Studies on the Constituents of Useful Plants. VI. 1) Constituents of the Calyx of Diospyros kaki (2), and Carbon-13 Nuclear Magnetic Resonance Spectra of Flavonol Glycosides

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Structure of substances XIV and XVIII, obtained previously among the constituents of the calyx of persimmons (Diospyros kaki, Ebenaceae), was examined, and XIV was determined as astragalin (kaempferol 3β,5-diglucosyranoside) and XVIII as n-butyl-β-D-fructopyranoside, although the latter was considered to be an artifact. Carbon-13 NMR spectra of kaempferol, quercetin, trifolin, astragalin, and hyperin were measured and their structure was proved from the assignment of NMR signals.

Keywords—Ebenaceae; Diospyros kaki Thunb.; astragalin; hyperin; trifolin; n-butyl-β-D-fructopyranoside; carbon-13 nuclear magnetic resonance spectra

Our previous report 2) described the isolation of higher fatty acids, aromatic acids, flavonols and their glycosides, steroids, and triterpenoids as a constituent of the calyx of persimmons (Diospyros kaki, Ebenaceae). In the present series of work, structure of substances XIV

1) A part of this work was presented at the Osaka Meeting of Japanese Pharmacognostic Society, October 1974 and at the 96th Annual Meeting of the Pharmaceutical Society of Japan, Nagoya 1976.
2) Location: Mishakara-Higashi, Gifu, 502, Japan.
and XVIII, among the constituents of the said calyx, was elucidated, and carbon-13 nuclear magnetic resonance (13C-NMR) spectra of flavonoids were examined.

Substance XIV was obtained as yellow crystals, mp 212—214°. It was positive to the ferric chloride, magnesium–hydrochloric acid, zinc–hydrochloric acid, and Molish–Udansky reactions, and gave kaempferol and glucose on hydrolysis. The position of the linkage of the sugar was considered to be the 3-position of kaempferol from the ultraviolet (UV) absorption spectrum and UV spectra of XIV after addition of various reagents. Consequently, XIV was assumed to be kaempferol 3-monoglucoside (astragalin) but the melting point of astragalin has been variously reported as 178°, 182—185°, 197—198°, 223°, and 225°, and the sample of astragalin we obtained melted at 224°. Therefore, peracetate of XIV was prepared and its NMR spectrum was measured, in which the signals for four CH₃ protons due to the acetoxyl bonded to the sugar were observed at 1.95—2.16 ppm, and those of three CH₃ protons due to the phenolic acetoxyl at 2.39—2.49 ppm. Therefore, this glucoside was established as kaempferol 3-monoglucoside, and identified with the authentic sample of astragalin.³

The crystalline substance XVIII of mp 149—151° was positive to the Molish–Udansky and Keller–Kiliani reactions, suggesting that it is a sugar derivative. Its composition corresponded to (C₁₀H₁₅O₃)₃ from its elemental analytical values and its mass spectrum of m/e 236 for M⁺ indicated that the composition would be C₁₀₂H₂₃₅O₉. Hydrolysis of XVIII with sulfulic acid produced fructose, and XVIII was assumed to be the fructoside of C₄H₈O₄. Infrared (IR) spectrum of XVIII exhibited absorptions due to the tetrahydropyran ring at 865 and 780 cm⁻¹, besides absorptions for OH, CH₃, and CH₂, suggesting that the fructose is a pyranose, not furanose. It is known that, when fructose takes a furanose–type conformation, a stable fragment ion of furan structure is produced by the liberation of a substituent at C-2, followed by cleavage between C-3 and C-6, whereas if it is a pyranose type, only the liberation of a substituent at C-2 occurs and a stable fragment ion pyran structure appears. In the case of pentaacetate of β-D-fructopyranose, m/e 331 for M⁺—O—COCH₃. The mass spectrum of the peracetate of XVIII showed the fragment ion formed by liberation of the butoxy group at m/e 381 (M⁺—O—CH₂), followed by the fragment ions at m/e 211, 169, and 109, formed by the rearrangement of the acetoxyl group. This was an evidence that XVIII is a pyranose-type fructoside.

In general, molecular optical rotation of glycosides is less affected by the substituents, compared to their anomers, according to Hudson’s rule for equioptical rotation. The optical rotation of XVIII, [α]D = -138°, is close to that of methyl-β-D-fructopyranoside of -173° (α-type, [α]D = +80°). Further, in 13C-NMR (in CD₃SO) spectrum of XVIII, signals due to β-, γ-, and δ-carbons appear respectively at 31.8, 18.9, and 13.4 ppm, and signals of fructose carbons at 100.0, 69.3, 68.9, 63.7, 62.1, and 59.4 ppm. The chemical shift of these signals for fructose carbons agree more with that of fructopyranose than that of fructofuranose.

