Chemical Studies on "Clover Sickness"

Part I. Isolation and Structural Elucidation of Two New Isoflavonoids in Red Clover

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Two new isoflavonoids were isolated from red clover as germination inhibitors for the same plant and their structures were determined as a glucoside of biochanin A (7-D- β -glucosyl-5,7-dihydroxy-4'-methoxyisoflavone) (II) and its 5-malonate (I), respectively. Besides these compounds the following substances were also isolated as inhibitors: trifolirhizin (III), ononin (IV), daidzein (V) and its 7-glucoside (VI), formononetin (VII), genistein (VIII) and biochanin A (IX).

Red clover (*Trifolium pratense* L.) has been well known to exhibit homogeneous allelopathy, so called "clover sickness". In the course of the research on the cause of this phenomenon, extracts of red clover were found to show considerable inhibitory action on the seed germination of the same plant.¹⁾ Then, isolation of the active principles contained in the extracts was undertaken by the present authors, and the results have been preliminarily reported in the previous paper.²⁾ Here the isolation and structural elucidation of two new isoflavonoids together with other various compounds obtained as germination inhibitors will be described in details.

At first, leaves and stems of red clover were extracted with methanol. Then ethyl acetate-soluble acidic and neutral fractions were separated from the methanol extract and treated as illustrated in Fig. 1a. The former acidic fraction was purified by silicic acid

partition chromatography and crystallized from aqueous methanol to give a biologically active principle (I) as colorless needles melting at 214~216°C. After evaporation of the solvent, the neutral fraction was applied to a charcoal column. As shown in Fig. 1b, successive elution with aqueous acetone, acetone and acetone-aqueous ammonia gave a new isoflavonoid (II) besides seven compounds which were identified as trifolirhizin³⁾ (III), ononin,⁴⁾ daidzein⁵⁾ (V) and its 7-glucoside⁶⁾ (VI), formononetin⁷⁾ (VII), genistein⁸⁾ (VIII) and biochanin A⁹⁾ (IX). The compound II was obtained from the eluate with 90% aqueous acetone as needles melting at 207~209°C. No inhibitor other than the nine compounds

3) J. B. Bredenberg and P. K. Hietala, *Acta Chem. Scand.*, **15**, 698 (1961).

4) F. Wessely and F. Lecher, *Monatsh.*, **57**, 395 (1931).

5) E. Walz, *Ann.*, **489**, 118 (1931).

6) S. Shibata, T. Murakami and Y. Nishikawa, *J. Pharm. Soc. Japan*, **79**, 757 (1957).

7) E. C. Bate-Smith, T. Swain and G. S. Pope, *Chem. and Ind.*, **1954**, 1127.

8) G. S. Pope and H. G. Wright, *ibid.*, **1953**, 1019.

9) S. Siddiqui, *J. Sic. and Ind. Research (India)*, **4**, 68 (1945).

1) S. Kumai, *Chemical Regulation of Plants*, **1**, 179 (1966).

2) S. Tamura, C. Chang, A. Suzuki and S. Kumai, *Agr. Biol. Chem.*, **31**, 1108 (1967).

mentioned above was isolated from red clover extracts in this experiment.

The molecular formula $C_{25}H_{24}O_{13}$ was assigned to the acidic substance I through elemental analysis and molecular weight de-

termination. Its IR spectrum (Fig. 2) reveals the presence of polyhydroxy and three carbonyl bands together with the presence of an isoflavone nucleus.¹⁰⁾ On mild hydrolysis with 0.01 N methanolic hydrochloric acid, I gave a

FIG. 1a. Isolation Procedure for 5-Malonyl-7-D- β -glucosyl-5,7-dihydroxy-4'-methoxyisoflavone (I).

* Treatment of this fraction is illustrated in Fig. 1b.

10) J. H. Looker and W. W. Hanneman, *J. Org. Chem.*, **27**, 381 (1962).

FIG. 1b. Isolation Procedure for 7-D- β -Glucosyl-5,7-dihydroxy-4'-methoxyisoflavone (II) and Other Compounds III-IX.

FIG. 2. IR Spectrum of 5-Malonyl-7-D- β -glucosyl-5,7-dihydroxy-4'-methoxyisoflavone (I) in Nujol.

neutral compound, C₂₂H₂₂O₁₀, completely identical with the compound II mentioned above.

