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SHORT COMMUNICATION

STEROLS OF *UROMYCES PHASEOLI* UREDOSPORES*

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Abstract—The major sterol of bean rust uredospores (*Uromyces phaseoli*) has been identified as 7,(*Z*)-24(28)-stigmastadien-3β-ol. A second sterol component appears to be 7-stigmasten-3β-ol. Both steroids are synthesized by the organism during the germination of the uredospores.

INTRODUCTION

SEVERAL reports of 7,24(28)-stigmastadien-3β-ol in higher plants have appeared[1–4] and this steroid has been found in flax rust uredospores.[5] In the cases where the configuration of the C-24(28) double bond has been determined,[3] or can be deduced from the information given,[4] the ethylidene group was found to be *trans* with respect to the carbon chain bearing the ring system, thus, at least in two cases, these steroids have been shown to have the (*Z*)-24(28) configuration. The major sterol of *Uromyces phaseoli* also has the (*Z*) configuration.

RESULTS AND DISCUSSION

From 3 g of spores about 6 mg of sterols were isolated by preparative TLC. After acetylation, the sterols were analyzed by GLC (column *A*) and found to contain at least three components. TLC with $AgNO_3$ impregnated plates provided for separation of the acetylated sterols into two bands (R_f 0·55 and 0·65). The upper band consisted of at least two components. The lower band contained only one component, which represented about 85% of the total sterols as determined by GLC. All components, as free sterols, were precipitable with digitonin, thus indicating that they were 3β-hydroxy steroids.

Sterol I. As its acetate derivative, sterol I was isolated from the mixed sterols by $AgNO_3$–TLC (R_f 0·55). After three crystallizations from acetone, a m.p. of 154–156° was observed. Free sterol I was obtained by transesterification of its acetate and crystallization from CH_3OH. It was determined to be at least 98% pure by GLC of its trimethylsilyl ether derivative (column *B*) and to have a m.p. of 146–148°.

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Since the acetate derivative exhibited a higher m.p. than sterol I, a double bond at the 7 position was indicated.[6] A Liebermann–Burchard reaction similar to other Δ^7 sterols[7] and a positive Tortelli–Jaffe reaction[8] were observed. The mass spectrum of sterol I acetate also indicated the presence of double bonds at both the 7 and 24(28) positions,[9–11] as the two largest peaks in the upper mass range were at *m/e* 356 and 313. In addition to the molecular ion, M, at 454 *m/e* (3·6%), strong peaks were observed at 439 (3·6%, M–CH_3); 394 (1·4%, M-acetate), 379 (3·1%, M–CH_3 and acetate); 356 (46·7%, M–C_7H_{14} of side chain); 341 (5·6%, M–C_7H_{14} and CH_3); 313 (100%, M-side chain-2H); 296 (5·7%, 356-acetate; 288 (7·9%, M-side chain and $C_{16,17}$); 281 (4·9%, 296–CH_3); 255 (12·4%, M-side chain and acetate); 213 (18·6%, M-side chain and 42 and acetate). Considering the different derivatives used, the fragmentation pattern of sterol I appeared to be similar to those observed for the 7,24(28) stigmastadienols identified previously[1,3–5,10] except that the relative intensity of the steroidal fragment corresponding to the peak at 356 *m/e* in our spectrum was lower, than those in the spectra where a comparison could be made.[1,4,10]

The presence of an ethylidene group at the 24-position also was indicated by the IR spectrum of sterol I acetate. Further evidence was obtained by germinating the uredospores on a medium containing methionine-methyl-^{14}C. During biosynthesis of sterol I, ^{14}C would be expected to be incorporated into the ethylidene group of the steroid if the group was present.[12] The resulting ^{14}C-labeled sterol I was isolated and oxidized with $KMnO_4$ and $NaIO_4$. After adding carrier acetic acid, the reaction mixture was steam distilled. Analysis of the distillate by GLC revealed that essentially all of the radioactivity of the distillate was associated with acetic acid. From the distillate, *p*-bromophenacyl acetate was prepared and recrystallized to a constant specific radioactivity and constant m.p. 86°. Calculations indicated that, approximately 80% of the radioactivity associated with sterol I was released at ^{14}C-acetic acid by the oxidation procedure.

