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LEGUMINOSAE

COUMESTANS IN DISEASED WHITE CLOVER

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Abstract—Four coumestans have been detected in white clover (*Trifolium repens*) infected with various foliar pathogens, and have been identified as coumestrol, 12-*O*-methylcoumestrol, trifoliol and 7,10,12-trihydroxycoumestan (*repensol*) by chromatographic and spectroscopic methods. Repensol has not previously been reported as a natural product.

INTRODUCTION

EIGHT coumestans, including coumestrol[1,2] (I), 12-*O*-methylcoumestrol[3] (II) and trifoliol[2] (IV) have been reported as minor constituents in alfalfa[4] (*Medicago sativa*), and later work has shown that the concentration level of these compounds in the plant is markedly increased by the presence of fungal infection.[4,5] Coumestrol and trifoliol have also been isolated by Bickoff and co-workers from large scale extractions of white clover (variety Ladino).[6,7] Their concentration levels however were normally very low, and they were usually not readily discernible on chromatograms of clover extracts. We have now found that when white clover is infected with the foliar pathogens *Pseudopeziza trifolii* and *Uromyces* sp. (rust), coumestrol and trifoliol increase markedly in concentration and are readily detectable

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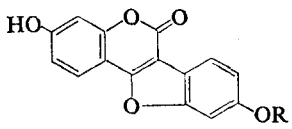
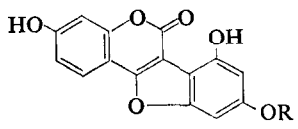


(I) R=H
(II) R=CH_3



(III) R=H
(IV) R=CH_3

by chromatographic means. In addition, two other coumestans, not previously reported as constituents of white clover, are found to be present. Their identification as 12-*O*-methylcoumestrol (II) and 7,10,12-trihydroxycoumestan (III) is reported in this communication. The latter was previously known only as a synthetic product,[7] the trivial name *repensol* is now given to this compound.

RESULTS

Healthy and fungal infected clover leaves were extracted by the usual procedure[8-10] and the ether-soluble material both before (fraction A) and after (fraction B) acid hydrolysis was examined by two-dimensional paper chromatography. The coumestans were found to be present in significant amounts (greater than 2μg/g) only in fraction A of the diseased plants. The four compounds were further purified by paper chromatography and the identities of coumestrol, 12-*O*-methylcoumestrol and trifoliol were established by means of direct chromatographic (Table 1) and spectroscopic (Table 2) comparisons with authentic materials.* The characterization of repensol as (III) was suggested by: (a) its chromatographic behaviour, which resembles that of coumestrol in the same way that trifoliol (IV)

TABLE 1. CHROMATOGRAPHIC PROPERTIES OF COUMESTANS FROM DISEASED WHITE CLOVER

Compound	R_f						Colour reaction‡ u.v.
	BeAW	30% HOAc	60% HOAc	2N NH_3	50% HOAc†	iPrOH-NH_3*†	
Coumestrol	0·32	0·25	0·29	0·23	0·48	0·66	bV-B
Repensol	0·35	0·23	0·25	0·20	0·49	0·61	B
12-*O*-Methyl-coumestrol	0·68	0·26	0·30	0·05	0·59	0·83	bB-V
Trifoliol	0·70	0·24	0·34	0·10	0·57	0·73	bV-B

* iPrOH–NH_3 = isopropyl alcohol-conc. NH_3 (2:1); for composition of other solvent systems see Ref. 10.
† TLC on silica gel.
‡ b = bright, B = blue, V = violet.

* Previously available by courtesy of Dr. R. M. Bickoff.

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TABLE 2. ULTRAVIOLET SPECTRAL PROPERTIES OF CLOVER COUMESTANS

Compound	λ_{max} (nm) in EtOH (85%)	λ_{max} (nm) in NaOH*
Coumestrol	344, 303, 244, 206	386, 320,† 281, 206
12-*O*-Methyl-coumestrol	342, 302, 243, 206	378, 311, 267, 245, 204
Repensol	354, 305,† 270, 213	400, 290, 207
Trifoliol	350, 303,† 269, 212	384, 294, 209

* *ca.* 0·003 N in 85% EtOH.
† Shoulder.

resembles 12-*O*-methylcoumestrol (II), and (b) its uv spectral properties, which closely resemble that of trifoliol (Table 2). The occurrence of a band at about 270 nm of greater optical density than the band at longer wavelength (*ca.* 350 nm) is a characteristic feature common to the spectra of these two compounds; this band is not found at such a long wavelength and at such a relative intensity for any of the other known alfalfa coumestans.[4,11] 7,10,12-Trihydroxycoumestan (III) has previously been synthesized as the parent phenol of trifoliol and its uv properties have been recorded.[7] Direct chromatographic and spectroscopic comparisons of repensol with an authentic sample of (III) kindly supplied by Dr. E. M. Bickoff confirmed the identification.

The contribution of these coumestans to the possible oestrogenicity of diseased white clover is under study.

EXPERIMENTAL

Plants

White clover plants (cv. Grasslands Huia) were grown in the glasshouse and inoculated with *Pseudopeziza trifolii* or *Uromyces* sp. Leaves of 60-day-old plants were harvested for analysis. Leaves of healthy plants were taken at the same time for comparison.

Extraction

Freeze-dried leaves (2 g) were extracted with EtOH and the extract processed essentially as previously described.[8,9] The Et_2O-soluble fractions before (fraction A) and after (fraction B) hydrolysis were separately studied.

Chromatography

The systems BeAW and 30% HOAc were used for 2-D chromatography. Spots were eluted and further purified by chromatography in 60% HOAc and 2N NH_3.

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