

a) The refinement of the equilibrium constants in systems with polynuclear complexes, where approximate values have been found graphically.

b) Calculation of consecutive equilibrium constants in systems with mononuclear complexes, where only the ligand number has been determined: even if linear transformations can be made<sup>3b</sup> the pit-mapping method seems a worth-while alternative. Even spectrophotometric data, which commonly give non-linear expressions may be attacked by this method.

c) Calculation of the parameters in the "two parameters" approximation<sup>5</sup> for equilibria, where the available data are not accurate enough for determining, independently, the formation constants for all the complexes  $MA$ ,  $MA_2$ ,  $MA_3$ , ...  $MA_N$ . For such systems, two ways of approximate calculations have been proposed:

1) When the standard deviation of an individual equilibrium constant comes out as greater than the value of this constant, Sullivan, Rydberg and Miller<sup>3b</sup> eliminate that particular constant from their equations, which from a chemical point of view is equivalent to assuming that the concentration of the related species is equal to zero.

2) In the "two parameters" approximation<sup>5</sup>, one assumes that all complexes exist and that the ratio between consecutive formation constants is a constant. — Neither of the approximations 1) or 2) can be completely true, but the second one may, we think, often come somewhat closer to chemical reality. Earlier, only the first approximation could be treated by computer programs<sup>3</sup>.

Our results hitherto like, in our opinion, those of Sullivan *et al.*<sup>3b</sup> indicate that with good data, the "least-squares" approach gives no gain in real accuracy, as compared with adequate graphical methods, using the experimental data directly. In problems with many constants to be determined, considerable time may however be gained by making the final refinement of the constants by means of the computer.

The results and the full description of our program, will be published in due time.

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## Investigation of the Structure of Trifolirhizin, an Antifungal Compound from *Trifolium pratense* L.

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In an investigation of the antifungal substances in red clover (*Trifolium pratense* L.) the isolation of one of the main components,  $C_{21}H_{24}O_{10}$ , m.p. 139–140°, has been reported<sup>1</sup>. This compound has now been investigated more closely. Since it is apparently a new compound, isolated so far only from the roots, the name trifolirhizin is proposed for it.

Trifolirhizin crystallizes from methanol as rods, m.p. 142–144° (decomp.),  $[\alpha]_D^{20}$  –183°, with one mole of methanol. The elementary composition of the compound has now been found to agree with  $C_{22}H_{22}O_{10}$ . It does not contain methoxyl or C-methyl groups and, according to the chemical and infrared data, is free from carbonyl and carboxyl groups. Upon catalytic hydrogenation it is recovered unchanged. It forms a tetraacetate,  $C_{30}H_{30}O_{14}$ , m.p. 188–189°,  $[\alpha]_D^{20}$  –126° in which all the hydroxyl groups are acetylated. Upon acid hydrolysis it forms glucose and ether-soluble substances which could not be characterized. Alkaline hydrolysis probably also releases glucose. Trifolirhizin is thus a glucoside.

Taking the glucose molecule into account, the empirical formula for the agly-

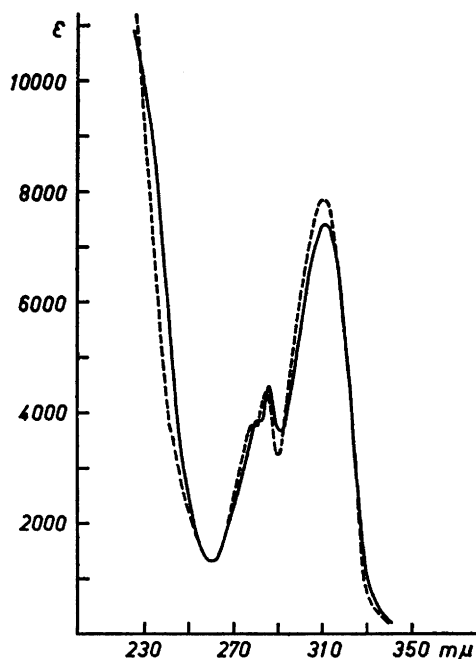


Fig. 1. Ultraviolet spectra in ethanol of pterocarpin (solid line) and trifolirhizin (broken line).

cone of trifolirhizin is  $C_{16}H_{12}O_5$ . One of the oxygen atoms is found in the glucoside bond. Since trifolirhizin contains four hydroxyl groups, all belonging to the glucose part, and since it does not have any carbonyl or carboxyl groups, the remaining four oxygen atoms in the aglycone must be of ether type. The infrared spectrum supports the presence of a methylenedioxy group<sup>2</sup>.

The presence of glucose, however, makes the normal colour test<sup>3</sup> for this group of little value.

