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# NEW TETRASACCHARIDE FLAVONOL GLYCOSIDE FROM *EPIMEDIUM ACUMINATUM*

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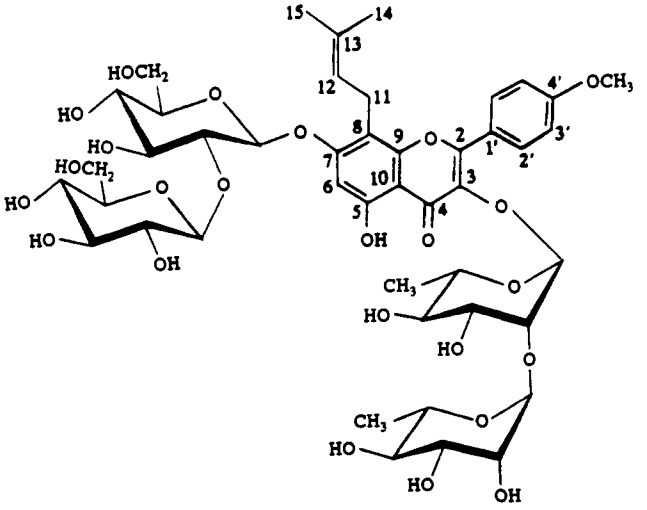
ABSTRACT.—A new tetrasaccharide flavonol glycoside was isolated from the aerial parts of *Epimedium acuminatum*, along with three known flavonoids. The structure of the new compound, named acuminatoside [**1**], was established to be anhydroicaritin-3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside by means of spectroscopic techniques (uv, eims, fdms, fabms, $^1$H nmr, $^1$H-$^1$H COSY, 2D-*J*, $^{13}$C nmr, APT, and $^1$H-$^{13}$C HETCOR) and chemical methods (acid hydrolysis, enzymatic hydrolysis, and tlc-densitometry). The known compounds were identified as icariin, epimedoside A, and kaempferitrin.

Some plants of the genus *Epimedium* (Berberidaceae) have been used as a tonic in traditional Chinese medicine. In a previous study, we investigated the constituents of the $CHCl_3$-soluble and EtOAc-soluble portions of the 95% EtOH extract from the aerial part of *Epimedium acuminatum* Franch (1). We report here the structure elucidation of a new flavonoid, acuminatoside [**1**], and the identification of three known flavonoids, isolated from the *n*-BuOH-soluble portion of the above extract.

## RESULTS AND DISCUSSION

Acuminatoside [**1**], a yellow amorphous powder, was positive to Mg-HCl and Molish tests. The uv spectrum showed absorption bands at 266, 311, and 338 sh nm, and bathochromic shifts were observed after adding certain reagents (NaOMe, $AlCl_3$/HCl, and NaOAc), which indicated that **1** was a flavonoid with the presence of a 5-hydroxyl group and the absence of free hydroxyl groups at C-3, C-4′, and C-7. The eims gave the molecular ion peak of the aglycone of **1** at *m/z* 368. The fragments (*m/z* 353, 313, 165, and 135) formed after retro-Diels-Alder cleavage suggested that **1** had a prenyl group in ring A and an MeO group in ring B.



Fabms data showed that **1** contained four sugar units. In the $^1$H-nmr spectrum (300 MHz, DMSO-$d_6$), four anomeric protons were observed and assigned to those of rhamnose δ 5.37 (br, s), glucose 5.00 (d, $J$ = 8.0 Hz), rhamnose 4.86 (br, s), and glucose 4.28 (d, $J$ = 8.0 Hz). The presence of two two-proton doublets at δ 7.87 ($J$ = 7.5 Hz) and 7.11 ($J$ = 7.5 Hz) and a one-proton singlet at 6.61, assignable to the proton at C-6 because of a glucosylation at C-7 (2), suggested that the aglycone was based on kaempferol with a substituent carbon linked at C-8. The characteristic signals based on a prenyl group as the substituent were observed at δ 1.66 (3H, s), 1.58 (3H, s), 3.34 (2H, m), and 5.14 (1H, br, t, $J$ = 5.0 Hz). The location of the prenyl group at the C-8 position was supported by the $^{13}$C-nmr spectrum of **1** because of the chemical shift value of the carbon atom at the C-6 position (δ 98.3) (3). In addition to those, the signal of an MeO group was observed at δ 3.83 (3H, s). Combined with the eims fragments, the aglycone was concluded to be anhydroicaritin.