The configuration of the ethylidene group was determined by the use of NMR. The NMR spectrum of sterol I acetate exhibited similar features as those reported for 7,(*Z*)-24(28)-stigmastadien-3β-ol[3,4]. The resonances were: δ0·535, singlet, CH_3-18; δ0·81, singlet CH_3-19; δ0·93 broad doublet $J = 7$, CH_3-21; δ0·97, doublet, $J = 7$, CH_3-26 and CH_3-27; δ1·58, doublet, $J = 7$, CH_3-29; δ2·01, singlet CH_3—CO; δ2·82, septet, $J = 7$, CH-25; δ4·72, broad, CH-3; δ5·18 (ca), broad, CH-7 and CH-28.

According to Frost and Ward[13] and Bates *et al.*,[14] steroids with an ethylidene group at the 24 position exhibit a C-25 proton resonance near δ2·82 if the group has the (*Z*)-configuration while the resonance is near δ2·2 if the (*E*)-configuration is present. Therefore the ethylidene group of sterol I must have the (*Z*)-configuration.

The C-18 and C-19 proton resonances for sterol I acetate were calculated from the additive chemical shifts of Zürcher.[15] A shift equal to that of a 17β-C_9H_{17} group was assumed for the $C_{10}H_{19}$ side chain of sterol I. For a 5α, 14α steroid, the values calculated were δ0·55 (C-18) and δ0·826 (C-19), while those found were δ0·535 (C-18) and δ0·81 (C-19).

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Frost and Ward[3] suggested that the contribution of the 17β-$C_{10}H_{19}$ side chain of 7,(*Z*)-24(28)-stigmastadienol should be δ-0·05 and δ-0·017 for the C-18 and C-19 proton resonances, respectively and our data support their conclusions. From these data we concluded that sterol I was 7,(*Z*)-24(28)-stigmastadien-3β-ol.

Other sterols. As mentioned above, the TLC band with an R_f of 0·65 in the $AgNO_3$-impregnated plate system was composed of at least two componenets. One of the components (sterol II) appears to be 7-stigmasten-3β-ol. Sterol II acetate was obtained by recrystallizations of the mixed steroids, which were isolated by $AgNO_3$–TLC. Sterol II acetate, however, was still contaminated with the other steroids (*ca.* 5–10%). The mass spectrum of sterol II acetate exhibited a molecular ion at 456 *m/e* (26·7%), and peaks at 441 (26%, M–CH_3); 396 (52%, M-acetate); 381 (27·4%, M-acetate and CH_3); 315 (17%, M-side chain and 2H); 288 (22·3%, M-side chain and $C_{16,17}$); 273 (14%, M-side chain and 42); 255 (100%, M-side chain and acetate); 213 (65·6%, M-side chain and 42 and acetate). The spectrum indicated that sterol II was a C_{29} steroid with a double bond at the 7-position.[10,11] The IR spectrum of sterol II acetate was similar to that of sterol I acetate except that the absorption band at 812 cm^{-1} was absent, thus indicating that sterol II does not possess a double bond at the 24(28)-position.[16] The presence of an ethyl group was indicated, since ^{14}C was incorporated into sterol II when uredospores were germinated on a medium containing methionine-methyl-^{14}C. After $KMnO_4$ and $NaIO_4$ oxidation more than 95% of the radioactivity of the labeled sterol remained in the non-volatile residue.

Sterol II appears to be the same steroid as one (stigmast-7-enol) isolated from flax rust uredospores.[5] Although the configuration of the ethylidene group in the 7,24(28)-stigmastadienol from flax rust[5] was not determined it probably has the same configuration as sterol I. Thus the steroids from both fungi appear to be quite similar.