When II was hydrolyzed with concentrated phosphoric acid, it afforded D-glucose and an isoflavone, and no other compound could be detected in the hydrolyzate. By comparison of UV and IR spectra as well as by chemical degradation according to the report of F. E. King *et al.*,¹¹⁾ the isoflavone was identified as biochanin A, 5,7-dihydroxy-4'-methoxyisoflavone,

which was first isolated from *Chana germ* by S. Siddiqui.⁹⁾ Thus, II was confirmed to be a new glucoside consisting of each one mole of D-glucose and biochanin A.

In the UV spectrum of II (Fig. 3), the absorption maximum at 263 m μ in neutral ethanol solution did not shift in alkaline conditions.¹²⁾ This suggests that the glucose moiety is attached to the C-7 of the isoflavone nucleus. The location of the linkage was further confirmed by the inaction of II with

11) F. E. King, M. F. Crundon and K. G. Neill, *J. Chem. Soc.*, **1952**, 4580.

12) L. Juro and R. M. Horowitz, *J. Org. Chem.*, **22**, 1618 (1957).

FIG. 3. UV Spectra of Biochanin A 7-glucoside (II).

— in EtOH, ---- in 0.01 N NaOH-EtOH.

FIG. 4. NMR Spectra of I and II in DMSO.

diazomethane.¹³⁾ In the NMR spectrum of II (Fig. 4), a 1H doublet at δ 5.07* was assigned to the C-1''H of the glucose moiety, and the coupling constant, 7.5 cps between C-1''H and C-2''H indicated the β -configuration. Since the signal at δ 5.07 was not deshielded by acetylation of II, C-7 and C-1'' should be connected with one another. Thus, II has been established to be 7-D- β -glucosyl-5,7-dihydroxy-4'-methoxy-isoflavone. This may be the first example for the isolation of a glucoside of biochanin A.

- (I) $R = \text{COCH}_2\text{CO}_2\text{H}$
 (II) $R = \text{H}$

When I was mildly hydrolyzed with dilute methanolic hydrochloric acid as mentioned earlier, carbon dioxide and acetic acid were produced besides II. Moreover, a trace amount of dimethyl malonate was detected, when hydrolyzate was treated with diazomethane and subjected to gas chromatography as shown in Fig. 5. The $\text{C}_3\text{H}_2\text{O}_3$ difference in the molecular formulas of I and II corresponds to the presence of a malonyl moiety in the former. The bands at 1698 and 1768 cm^{-1} in the IR spectrum of I can be respectively assigned to the two carbonyls of the malonyl residue constituting respectively the free carboxyl and the ester bond with phenol. Further, a 2H singlet at δ 3.35 in the NMR spectrum of I (Fig. 4) should be assigned to the methylene group in the malonyl moiety. Thus, the structure of I has been established as 5-malonyl-7-D- β -glucosyl-5,7-dihydroxy-4'-methoxyisoflavone, which is a novel hemi-

Fig. 5. Gas Chromatogram of Ether-Soluble Acidic Fraction Derived from Hydrolysis of I.

Column (5×225 mm): diethylene glycol succinate (Shimalite, 60~80 mesh, 15%); Column temp.: 120°C ; Sample chamber temp.: 145°C ; Detection temp.: 170°C ; Carrier gas: N_2 30 ml/min.

malonate among the isoflavonoids isolated so far.

The compounds reported here showed inhibitory activity on germination of red clover seeds at the concentrations of 30 to 1000 ppm. The details of the biological activities will be reported elsewhere.

EXPERIMENTAL

IR measurements were made with a JASCO-S KCl spectrophotometer, and UV spectra were measured with a Cary-PM spectrophotometer. NMR spectra were measured with a JNM-4H-100 spectrometer. Gas chromatography was performed on a Shimadzu Gas Chromatograph GC-1B.

Isolation of an acidic isoflavonoid (I), 5-malonyl-7-D- β -glucosyl-5,7-dihydroxy-4'-methoxyisoflavone. As indicated in Fig. 1a, 50 kg of leaves and stems of red clover were dried in shade and extracted with three 50 l-portions of methanol. The combined extracts were evaporated under reduced pressure to 5 l of the aqueous residue, which was shaken with 4 l of hexane. The aqueous phase was adjusted to pH

13) T. A. Geissman and R. O. Clinton, *J. Am. Chem. Soc.*, **86**, 697, 700 (1964).

* Chemical shifts are expressed in δ -values (ppm) from tetramethylsilane as an internal standard.

