J. Biochem. Mol. Biol. Vol. 29, No. 5 pp. 423~428 September 30, 1996

Structural Characterization of a Flavonoid Compound Scavenging Superoxide Anion Radical Isolated from *Capsella bursa-pastoris*

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(Received April 26, 1996)

Abstract: A superoxide anion radical scavenger isolated from *Capsella bursa-pastoris* was characterized by infrared (IR) spectroscopy, sugar analysis, ultraviolet (UV) spectroscopy, ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopies, and fast atom bombardment (FAB) mass analysis. The compound was assumed to be a flavonoid-O-glycoside from IR spectrum and UV absorption maxima. When the sugar composition of the compound was examined by thin layer chromatography (TLC) and gas chromatography (GC) of the acid hydrolysate, only glucose was detected. According to the results of UV spectrotroscopy by using shift reagents, the compound was supposed to be luteolin (5,7,3',4'-tetrahydroxy flavone) or chrysoeriol (5,7,4'-trihydroxy-3'-methoxy flavone) with glucose. Based on ^{1}H- and ^{13}C-NMR spectroscopies, the compound was deduced as 7,4'-dihydroxy-5,3'-dimethoxy-α-6-*c*-glucosyl-β-2″-*o*-glucosyl flavone. In FAB mass analysis the compound was finally characterized as 7,4'-dihydroxy-5,3'-dimethoxy-α-6-*c*-glucosyl-β-2″-*o*-glucosyl flavone ($C_{29}H_{34}O_{16}$, M.W.=638).

Key words: *Capsella bursa-pastoris*, 7,4'-dihydroxy-5,3'-dimethoxy-α-6-*c*-glucosyl-β-2″-*o*-glucosyl flavone, superoxide anion radical scavenger.

The screening of antioxidants which had little toxic effect has been performed from natural products (herbs, foods and etc.). In Korea, gingseng, sea weeds, *Codonopsis ianceolata*, pueraria root, *Crataegus pinnatifida*, *Terminalia chebula*, garlic, and *Us javanica* are known to have antioxidant activities (Choi *et al.*, 1992). Natural antioxidants which were recorded are as follows: vitamins such as ascorbic acid and tocopherols, phenolic compounds such as phenolic acids, flavonoids and tannins, carotenoids and Maillard reaction products (Larson, 1988). In addition, antioxidant effects were also found in some peptides, alkaloids and phospholipids (Kawashima *et al.*, 1989).

Capsella bursa-pastoris is a Korean wild plant that belongs to the family *Cruciferae* (Dicotyledoneae) and the young leaves and roots have been used as an edible vegetable in Korea. It has been also used as a traditional crude drug for diabetes and fever. We recently found that ethanol extracts from *Capsella bursa-pastoris* had not only antioxidant effects but also scavenging effects on some radicals (Hong *et al.*, 1995; Kwak *et al.*, 1995). We purified (Kwak *et al.*, 1996) and characterized a scavenger compound of superoxide anion radical from the plant. The present paper describes the structural characterization of the scavenger compound named as CBP-KI01 isolated from *Capsella bursa-pastoris*.

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Materials and Methods

Chemicals

Deuterium oxide (D_2O), potassium bromide (KBr), sodium 3-trimethylsilyl propane-1-sulphonate-d_4 (TSP) and sodium borohydride ($NaBH_4$) were purchased from Sigma (St. Louis, USA). Trifluoroacetic acid (TFA) and standard sugars were from Aldrich (Milwaukee, USA), and cellulose-coated plastic sheet from Merck (Woodbridge, USA).

Infrared (IR) spectroscopy

About 3 mg of purified compound was mixed with 200 mg of pure potassium bromide (KBr) and pressed

into a disk pellet. The whole IR spectra (4,000~450 cm^{-1}) were recorded with FT/IR spectrometer (Bomem Michelson Series MB 102-C15).

Sugar analysis of glycoside

About 5 mg of the compound was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 121℃ for 2 h and was divided into aglycone and sugar. TFA was removed through evaporation and the water layer was obtained from solvent fractionation. One portion of the water layer was analyzed by thin layer chromatography (TLC) on cellulose-coated plastic sheets (Merck, 5577) with developing solvent of ethyl acetate : pyridine : water : acetic acid (5 : 5 : 1 : 3). Sugars were detected with alkaline silver nitrate and were compared with standard sugars (Chaplin, 1987).

