Phytochemistry, Vol. 31, No. 2, pp. 503–506, 1992
Printed in Great Britain.

0031 9422/92 $5.00+0.00

MICROBIOLOGICAL PREPARATION OF ATISENOLIDES BY *GIBBERELLA FUJIKUROI*

BRAULIO M. FRAGA, RICARDO GUILLERMO and JAMES R. HANSON*

Instituto de Productos Naturales y Agrobiología, La Laguna, 38206-Tenerife, Canary Islands, Spain; *School of Molecular Sciences, University of Sussex, Brighton, U.K.

(*Received* 31 *May* 1991)

Key Word Index *Gibberella fujikuroi*; diterpenes; kaurenolides; atisenolides; gummiferolic acid.

Abstract—The microbiological transformation of *ent*-19-hydroxyatis-6,16-diene into 3α,7β-dihydroxyatisenolide and 7β,18-dihydroxyatisenolide, and of *ent*-atis-6,16-dien-19-oic acid into 7β-hydroxyatisenolide, 3α,7β-dihydroxyatisenolide and 7β,18-dihydroxyatisenolide, using the fungus *Gibberella fujikuroi* have been carried out. The substrates have been prepared from gummiferolic acid.

INTRODUCTION

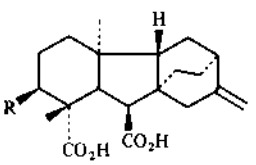
In previous work we have examined the microbiological transformation of *ent*-7α-hydroxyatis-16-en-19-oic acid (**5**) by *Gibberella fujikuroi* and shown that it is converted to the atisene analogues of gibberellins A_{12} and A_{14} (**1** and **2**). However, no atisagibberellin metabolites oxygenated at C-20 or in the C-19 series were isolated [1]. This suggested that there were some constraints on the biotransformation because of the difference between the carbon skeleta of the *ent*-atisene and the natural *ent*-kaurene substrate. Whereas *ent*-7α-hydroxykaur-16-en-19-oic acid (**3**) is the substrate for ring contraction, *ent*-kaur-6,16-dien-19-oic acid (**4**) is converted into the kaurenolides [2, 3]. We have, therefore, prepared *ent*-atis-6,16-dien-19-ol (**9**) and *ent*-atis-6,16-dien-19-oic acid (**10**), and examined their biotransformation by *G. fujikuroi* in order to test the skeletal substrate:specificity of the kaurenolide pathway.

RESULTS AND DISCUSSION

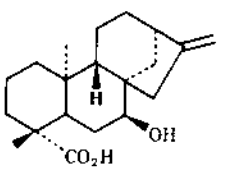
ent-Atis-6,16-dien-19-ol (**9**) was obtained in the following way: methyl *ent*-7α-hydroxyatis-16-en-19-oate (**6**), obtained from gummiferolic acid (**8**) [4], was dehydrated with thionyl chloride and then reduced with lithium aluminium hydride to *ent*-atis-6,16-dien-19-ol (**9**). This alcohol was separated from a small amount of the 7-chloro compound (**13**). The diene showed the expected ^{1}H NMR signals for H-6 and H-7 (δ 5.70 *dd*, $J=10$ and 2 Hz; 5.30, *dd*, $J=10$ and 3 Hz) in which H-7 showed a long-range coupling to H-5.

ent-Atis-6,16-dien-19-oic acid (**10**) was prepared by hydrolysis of the corresponding methyl ester (**11**), which had been obtained by solvolysis of the mesylate **7**, a transformation product of gummiferolic acid [5].

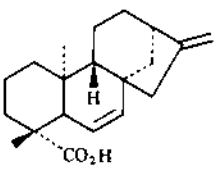
The fungus *G. fujikuroi* was grown in shake culture in the presence of AMO 1618 to inhibit the formation of *ent*-kaurene and consequently its metabolites [6, 7]. Incubation of *ent*-atis-6,16-dien-19-ol (**9**) with this culture gave a low yield of a mixture of atisenolides which were separated as their acetates. Both 7β,18-diacetoxyatisenolide (**16**) and 3α,7β-diacetoxyatisenolide (**17**) showed ^{1}H NMR signals for H-5, H-6 and H-7 (δ 2.01, *d*, $J=5$ Hz; 4.70, *dd*, $J=5$ and 7 Hz; 5.38, *d*, $J=5$ Hz for **16**; 2.01, *d*, $J=5$ Hz; 4.67, *t*, $J=5$ Hz; 5.36, *d*, $J=5$ Hz for **17**) comparable to those of kaurenolides. 7β,18-Diacet-



