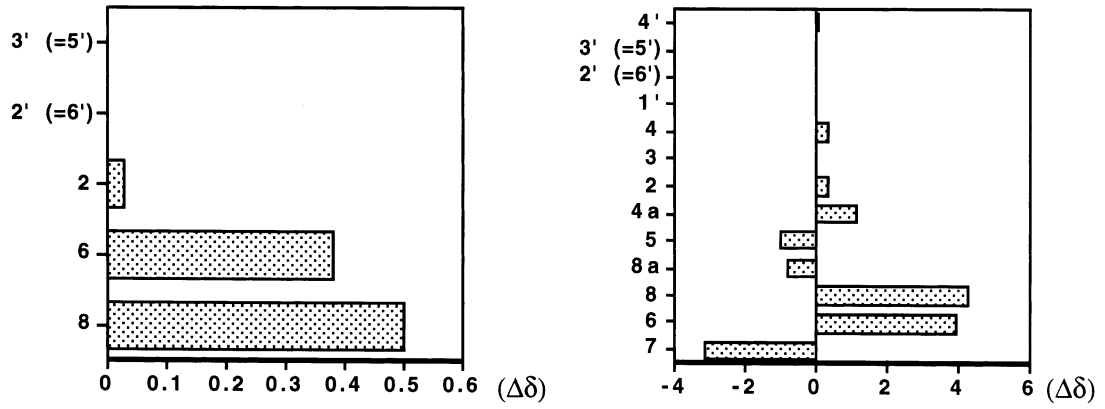

Figure 2. Chemical shift difference of ^{1}H NMR (left) and ^{13}C NMR (right) between Genistein (**3**) and compound **4**.

mass spectrum of **2** showed its molecular peaks with negative FAB/MS at *m/z* 333.0185 (M−H) calcd. 333.0069 for sulfate $C_{15}H_{10}O_7S_1$ or 333.0164 for phosphate $C_{15}H_{11}O_7P_1$ with technical errors being *m/z* ± 0.01. Similarly, mass spectrum of **3** (*m/z* 349.0146, M−H) cannot distinguish the Genistein derivative from the corresponding sulfate (*m/z* 349.0018) or phosphate (*m/z* 349.0113) except the fact from sulfatase digestion.

So the next question was the linking position of the substituted hydroxy group either at the 4′ (or 5) or 7 position. Assignment of the ^{1}H and ^{13}C NMR signals with all of these compounds Daidzein (**1**), **2**, Genistein (**3**) and **4** is of great significance to determine without ambiguity. NMR spectra of **1** and **2** with the ^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY and HMBC made complete assignments of all the proton and carbon signals as shown in Table 1 with **1** and **2**, and in Table 2 with **3** and **4**. The chemical shifts are usually observed to go down field when acylated either as sulfate or phosphate. Simple comparison of chemical shifts of the corresponding ^{1}H and ^{13}C signals are summarized in Figs. 1 and 2. The chemical shift difference in Fig. 1 clearly indicates that no signal has changed at all around the position 4′, but signals around the H-6 and H-8 show significant changes as well as those around the C-6, C-7 and C-8 positions. These results led us to conclude that the acylation of compound **2** happened at the 7-*O*-position of Daidzein.

Genistein has three hydroxy groups at the 4′-, 5- and 7-positions on isoflavone skeleton. So it was a little bit more difficult to determine the acylated position. Using the same 2D spectroscopic methods as the previous case, the ^{1}H and ^{13}C NMR signals of **3** and **4** were unambiguously assigned. The results are summarized in Table 2. The critical assignment of the ^{13}C-8a was concluded from the correlation with H-2 through 2-bond HMBC coupling. The signal corresponding to the hydroxy group at the C-5 gave sharp peak due to a hydrogen bonding with the carbonyl group at the C-4 position.

Fig. 2 shows the chemical shift difference of ^{1}H NMR (left) and ^{13}C NMR of Genistein **3** and Compound **4**. From both of ^{1}H and ^{13}C NMR data, the position 4′ is not acylated. The 5-OH should remain in free form without acylation since no significant change occurred around C-4a or C-5. On the other hand, the C-6, -7 and -8 show very similar chemical shift changes as the case of Daidzein (**1**) and **2**. Therefore, we have concluded that the acylation happens at the 7-*O*-position of Genistein.