An another portion of water layer was reduced with sodium borohydride ($NaBH_4$) to aldonic acid and was converted into alditol acetates with acetic anhydride to be identified by GLC (Tones and Albersheim, 1972). FID/GC (Flame ionization detector/gas chromatography, Shimadzu GC-6A) analysis for the alditol acetate was conducted on a stainless column (0.3 i.d.×200 cm) packed with 3% silicone OV-225 at 225~230℃. Nitrogen was used as carrier gas at a flow rate of 1.85 Kg/cm^2.

Ultraviolet (UV) spectroscopy

As shift reagents [sodium methoxide (NaOMe), $AlCl_3$ HCl and sodium acetate (NaOAc)/H_3BO_3] were added to a methanol solution of the scavenger, UV spectra changed by shift reagents were recorded. UV spectra (190~500 nm) were recorded on Uvicon-930 spectrophotometer at a scan speed of 500 nm/min.

Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra of the scavenger were recorded on Superconducting Fourier-Transform NMR spectrometer DRX 300 at 25℃. One dimensional ^1H spectra were acquired at 300 MHz and ^{13}C spectra at 75 MHz. The scavenger was dissolved in deuterium oxide (D_2O) and chemical shifts were expressed relative to that of an internal standard, sodium 3-trimethylsilyl propane-1-sulphonate-d_4 (TSP).

Fast atom bombardment (FAB) mass analysis

Molecular weight of the scavenger was determined with positive ion FAB mass (VG 70-VSEQ) system. FAB source was 35 Kev Cs^+ ion beam and matrix was glycerol and 3-nitrobenzyl alcohol.

Results and Discussion

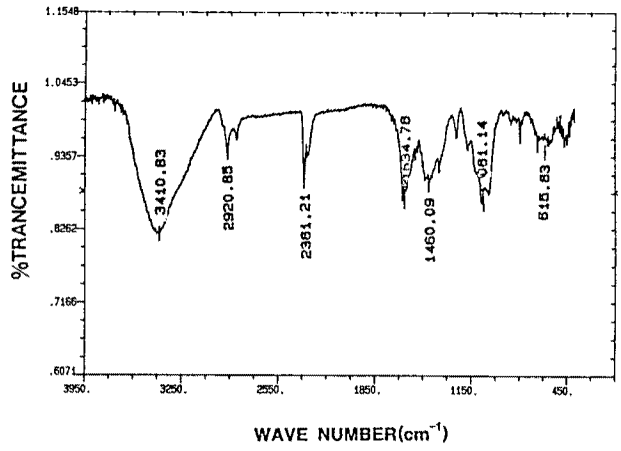


Fig. 1. IR spectrum of compound CBP-KI01 from *Capsella bursa-pastoris*. The spectrum was recorded on Bomen Michelson Series IR spectrometer using a potassium bromide.

Infrared (IR) spectroscopy

As the compound CBP-KI01 had been assumed to be a phenolic glycoside from the physicochemical properties (Kwak *et al.*, 1996), IR spectroscopy was carried out to confirm functional groups. IR spectrum presented in Fig. 1 showed typical absorption spectra of phenolic compounds such as O-H stretching (3,410 cm^{-1}), aromatic C-H stretching (above 3,000 cm^{-1}) and aromatic C=C stretching (1,600-1,400 cm^{-1}). And a glycoside absorptions such as aliphatic C-H stretching (2,920 cm^{-1}) and stretching of ether [υ(C-O), 1,081 cm^{-1}] indicating glycosidic bond were observed. The stretching of carbonyl group [υ(C=O), 1,635 cm^{-1}] was also detected, reflecting the compound was a flavonoid-*O*-glycoside.

Sugar analysis of glycoside

It is generally known that sugar moieties of flavonoid glycosides are composed of pentoses (such as D-apiose, L-arabinose, L-rhamnose and D-xylose), hexoses (such as D-allose, D-galactose, D-glucose and D-mannose) and uronic acid (such as D-galacturonic acid and D-glucuronic acid). The most widely distributed sugar is glucose (Harborne and Mabry, 1982). When the sugar compositions of the compound were examined with TLC and GLC methods, only glucose was detected (Fig. 2). When this compound was even left for long time at room temperature, the scavenging activity was not reduced. In addition, the compound was more soluble in water than in organic solvents such as butanol, chloroform and hexane. These properties of the compound coincide with the report (Iio *et al.*, 1985) that glycosylation of a flavonoid makes it less reactive and more water soluble.

