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FORMATION OF 2-CYANOMETHYLENECYCLOHEXYL ACETATE BY INCUBATION OF ACETATE WITH SLICES OF JOJOBA COTYLEDONS

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Key Word Index—*Simmondsia chinensis*; Simmondsiaceae; jojoba; cotyledons; incubation with acetate; simmondsin; simmondsyl acetate; E-2-cyanomethylene-4,5-dimethoxy-3-hydroxycyclohexyl acetate.

Abstract—E-2-cyanomethylene-4,5-dimethoxy-3-hydroxycyclohexyl acetate was isolated and characterized from the incubation of either developing or germinating jojoba (*Simmondsia chinensis*) cotyledon slices with acetate. This compound is a derivative of simmondsin, with the glucose group replaced by acetate, and is formed during the incubation. Its presence could not be detected in fresh tissue.

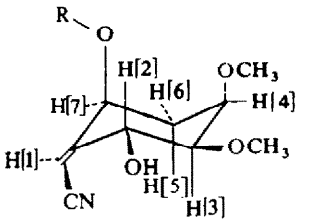
INTRODUCTION

Interest in the jojoba [*Simmondsia chinensis* (Link) C. K. Schneid.], a desert shrub native to the southwestern arid lands of North America, as a commercial crop stems from the liquid wax which can be pressed from the seed. The wax esters, composed principally of C_{38}–C_{44} homologues derived predominantly from C_{20} and C_{22} monoenoic acids and alcohols, are obtained in a high yield and purity, and are a potential replacement for spermaceti [1–3]. The meal left after wax expression had certain toxic properties when tested as an animal feedstuff [4,5]. The principal toxic component was isolated and identified as E-2-cyanomethylene-4,5-dimethoxy-3-hydroxycyclohexyl β-D-glucoside (**1a**), and was named simmondsin [4].

Investigations into the biosynthesis of long chain fatty acids and alcohols and their esterification to form waxes in the jojoba are underway in our laboratory. During the preliminary work [1-^{14}C]acetate was incubated with developing cotyledon slices, and the radioactivity incorporated into wax esters, free alcohols, and a novel polar material [6]. This paper reports the identification of this novel compound as a derivative of simmondsin.

RESULTS AND DISCUSSION

Incubations with immature jojoba seed slices and low concentrations of high specific activity [1-^{14}C]acetate gave greater than 50% ^{14}C-incorporation into polar lipids (i.e. material remaining at the origin after TLC in a petrol–Et_2O–HOAc (80:20:1) solvent system) [6]. TLC of the polar lipid fraction showed over 90% of the ^{14}C-activity to be associated with a mobile band with neither strongly acidic nor basic properties of (R_f 0.81 in $CHCl_3$–MeOH–H_2O (65:25:4); R_f 0.79 in $CHCl_3$–MeOH–H_2O–HOAc (80:15:2:5); R_f 0.78 in $CHCl_3$–MeOH–7M aqueous NH_4OH (70:20.2); R_f 0.46 in diisobutyl ketone–HOAc–H_2O) (40:25:3.7). Base hydrolysis of this band showed little of the ^{14}C radioactivity to be associated with fatty acid groups. The labelled 'polar lipid' was a minor component in terms of mass and could not be characterized by its TLC behaviour. Nevertheless since it represented the major ^{14}C-labelled fraction, it required identification. From several large-scale incubations with high concentrations of low specific activity [1-^{14}C]acetate 11 mg (10 μCi) of this pure polar material were isolated and it was shown to be E-2-cyanomethylene-4,5-dimethoxy-3-hydroxycyclohexyl [1-^{14}C]acetate (**1b**). Thus its structure is related to that of simmondsin (**1a**) by replacement of β-D-glucose by acetate. We propose that the diol derivative Z-2-cyanomethylene-1,3-dihydroxy-4,5-dimethoxycyclohexane (**1c**) be called simmondsol and thus compound **1b** would be simmondsyl acetate.



1b

1a R = Glc
1b R = Ac
1c R = H

The UV, IR and PMR spectra of **1b** are consistent with its proposed structure, and have been discussed with respect to simmondsin (**1a**) by Elliyer *et al.* [4]. PMR spectroscopy provided the most conclusive evidence for the correct identification of **1b**. Using specific spin-decoupling studies Elliger *et al.* were able to define the relative configurations and the conformation of the aglucone ring of simmondsin [4], and the very similar PMR spectrum of simmondsyl acetate indicates an identical configuration and conformation. The principal differences in the PMR spectrum of **1b**, compared to **1a**,