3.0 with dilute hydrochloric acid and extracted with three 2.5 l-portions of ethyl acetate. The extracts were concentrated to 3 l under reduced pressure and stirred with three 2 l-portions of saturated aqueous sodium bicarbonate. The combined aqueous layers were acidified to pH 3.0 and extracted with three 3 l-portions of ethyl acetate. The extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure to yield 100 g of a residual syrup. Ten grams of the syrup was dissolved in 50 ml of ethyl acetate and applied to a column by using 150 g each of silicic acid (Mallinckrodt) and 0.1 N phosphate buffer (pH 6.5). The column was successively eluted with 2 l-portions of ethyl acetate containing 1.0, 2.0 or 3.0% butanol saturated with the above-mentioned phosphate buffer. The eluate with ethyl acetate containing 3% butanol afforded 1.5 g of a yellow oil, which was further applied to a column of 20 g of celite impregnated with the upper layer of the mixture of chloroform-60% ethanol-ethyl acetate (2:2:1, v/v). The column was successively eluted with 50 ml-portions of the lower layer of the above mentioned solvent system. Fractions No. 5~8 were combined and evaporated under reduced pressure to give 10 mg of I. Recrystallization from aqueous methanol afforded colorless needles, mp 214~216°C. *Anal.* Found: C, 56.07; H, 4.43. *Calcd.* for $C_{25}H_{24}O_{13}$: C, 56.39; H, 4.54%. UV $\lambda_{\text{max}}^{\text{EtOH}}$ $m\mu(\epsilon)$: 263 (29,000), 335 (3500). IR $\nu_{\text{max}}^{\text{Nujol}}$ cm^{-1} : 3200, 1768, 1698, 1660, 1615, 1580, 1518.

Isolation of a neutral isoflavonoid (II), 7-D- β -glucosyl-5,7-dihydroxy-4'-methoxyisoflavone. As indicated in Fig. 1b, the remaining ethyl acetate solution after separation of the acidic fraction by stirring with saturated aqueous sodium bicarbonate was evaporated under reduced pressure to give 150 g of a yellow syrup. Ten grams of the syrup was applied to a 100 g-charcoal column and the column was successively eluted with 2 l-portions of water containing acetone increased by ten percent steps. The eluate with 90% aqueous acetone was evaporated under reduced pressure to give 1.2 g of a yellow powder, which was further applied to a 15 g-charcoal column with the same manner as mentioned above. The fraction with 90% aqueous acetone was evaporated under reduced pressure to give 150 mg of crude II. Recrystallization from aqueous ethanol afforded colorless needles, mp 207~209°C. *Anal.* Found: C, 58.95; H, 4.82. *Calcd.* for $C_{22}H_{22}O_{10}$: C, 59.19, H, 4.97%, UV $\lambda_{\text{max}}^{\text{EtOH}}$ $m\mu(\epsilon)$: 263 (30,000), 336 (3000). IR $\nu_{\text{max}}^{\text{Nujol}}$ cm^{-1} : 3200, 1660,

1580, 1520, 1493.

Isolation and identification of compounds III-IX. As shown in Fig. 1b, trifolirhizin (III) and ononin (IV) were obtained from the eluate with 60 and 70% aqueous acetone from the above mentioned charcoal column in yields of 45 and 210 mg, respectively. Further, 10 mg of daidzein (V), 2 mg of its 7-glucoside (VI), 350 mg of formononetin (VII), 15 mg of genistein (VIII) and 200 mg of biochanin A (IX) were obtained respectively.

III and VII were assigned by the IR and UV spectra and melting points which were identical with reported data for the compounds. Other five compounds were proved by comparisons of their melting points and chemical reactions with those in the earlier reports. Moreover, these assignments were supported by the following reactions. When daidzein glucoside (VI) was hydrolyzed with concentrated phosphoric acid, it gave free daidzein (V). Treatment of VI with diazomethane afforded ononin (IV). Further, hydrolysis of IV with concentrated phosphoric acid yielded formononetin (VII). When V and VII were treated with diazomethane, they afforded 4',7-dimethoxy-isoflavone. In a similar way genistein (VIII) and biochanin A (IX) gave 4',7-dimethoxy-5-hydroxy-isoflavone.