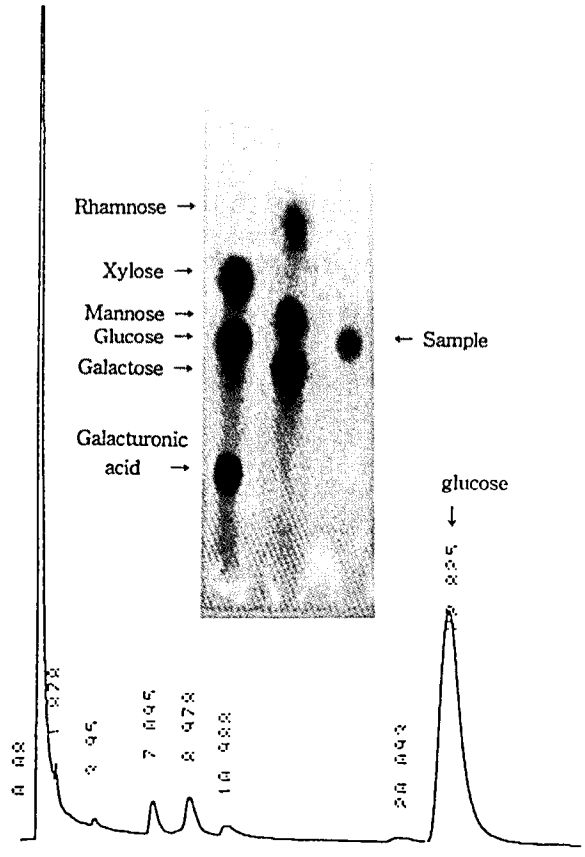
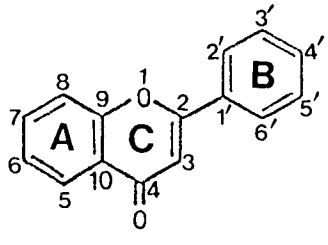


Fig. 2. Sugar analysis of compound CBP-KI01 using TLC and GLC. TLC was carried out on cellulose-coated plastic sheets (Merck, 5577) with a devoloping solvent of ethyl acetate : pyridine : water : acetic acid (5 : 5 : 1 : 3). The alditol acetate derivative converted from the acid hydrolysate of the compound was analyzed by gas liquid chromatography.

Ultraviolet (UV) spectroscopy

Spectra of the flavonoids typically consist of two absorption maxima in the ranges of 240~280 nm (band II) and 300~550 nm (band I) (Markham, 1982). Changes in the substitution of the A-ring tend to be reflected in the band II absorption while alterations in the substitution of the B- and C- rings tend to be more apparent from band I absorption. Additional oxygenation (especially hydroxylation) generally causes a shift of the appropriate band to the longer wavelengths. Methylation and glycosylation cause band shift to shorter wavelengths.



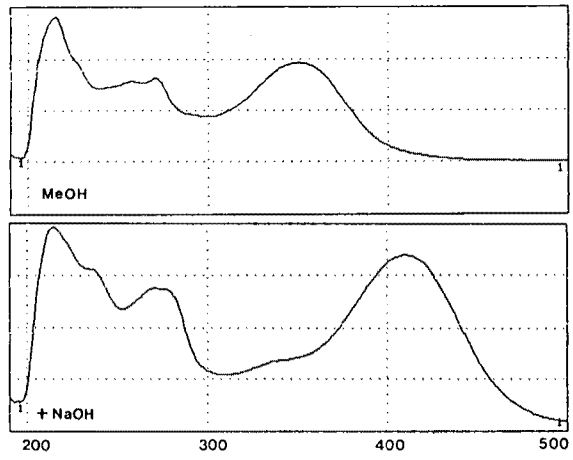


Fig. 3. NaOMe spectrum of the compound CBP-KI01 from *Capsella bursa-pastoris*. Change in UV absorption of the compound by addition of NaOH was recorded on Uvicon-930 spectrophotometer at a scan speed of 500 nm/min.

NaOMe spectrum

Fig. 3 shows the UV spectrum in methanol solution of the compound, which consists of absorption maxima at 260, 269 nm (band II) and 347 nm (band I) and a shoulder in 264 nm. No peak was detected in visible the regions, reflecting that the compound was not expected to be an anthocyanin (Chandra *et al*).

A small peak occurred at 330 nm on addition of NaOMe, which was indicative for a free hydroxyl group at C-7 position of flavonoid (Fig. 3). A bathochromic shift of 60 nm of band I and hyperchromic effect were also observed, which means a free hydroxyl group at C-4′. And the intensity of band I was continually been reduced. It was due to the rapid degradation of alkali-sensitive *o*-dihydroxyl group (Markham, 1982).