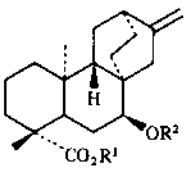
	R
1	H
2	OH



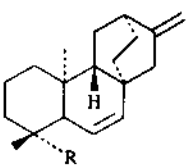
3



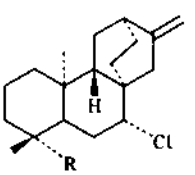
4



	R^1	R^2
5	H	H
6	Me	H
7	Me	Ms
8	H	Ang

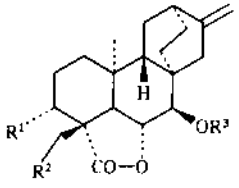


	R
9	CH_2OH
10	CO_2H
11	CO_2Me

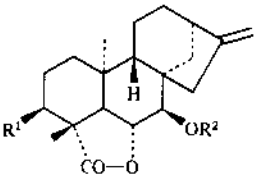


	R
12	CO_2Me
13	CH_2OH

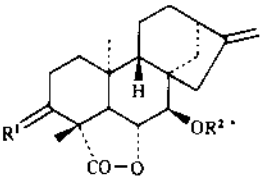
oxyatisenolide (**16**) lacked the lower-field C-Me resonance but contained an additional CH_2O- AB system (δ5.09 and 4.18, $J=11$ Hz). The multiplicity of the additional CHOAc signal in **17** ($J=5$ and 9 Hz) and the study of the ^{13}C NMR spectrum (Table 1) suggested that the second metabolite was 3α,7β-diacetoxyatisenolide (**17**). The coupling constant of the H-3α in the 3β,7β-diacetoxykaurenolide (**20**) was 6 Hz [8]. With a view to comparing the NMR spectra of our product with those corresponding to the *ent*-kaurene series, we have now synthesized 3α,7β-dihydroxykaurenolide (**27**) starting from the 3β-epimer (**21**). Acetylation of **21** in the usual way gave the diacetate **20**. Partial hydrolysis of this compound afforded the monoacetates **22** and **23**, the latter being oxidized with Jones reagent to give the 3-oxo-derivative **24**. Reduction of **24** with sodium borohydride gave the required 3α-epimer (**25**), which by acetylation formed the diacetate **26**. The coupling constant of the H-3β in its ^1H NMR spectrum ($J=6$ and 10 Hz) was similar to that of **17**, confirming the structure assigned to this atisenolide. Hydrolysis of **26** afforded 3α,7β-dihydroxykaurenolide (**27**).



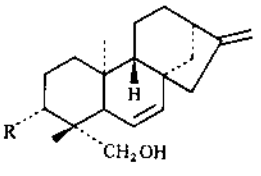
	R^1	R^2	R^3
14	H	OH	H
15	OH	H	H
16	H	OAc	Ac
17	OAc	H	Ac
18	H	H	H



	R^1	R^2
19	H	Ac
20	OAc	Ac
21	OH	H
22	OAc	H
23	OH	Ac



	R^1	R^2
24	O	Ac
25	α-OH,β-H	Ac
26	α-OAc,β-H	Ac
27	α-OH,β-H	H



	R
28	H
29	OH

The incubation of *ent*-atis-6,16-dien-19-oic acid (**10**) also gave the compounds **14** and **15**, and the 7β-hydroxyatisenolide (**18**), the precursor of the dihydroxy-atisenolides. In this biotransformation the yields were higher, as expected. The atisenolide **18** was identical with that prepared synthetically [4].

The isolation of 3α,7β-dihydroxyatisenolide (**18**) and not the corresponding 3β-derivative as occurs in the kaurenolide biosynthetic pathway [8] is noteworthy. We have previously obtained a 3α-hydroxylation in the microbiological transformation of 18-hydroxy-kaur-6,16-diene (**28**) by *G. fujikuroi* to afford (**29**) [9].