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These evidences suggest that XVIII is \( n \)-butyl-\( \beta \)-\( d \)-fructopyranoside, and it was found to be identical with an authentic specimen of \( n \)-butyl-\( \beta \)-\( d \)-fructoside synthesized by the reaction of \( \beta \)-\( d \)-fructose and \( n \)-butanol at room temperature, in the presence of a dehydrating agent.\(^{17}\) However, there has been no example of the presence of a \( n \)-butyl-glycoside in nature and it was assumed that XVIII is an artifact formed during the process of extraction by the condensation of fructose contained in the crude drug with \( n \)-butanol used for extraction of the constituents.

In recent years, \(^{13}\)C-NMR spectrometry is being used increasingly for the structural determination of flavonoids. Kingsbury and Looker\(^{18}\) measured \(^{13}\)C-NMR of flavone (A) (in CDCl\(_3\)) and assigned the signals as shown in Table I.

On the other hand, Stothers\(^{19}\) proposed the additivity with respect to the chemical shift produced by the introduction of a substituent in the benzene ring, and stated that, when the substituent was a hydroxyl, signal of the carbon into which OH group was substituted shift to a lower field by 27 ppm (\( \beta \)-effect) and that of the two carbon atoms ortho to the carbon atom substituted with OH group shifted to a higher field by 12.6 ppm. The \(^{13}\)C-NMR spectra of kaempferol (B) and quercetin (C)\(^ {20}\) were measured (in CDCl\(_3\)) and the signals were assigned according to Stothers' additive rule (cf. Table I).

These \(^{13}\)C-NMR data of kaempferol and quercetin were in good agreement with the assignment for 3',4',5,7-tetramethoxyflavonol (D) (in CDCl\(_3\)) made by Kingsbury and

### Table I. \(^{13}\)C Chemical Shifts of Flavone (A), Kaempferol (B), Trifolin (E), Astragalin (F), and Hyperin (G)

<table>
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<tr>
<th>Aglycone moieties</th>
<th>( \text{A}^{a)} )</th>
<th>( \text{B} )</th>
<th>( \text{E} )</th>
<th>( \text{F} )</th>
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<tbody>
<tr>
<td>C(^{-2})</td>
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<td>146.7</td>
<td>156.4</td>
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Except A all spectra were measured in CDCl\(_3\), SO.

\( a) \text{ In CDCl}_3, \quad b) \text{Tentative assignment only. These pairs may be interchanged.}

\(^{17}\) Org. Syntheses, Collected Vol. 1, 364.
Looker.\textsuperscript{18} This fact indicates that the structure of hydroxyflavones can be assigned from their $^{13}$C-NMR \textit{per se}, without methylation of their hydroxyl groups, and is a valuable experimental result in the structural elucidation of flavonoids.

These $^{13}$C-NMR data were then used to determine the kind and position of the sugar attached to flavonols by application to flavonoids. The signal patterns of trifolin and astragalin\textsuperscript{21}) (Fig. 1) agree respectively with the overlapped signals of kaempferol and sugar portion of methyl-$\beta$-$\alpha$-galactopyranoside\textsuperscript{22}) and those of kaempferol and sugar portion of methyl-$\beta$-$\alpha$-glucopyranoside,\textsuperscript{22}) except for the shift of the signal at 135.7 ppm (C-3) in kaempferol to a higher field by about 2.8 ppm, and a shift of the signal at 146.7 ppm (C-2) to a lower field by about 10 ppm to overlap with the signal of carbon at 5-position in kaempferol at 156.3 ppm.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Correlation of the $^{13}$C-NMR Spectra of Trifolin, Kaempferol and Astragalin}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Correlation of the $^{13}$C-NMR Spectra of Hyperin and Quercetin}
\end{figure}


These results indicate that trilolin is kaempferol 3-β-D-galactopyranoside and that astragalin is kaempferol 3-β-D-glucopyranoside. The signal pattern of hyperin (Fig. 2) agrees completely with that of quercetin except for the shift of the signal of carbon at 3-position in quercetin to a higher field by about 2.6 ppm, and the shift of the signal of carbon in 2-position to a lower field by about 10 ppm, thereby overlapping with that of carbon at 5-position, same as trilolin. In the signals of the sugar moiety, however, there are signals of methyl-β-D-glucopyranoside besides those of methyl-β-D-galactopyranoside, although that of the former is weaker than that of the latter, and the content must be small. This is in accordance with the fact that sugars other than D-galactose or other glycosides were not detected in paper chromatography of the sugar in the hydrolysate of hyperin. Consequently, hyperin isolated in the present work is proved to be quercetin 3-β-D-galactopyranoside, and a small amount of quercetin 3-β-D-glucopyranoside is contaminated in this hyperin.

