Isolation and Structure of Medicarpin-β-D-glucoside in Alfalfa

Youji Sakagami, Sumio Kumai* and Akinori Suzuki

Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo 113
*Grassland Research Institute, Nishinasuno, Tochigi-ken 329-27
Received December 12, 1973

Medicarpin-β-D-glucoside(II) was isolated from roots of alfalfa, Medicago sativa L. Its structure was established by chemical and physicochemical evidences.

Medicarpin (3-hydroxy-9-methoxypterocarpan, I) was first isolated from heart wood of a tropical tree, Swarzia madagascariensis. Later, the compound was obtained as a phytoalexin from alfalfa, M. sativa L., and also from red clover, Trifolium pratense L.

During our investigation on the constituents in roots of alfalfa, we isolated a new glucoside, medicarpin-β-D-glucoside, and established its structure as (−)-3-β-D-glucosyl-9-methoxypterocarpan (II). In this paper we wish to report our experimental results leading to the structure elucidation.

Roots (12 kg) of alfalfa, M. sativa L. cultivar "Rhizoma" were harvested in November, 1972 at Nishinasuno, Tochigi Prefecture and macerated with methanol. The methanol extract thus obtained was concentrated under reduced pressure, and the resulting aqueous solution was re-extracted with ethyl acetate at pH 2. After being washed with aqueous sodium bicarbonate, the extract was concentrated under reduced pressure to give 66 g of a neutral fraction. This fraction was applied to charcoal column chromatography by use of water-acetone as eluant, increasing stepwise the content of acetone. Evaporation of an eluate with 100% acetone gave an oil containing white powdery crystals. The crude crystals collected on a glass filter were washed successively with ethyl acetate, methanol and benzene to give 1.5 g of pure crystals (II), mp 270–272°C.

In the NMR spectrum of II (Fig. 1), signals composed of two overlapping AKM systems were observed at δ 6.30–7.50 ppm. This suggests the presence of two tri-substituted aromatic rings. Further, the signals at δ 3.69 ppm (3H, s) indicated the presence of an aromatic methoxyl. These data along with the IR spectrum of II (Fig. 2) strongly suggested that II should be a glycosidic derivative of an aromatic compound.
On acetylation with acetic anhydride in pyridine, II yielded tetraacetyl derivative of II (III), whose molecular formula was determined as C_{30}H_{32}O_{13} by high resolution mass spectrometry and elementary analysis. Comparison of the IR spectrum of III with that of II revealed that all of the hydroxyl groups in II had been acetylated in III. Signals at $\delta$ 2.02 ppm (3H, s), $\delta$ 2.03 ppm (6H, s) and $\delta$ 2.05 ppm (3H, s) in the NMR spectrum of III showed the presence of four acetyl groups. Thus, the molecular formula of II was established as C_{22}H_{24}O_{9}. In the high resolution mass spectrum of III, fragment ions at m/e 331.1019 (calcd. for C_{14}H_{19}O_{9}: 331.1026) were ascribed to the tetraacetyl hexose moiety, and ions at m/e 270.0885 (calcd. for C_{16}H_{14}O: 270.0890) to the aglycon.

Then, the acid hydrolysis of II was attempted with 1N hydrochloric acid in dioxane-water. At the end of the reaction, water was added to the mixture, which was then extracted with ethyl acetate. The residual aqueous solution was evaporated to dryness under reduced pressure, and a small portion of the solid residue was analyzed by GLC after trimethylsilylation. The chromatogram revealed that glucose was a sole constituent sugar in II. The stereochemistry of the glucose was determined to be D-form by measurement of ORD spectrum.