Elemental analysis of **2** with X-ray micro-analyzer equipped with EDS detector (this analysis is not quantitative) proved the presence of phosphorous atom but no sulfur atom was detected. This result did not meet the result of sulfatase digestion. There exists, however, only 0.01 mass unit difference between a sulfate and a phosphate. So it may not be possible to conclude either of them through mass spectrometric method except that they have monoacylated structure (see mass data above). We focused our efforts to collect further evidence that the ester **2** and **4** would be phosphates instead of sulfates. A ^{31}P NMR would suggest

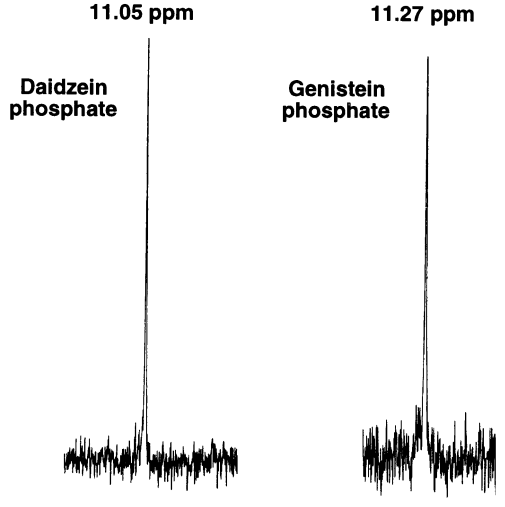

Figure 3. ^{31}P NMR of **2** and **4**.

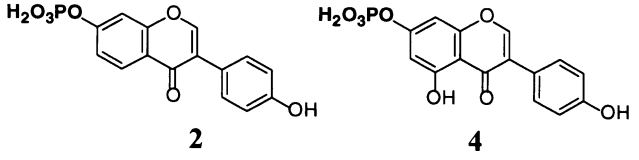

Figure 4. Structure of Daidzein-7-*O*-phosphate (**2**) and Genistein 7-*O*-phosphate (**4**).

the structure, not only from its signal but also from the coupling with ^{13}Cs.

In fact, the ^{31}P NMR with both **2** and **4** gave signals at 11.05 and 11.27 ppm, respectively, as shown in Figs. 3 and 4. This is the first example of the isoflavonoid having a mono phosphate at the 7-*O*-position. Even isoflavone sulfates, that have been previously reported, are located at the 4′-*O*-sulfate but not at the 7 position. We tried to confirm the position by measuring the coupling effect between the atoms in the vicinity of these isoflavone phosphates in NMR spectra. Very clear couplings between ^{13}C and ^{31}P were observed between those carbon signals at the 6, 7 and 8 positions and phosphorous atoms as summarized in Fig. 5 with compound **2** and Fig. 6 with compound **4**, respectively.[9] These results have double checked the linking position of the phosphate; thus confirming it is at the 7-*O*-position.

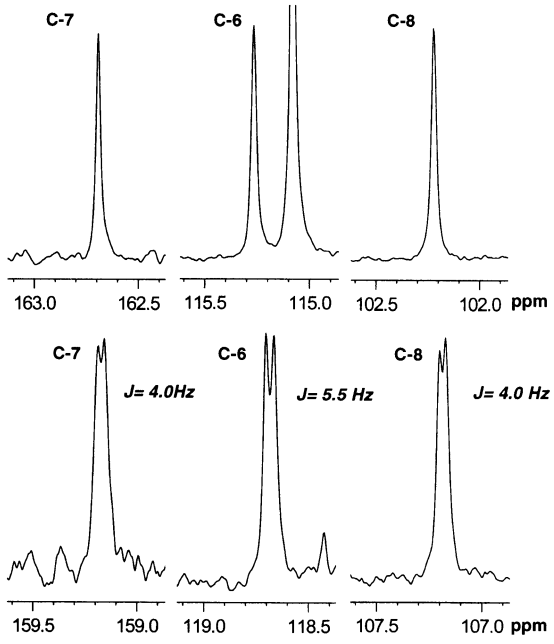

Figure 5. ^{13}C–^{31}P coupling of C-6,7,8 position of Daidzein phosphate.