This work, which complements our earlier study on the atisagibberellins [1], shows that the kaurenolide pathway in the fungus can also metabolize the *ent*-atisene skeleton.

EXPERIMENTAL

Mps: uncorr. IR: $CHCl_3$; NMR: $CDCl_3$; MS: 70 eV (probe); CC: silica gel (0.063–0.2 mm). The substances were crystallized from petrol–EtOAc except where otherwise indicated.

Table 1. ^{13}C NMR spectral data of compounds **9**,**16**–**20** and **26**

C	9	16	17	18	26	19*	20*
1	36.52	31.12	31.15	32.02	36.05	37.3	34.9
2	18.04	16.32	23.22	16.41	24.16	17.3	23.8
3	35.94	22.65	68.29	26.92	72.61	28.5	68.4
4	36.52	45.81	45.15	41.68	44.65	41.2	44.7
5	51.42	52.05	53.11	52.95	53.29	51.7	51.5
6	125.11	82.74	81.83	84.71	79.49	80.1	80.2
7	134.64	77.37	77.29	76.98	73.51	73.2	72.8
8	n.o.	37.40	37.62	37.60	44.37	44.3	44.3
9	55.72	53.04	56.36	55.52	55.36	55.5	55.7
10	38.05	34.03	33.69	33.92	34.25	34.1	34.0
11	28.35	29.40	29.50	30.14	17.06	17.1	17.1
12	36.64	36.31	36.21	36.55	32.38	32.5	32.3
13	26.92	26.24	26.17	26.35	37.19	37.3	37.2
14	28.06	27.26	27.16	27.06	34.25	34.2	34.0
15	45.65	41.23	41.23	40.28	43.33	43.2	43.1
16	152.01	150.46	150.36	151.06	158.03	158.2	157.9
17	106.17	107.98	107.97	107.33	107.82	107.7	107.8
18	26.43	68.20	19.66	25.14	23.52	25.7	21.4
19	66.00	177.63	179.36	182.47	175.66	181.4	179.0
20	14.31	18.94	18.57	18.69	19.67	20.3	19.1

n.o. Values not observed.
*Values obtained from ref. [10].

Dehydration of ent-7α-*hydroxyatis*-16-*en*-19-*oic acid methyl ester* (**6**) The methyl ester **6** [4] was dissolved in dry pyridine (9 ml) at 0° and freshly distilled $SOCl_2$ (0.6 ml) added dropwise. The mixture was left for 3 hr at this temp., then poured over HCl soln (2%) and extracted with EtOAc as usual. Chromatography of the residue, eluting with petrol–EtOAc (30:1), gave the dehydrated compound **11** and the chloro derivative **12**.

Reduction of compounds **11** *and* **12**. The mixture of **11** and **12** (300 mg) in dry THF (6 ml) was added dropwise to a suspension of $LiAlH_4$ (100 mg) in the same solvent (6 ml). After refluxing for 12 hr the excess of reagent was destroyed by dropwise addition of EtOAc. The reaction mixt. was washed with 3% HCl aq and extracted with EtOAc. Dry CC of the residue, eluting with petrol–EtOAc (9:1), afforded *ent*-19-hydroxyatis-6,16-diene (**9**) (160 mg), mp 105–107° (from EtOAc), $[M]^+$ at *m/z* 286.2324. $C_{20}H_{30}O$ requires 286.2295; ^1H NMR (200 MHz): δ 0.88 and 0.99 (each 3H, *s*), 3.53 and 3.65 (each 1, *d*, $J = 11$ Hz, H-19), 4.59 and 4.75 (each 1H, *br s*, H-17), 5.30 (1H, *dd*, $J = 10$ and 3 Hz, H-6), 5.70 (1H, *dd*, $J = 10$ and 2 Hz, H-7); EIMS *m/z* (rel. int.): 286 $[M]^+$ (20), 271 (25), 255 (100), 253 (100), 215 (24), 199 (79), 185 (37), 171 (28), 169 (32), 155 (69), 143 (87), 131 (70). Further elution gave *ent*-7β-chloro-19-hydroxyatis-16-ene (**13**) (6 mg); $[M]^+$ at *m/z* 324.1981. $C_{20}H_{31}O^{37}Cl$ requires 324.2034; $[M]^+$ at *m/z* 322.2069. $C_{20}H_{31}O^{35}Cl$ requires 322.2063; ^1H NMR (200 MHz): δ 0.97 and 1.23 (each 3H, *s*), 3.47 and 3.68 (each 1H, *d*, $J = 11$ Hz, H-19), 3.73 (1H, *dd*, $J = 4$ and 12 Hz, H-7), 4.58 and 4.74 (each 1H, *dd*, $J = 2$ Hz, H-17); EIMS *m/z* (rel. int.): 324 $[M]^+$ (2) 322 (7), 291 (42), 287 (14), 286 (15), 256 (26), 255 (100), 243 (32), 199 (17), 157 (15), 147 (21), 131 (30).