As the changes of band I by NaOMe were usually overserved in flavones, flavonols, chalcones or aurones (Bros and Saran, 1987), this compound was supposed to be 7,4′-dihydroxy flavone, flavonol, chalcone or aurone with *o*-dihydroxyl groups in A-ring or B-ring.

NaOAc and NaOAc/H_3BO_3 spectra

Sodium acetate is used primarily to detect the presence of free 7-hydroxyl groups because it causes significant ionization of only the most acidic of the flavonoid hydroxyl groups. And NaOAc/H_3BO_3 bridges the two hydroxyls in an *o*-dihydroxy group, so it is used to detect their presence (Markham, 1982).

As shown in Fig. 4, a bathochromic shift of 5 nm of band II on addition of NaOAc and a bathochromic shift of 30 nm of band I on addition of NaOAc/H_3BO_3 were observed (Fig. 4). The former was indicative of 7-hydroxyl in flavone, flavonol or isoflavone, the latter was indicative of *o*-dihydroxy group in flavone, flavonol,

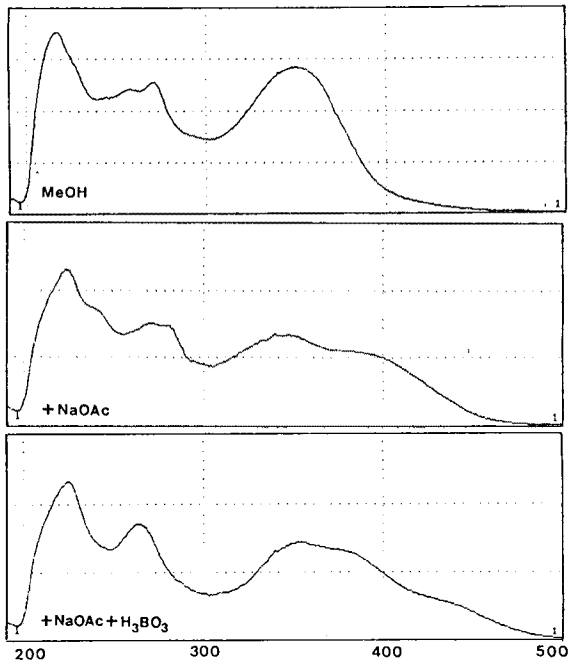


Fig. 4. NaOAc spectrum and NaOAc/H_3BO_3 spectra of compound CBP-KI01 from *Capsella bursa-pastoris*. Changes in UV absorption of the compound by addition of NaOAc and H_3BO_3 were recorded on Uvicon-930 spectrophotometer at a scan speed of 500 nm/min.

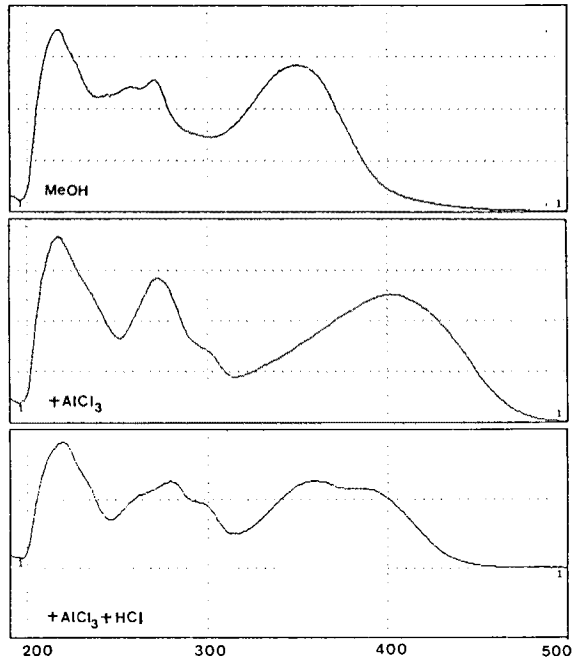


Fig. 5. $AlCl_3$ spectrum and $AlCl_3$/HCl spectra of $O_2\cdot^-$ scavenger from *Capsella bursa-pastoris*. Changes in UV absorption of the compound by addition of $AlCl_3$ and HCl were recorded on Uvicon-930 spectrophotometer at a scan speed of 500 nm/min.

chalcone or aurone. In comparison with NaOMe spectrum, the compound was proposed to be 7,4′-dihydroxy flavone or flavonol with *o*-dihydroxy group in A-ring or B-ring.

