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(+)-7-ISO-JASMONIC ACID AND RELATED COMPOUNDS FROM *BOTRYODIPLODIA THEOBROMAE*

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Key Word Index—*Botryodiplodia theobromae*; Sphaeropsidaceae; (+)-7-iso-jasmonic acid; (+)-9,10-dihydro-7-iso-jasmonic acid; (+)-11,12-didehydro-7-iso-jasmonic acid; (+)-cucurbic acid.

Abstract—Metabolites produced by *Botryodiplodia theobromae* (synonym *Lasiodiplodia theobromae*) possessing plant growth regulating activities were shown to be the 7-epimer of jasmonic acid and its derivatives.

INTRODUCTION

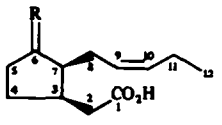
Cultures of the fungus *Botryodiplodia theobromae* Pat. (synonym *Lasiodiplodia theobromae* Griff. & Maubl.) showing plant growth inhibitory effects were reported in 1971, and the active metabolite was identified as jasmonic acid (**4**) [1]. This acid and 9,10-dihydrojasmonic acid (**8**), conjugated with isoleucine, were isolated from the culture filtrate of the fungus *Gibberella fujikuroi* (Saw.) Wr. [2]. Methyl jasmonate (4-Me ester) was isolated and structurally elucidated for the first time as an important odoriferous constituent of the essential oils of *Jasminum grandiflorum* L. [3, 4]. Recently, jasmonic acid and/or its methyl ester have been shown to be of general distribution in plants [5] and are considered to be representatives of a new type of endogenous plant growth regulator [6].

With the aim of studying the biosynthesis of jasmonic acid (**4**) using a suitable microbial system, we reinvestigated the production of this acid by the fungus *B. theobromae*. However, using a strain isolated from Cuban oranges, we were unable to isolate jasmonic acid although three related C_{12}-cyclic fatty acids with opposite stereochemistry at C-7 were isolated.

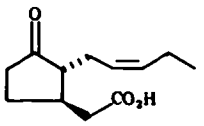
RESULTS AND DISCUSSION

From a number of strains of *B. theobromae* examined, one (D 7/2) isolated from Cuban oranges was the most effective in the production of cyclic fatty acids. After 7 days of growth at 30° in surface culture, the entire broth was lyophilized and the acidic compounds were extracted under carefully controlled pH conditions (see Experimental). Further purification and separation by silica gel column chromatography, preparative HPLC and TLC yielded four compounds identified as (+)-7-iso-jasmonic acid (**1**, also named (+)-2-iso- or (+)-2-epi-jasmonic acid [7–9]), (+)-9,10-dihydro-7-iso-jasmonic acid (**5**), (+)-11,12-didehydro-7-iso-jasmonic acid (**9**) and (+)-cucurbic acid (**2**) [10].

The compounds isolated were characterized by their ORD values as well as by TLC, GC and/or HPLC of the free acids and their respective methyl esters. The structures of **1** and **2** were confirmed by comparison with authentic samples, and the structures of **5** and **9** were established from their IR, NMR and mass spectra and by some chemical transformations. Thus the cyclopentanoneacetic acids with *cis*-stereochemistry of the side chains can be rapidly isomerized by treatment with dilute hydrochloric acid or potassium hydroxide into the more stable *trans*-stereoisomers leading to a mixture of *trans/cis*-isomers in the ratio 9:1 [11, 12]. In this manner, (+)-7-iso-jasmonic acid (**1**) was isomerized to a mixture consisting mainly (−)-jasmonic acid (**4**), and the (+)-7-iso-jasmonic acid derivatives **5** and **9** were transformed into mixtures containing mainly (−)-9,10-dihydrojasmonic acid (**8**) and (−)-11,12-didehydrojasmonic acid (**10**), respectively. The identity of the products was proved, where possible, by comparison with authentic samples. Catalytic hydrogenation of the C=C bonds in



1 R = O
2 R = α-OH, β-H
3 R = α-H, β-OH



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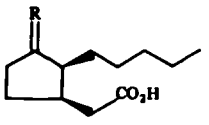
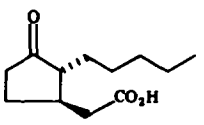


5 R = O
6 R = α-OH, β-H
7 R = α-H, β-OH



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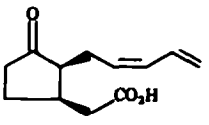

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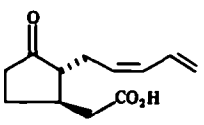

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the methyl esters of **1** and **9** yielded, in both cases, methyl (+)-9,10-dihydro-7-iso-jasmonate (**5**-methyl ester). Reduction of **1** with sodium borohydride in sodium hydrogen carbonate solution gave the two hydroxy acids (+)-cucurbic acid (**2**) and its 6-epimer (**3**). Similarly, reduction of **5** gave the 6-epimeric hydroxy acids **6** and **7**. The methyl ester of compound **6** was also obtained by catalytic hydrogenation of methyl (+)-cucurbate (**2**-methyl ester). The position of the second (conjugated) double bond in **9** was determined by high-resolution ^{1}H NMR spectroscopy.