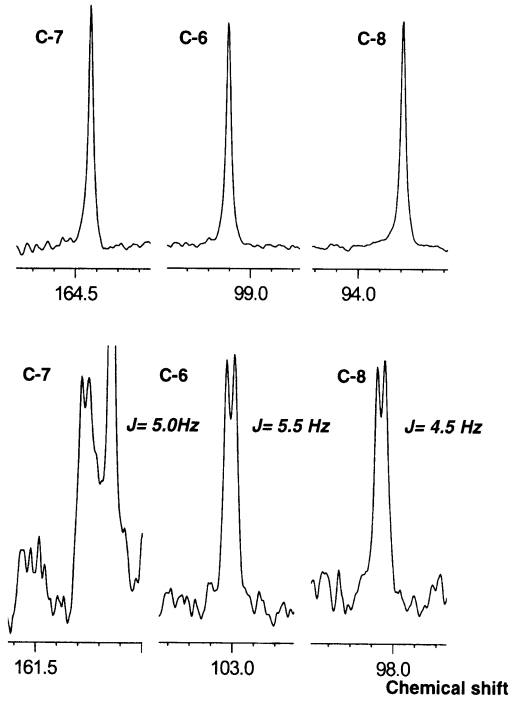

Figure 6. C–P coupling of C-6,7,8 position of Genistein phosphate.

3. Experimental

3.1. Bacterial strain and culture conditions

The bacterial strain used in this study was *B. subtilis* NCI-21011. The bacteria were grown in the standard broth containing 0.5 mg/mL isoflavonoids (Daidzein or Genistein purchased from Nakahara Kagaku, Gifu, Japan) at 30°C for 6–8 d, and then the supernatant of the broth was prepared by centrifugation.

3.2. Purification of compounds

Ethanol was added to the supernatant and the mixture was centrifuged. After evaporation of the supernatant, the sediment obtained from the evaporated residue by ethanol precipitation several times was diluted in the solution containing 70% ethanol and 0.1% TFA. After filtration with 0.45É m filter, the solution was applied to HPLC system. HPLC was conducted on an ODS column (Tosoh, ODS-80 Ts; 21.5×300 mm^2) with UV detector (254 nm) under the gradient condition (5–75% ethanol containing 0.1% TFA for 170 min) at 5 mL/min. After evaporation of the fraction composed by the transformation product, the white powder was obtained finally.

Proton NMR spectra were recorded on a Bruker AM-600 at 600 MHz. Chemical shifts (δ) are given in parts per million relative to DMSO–d_6 (δ 2.49) as internal standard and coupling constants (J) in Hz. Carbon NMR were recorded on a Bruker AMX-600 at 150.9 MHz. Chemical shifts are (δ) given in parts per million relative to DMSO-d_6 (δ 39.7) as internal standard. Coupling constants (J) are given in Hz. Phosphorus NMR spectra were recorded on a Bruker ARX-400 for 162 MHz and triphenylphosphine (δ 0.00 ppm) was employed as an external standard.

3.2.1. Daidzein 1. ^{1}H NMR (600 MHz, DMSO-d_6) δ 6.79 (2H, d, J=8.4 Hz, H-3′, -5′), 6.84 (1H, d, J=2.1 Hz, H-8), 6.92 (1H, dd, J=8.8, 2.1 Hz, H-6), 7.37 (2H, d, J=8.4 Hz, H-2′, 6′), 7.95 (1H, d, J=8.8 Hz, H-5), 8.27 (1H, s, H-2), 9.52* (1H, s, H-4′), 10.75* (1H, s, H-7).(* interchangeable)

^{13}C NMR (150 MHz) δ 102.2 (C-8), 115.1 (C- 3′, 5′), 115.2 (C-6), 116.8 (C-4a), 122.7 (C-1′), 123.6 (C-3), 127.4 (C-5), 130.2 (C-2′, 6′), 152.9 (C-2), 157.3 (C-4′), 157.6 (C-8a), 162.7 (C-7), 174.8 (C-4).