Acid hydrolysis of **1** afforded D-glucose, L-rhamnose, and the aglycone. The molar ratio of aglycone to glucose to rhamnose from **1** was 1:2:2, which was determined by tlc-densitometry after acid hydrolysis. Partial acid hydrolysis of **1** produced a compound which was identified as anhydroicaritin-7-*O*-β-D-glucopyranoside by spectroscopic analysis.

Enzymatic hydrolysis of **1** gave a product. The uv spectrum of this product and the shift values after adding some reagents showed that the hydroxyl group at C-7 was free. The fabms revealed that the product contained two rhamnose units. Combined with the uv and $^1$H-nmr results, the disaccharide moiety was attached at C-3. The interlinkage of the biose was determined to be α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranoside by a key $^{13}$C-nmr signal (75.5 ppm) and was confirmed by the $^1$H-$^{13}$C HETCOR spectrum which showed that the proton (δ 4.14) at the C-2 of the rhamnose attached to the aglycone had a cross peak with the C-2 (δ 75.5), indicating that the carbon signal was shifted downfield δ 5.4 by the glycosylation of the terminal rhamnose. The enzymatic hydrolysis product was, therefore, identified as anhydroicaritin 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranoside.

So far, the above data revealed that **1** contained the structure of anhydroicaritin-3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranoside. Further, the $^{13}$C-nmr spectrum of **1** showed four anomeric carbon signals of the sugar moieties at δ 101.8, 100.8, 100.7, and 97.0. The location of the other glucose unit of **1** was determined to be attached to the glucose unit at the C-7 position by its $^{13}$C-nmr and $^1$H-$^{13}$C HETCOR spectra. The chemical shift value at the C-2 (δ 82.0) of the glucose unit attached to aglycone was shifted downfield (δ 8.5), but those at the C-1 (δ 97.0) and C-3 (δ 76.6) showed upfield shifts (ca. δ 2.4 and 0.2, respectively), demonstrating that the terminal glucose was linked to the glucose unit by a (1→2) linkage (4).

From these data, the structure of **1** was concluded to be anhydroicaritin-3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside, and was named acuminatoside. In addition, the three known compounds were identified as icariin, epimedoside A, and kaempferitrin on the basis of spectroscopic analysis and comparison with authentic samples.

## EXPERIMENTAL

GENERAL EXPERIMENTAL METHODS.—The $^1$H-nmr and $^{13}$C-nmr spectra measured with TMS as internal reference were run on FX-100, XL-VXR 300, and JNM-GX 400 nmr spectrometers, respectively. Eims and fdms were measured on an MAT 711 mass spectrometer. Fabms were recorded on a KYKY ZhP-5 mass spectrometer. Uv spectra were measured on a Philips PYS Unicam PU 8800. Sephadex LH-20

(Pharmacia), Polyclar A T (Serva), and cellulase (Sigma) were used. Polyamide films were produced by the Huang-Yan Chemical Factory, Zhejiang Province, China. Si gel was the product of the Qingdao Marine Chemical Factory, Shandong Province, China.

PLANT MATERIAL.—The aerial parts of *E. acuminatum* were collected on the E-mei Mountain, Sichuan Province, China in July, 1985. The material was identified by Prof. Wen-yan Lian, Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences. A voucher specimen has been deposited in the herbarium of the Institute.

EXTRACTION AND ISOLATION.—The dried aerial parts (2 kg) were extracted 4 × with 95% EtOH (24 liters) under reflux for 2 h. The combined extracts were concentrated at reduced pressure. The residue (292 g) was suspended in $H_2O$ and extracted successively with $CHCl_3$, EtOAc, and *n*-BuOH. The *n*-BuOH portion (49 g) was chromatographed on a Si gel Column (eluent: $CHCl_3$-MeOH gradient) and separated into seven parts. Compound **1** (1.5 g) from part five, icariin (2.0 g) from part three, and epimedoside A (100 mg) and kaempferitrin (50 mg) from part four were obtained by the combination of Polyclar AT CC and purification with a Sephadex LH-20 column.