Thus, contrary to a former investigation [1], *B. theobromae* does not biosynthesize (−)-jasmonic acid (**4**), but rather its 7-iso-stereomer **1**, as the main cyclic fatty acid. This is confirmed by the isolation of the minor compounds **2**, **5** and **9**, which also belong to the 7-iso-*cis*-series and is in agreement with previous studies on the biosynthesis of cyclic fatty acids of the jasmonic acid type [13, 14]. The presence of the acid **1** or its methyl ester, in addition to variable amounts of **4** or its methyl ester, has also been demonstrated in higher plants: in lemon fruits ([9] see also ref. [8]) and young fruits of *Vicia faba* L. [15].

EXPERIMENTAL

Chromatographic methods. TLC (silica gel): (a) $CHCl_3$–MeOH–HOAc (70:10:0.5); (b) *n*-hexane–EtOAc–HOAc (60:40:1); detection by anisaldehyde reagent and heating for 5–10 min at 120° [16]; analytical HPLC: Si 100 Polyol RP 18 (25 × 0.46 cm) mobile phase MeOH–H_2O–H_3PO_4 (55:45:0.1), flow rate 1 ml/min, detector set at 228 nm.

Fermentation. The fungus (strain D7/2) was isolated from Cuban *Citrus sinensis* Osbeck cv. Valencia and pre-cultured on malt agar. The mycelium was homogenized with distilled H_2O and used for inoculation of the production medium. The fungus was grown in surface culture for 7 days at 30° in flasks (400 ml) containing 100 ml of the following medium: 30 g sucrose, 5 g soya flour, 15 ml corn steep liquor and 10 ml of mineral salt soln, adjusted to 1 l. with water and to pH 5.4–5.6 with 1 M NaOH and sterilized at 120° for 20 min. The mineral salt soln used contained 0.5 g KH_2PO_4, 0.3 g $MgSO_4 \cdot 7\,H_2O$, 10 mg $FeSO_4 \cdot 7\,H_2O$, and 8.8 mg $ZnSO_4 \cdot 7\,H_2O$/l.

Isolation procedure. The contents from 35 culture flasks were lyophilized and extracted with EtOAc (3 × 1 l.). Acidic compounds were separated by extraction into saturated $NaHCO_3$ soln (2 × 100 ml) and back extraction of the aq. phase with $CHCl_3$ (2 × 50 ml) after acidification to pH 3.5 with 4 M HCl. The extract was dried (Na_2SO_4) and evaporated. The crude gum was purified by CC (600 × 20 mm) on silanized silica gel prepared by treatment of silica gel with TMCS in C_6H_6; elution was with a stepwise gradient of EtOAc in $CHCl_3$. Fractions eluted with $CHCl_3$–EtOAc (9:1) gave a crude mixture which on prep. HPLC on RP 8 (310 × 25 mm) with MeOH–H_2O–H_3PO_4 (60:40:0.1), flow rate 2 ml/min, gave:

(+)-11,12-*Didehydro-7-iso-jasmonic acid* [(1R,2S)-3-*oxo*-2-(2Z, 4-*pentadienyl*) *cyclopentane*-1-*acetic acid*] (**9**). 15 mg; R_t: 100–130 min; R_f (TLC system a) 0.20; ORD (MeOH, *c* 0.1): $[\alpha]^{25}$ (nm) −876 (272), +811 (318), +50 (589); IR ν_{max} cm^{-1}: 3000, 1738, 1650; UV λ_{max}^{MeOH} nm: 228 (log ε 3.04); ^{1}H NMR (200.13 MHz, $CDCl_3$, TMS as internal standard): δ 5.13 (1 H, *d*, *J* = 10.5 Hz, 12-H), 5.21 (1H, *d*, *J* = 17 Hz, 12-H'), 5.37 (1H, *dt*, *J* = 10.5 Hz, *J'* = 7.9 Hz, 9-H), 6.07 (1H, *t*, *J* = 10.5 Hz, *J'* = 11.5 Hz, 10-H), 6.60 (1H, *m*, 11-H); MS (70 eV) *m/z* (rel. int.): 222 $[M]^+$ (20), 204 (14), 193 (14), 191 (17), 179 (11), 167 (17), 149 (88), 130 (66), 107 (86), 91 (71), 83 (57), 79 (100). The ^{1}H NMR signals of H-9, H-10, H-12 and H-12' were broadened ($\Delta\nu_{1/2}$ *ca* 3–5 Hz), probably due to further unresolved couplings (which were not determined). The accuracy of the coupling constants was *ca* ±0.5 Hz.