Hydrolysis of the methyl ester of ent-*atis*-6,16-*dien*-19-*oic acid* (**11**). The methyl ester **11** (590 mg) [5] in DMSO (15 ml) was treated with K *t*-butoxide (400 mg) and stirred under N_2 at 90° for 5 hr. The reaction mixture was poured into water, acidified with dil. HCl (3%) and extracted with EtOAc. Evapn of the solvent and chromatography of the residue eluting with petrol–EtOAc (17:3), gave the acid **10** (320 mg), $[M]^+$ at *m/z* 300.2079. $C_{20}H_{28}O_2$ requires 300.2089; ^1H NMR (200 MHz): δ 0.84 and 1.29 (each 3H, *s*), 4.61 and 4.77 (each 1H, *br s*, H-17), 5.32 (1H, *dd*, $J = 10$ and 3 Hz, H-6), 5.99 (1H, *dd*, $J = 10$ and 1.5, H-7); EIMS *m/z* (rel. Int.): 300 $[M]^+$ (100), 285 (24), 272 (44), 255 (85), 239 (62), 229 (71), 211 (32), 157 (36), 105 (35).

Incubation experiments. The fungus *Gibberella fujikuroi* (ACC 917), inhibited with 5×10^{-5} M AMO 1618, was grown in shake culture at 25° for 1 day in 90–75 conical flasks (250 ml) each containing sterile medium (50 ml) [11]. The substrate (see below) in EtOH (19–16 ml) was distributed equally between the flasks and the incubation allowed to continue for a further 6 days. The broth was filtered, adjusted to pH 2 with dil HCl, and extracted with EtOAc. The extract was sepd into acidic and neutral frs with $NaHCO_3$. The acidic fr. was methylated with CH_2N_2 and chromatographed.

The incubation of *ent*-19-hydroxyatisa-6,16-diene (**11**) was carried out with 300 mg. The chromatography of the neutral fr, eluting with petrol–EtOAc (25:2), gave starting material (140 mg), and further elution with petrol–EtOAc (1:1) gave a mixt. of the atisenolides **14** and **15** (11 mg). This mixt. was acetylated with Ac_2O–pyridine in the usual way. The mixt. of less acetates obtained was chromatographed eluting with petrol–EtOAc (5:1) to afford 7β,18-diacetoxyatisenolide (**16**) (6 mg) and 3α,7β-diacetoxyatisenolide (**17**) (3 mg).

The incubation of *ent*-atis-6,16-dien-19 oic acid (**10**) (245 mg) gave 7β-hydroxyatisenolide (35 mg) (**18**) and the mixt. of **14** and **15** (45 mg), which was resolved by acetylation and chromatography of the acetates mixt. as above.

7β,18-*Diacetoxyatisenolide* (**16**), Mp 162–165°, $[M]^+$ at *m/z* 416.2204. $C_{24}H_{32}O_6$ requires 416.2198; ^1H NMR (200 MHz): δ 1.06 (3H, *s*, H-20), 1.96 (1H, *d*, $J = 7$ Hz, H-5), 2.10 and 2.13 (each 3H, *s*), 2.35 (1H, *br s*, H-12), 4.09 and 4.18 (each 1H, *d*, $J = 11$ Hz, h-18), 4.70 and 4.82 (each 1H, *br s*, H-17), 4.70 (1H, *dd*, $J = 5$ and 7 Hz, H-6), 5.38 (1H, *d*, $J = 5$ Hz, H-7); EIMS *m/z* (rel. int.): 416 $[M]^+$ (2), 374 (4), 356 (5), 296 (8), 268 (9), 253 (80), 171 (5), 149 (20), 107 (15).