Our attempts to isolate the aglycon from an ethyl acetate extract of the acid hydrolyzate was unsuccessful. After many trials, the glycosidic linkage in II was hydrolyzed by use of a commercial crude enzyme syrup from snail digestive fluid. A phenolic fraction was separated from enzyme hydrolyzate through the conventional procedure and purified by use of preparative silicagel TLC. Recrystallization from chloroform-hexane gave crystals of the aglycon (I), mp 131~132°C. The molecular formula of I was determined as C_{16}H_{14}O_{4} from mass spectrometry. The features of IR, UV, NMR and mass spectra of I showed close similarities with those of pterocarpanoids. Finally, I was confirmed to be medicarpin, (−)-3-hydroxy-9-methoxysterocarpan through comparison of IR, NMR and UV spectra, and optical rotation with the data* reported in the literatures.

The next problem to be clarified was the determination of stereochemistry of the glycosidic linkage. On the NMR spectrum of II in dimethylsulfoxide (Fig. I), signals due to an anemic proton could not be easily assignable, because signals originating from hydroxyl protons in the glucose moiety appeared in the region of anemic proton. Addition of fairly large amount of D_{2}O to the dimethylsulfoxide solution shifted the signals of hydroxyl protons resulting in simplification of the spectrum. Thus, anemic proton signals were observed at $\delta$ 4.83 ppm (1H, d, J=6.5 Hz). The NMR spectra of phenyl-α- and β-glucosides measured under the same conditions were ex-

* The copies of IR, UV and NMR spectra of an authentic medicarpin were kindly supplied by Dr. D. G. Smith.
Medicarpin-β-D-glucoside in Alfalfa

Amined, and the anomeric proton signals of α- and β-glucosides were observed at δ 5.36 ppm (1H, d, J = 3.4 Hz) and δ 4.85 ppm (1H, d, J = 6.8 Hz), respectively. These results unequivocally established the stereochemistry of the glycosidic linkage in II as β. Thus the structure of II was confirmed to be (−)-3-β-D-glucosyl-9-methoxypterocarpan.

To our knowledge, only trifolirhizine and sophojaponicin have been known as glycosides of pterocarpanoids. From the viewpoint of phytopathology, it seems interesting that the roots of alfalfa autonomously contained a large amount of II, because trifolirhizin is considered to act as a phytoalexin.

EXPERIMENTALS

MELTING POINTS were uncorrected. Optical rotation were measured with a JASCO DIP-S polarimeter and ORD spectra were obtained with a JASCO J-20 spectropolarimeter. IR spectra were run on a JASCO IR-S spectrometer and NMR spectra were recorded with a JEOL-JNM-4H-100 spectrometer using tetramethylsilane as an internal standard. Mass spectra were measured with a Hitachi RMU-6L mass spectrometer being operated with ionization energy of 70 eV, and high resolution mass spectra were obtained with a Hitachi RMH-2 mass spectrometer equipped with a Hitachi 002 dataizer. UV spectra were run on a Cary Spectrometer Model 14.

Isolation of medicarpin-β-D-glucoside (II) from roots of alfalfa

Roots (12 kg) of alfalfa, Medicago sativa L. cultivar "Rhizoma," were soaked in methanol (60 liters) for two weeks and this procedure was repeated again. The combined solution were concentrated under reduced pressure, and the aqueous residue was adjusted at pH 2 with 3N hydrochloric acid. After repeated extraction with ethyl acetate, the combined organic layer was washed carefully with saturated aqueous sodium bicarbonate and dried over anhydrous sodium sulfate. Evaporation under reduced pressure gave 66 g of a neutral fraction, which was dissolved in 200 ml of acetone and mixed with acid-washed Celite (200 g). After being dried in vacuo, the impregnated Celite was charged onto a charcoal column (charcoal, 700 g; φ 7.5 cm × 105 cm). The column was eluted with a solvent system of acetone-water. After successive elutions with 40, 60, and 80% acetone (12, 27 and 11 liters, respectively), the column was eluted with 100% acetone (11 liters), which was evaporated under reduced pressure to yield 11 g of oil. White powdery crystals deposited in this oil were separated on a glass filter by filtration and successively washed with ethyl acetate, methanol and benzene to give pure crystals of II (1.48 g).