are the prominent acetoxy resonance (δ 2.07 ppm) and the downfield shift of the H(7) resonance from δ 4.96 ppm in **1a** to δ 5.87 ppm in **1b** caused by the stronger deshielding influence exerted by the ester carbonyl group. The three major ions in the mass spectrum of **1b** can be interpreted in terms of the following ions (*m/e*): CH_3CO^+ (43), $CH(OH)CH(OMe)^+$ (74) and $CH(OMe)CH(OMe)^+$ (88). The base peak ion at *m/e* = 88 is a typical fragment from permethylated sugars. Ions corresponding to the loss of MeOH (223), HOAc (195) and MeOH + HOAc (163) were also observed. The high resolution mass determination of M^+ and $(M-32)^+$ ions was consistent with their proposed molecular formulae. The GLC retention time of **1b** on an apolar SE-30 column also indicated that its MW lay in the 250 region. Simmondsyl acetate has MW 255 and an ECL of 15.6, whereas the GLC standard methyl pentadecanoate has MW 256 and an ECL of 15.0. Trimethylsilylation of **1b** gave a compound of lower polarity, as judged by TLC, with an ECL of 15.0 on a SE-30 GLC column. GC–MS (70 eV) of the TMSi ether of **1b** failed to produce a molecular ion fragment or a diagnostic spectrum.

Alkaline hydrolysis of ^{14}C-simmondsyl acetate showed the ^{14}C-label to be associated entirely with the acetyl group and not with the cyclohexyl ring. The specific activity of **1b** was 0.21 Ci/mol, which compared with the value of 0.26 Ci/mol for the [1-^{14}C]acetate used in the preparative incubations. These measurements indicate that simmondsyl acetate is derived entirely from exogenous acetate during the incubation, and does not represent even a minor constituent in jojoba cotyledons. This was confirmed by direct extraction of jojoba cotyledons with $CHCl_3$–MeOH (1:1), when simmondsyl acetate could not be detected by either GLC or UV spectroscopy. The estimated detection limits for these analyses of 5 and 13 μg respectively per g of fresh, developing cotyledon indicated that compared with the mass of ^{14}C-simmondsyl acetate isolated after the incubation with ^{14}C-acetate (~100 μg/g tissue) any endogenous simmondsyl acetate present was at least an order of magnitude less in amount. Simmondsol (**1c**) was not detected during these analyses. Although the authentic compound was not available for comparison its TLC, GLC and UV characteristics were predicted as being the following: an R_f value between that of simmondsyl acetate and of simmondsin 2'-ferulate; a RR_t on a SE-30 GLC column less than the value for simmondsyl acetate; and a UV absorption maxima and extinction coefficient similar to **1a** and **1b**. Assuming a reasonably quantitative extraction maximum limits for simmondsol concentration in fresh, developing cotyledons were 13 μg/g tissue (by UV spectroscopy) and 3 μg/g tissue (by GLC).

Therefore, biosynthesis of simmondsyl acetate occurs during the incubation of acetate with chopped cotyledon tissue, either by direct acetyl transfer to simmondsin or by hydrolysis of simmondsin to simmondsol with subsequent acetylation. Further incubations with slices or homogenates of jojoba cotyledons without exogenous acetate may be helpful in determining whether simmondsol is released by hydrolysis or not. The following aspects of the reaction are noteworthy: (a) it occurs in both developing and germinated cotyledonous tissue [6], (b) labelled simmondsin and simmondsyl acetate were not detected (< 5% of the total ^{14}C activity) when incubations employed [U-^{14}C]glucose as substrate [6]; and (c) incubations with ^{14}C-acetate where homogenates replaced tissue slices resulted in almost exclusive incorporation of activity into simmondsyl acetate. The physiological significance, if any, of the reaction is unclear, as indeed is the role of simmondsin itself. However, the formation of simmondsyl acetate has now been documented; future investigators employing ^{14}C-acetate with developing jojoba seeds should be cognizant of the extensive formation of this compound.

EXPERIMENTAL

General procedures. TLC was done on Si gel absorbant. For analytical TLC mass was detected by charring at 200° after spraying with 50% aqueous H_2SO_4. For preparative TLC 1 mm thick plates were used, with detection by scanning for radioactivity or by visualizing the spots in I_2 vapour. Material was recovered from the Si gel by eluting with several portions of $CHCl_3$–MeOH (1:1). For GLC the operating parameters were as follows: He carrier gas at 60 ml/min, injector temperature 220°, mass detection by a TC unit at 250°, and radioisotope detection by a Nuclear Chicago Biospan No. 4998 flow proportional counter at 250° in series with the TC detector.