TABLE 1. $^{13}$C-nmr Spectra[a] of Compound **1** and Its Enzymatic Hydrolysis Product.

| Carbon | Compound | |
|---|---|---|
| | **1** | Enzymatic hydrolysis product of **1** |
| C-2 | 157.3 | 156.7 |
| C-3 | 134.6 | 134.4 |
| C-4 | 178.2 | 177.9 |
| C-5 | 160.6 | 161.9 |
| C-6 | 98.3 | 98.4 |
| C-7 | 161.5 | 161.3 |
| C-8 | 108.4 | 106.0 |
| C-9 | 153.0 | 153.8 |
| C-10 | 105.7 | 104.2 |
| C-11 | 21.7 | 21.2 |
| C-12 | 122.2 | 122.3 |
| C-13 | 131.2 | 131.3 |
| C-14 | 25.7 | 25.4 |
| C-15 | 18.1 | 17.8 |
| C-1′ | 122.2 | 122.3 |
| C-2′, -6′ | 130.7 | 130.4 |
| C-3′, -5′ | 114.2 | 114.1 |
| C-4′ | 159.1 | 158.9 |
| OMe | 55.7 | 55.5 |
| Rha | | |
| C-1 | 100.7 101.8 | 100.7 101.6 |
| C-2 | 75.7 70.3 | 75.5 70.1 |
| C-3 | 70.4 70.6 | 70.5 70.7 |
| C-4 | 72.1 71.5 | 72.0 71.4 |
| C-5 | 69.8 69.0 | 70.3 68.8 |
| C-6 | 17.9 17.8 | 17.6 17.5 |
| Glu | | |
| C-1 | 97.0 100.8 | |
| C-2 | 82.0 73.5 | |
| C-3 | 76.7 76.9 | |
| C-4 | 70.0 69.4 | |
| C-5 | 77.3 77.3 | |
| C-6 | 61.4 60.8 | |

[a]Chemical shifts were expressed in ppm from TMS. Solvent DMSO-$d_6$; **1** (75.45 MHz), enzymatic hydrolysis product (100.6 MHz). Assigned on the basis of $^1$H-$^1$H COSY and $^1$H-$^{13}$C HETCOR spectra.

IDENTIFICATION OF KNOWN FLAVONOIDS.—Icariin: yellow needles; mp 245–247°; Mg-HCl test positive; Molish test positive. Icariin was identified by uv, eims, $^1$H nmr, and direct comparison with an authentic specimen (ir and tlc). Epimedoside A: yellow amorphous powder; mp 219–221°; Mg-HCl test positive; Molish test positive. Epimedoside A was identified by uv, fdms, eims, $^1$H nmr, and $^{13}$C nmr. Kaempferitrin: yellow needles; mp 196–196.5°; Mg-HCl test positive; Molish test positive. Kaempferitrin was identified by uv, fdms, eims, $^1$H nmr, and $^{13}$C nmr.

ACID HYDROLYSIS OF **1**.—A 10% HCl solution (5 ml) of **1** (25 mg) was heated under reflux for 10 h. The solution was neutralized with $Ag_2CO_3$, and the filtrate was subjected to Si gel tlc [developing solvent $CHCl_3$-MeOH-$H_2O$ (13:7:2) lower phase], to result in identification of glucose and rhamnose by the usual procedures.

To determine the molar ratio of the sugars and the aglycone of **1**, a 10% HCl solution of **1** was heated under reflux for 10 h. The solution was evaporated in vacuum, and the residue was prepared for quantitative determination by tlc densitometry (5). For partial acid hydrolysis of **1** a 15% HOAc solution (10 ml) of **1** (100 mg) was heated under reflux for 10 h. The precipitate was purified by a Sephadex LH-20 column and recrystallized to produce the partial hydrolysis product.

ENZYMATIC HYDROLYSIS OF **1**.—A 0.2 M NaOAc/HOAc buffer solution (pH 5) (50 ml) that contained **1** (300 mg) and cellulase (300 mg) was incubated at room temperature for 48 h. The precipitate was chromatographed on a polyamide column and purified with Sephadex LH-20, giving a yellow powder of the enzymatic hydrolysis product.