The low hydrogen content and resistance to hydrogenation can only be explained if two benzene rings are assumed to be present. The very peculiar ultraviolet spectrum (Fig. 1) can be interpreted according to this assumption: the maxima at 280 and

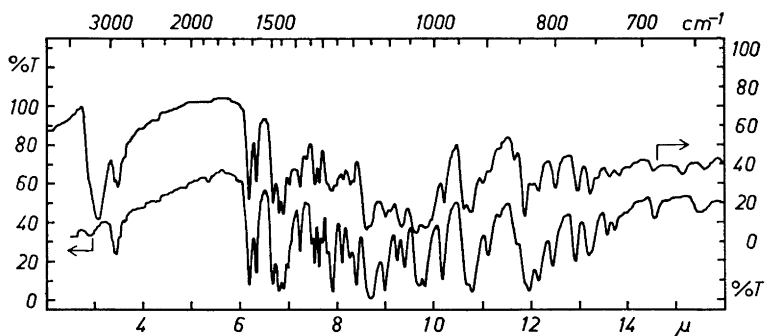
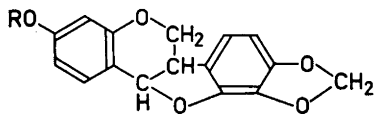


Fig. 2. Infrared spectra of trifolirhizin (above, right scale, 2.2 mg/300 mg KBr) and pterocarpin (below, left scale, 2.0 mg/300 mg KBr).

285  $m\mu$  are formed from a slightly substituted benzene ring and the maximum at 310  $m\mu$  from a strongly substituted benzene ring. The latter maximum, however, occurs at a surprisingly high wavelength.

Taking into account the occurrence of isoflavones in clover<sup>4-6</sup> and their antifungal properties<sup>7-9</sup> a compound of pterocarpin (I)<sup>10</sup> type could be assumed to be present. When the ultraviolet and infrared spectra of a sample of pterocarpin, kindly supplied by Dr. W. B. Whalley, of Liverpool, were compared with those of trifolirhizin, a striking similarity was observed (Figs. 1 and 2). The ultraviolet spectra show that the compounds have identical chromophores. Since pterocarpin is methylated at the 7-position and trifolirhizin does not contain any methoxyl group, the glucose is evidently bound to the aglycone at this point. This would also explain<sup>11</sup> the suspected alkali lability of trifolirhizin. On the basis of the above results the structure II is therefore tentatively suggested for trifolirhizin.



I: R = CH<sub>3</sub>

II: R = Glucose

It is of interest to note the recent report<sup>12,13</sup> of the isolation of an antifungal compound from *Pisum sativum* L. which seems to bear a close relationship to the above-mentioned compounds. Lack of details, however, precludes further comparison.

**Experimental.** For details of the isolation, see Hietala<sup>1</sup>. A good concentrate of the compound can be obtained by analogous operation in a series of about ten separatory funnels. Trifolirhizin crystallizes well from methanol, forming rods, m.p. 142–144° (decomp.),  $[\alpha]_D^{20}$  –183° (ethanol, *c* 1.5). (Found: C 57.89; H 5.58; O 35.96; O–CH<sub>3</sub> 5.51, 7.35; C–CH<sub>3</sub> 0.00. Calc. for C<sub>22</sub>H<sub>22</sub>O<sub>10</sub> · CH<sub>3</sub>OH: C 57.73; H 5.48; O 36.78; O–CH<sub>3</sub> 6.5. Crystallization from benzene gave C 59.46; H 5.23; O 33.52; O–CH<sub>3</sub> 0.32. Calc. for C<sub>22</sub>H<sub>22</sub>O<sub>10</sub>: C 59.19; H 4.97; O 35.84.) The compound is difficultly soluble in benzene, easily soluble in warm methanol. It does not react with 2,4-dinitrophenylhydrazine under normal conditions. Hydrogenation with

platinum in ethanol or acetic acid at room temperature gave unchanged starting material (mixed m.p., IR). **Acetate:** The compound was acetylated with acetic anhydride and pyridine (30 min, water bath) and crystallized from methanol, cluster of needles, m.p. 188–189°,  $[\alpha]_D^{20}$  –126° (acetone, *c* 1). (Found: C 58.49, 58.23; H 4.94, 5.07; O 35.76, 36.52; O–CH<sub>3</sub> 0.00; Acetyl 27.74%. Calc. for C<sub>30</sub>H<sub>30</sub>O<sub>14</sub>: C 58.63; H 4.92; O 36.45; Acetyl (4–OH) 28.0%.) The infrared spectrum had no absorption in the hydroxyl stretching range. **Hydrolysis experiments:** The compound (51 mg) was dissolved in acetic acid (10 ml) and conc. HCl (1 ml) was added. The solution was kept at room temperature for 20 h and was then warmed on a water bath for 30 min. After addition of water the solution was extracted with ether. The extracted aqueous solution was poured through a column of IR-120 in H-form and the column washed with water. The eluate was repeatedly evaporated to a small volume and rediluted with water; finally it was evaporated to dryness. The residue was extracted with hot ethanol and the ethanol solution evaporated. Paper chromatography of the evaporation residue in three different solvent systems (phenol-NH<sub>3</sub>, butanol-acetic acid-water, ethyl acetate-pyridine-water) gave spots with the same *R<sub>F</sub>* values as glucose. The osazone, prepared in the usual way, melted at 202–204° and gave no depression with authentic material. Upon evaporation the ether solution gave a dark, semisolid mass (27 mg), with a maximum in the ultraviolet at 286  $m\mu$ . No crystalline material could be obtained from this residue. Alkaline hydrolysis of trifolirhizin with boiling 1 N NaOH in methanol for 1.5 h yielded, after acidification and extraction as above, upon paper chromatography of the water phase, a spot with the same *R<sub>F</sub>* value as glucose (butanol-acetic acid-water). No crystalline material could be obtained from the ether phase.