Mild acid hydrolysis of I

Formation of carbon dioxide—To 5 ml of methanol containing 0.05 ml of 1 N hydrochloric acid was added 50 mg of I, and the mixture was refluxed at 50°C for 20 min in a stream of nitrogen purified with acid, alkali and aqueous potassium permanganate successively. Carbon dioxide evolved was passed through 20 ml of aqueous solution containing 0.8 g of barium hydroxide and 0.4 g of barium chloride. The precipitated barium carbonate was separated, washed successively with water, 95% ethanol and ether and dried under reduced pressure. The yield was 17.3 mg which corresponds to 3.7 mg of carbon dioxide.

Formation of acetic and malonic acids—After the end of the hydrolysis, the reaction mixture was neutralized with dilute sodium hydroxide and the solvent was evaporated under reduced pressure. Then, water was added to the residue to amount to 50 ml, and the resulting solution was treated as follows:

a) One half of the solution was subjected to steam distillation, and 200 ml of the distillate was neutralized with 0.68 ml of 0.01 N sodium hydroxide which corresponds to 2.73 ml of acetic acid assuming that it

should be the predominant organic acid. The remaining aqueous solution after steam distillation was used for the isolation of II.

b) Another half of the solution was acidified with dilute hydrochloric acid and extracted with three 20 ml- portions of ether. The aqueous layer was used for the isolation of II. The combined ether extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was treated with 2 ml of ethereal diazomethane and the solvent was removed to yield an oily mixture of methyl esters, which was subjected to gas chromatography to reveal the presence of a minute amount of dimethyl malonate as shown in Fig. 5.

Formation of the isoflavonoid (II)—The above mentioned aqueous residues were combined and evaporated to a solid, which was then dissolved in 1 ml of ethanol. After separation of insoluble matter, the solution was evaporated under reduced pressure to give 40 mg of the isoflavonoid (II). Recrystallization from methanol afforded colorless needles, mp 207~209°C.

Hydrolysis of II and its identification as biochanin A glucoside. To a solution of 200 mg of II in 10 ml of 50% aqueous ethanol was added 1 g of concentrated phosphoric acid. The mixture was heated at 115°C for 3 hr in a sealed tube and then poured into 100 ml of ice-water to give crystals. Recrystallization from aqueous methanol afforded 110 mg of colorless needles, mp 212~214°C, which were identified as biochanin A based on the following data. *Anal.* Found: C, 67.72; H, 4.10. Calcd. for $C_{18}H_{12}O_5$: C, 67.60; H, 4.26%. UV $\lambda_{\max}^{EtOH}$ $m\mu(\epsilon)$: 263 (30,000), $\lambda_{\max}^{EtOH-NaOH}$ $m\mu(\epsilon)$: 274 (30,000), 336 (9000). IR $\nu_{\max}^{Nujol}$ cm^{-1} : 3475, 3380, 1660, 1631, 1573, 1532, 1300, 1258, 1190. By alkali fusion, the crystals afforded 2,4,6-trihydroxybenzoic acid and *p*-hydroxybenzoic acid. In addition, treatment with hot aqueous barium hydroxide gave anisic

acid.

The filtrate, obtained after separation of biochanin A, was concentrated to 10 ml under reduced pressure and applied to a column of 25 ml of IR-120 in H form. The column was eluted with 100 ml of water, and the eluate was concentrated to 10 ml. This was further applied to a column of 25 ml of IR-45 in OH form and eluted with 100 ml of water. The eluate was evaporated under reduced pressure to afford 80.1 mg of a syrup, which was subjected to paper chromatography in different solvent systems such as phenol-water (95:5, v/v), collidine and butanol-acetic acid-water (4:1:5, v/v). In each case a spot corresponding to D-glucose was obtained. Further, phenylhydrazone of D-glucose obtained from the natural source melted at 206~208°C and gave no depression in the melting point mixed with an authentic sample. Thus, II was identified as biochanin A glucoside.

Pentaacetate of II. After treatment of 50 mg of II with 5 ml of pyridine and 3 ml of acetic anhydride at 100°C for 1 hr, the reaction mixture was poured into 50 ml of ice-water. The resulting crude crystals were dissolved in 20 ml of ethyl acetate, and the solution was washed with saturated aqueous sodium bicarbonate, dried over anhydrous sodium sulfate and evaporated under reduced pressure to give crystals. Recrystallization from ethyl acetate-hexane afforded 45 mg of pentaacetate of II. Mp 204~206°C. *Anal.* Found: C, 58.76; H, 4.92. Calcd. for $C_{32}H_{32}O_{15}$: C, 58.53; H, 4.91%. IR $\nu_{\max}^{Nujol}$ cm^{-1} : 1778, 1752, 1293, 1258, 1223.

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