Properties of medicarpin-β-D-glucoside (II)

Mp 270~272°C. [α]_D^23 = −149° (c = 0.9, pyridine). Soluble in pyridine and dimethylsulfoxide, slightly soluble in dioxane, and hardly soluble in other common organic solvents and water. Anal. Found: C, 60.56; H, 5.47. Caled. for C_{22}H_{24}O_9: C, 61.11; H, 5.59%. UV _max nm (ε): 287 (10253). IR _max cm⁻¹: 3560~3080, 1620, 1575, 1284, 1072, 955, 832 (Fig. 2). NMR (in D_6-dimethylsulfoxide+D_2O) δ TMS ppm: 3.69 (3H, s, 9-OCH_3), 4.28 (1H, m, 6-Heq), 4.83 (1H, d, J = 6.5 Hz, 1-L-H), 5.60 (1H, d, J = 7.0 Hz, 11a-H), 6.41 (1H, s, 10-H), 6.45 (1H, dd, J = 2.2, 8.7 Hz, 8-H), 6.55 (1H, d, J = 2.5 Hz, 4-H), 6.71 (1H, dd, J = 2.5, 8.3 Hz, 2-H), 7.24 (1H, d, J = 8.7 Hz, 7-H), 7.38 (1H, d, J = 8.3 Hz, 1-H) (Fig. 1).

Tetraacetyl medicarpin-β-D-glucoside (III)

II (140 mg) was dissolved in a mixture of pyridine (18 ml) and acetic anhydride (6 ml) and allowed to stand at room temperature for 6 days. After completion of the reaction, the mixture was poured onto ice-cold conc. hydrochloric acid (20 ml) and extracted repeatedly with ethyl acetate. The combined extracts were washed with aqueous sodium bicarbonate and dried over anhydrous sodium sulfate. Evaporation of the solvent under reduced pressure afforded crude crystals, which were recrystallized from ethyl acetate-methanol. Yield 142 mg. Mp 183~184°C. Anal. Found: C, 59.94; H, 5.24; O, 34.9. Caled. for C_{30}H_{32}O_{13}: C, 59.99; H, 5.37; O, 34.6%. [α]_D^23 = −109° (c = 1.0, ethyl acetate). UV _max nm (ε): 284 (10270). IR _max cm⁻¹: 1750, 1618, 1595, 1495, 1284, 1180, 1083, 1032, 955, 832. NMR δ ppm: 2.02 (3H, s, CH_3CO), 2.03 (6H, s, 2×CH_3CO), 2.05 (3H, s, CH_3CO), 3.50~3.72 (2H, m, 6a-H, 6-Hx), 3.73 (3H, s, 9-OCH_3), 3.80~3.96 (1H, m, 5-L-H), 4.15~4.27 (3H, m, 6-, 6-Hx), 4.99~5.30 (4H, m, 1-, 2-, 3-, 4'-H), 5.46 (1H, d, J = 7.0, 11a-H), 6.36~6.47 (2H, m, 8-, 10-H), 6.54 (1H, d, J = 2.5 Hz, 4-H), 6.66 (1H, dd, J = 2.5, 8.0 Hz, 2-H), 7.07 (1H, d, J = 8.8 Hz, 7-H), 7.39 (1H, d, J = 8.0 Hz, 1-H). MS m/e: 600 (M⁺), 331, 270, 169, 109, 43 (base peak).

Acid hydrolysis of II

II (13 mg) was suspended in a mixture of 2 N hydrochloric acid (2.5 ml) and dioxane (2.5 ml) and refluxed at 120~130°C for 2 hr. At the end of the end of the reaction, 5 ml of water was added to the reaction mixture, which was extracted repeatedly with ethyl acetate. The aqueous layer was evaporated...
in vacuo with repeated additions of water, and the residue was dried over sodium hydroxide in a vacuum desiccator to give 5.8 mg of crude glucose.