Preparation and isolation of **1b**. In a typical preparative incubation 50 g of finely sliced developing cotyledons from jojoba were incubated with 1 mM [1-^{14}C] sodium acetate (15 μCi) in 0.1 M phosphate buffer (100 ml) at pH 7 and at 25°. The reaction was terminated after 4 hr by adding *iso*-PrOH (100 ml) and heating at 80° for 15 min. The mixture was homogenized in a blender with $CHCl_3$–MeOH (1:1) (250 ml) and left to stand overnight. Addition of 0.7% saline gave the chloroform layer containing the extracted lipids. Pooled extracts from several preparative incubations gave 23 g (21 μCi) of lipid, from [1-^{14}C]-acetate of an averaged sp. act. = 0.26 Ci/mol. A 'polar lipid' concentrate (0.8 g 11.1 μCi) was obtained by silicic acid column chromatography, and further preparative TLC separations in $CHCl_3$–MeOH–7M aqueous NH_4OH (90:10:1) and then $CHCl_3$–MeOH–H_2O (65:25:4) solvent systems gave 11 mg (10.0 μCi) of **1b**, of purity >95%. GLC of **1b** gave single mass and radioactivity peaks (ECL = 15.6) on a 1.8 m × 5 mm packed column with 1.5% SE-30 at 160°. TLC of **1b** in the solvent systems described at the beginning of the 'Results and Discussion' section showed single mass and ^{14}C-radioactive spots.

Identification of **1b**. IR spectrum, $\gamma_{max}^{CHCl_3}$ cm^{-1}: 3450, *s* (O—H), 2220, *m* (conjugated C≡N); 1735, *s* (ester C=O). UV spectrum, λ_{max}^{EtOH} nm (log ε): 226 (3.8), —CH=CH—C≡N chromophore. PMR spectrum (100 MHz, $CDCl_3$), with assignments given according to the hydrogen atom labelling of **1b** shown: δ 1.53 (1H, *d*, $J_{5,6}$ = 15.6 Hz, *t*, *J* = 3.5 Hz, H(5)), δ 2.07 (3H, *s*, $C\underline{H}_3CO$—), δ 2.61 (1H, *d*, $J_{5,6}$ = 15.9 Hz, *t*, *J* = 3.8 Hz, H(6)), δ 3.10 (1H, *dd*, $J_{2,3}$ = 9.3 Hz, $J_{3,4}$ = 3.2 Hz, H(3)), δ 3.1 (1H, broad *s*, —O$\underline{H}$), δ 3.40 (3H, *s*, —OC$\underline{H}_3$), δ 3.49 (3H, *s*, —OC$\underline{H}_3$), δ 3.91 (1H, *q*, $J_{3,4}$ = $J_{4,5}$ = $J_{4,6}$ = 3.5 Hz, H(4)), δ 4.75 (1H, *dd*, $J_{1,2}$ = 1.9 Hz, $J_{2,3}$ = 9.3 Hz, H(2)), δ 5.76 (1H, *d*, $J_{1,2}$ = 1.8 Hz, H(1)), δ 5.87 (1H, *t*, $J_{6,7}$ = $J_{5,7}$ = 3.7 Hz, H(7)). MS (probe, 150°), 70 eV: *m/e* (rel. int.): 255 [M^+] (0.13), 254 (0.09), 223 (0.71), 195 (8), 181 (6), 163 (14), 150 (16), 134 (25), 89 (10), 88 (100), 75 (17), 74 (60), 73 (13), 71 (19), 45 (25), 43 (97) and 41 (15). High resolution MS gave M^+ as 255.11058 amu and $(M-32)^+$ as 223.08484 amu, compared with calcd values of 255.11067 amu and 223.08446 amu for $C_{12}H_{17}O_5N$ and $C_{11}H_{13}O_4N$ respectively.

Base hydrolysis of ^{14}C-labelled **1b** by refluxing in 1 M KOH 50% aq. MeOH for 1 hr gave, on acidification and extraction with Et_2O, 3% of the radioactivity in the organic layer, and 97% in the aqueous phase, which was lost by evap. under acidic but not basic pH. The aq. ^{14}C activity eluted with cold acetate (R_f 0.5) in a PrOH–7 M aq. NH_4OH (7:3) TLC solvent system. The acetate content of the aqueous phase was assayed by GLC pyrolysis of the benzyldimethylphenylammonium acetate salt according to the method of Richards *et al* [7].

1b was silylated by heating with BSTFA plus 1% TMCS

(30 μl) for several min at 50°. GLC of the product (1.5% SE-30, 160°) gave an ECL value of 15.0, while TLC dual development in petrol:Et_2O (1:1) gave R_f 0.24 (R_f of **1b** 0.04).

Analysis of the lipid extract from fresh seeds for **1b**. The polar lipid fraction from silicic acid column chromatography of a $CHCl_3$–MeOH (1:1) extract of immature cotyledon tissue (15 g, yielding 2.2 g of oil) was subjected to preparative TLC in $CHCl_3$–MeOH–H_2O (65:25:4). All the bands between simmondsyl acetate (R_f 0.83), **1b** and simmondsin (R_f 0.28), **1a** inclusive were recovered and examined by GLC (SE-30) and UV spectroscopy. Estimates of simmondsin and simmondsin 2′-ferulate (R_f 0.45) concentrations of 550 and 410 μg per g of cotyledon tissue respectively were made, according to the UV data reported by Elliger *et al.* [8].

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