$AlCl_3$ and $AlCl_3$/HCl spectra

By forming acid-stable complexes between hydroxyls and neighboring ketones, and acid-labile complexes with o-dihydroxy groups, these reagents can be used to detected both groupings. The "$AlCl_3$" spectrum thus represents the sum effect of all complexes on the spectrum, while the "$AlCl_3$/HCl" spectrum represents the effect only of the hydroxy-keto complexes (Harbone, 1982).

As shown in Fig 5, a bathochromic shift of 52 nm of band I on addition of $AlCl_3$ means that complexes are formed between hydroxyl group and neighboring ketone with Al^{3+} ion. The position of hydroxyl groups which formed complexes was proposed to be C-3 or C-5. According to the report (Harnorne and Mabry, 1982) that 60 nm shift was formed in case of 3-hydroxyl and 35~55 nm shift was formed in case of 5-hydroxyl, the compound was regarded as 5-hydroxyl, which was indicative for flavone. On addition of acid, the shift of band I decreased, indicating that it was caused by separation of Al^{3+} complex from *o*-dihydroxy group in B-ring. So the hydroxylation position of the compound was supposed to be in 5,3′ and 4′ of flavonoid.

Table 1. ^{1}H-NMR data of compound CBP-KI01 from *Capsella bursa-pastoris*

Position	Chemical shifts, δ (ppm)[a]
Aglycone	
H-3	6.13
H-8	6.13
H-2'	6.96
H-5'	6.63
H-6'	6.96
Di Ar-OCH_3	3.65, 3.60
C-Glucosyl H_1	4.92
C-Glucosyl protons	2.94~3.58
O-Glucosyl H_1	4.46
O-Glucosyl protons	3.68~4.02

[a]Relative to the internal standard, sodium 3-trimethylsilyl propane-1-sulphonate-d_4 (TSP).

Based on the results of UV spectra by using shift reagents, the identity of the compound was narrowed to luteolin (5,7,3′,4′-tetrahydroxy flavone) or chrysoeriol (5,7,4′-trihydroxy-3′-methoxy flavone) with glucose. Diosmetin (5,7,3′-trihydroxy-4′-methoxy flavone) was excluded because the hyperchromic effect of band I in NaOMe spectrum depended on free 4′-hydroxyl

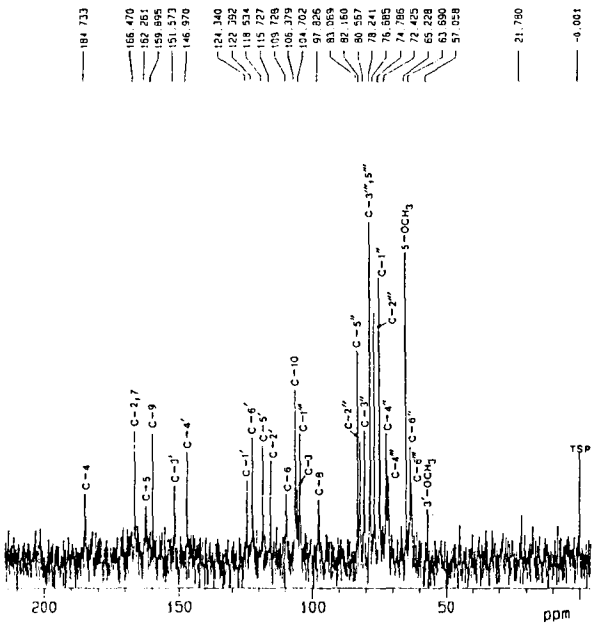


Fig. 6. ^{13}C-NMR spectrum of compound CBP-KI01 from *Capsella bursa-pastoris*. Chemical shifts were expressed relative to that of an internal standard, sodium 3-trimethylsilyl propane-1-sulphonate-d_4 (TSP).

group (Markham, 1982).

Nuclear magnetic resonance spectroscopy

^{1}H-NMR spectroscopy: ^{1}H-Chemical shifts of the compound CBP-KI01 relative to TSP are presented in Table 1. The peak at 6.96 ppm was regarded as superimposed H-6′ and H-2′, that at 6.63 as H-5′, and that at 6.13 as superimposed H-3 and H-8. As for aliphatic protons, two glucosyl H_1 peaks (4.92 ppm (α-linkage) and 4.46 ppm (β-linkage), *O*-glucosyl protons (3.68~4.02 ppm), *C*-glucosyl protons (2.94~3.58 ppm) and two methoxyl protons (3.65 and 3.60 ppm) were detected (Casu, 1985).