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## A Physico-chemical Study on the Haemolytic Activities of *Staphylococcus aureus*

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In order to obtain good yields of haemolysins from *Staphylococcus aureus* (238) the cells have to be cultivated for rather long times. In the process heavily charged acid polyelectrolytes appear in the medium, most probably due to lysis of part of the cells. Whether the haemolytic activities are to be purified from the cells or, as in the following report, from the medium, these acid polyelectrolytes will interfere with the purification procedure<sup>1,2</sup>. Some results of a characterization by means of counter flow electrophoresis<sup>2</sup> and chromatography will be reported.

*Materials and methods. Medium I.* 1) Heat 1 800 g of freshly ground horse meat in 3 000 ml water to 75°C. 2) Add 3 000 ml 0.1 M sodium carbonate. 3) Allow to cool to 45–50°C. 4) 5 g pancreatine and 30 ml CHCl<sub>3</sub> are added. 5) Let stand for 3.5 h at 37°–40°C. 6) Add 40 ml conc. HCl. 7) Heat to boiling. 8) Filter through coarse filter paper (not cotton wool). 9) Adjust

pH to 8.2–8.3 with 0.1 M HCl and 0.1 M NaOH. 10) Heat to boiling and filter again. 11) Add agar to 1.2 % W/v. 12) Autoclave for 30 min at 119°C. 13) Filter. 14) Autoclave for 20 min at 119°C. 15) Dissolve one part agar medium (I) in 1.34 parts broth medium (II, see below). This step gives a final agar concentration in medium I of 0.85 %. 16) Dispense and autoclave for 15 min at 119°C.

*Medium II.* 1) Autoclave together 2 500 g of freshly ground horse meat and 6 000 ml 0.25 M NaCl for one hour at atmospheric pressure and filter through cheese cloth and coarse filter paper. 2) Make up volume to 6 000 ml. 3) Add peptone (Merck) to give 4 % W/v. 4) Adjust pH to 7.4 (see step 9 for medium I). 5) Heat to boiling and filter through coarse filter paper. 6) Add 12 g KH<sub>2</sub>PO<sub>4</sub> per litre. 7) Adjust pH to 7.4. 8) Boil the broth for 30 min, filter and autoclave for 20 min at 119°C. 9) Dispense and autoclave for 45 min at 100°C.

*Cultivation technique.* The cells were first cultivated on 10 % ox blood agar plates for 24 h at 37°C. No carbon dioxide was added to the air. The cells were transferred to a 10 % horse serum broth and cultivated for 24 h under the same atmospheric conditions.

From this culture 5 ml were inoculated into 400 ml of the semisolid medium I, contained in a 2 litre Fernbach bottle. After 72 h, 600 ml of medium II were added to the bottle, and the cultivation was continued for another 72 h. The temperature was 37°C and the gas phase consisted of CO<sub>2</sub>:O<sub>2</sub> in the proportions 2:5. The gas phase was regulated by two narcose flowmeters (AGA 67827K and AGA 67828K, respectively) coupled in parallel.

*Harvesting procedure.* The culture was cooled rapidly to +1°C and was centrifuged at +1°C for 90 min at 7 000 × g average.

*Concentration of the medium.* Different methods were tried. Freeze drying, pervaporation, the polyethylene glycol method of Kohn<sup>3</sup> and the technique of Palmstierna<sup>4</sup> were used. In our hands the last mentioned method gave the best results. Very small, if any, losses in haemolytic (*in vitro*<sup>5</sup>) and lethal activity (mice) were found. The lethal activity was tested on 12–14 g mice (Sigtuna strain) by giving 0.25 ml intraperitoneally. Serial dilutions were made in saline.

*Chemical methods.* In order to isolate the proteins from the acid polyelectrolytes under as mild conditions as possible, the counter flow electrophoresis (CFE) method of Eliasson *et al.*<sup>2</sup> was used. In this case the proteins were sucked off from the top of the column and pumped into a jacketed and cooled DEAE ion exchanger column<sup>6</sup> (2 × 20 cm) *via* a modified hose pressure micropump<sup>2</sup>. The electrophoresis