Confirmation of the Structure of Trifolirhizin

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In a recent communication [1], structure I was tentatively suggested for trifolirhizin, an antifungal glucoside of red clover. Since the structure of pterocarpin, on which the tentative structure was based, now has been revised to II[2], the corresponding change has to be made in the structure of trifolirhizin (III). This structure has now been confirmed by the isolation of the aglycone (IV) and its methylation to pterocarpin.

Acid hydrolysis of trifolirhizin probably releases the aglycone. Unfortunately, the small amount of starting material available and the instability of the aglycone to acid treatment made its isolation difficult. The low solubility of trifolirhizin in water also hindered the enzymatic cleavage of the pure compound. It was therefore decided to isolate the aglycone from the plant after enzymatic cleavage *in situ*. The whole roots of red clover were crushed in water and left for the enzymatic reaction to take place. The ethanol-soluble part was distributed between light petroleum — *n*-butanol/1 N ammonia. When the extracts were worked up, no trifolirhizin was found. Instead, the aglycone m.p. 179.5–180° $[\alpha]_D^{20}$ −214°, was obtained at the expected place. The infrared spectrum of the aglycone resembled that of pterocarpin but showed hydroxyl band absorption in the stretching range. The ultraviolet spectrum in ethanol was almost identical in shape with those of pterocarpin and trifolirhizin [1].

Methylation of the compound with diazomethane gave, in high yield, a neutral compound identified as pterocarpin, thus confirming structure IV for the aglycone and III for trifolirhizin.

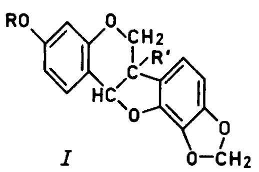
When the ultraviolet spectrum of the aglycone is taken in alkaline solution, a bathochromic shift to about 300 mμ is observed for the maxima at 281 and 287 mμ, which shows that these maxima belong to ring A. The maximum at 310 mμ is not influenced and thus belongs to ring B. This is essentially in agreement with the previously made interpretation of the ultraviolet spectrum of trifolirhizin [1].

Application of Klyne's method [3] for steroid glycosides shows that trifolirhizin is a β-glucoside. (The molecular rotation difference between trifolirhizin and the aglycone is −209; phenyl glucosides [4]: α-form + 402, β-form −182. The acetates give −166 for trifolirhizin, and + 688 and −123 for the respective phenyl glucosides.)

Biogenetical considerations would make structure V an attractive formula for the newly found antifungal substance pisatin [5].

Experimental. All m.p.'s are corrected. The ultraviolet spectra were measured with a Beckman DU apparatus. The infrared spectra were measured with a Perkin-Elmer No. 21 spectrometer.

Isolation of the aglycone. 550 g of roots of red clover were sliced and homogenised in a Waring Blendor apparatus with addition of 300 ml of water. The pulp was left at room temperature for 18 h, after which ethanol was added until a concentration of 70 % was reached. The next day the solution was filtered and the residue exhaustively extracted with 2 liter of 96 % ethanol. The extracts were filtered cold and evaporated *in vacuo*. The residue was suspended in 100 ml of 1 N ammonia and extracted with three portions of 100 ml of light petroleum (40–60°). The light petroleum extract was checked for the absence of the aglycone by ultraviolet spectrophotometry, and



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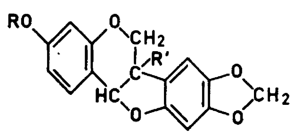

II – V

I and III: R = Glucose, R' = H; *II:* R = CH_3, R' = H;
IV: R = H, R' = H; *V:* R = CH_3, R' = OH.

discarded. A Craig countercurrent distribution in six tubes was performed between 1 N ammonia and a mixture of light petroleum and n-butanol (v/v 3:1) (after the fundamental distribution, all upper phases were withdrawn and combined, extract A) followed by a distribution in the same way with the solvent mixture 1 N ammonia — mixture of light petroleum and n-butanol (v/v 1:3) (extract B). Extract B yielded 40 mg of pratol (IR, m.p.) but no trifolirhizin could be isolated. Extract A was evaporated *in vacuo* and the residue again distributed between the former solvent system as before. The upper phase was evaporated *in vacuo*, the residue dissolved in 200 ml of methanol and 20 ml of water and the solution filtered.The filtrate was extracted with a small amount of light petroleum, which was discarded. Upon addition of 200 ml of water, an oil slowly separated out and after a time crystallised as tiny needles. The crystals were filtered, washed with aqueous methanol and water, and dried, yield 204 mg, m.p. 167—172°. Crystallisation from light petroleum gave a fine crystalline powder which sublimed at 150°/0.1 mm as long, fine needles, m.p. 179.5—180°, $[\alpha]_D^{20}$ —214° (ethanol, c 1 %). (Found C 66.84, H 4.11, O—CH_3 0.00; Calc. for $C_{16}H_{12}O_5$ C 67.59, H 4.26.) The compound is sparingly soluble in light petroleum and readily soluble in methanol. Ultraviolet spectra: ethanol, λ_{max} 281, 287, 310 mμ (logε 3.58, 3.63, 3.83), λ_{min} 260, 283, 291 mμ (3.18, 3.575, 3.57); 0.1 N sodium hydroxide in 50 % ethanol, λ_{max} 249, 300—303 mμ (logε 4.11, 3.92), inflexion 309 mμ (3.89), λ_{min} 233, 270 mμ (3.93, 3.43). Infrared spectrum: KBr, 3 530, 3 330, 1 628, 1 598, 1 506 cm^{-1}. *Methylation.* The aglycone (20 mg) was methylated with an excess of diazomethane in dry ether for three days at + 4°. The excess of diazomethane was decomposed by a drop of acetic acid and the solution was extracted with 1 N sodium hydroxide solution. The extracted ether solution was evaporated and the residue (18 mg) crystallised from light petroleum and sublimed in a high vacuum. The sublimate, m.p. 162—163°, $[\alpha]_D^{20}$ —200° (ethanol, c 0.2 %) gave no depression with authentic pterocarpin, m.p. 163—164°, ($[\alpha]_{5461}^{20}$ —207.5° (chloroform)) [6], kindly provided by Dr. W. B. Whalley, Liverpool. The ultraviolet and infrared spectra were identical. There was no change in the ultraviolet spectrum upon addition of alkali.

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A Qualitative Chemical Method for the Demonstration of "Pauly-positive" Bases in Rumen Contents

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Many methods have been described for the demonstration of physiologically active amines in biological material. Few methods have been applied to rumen contents [1]. Since histamine and tyramine, so-called Pauly-positive bases, are among compounds of interest in ruminant metabolism [2], a relatively simple chemical method was developed for demonstrating these substances in rumen contents, care being taken to avoid secondary processes during the course of the analysis.

To one volume rumen contents, with and without added histamine, tyramine, and histidine, were momentarily added 4 volumes of a 99:1 (volume) mixture of ethanol and acetic acid [3]. The mixture was centrifuged at 2 000 g for 15 min, and the supernatant adjusted to pH 7.0 with sodium hydroxide. Any precipitate formed was removed by centrifugation. The clear solution was treated with 2.5 volumes Amberlite IRC 50 and the bases adsorbed were eluted with 0.1 N hydrochloric acid [4]. The eluate was evaporated to dryness in a water bath and the residue was washed