Previously the compound was supposed to be a luteolin or chrysoeriol with glucose from UV spectroscopy. ^{1}H-NNR data confirmed the compound to be 4′-hydroxy-3′-methoxy flavone because there are no protons excepting H-2′,5′ and 6′ in B-ring. Only H-8 peak was found in A ring, and this corresponded with the results of UV spectroscopy (Fig. 3, 4, and 5) that the compound required hydroxylation at C-5 and free hydroxyl group at C-7. These explained that free hydroxyl group was bound to C-7 position, methoxyl group to C-5, C-glucose to C-6 and *O*-glucose to *C*-glucose carbon. Thus the compound was supposed to be 7,4′-dihydroxy-5,3′-dimethoxy-α-6-*c*-glucosyl flavone with *O*-glucose bound to *C*-glucose.

^{13}C-NMR spectroscopy

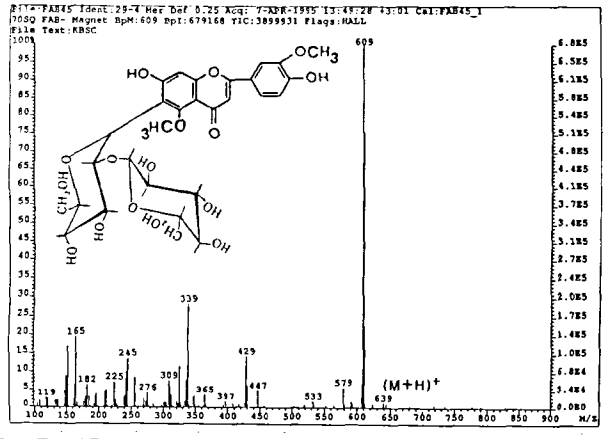


Fig. 7. FAB mass spectrum and structure of compound CBP-KI01 from *Capsella bursa-pastoris*.

In ^{13}C-NNR spectrum of this compound (Fig. 6), C-3 peak of 105.5 ppm is indicative for flavone of flavonoid types, C-1″ peak at 74.79 ppm means α-linked *C*-glucoside, and C-1″ peak at 104.70 ppm is indicative for β-linked *O*-glucoside. In case of *O*-glucosides, α-C-1 appears at 100 ppm and β-C-1 at 104 ppm, in contrast to *O*-glucosides, the signal for C-1 in *C*-glucosides appears in the same region as do the other oxymethine carbon resonances owing to the presence of only one oxygen substituent on this carbon. With the exception of the 6-deoxy derivatives e.g. rhamnosyl and fucosyl, all carbons of the glycosyl moiety resonate between 60.0 to 85.0 ppm (Agrawal, 1989). Thus, it is resonable that the peak at 74.79 ppm is C-1″ signal of *C*-glucoside. As chemical shift of *C*-glucosyl C-2″ showed downfield shift in comparison with general ^{13}C-NMR pattern of flavonoids, *O*-glucose might bind to C-2″ position of *C*-glucose. With regard to the downfield shifts and reduced intensities, each C-5 and C-3′ seems strongly to have methoxyl group.

Thus the compound was strongly deduced as 7,4′-dihydroxy-5,3′-dimethoxy-α-6-*c*-glucosyl-β-2″-*o*-glucosyl flavone ($C_{29}H_3O_{16}$, M.W.=638), which agreed with that of UV spectroscopy.

Fast atom bombardment (FAB) mass analysis

As shown in Fig. 7, molecular weight of the compound CBP-KI01 was determined as 638 in FAB mass ananlysis and this clearly coincided with UV and NMR studies. It was suggested that the base peak of 609 resulted from α-cleavage of 3′-methoxyl. As for molecular cleavage pattern of each peak, 429 was indicative for the detachment of a glucose, 339 for that of two glucoses, and 245 for that of two glucoses and two methoxyl groups. And the peak around 150 means the detachment of B-ring of flavonoid. Consequently, this compound was exactly characterized as 7,4′-dihyd-

roxy-5,3′-dimethoxy-α-6-c-glucosyl-β-2″-o-glucosyl flavone ($C_{29}H_{34}O_{16}$, M.W.=638).

This is the first flavonoid isolated and characterized from *Capsella bursa-pastoris*.

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