It has been suggested that the flax rust uredospore is unable to synthesize its own sterol requirement and therefore is dependent upon the host plant for sterols. We have found that, in addition to the ability to add an ethylidene or ethyl group at C-24, germinating bean rust uredospores also were able to synthesize the steroids from acetate-1-^{14}C acid. The steroids isolated from uredospores that were germinated on ^{14}C-acetate was oxidized with $KMnO_4$ and $NaIO_4$, and approximately 97% of the radioactivity was retained by the oxidized steroid nucleus. Thus, the germinating bean rust uredospore apparently has the enzymes necessary for the synthesis of steroids from such simple precursors as acetate and methionine. Nevertheless, this does not provide direct evidence concerning the synthesis of steroids during the phase when the uredospores are being formed on the host plant.

EXPERIMENTAL

Bean rust uredospores, *Uromyces phaseoli* (pers.) Wint. var. *typica* Arth., were produced as described by Trocha and Daly.[15] Spores broken with a high speed oscillating shaker and steel balls, were extracted with $CHCl_3$–CH_3OH (2:1, v/v). After concentration, the extract was chromatographed by preparative TLC[18] (Silica Gel G) with a solvent of Et_2O–heptane–HOAc (70:30:1, v/v/v). Steroids were extracted from the TLC adsorbent with $CHCl_3$ and rechromatographed (preparative TLC) with a solvent of 0·5% HOAc in $CHCl_3$. After acetylation, the steroids were subjected to the same chromatography steps again. Chromatography with $AgNO_3$ (12·5%) impregnated silica Gel G plates was performed with 0·5% HOAc in $CHCl_3$ as a solvent.

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The germination of spores on media containing L-methionine-methyl-^{14}C ($3{\cdot}4 \times 10^5$ dpm/ml) or NaOAc-1-^{14}C ($1{\cdot}1 \times 10^5$ dpm/ml) and the extraction of germinated spores has been described.[18] Purification of ^{14}C-labelled steroids was the same as that described above.

$NaIO_4$ and $KMnO_4$ oxidation was performed by modification of the von Rudloff method.[19] The sample (*ca.* $1{\cdot}5 \times 10^6$ dpm) and 3 mg stigmasterol acetate was dissolved in 60 ml *t*-BuOH. Then 20 ml of a 97·5 mM $NaIO_4$, 2·5 mM $KMnO_4$ solution, 10 ml of 0·5% K_2CO_3, and 10 ml H_2O were added. After the reaction mixture was shaken at room temp. for 48 hr, 1 ml of HOAc was added as a carrier, and the mixture was acidified with HCl, decolorized with $NaHSO_3$, and steam distilled. The volatile fatty acids in the distillate were analyzed by GLC (column C), and GLC fractions were trapped[20] and counted for radioactivity. From the distillate *p*-bromophenacyl acetate was prepared. After the second crystallization, the m.p. 86°, and specific radioactivity were constant.

Three GLC procedures were used: Column *A*, 2·4 m × 3 mm stainless steel, was packed with 1·5% SE-30 on Anakrom ABS, 90–100 mesh, operated at 235° with a flow rate of 60 ml/min, and used with a F & M model 5750 instrument. Column *B*, 3·6 m × 3 mm stainless steel, was packed with 1% QF-1 on Anakrom ABS 90–100 mesh, operated at 230° with a flow rate of 60 ml/min, and used with the F & M instrument. Column *C*, 1·8 m × 3 mm stainless steel, was packed with 20% Carbowax-20m, operated at 135° with a flow rate of 30 ml/min, and used with a Jarrel-Ash model 700 instrument.

The 100 MHz NMR spectra were obtained from a 50 × 2 sec accumulation with a Fourier attachment on a Varian Associates HA 100 spectrometer. A solvent of $CDCl_3$ and standard of TMS was employed. IR spectra were recorded with a Perkin–Elmer model 621. Samples were incorporated into KBr discs. A Hitachi model RMU-60 spectrometer, operated with an ionizing potential of 80 eV, was used for the mass spectra. Corrected m.p's have been reported.

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