Identification of d-glucose

A small amount of crude glucose was treated by the method of Sweeley et al.\textsuperscript{8} to give trimethylsilyl derivative, which, without further purification, was applied to GLC analysis under the following condition: Instrument, Hitachi K–53 Gas-chromatograph equipped with FID; Column, a glass column (\(\phi 3\) mm × 200 cm) packed with 3\% OV–1 on Chromosorb; Column temperature, 143°C; Flow rate, 35 ml/min; Carrier gas, N\textsubscript{2}. Retention times (7.7 and 12.8 min) of the sample was completely identical with those of an authentic trimethylsilyl derivative of glucose. Further identification was carried out by co-chromatography on GLC.

The ORD spectrum of the glucose obtained from II showed a positive plane curve in water.

Isolation of medicarpin (I)

One hundred mg of II was finely powdered by use of a homogenizer and suspended in 20 ml of 1M sodium citrate buffer (pH 5.1). After treatment with a supersonic generator, the suspension was added to 1 ml of crude enzyme syrup prepared from snail digestive fluid (Sue D’Helix Pomatia, Industrie Biologique Francaise), and the mixture was shaken at 37°C for 24 hr. At the end of the reaction, the unaltered II was precipitated by centrifugation, and the precipitates were washed successively with water and acetone. The supernatant solution and the wash water were combined and extracted with ethyl acetate, from which a phenolic fraction was separated through the conventional procedure. The fraction was combined with acetone used for washing and evaporated under reduced pressure to give an oil (28 mg). The oil was applied onto Silicagel GF254 preparative TLC (thickness, 0.5 mm) using benzene-ethyl acetate (5:1, v/v) as a developing solvent. The band observed at R\textsubscript{F} 0.45–0.50 under a UV lamp was scraped off and extracted with acetone. After evaporation of the solvent, the residue was recrystallized from chloroform-hexane to afford 5.3 mg of I as pure crystals.

The properties of I were as follows: Mp 131–132°C. MS m/e: 270 (M\textsuperscript{+}), 255, 197, 161, 148, 147, 135. UV \(\lambda\text{MeOH}\) nm (\(\epsilon\)): 282 (4977), 287 (5526). IR \(\nu\) cm\textsuperscript{-1}: 3390, 1619, 1588, 1493, 1447, 1385, 1335, 1322, 1294, 1276, 1187, 1146, 1128, 1114, 1085, 1035, 1018, 992, 895, 847, 830, 793, 740. NMR \(\delta\text{TMS}\) ppm: 3.49–3.75 (2H, m, 6a-H, 6-Hx), 3.76 (3H, s, 9-OCH\textsubscript{3}), 4.17–4.29 (1H, m, 6–Hec), 5.48 (1H, d, \(J=6.0\) Hz, 11a–H), 6.39–6.53 (3.5 Hz, m, 4–, 8–, 10–H, 2–0.5 H), 6.59 (0.5H, d, \(J=2\) Hz, 2–0.5 H), 7.17 (1H, d, \(J=9.0\) Hz, 7–H), 7.37 (1H, d, \(J=8.0\) Hz, 1–H). ORD 600–330 nm (\(c=0.050\), MeOH), 330–240 nm (\(c=0.0083\), MeOH) \([\alpha]\text{MeOH}\): −120 (590), −440 (T, 343), +144 (300), +324 (P, 295), −384 (285), −6480 (T, 277), −5760 (P, 269), −14280 (T, 243).

Acknowledgement. The authors express their gratitude to Prof. S. Tamura of The University of Tokyo for his unfailing guidance throughout the course of this work. They are indebted to Dr. D. G. Smith of Atlantic Regional Laboratory, Halifax, Canada for supplying the copies of IR, UV and NMR spectra of an authentic medicarpin and Dr. D. Donnelly of University College, Dublin, Ireland for his gift of an authentic homopterocarpin. They wish to express their thanks to Prof. S. Shibata of The University of Tokyo and Dr. Y. Nishikawa of Kanazawa University for their gifts of sopho japonicin and its acetate. They are indebted to Mr. K. Furihata of Institute of Applied Microbiology, The University of Tokyo for measurement of NMR spectra and members of Analytical Laboratory of the same Institute for elemental analyses. They thank Dr. K. Aizawa and his colleagues of their Department for instrumental analyses.

REFERENCES