Thus, the structure of substances XIV and XVIII, obtained as the constituent in the calyx of kaki, was determined as astragalin and n-butyl-β-D-fructopyranoside. At the same time, 13C-NMR spectra of flavonol glycosides (trilolin, astragalin, and hyperin) were measured to determine the kind, position, and manner of bonding of the sugar to flavonols in these compounds.

**Experimental**

All melting points are uncorrected. Toyo Roshi No. 50 was used for the paper chromatography and Kieselgel G (Merck) for thin-layer chromatography (TLC). The coloring agent was 10% H2SO4 for TLC, and 3% Mg(OAC)2 for flavonoids and aniline hydrophthalate for sugar in paper chromatography. IR spectra were taken with JASCO Model IRA-1, NMR with Hitachi-Perkin-Elmer (60 MHz), mass spectra (MS) with Hitachi RMU-6D, and UV with Shimadzu Model MPS-50L.

**Astragalin (XIV)**—XIV was obtained as pale yellow needles (from MeOH), mp 212—214°, 110 mg. Zn–HCl reaction, orange; Molish–Udresky reaction, positive; zircon–citric acid reaction, negative. Identified by mixed fusion with authentic astragalin, mp 223°, and comparison of IR spectra and paper chromatograms. UV: λmax nm: 267, 300 (sh), 352; λmax nm: 276, 307, 370; λmax nm: 296, 296 (sh), 306, 352, 398; λmax nm: 276, 296 (sh), 304, 347, 398; λmax nm: 275, 327, 400. Paper chromatography: RF 0.70 (astragalin, RF 0.70 (15% AcOH)). Astragalin peracetate: mp 212—214° (MeOH). NMR (CDCl3): δ 1.85—2.16 (sugar, OC–CH2–X), 2.30—2.49 (phenolic –CO–CH2–X), 4.00, 5.05—5.40 (each 3H, br, aliphatic H, on glucose H), 5.53 (1H, d, J = 6.0 Hz, anemic H), 6.90 (1H, d, J = 2.0 Hz, C(6)-H), 7.30 (2H, d, J = 8.7 Hz, C(3′,5′)-H), 7.44 (1H, d, J = 2.0 Hz, C(8)-H), 8.13 (2H, d, J = 8.7 Hz, C(2′,6′)-H).

Hydrolysis of Astragalin: A mixture of 20 mg of astragalin and 10 ml of 5% H2SO4 was heated for 2 hr, cooled, and yellow crystals that precipitated out were collected and recrystallized from AcOEt to the aglycone, mp 272—273°. The aglycone was obtained by kmpermixed fusion and paper chromatography. RF 0.47 (60% AcOH), 0.16 (15% AcOH). Mother liquor of hydrolysate, left after collection of the aglycone, was neutralized with BaCO3 and submitted to paper chromatography, from which the sugar was identified as glucose. RF 0.38 (80% phenol) (glucose, RF 0.38).

**n-Butyl-β-D-fructopyranoside (XVIII)**—XVIII was obtained as colorless needles (from AcOEt), mp 149—150°, 20 mg. Molish–Udresky reaction, positive; Keller–Kiliani reaction, positive. [α]D −138° (MeOH). Anal. Calcd for C11H22O11: C, 50.74; H, 8.43. Found: C, 50.83; H, 8.53. MS, m/z: 236 (M+), 205, 149, 103, 77. IR νmax cm−1: 3400, 2950, 1400, 1120, 1060, 912, 890, 865, 780, 605. NMR (CD2OD): δ 0.95 (3H, t, –CH2–CH3), 1.51 (4H, br, –CH2–CH2–), 3.55 (2H, t, –O–CH2), 3.35—3.92 (7H).

Hydrolysis of astragalin: A mixture of 20 mg of astragalin and 10 ml of 5% H2SO4 was heated for 2 hr, neutralized with BaCO3, and fructose was identified from the hydrolysate solution by paper chromatography. RF 0.31 (80% phenol) (fructose, RF 0.31).

**Synthesis of n-Butyl-β-D-fructoside**—A solution of 5 g of dried fructose dissolved in 50 ml of 0.5% HCl in n-BuOH was stirred at room temperature for 30 hr. The reaction mixture evaporated to dryness and
the residue was purified by chromatography (developed with CHCl₃–MeOH (5:1)) over a silica gel column, to give 1.0 g of a product with mp 149—151° (from AcOEt). TLC: Rf 0.66 (CHCl₃–MeOH = 5:1).

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