3α,7β-*Diacetoxyatisenolide* (**17**). Mp 195–197° (from $CHCl_3$), $[M]^+$ at *m/z* 416.2216. $C_{24}H_{32}O_6$ requires 416.2198; ^1H NMR (200 MHz): δ 1.08 (3H, *s*, H-20), 1.35 (3H, *s*, H-18), 2.01 (1H, *d*, $J = 7$Hz, H-5), 2.07 and 2.12 (each 3H, *s*), 2.34 (1H, *br s*, H-12), 4.67 (1H, *t*, H-6), 4.68 and 4.81 (each 1H, *d*, $J = 2$ Hz, H-17), 5.36 (each 1H, *d*, $J = 5$ Hz, H-7), 5.53 (1H, *dd*, $J = 5$ and 9 Hz, H-3); EIMS *m/z* (rel. int.): 416 $[M]^+$ (85), 374 (47), 356 (67), 314 (81), 296 (100), 286 (78), 268 (82), 253 (71), 197 (72), 135 (41), 91 (99).

7β-*Hydroxyatisenolide* (**18**). $[M]^+$ at 316.2036 *m/z* $C_{20}H_{28}O_3$ requires; ^1H NMR (200 MHz): δ 0.92 and 1.28 (3H, *s*, H-18 and H-20), 1.77 (1H, *d*, $J = 7$ Hz, H-5), 3.99 (1H, *d* $J = 5$ Hz, H-7), 4.71 (1H, *dd*, $J = 7$ and 5 Hz, H-6), 4.67 and 4.79 (each 1H, *br s*, H-17); EIMS *m/z* (rel. int.): 316 $[M]^+$ (20), 298 (34), 286 (28), 257 (14), 255 (23), 239 (18), 199 (15), 137 (31).

Partial hydrolysis of 3β,7β-*diacetoxykaurenolide* (**20**). A soln of the diacetate (**20**) (250 mg) in methanolic KOH 4×10^{-3} (8 ml) was left at room temp. for 30 min. Usual work-up and chromatography of the residue afforded starting material (60 mg); further elution gave 3β-acetoxy-7β-hydroxykaurenolide (**22**) (60 mg), mp 213–214° (from MeOH), ^1H NMR (200 MHz): δ 0.96 and 1.35 (each 3H, *s*), 1.98 (1H, *d*, $J = 6.5$ Hz, H-5), 2.08 (3H, *s*), 4.36 (1H, *br s*, H-7), 4.63 (1H, *t*, $J = 6.5$ Hz), 4.88 and 5.02 (each 1H, *br s*, H-17), 5.41 (1H, *dd*, $J = 5$ and 8 Hz, H-3); EIMS *m/z* (rel. int.): 356 $[M-18]^+$ (33), 341 (6), 314 (62), 296 (11), 286 (19), 255 (17), 190 (14), 145 (20), 119 (28). Further elution afforded 3β-hydroxy-7β-acetoxykaurenolide (**23**) (32 mg), ^1H NMR (200 MHz): δ 1.05 and 1.34 (each 3H, *s*), 1.98 (1H, *d*, $J = 7.5$ Hz, H-5), 2.09 (3H, *s*), 4.39 (1H, *br d*, H-3), 4.71 (1H, *t*, $J = 7.5$ Hz, H-6), 4.88 and 5.02 (each 1H, *br s*, H-17), 5.73 (1H, *d*, $J = 7.5$ Hz, H-7); EIMS *m/z* (rel. int.): 374 $[M]^+$ (15), 356 (3), 346 (15), 332 (14), 314 (100), 296 (13), 286 (17), 255 (31), 215 (10), 190 (15), 147 (15), 119 (20). Further elution gave 3β,7β-dihydroxykaurenolide (**20**) (36 mg).