(+)-7-*iso-Jasmonic acid* [(1R,2S)-3-*oxo*-2-(2Z-*pentenyl*)-*cyclopentane*-1-*acetic acid*] (**1**). 1.3 g; R_t: 135–158 min; R_f (TLC system a) 0.22; ORD (MeOH, *c* 0.1): $[\alpha]^{25}$ (nm) −1070 (272), +1490 (310), +64 (589); MS identical with **4** [17]; the compound was shown to be identical with authentic **1** [7].

(+)-9,10-*Dihydro*-7-*iso-jasmonic acid* [(1R,2S)-3-*oxo*-2-*pentylcyclopentane*-1-*acetic acid*] (**5**). 20 mg; R_t: 200–225 min; R_f (TLC system a) 0.23; ORD (MeOH, *c* 0.1); $[\alpha]^{25}$ (nm) −295 (272), +228 (314), +10.5 (589); MS identical with **8** [2, 3].

Fractions eluted with $CHCl_3$–EtOAc (4:1) were further purified by prep. TLC on silica gel (1 mm) to give:

Cucurbic acid [(1R,2S,3S)-3-*hydroxy*-2-(2Z-*pentenyl*)-*cyclopentane*-1-*acetic acid*] (**2**). 11 mg; R_f values in solvent systems a (0.13) and b (0.27). See ref. [10]. The total yield of cyclic fatty acid produced was approximately 1.35 g (385 mg/l.).

Isomerization of the isolated 7-*iso-oxo acids.* 2 mg of **1**, **5** and **9**, respectively, were heated with 1 ml 1 M KOH. After 5 hr at 60°, 1.5 ml 1 M HCl was added and the solns were extracted (× 3) with $CHCl_3$. Prep. TLC with solvent system a yielded 1.8 mg **4** (R_f 0.28), **8** (R_f 0.29) and **10** (R_f 0.25), respectively. **4** was shown to be identical with authentic **4** isolated from *V. faba* [17] and **8** with authentic (±)-**8** obtained by hydrolysis of commercial (±)-**8**-methyl ester.

Catalytic hydrogenation of **1**- *and* **9**-*methyl ester.* Adams catalyst (2 mg) in 5 ml EtOH was saturated with H_2 and 3 mg **1**-methyl ester or **9**-methyl ester was added. After 20 min the mixtures took up *ca* 0.30 ml and 0.55 ml hydrogen, respectively. Recovery and separation by HPLC yielded in both cases *ca* 1.5 mg **5**-methyl ester (R_t 19.6 min).

Reduction of the 7-*iso acids* **1** *and* **5** *with* $NaBH_4$. 5 mg of **1** or **5** was dissolved in a soln of 17 mg $NaHCO_3$ in 1 ml H_2O and treated with 10 mg $NaBH_4$ for 30 min at room temp. After acidification to pH 3 with 4 M HCl; extraction with $CHCl_3$, drying of the chloroform extract with Na_2SO_4 and evaporation of the solvent, prep. TLC using solvent system b yielded 1.8 mg **2** (R_f 0.27) and 1.4 mg **3** (R_f 0.42) from **1** [7] and 2 mg **6** (R_f 0.26) and 1.8 mg **7** (R_f 0.41) from **5**. Compound **2** was shown to be identical with the isolated sample of (+)-cucurbic acid; see ref. [10].

Methylation of carboxylic acids. The methyl esters of all the carboxylic acids were prepared by treatment with ethereal CH_2N_2 and analysed by GC. The methyl esters of the 6-oxo acids separated under the following conditions: stainless steel column (3 m × 4 mm), 10% EG SS-X on Gas Chrom P (125–150 μm), N_2 50 ml/min, column temp. 170°, FID; for separation of the methyl esters of the 6-hydroxy acids the following conditions were used: stainless steel column (2 m × 4 mm), 3% OV 225 on Gas Chrom Q (100–120 mesh), N_2 110 ml/min, column temp. 150°, FID; R_t (min): EG SS-X, **1** 47.0, **4** 38.5, **5** 36.0, **8** 30.5, **9** 69.0, **10** 67.5; OV 225, **1** 17.8, **4** 15.0, **5** 15.9, **8** 13.6, **2** 21.1, **3** 18.3, **6** 19.2, **7** 16.5. Combined GC/MS was performed with an 80 eV mass spectrometer and a glass column (1.80 m × 2 mm) containing 10% EG SS-X on Gas Chrom P (100–120 μm).

Catalytic hydrogenation of **2**-*methyl ester.* Using Adams catalyst, catalytic hydrogenation as described above yielded **6**-methyl ester, identified by GC.

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