ACUMINATOSIDE [**1**].—A yellow amorphous powder: uv λ max (MeOH) nm 266, 311, 338 sh, (+ NaOMe) 268, 294 sh, 350, (+$AlCl_3$) 276, 303, 340, 405, (+ $AlCl_3$/HCl) 274, 301, 336, 404, (+ NaOAc) 268, 312, 336 sh, (+ NaOAc/$H_3BO_3$) 266, 312, 336 sh; fdms *m/z* $[M+Na+2H]^+$ 1009, $[M-glu+Na+2H]$ 847, $[M-2\,glu+Na+2H]^+$ 685, $[aglycone+glu+H]^+$ 531, $[aglycone]^+$ 368; eims *m/z* 368 (100), 353 (32.5), 339 (2.2), 325 (2.0), 313 (74.2), 300 (5.6), 284 (2.0), 184 (7.2), 165 (7.3), 157 (17.0), 135 (10.7), 128 (6.9), 107 (5.3); $^1$H nmr (300 MHz, DMSO-$d_6$) δ 0.79 (3H, d, $J$ = 6.0 Hz, rha-Me′), 1.08 (3H, d, $J$ = 6.0 Hz, rha-Me), 1.58, 1.66 (6H, s x2, Me-14, -15), 3.34 (2H, m, H-11), 3.83 (3H, s, 4′-OMe), 4.10 (1H, br, rha-H-2), 4.28 (1H, d, $J$ = 8.0, glu-H-1′), 4.86 (1H, br, s, rha-H-1′), 5.00 (1H, d, $J$ = 8.0 Hz, glu-H-1), 5.14 (1H, br, t, $J$ = 5.0 Hz, H-12), 5.37 (1H, br, s, rha-H-1), 6.61 (1H, s, H-6), 7.11 (2H, d, $J$ = 7.5 Hz, H-3′; 5′), 7.87 (2H, d, $J$ = 7.5 Hz, H-2′; 6′), 12.60 (1H, s, 5-OH); $^{13}$C nmr see Table 1.

PARTIAL ACID HYDROLYSIS PRODUCT OF **1**.—A yellow powder; uv λ max (MeOH) nm 266, 320, 369, 420 sh, (+ NaOMe) 261, 419, (+ $AlCl_3$) 264, 296 sh, 354, 432, (+ $AlCl_3$/HCl) 262, 294 sh, 351, 430, (+ NaOAc) 266, 321, 372, (+ NaOAc/$H_3BO_3$) 267, 321, 369; fabms *m/z* [aglycone + glu + 1]$^+$ 531, [aglycone + 1]$^+$ 369.

ENZYMATIC HYDROLYSIS PRODUCT OF **1**.—A yellow powder: uv λ max (MeOH) nm 266, 284 sh, 334 sh, (+ NaOMe) 278, 376, (+ $AlCl_3$) 275, 304, 342, 402 sh, (+ $AlCl_3$/HCl) 276, 300, 338, 400 sh, (+ NaOAc) 276, 352, (+ NaOAc/$H_3BO_3$) 266, 284 sh, 332 sh; fabms *m/z* [aglycone + 2 rha + 1]$^+$ 661, [aglycone + rha + 1]$^+$ 515, [aglycone + 1]$^+$ 369, $^1$H nmr (400 MHz, DMSO-$d_6$) δ 0.83, (3H, d, $J$ = 6.0 Hz, rha-Me′), 1.13 (3H, d, $J$ = 6.0 Hz, rha-Me), 1.64, 1.69 (6H, s x2, Me-14, -15), 3.32, 3.44 (each 1H, br, d, $J$ = 14.0 Hz, H-11), 3.71 (1H, br, s, rha-H-2′), 3.87 (3H, s, 4′-OMe), 4.14 (1H, br, s, rha-H-2), 4.91 (1H, br, s, rha-H-1′), 5.17 (1H, br, t, $J$ = 5.0 Hz, H-12), 5.39 (1H, br, s, rha-H-1), 6.34 (1H, s, H-6), 7.13 (2H, d, $J$ = 9.0 Hz, H-3′, -5′), 7.87 (2H, d, $J$ = 9.0 Hz, H-2′, -6′); $^{13}$C nmr see Table 1.

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