3.2.2. Daidzein-7-*O*-phosphate 2. ^{1}H NMR (600 MHz, DMSO-d_6) δ 6.80 (2H, d, J=8.5 Hz, H-3′, 5′), 7.22 (1H, d, J=8.4 Hz, H-6), 7.37 (2H, d, J=8.5 Hz, H-2′, 6′), 7.40 (1H, s, H-5), 7.97 (1H, d, J=8.4 Hz, H-5), 8.31 (1H, s, H-2).

^{13}C NMR (150 MHz) δ 107.2 (d, J=4.0 Hz, C-8), 115.1

(C-3′, 5′), 118.3 (C-4a), 118.7 (d, *J*=5.5 Hz, C-6), 122.6 (C-1′), 123.7 (C-3), 126.4 (C-5), 130.2 (C-2′, 6′), 153.3 (C-2), 156.9 (C-8a), 157.4 (C-4′), 159.2 (d, *J*=4.0 Hz, C-7), 175.1 (C-4). ^{31}P NMR (162 MHz, DMSO-d_6) δ 11.05 (s).

HRMS (FAB) calcd for $C_{15}H_{10}O_7P$ 333.0164 $(M-H)^-$, found 333.0185 $(M-H)^-$.

3.2.3. Genistein 3. ^{1}H NMR (600 MHz, DMSO-d_6) δ 6.21 (1H, d, *J*=2.1 Hz, H-6), 6.37 (1H, d, *J*=2.1 Hz, H-8),6.81 (2H, dbt, *J*=8.4 Hz, H-3′, -5′), 7.36 (2H, dbt, *J*=8.4 Hz, H-2′, -6′), 8.31 (1H, s, H-2), 9.56* (1H, s, H-4′), 10.86* (1H, s, H-7), 12.94 (1H, s, H-5). (* interchangeable).

^{13}C NMR (150 MHz) δ 93.8 (C-8), 99.1 (C-6), 104.6 (C-4a), 115.2 (C-3′, 5′), 121.4 (C-1′), 122.4 (C-3), 130.3 (C-2′, 6′), 154.1 (C-2), 157.6 (C-4′), 157.7 (C-8a), 162.1 (C-5), 164.4 (C-7), 180.4 (C-4).

3.2.4. Genistein-7-*O*-phosphate 4. ^{1}H NMR (600 MHz, DMSO-d_6) δ 6.59 (1H, s, H-6), 6.80 (2H, d, *J*=8.4 Hz, H-3′, -5′), 6.87 (1H, s, H-8), 7.36 (2H, d, *J*=8.4 Hz, H-2′, -6′), 8.34 (1H, s, H-2).

^{13}C NMR (150 MHz) δ 98.1 (d, *J*=5.0 Hz, C-8), 103.0 (d, *J*=5.5 Hz, C-6), 105.7 (C-4a), 115.2 (C-3′, 5′), 121.3 (C-1′), 122.4 (C-3), 130.2 (C-2′, 6′), 154.4 (C-2), 156.9 (C-8a), 157.6 (C-4′), 161.1 (C-5), 161.3 (d, *J*=4.5 Hz, C-7), 180.6 (C-4). ^{31}P NMR (162 MHz, DMSO-d_6) δ 11.27 (s).

HRMS (FAB) calcd for $C_{15}H_{10}O_8P$ 349.0113 $(M-H)^-$, found 349.0146 $(M-H)^-$.

4. Summary

We have concluded that the Daidzein and Genistein analogs **2** and **4** were deduced to be Daidzein-7-*O*-phosphate and Genistein 7-*O*-phosphate, respectively. Although these phosphates did indeed undergo hydrolysis with sulfatase to give the corresponding isoflavone molecules **1** and **3**, these facts do not give final evidence of the presence of sulfate group. Several papers that have reported the structure(s) of Daidzein sulfate(s) and Genistein sulfate(s) based on the results of enzymic hydrolysis, are to be reexamined. A new ultra-micro analytical method to determine either phosphate(s) or sulfate(s) is awaited beside ^{13}C, ^{31}P NMR spectroscopy.

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