Oxidation of compound **23**. Compound **23** dissolved in Me_2CO (10 ml) was treated dropwise with Jones reagent (0.2 ml) and left at 0° for 20 min. MeOH was added to destroy the excess reagent. The solvent was partially evapd and the reaction mixture diluted with H_2O and worked-up, yielding 3-oxo,7β-acetoxykaurenolide (**24**), ^1H NMR (200 MHz): δ0.98 and 1.53 (each 3H, *s*), 2.10 (3H, *s*), 2.36 (1H, *d*, $J = 7$ Hz, H-5); 4.64 (1H, *t*, $J = 7$ Hz, H-6); 4.93 and 5.06 (each 1H, *br d*, H-17); 5.83 (1H, *d*, $J = 7$ Hz, H-7); EIMS *m/z* (rel. int.): 372 $[M]^+$ (22), 330 (24), 312 (100), 285 (8), 271 (10), 269 (20), 256 (7), 202 (23), 190 (11), 159 (11), 147 (20), 123 (21).

Reduction of compound **24**. The ketone **24** (50 mg) in MeOH (6 ml) was treated with $NaBH_4$ (30 mg) at room temp for 90 min. The soln was acidified, concd *in vacuo* and poured into H_2O. The product was recovered in EtOAc and chromatographed on silica gel to afford 3α-hydroxy-7β-acetoxykaurenolide (**25**) (45 mg), ^1H NMR (200 MHz): δ 1.03 and 1.52 (each 3H, *s*), 1.83 (1H, *d*, $J = 6$ Hz, H-5), 2.11 (3H, *s*), 3.60 (1H, *dd*, $J = 6.3$ and 10.5 Hz, H-3), 4.73 (1H, *t*, $J = 6$ Hz, H-6), 4.91 and 5.03 (each 1H, *br s*, H-17), 5.90 (1H, *d*, $J = 6$ Hz, H-7); EIMS *m/z* (rel. int.): 374 $[M]^+$ (3), 3.56 (3), 332 (5), 314 (100), 296 (10), 268 (23), 255 (21), 225 (11), 201 (12), 159 (17), 147 (30). Diacetate **26**, mp 225–228° (from MeOH); $[M]^+$ at *m/z* 416.2208. $C_{24}H_{32}O_6$ requires 416.2198; ^1H NMR (200 MHz): δ 1.09 and 1.37 (each 3H, *s*), 1.86 (1H, *d*, $J = 6$ Hz, H-5), 2.09 and 2.15 (each 3H, *s*), 4.67 (1H, *t*, $J = 6$ Hz, H-6), 4.94 (1H, *dd*, $J = 6$ and 10 Hz, H-3), 4.90 and 5.03 (each 1H, *br s*, H-17), 5.88 (1H, *d*, $J = 6$ Hz, H-7); EIMS *m/z* (rel. int.): 416 $[M]^+$ (6), 374 (14), 356 (79), 315 (24), 314 (18), 147 (26).

Hydrolysis of compound **26**. The diacetate **26** (15 mg) in a methanolic soln of KOH (2%) was refluxed for 3 hr. The soln was acidified and the product recovered with EtOAc in the usual way to afford, 3α,7β-dihydroxykaurenolide (**27**) (10 mg), mp 222–223° (from MeOH), ^{1}H NMR (200 MHz): δ 0.92 and 1.51 (each 3H, *s*), 3.60 (1H, *dd*, $J = 6.7$ and 10.8, H-3), 4.50 (1H, *d*, $J = 5.5$ Hz, H-7), 4.69 (1H, *t*, $J = 5.5$ Hz, H-6), 4.88 and 5.01 (each 1H, *br s*, H-17); EIMS *m/z* (rel. int.): 332 $[M]^+$ (1), 314 (100), 299 (4), 296 (7), 286 (8), 268 (12), 255 (23), 225 (5), 201 (9), 190 (11), 159 (8), 145 (11).

Acknowledgements—This work has been supported for the CICYT (Madrid). We thank Prof. B. Rodriguez (CSIC, Madrid) for a gift of extracts of *Margotia gummifera* and Drs M. G. Hernández and P. González for their help and interest in this work. R.G. thanks the ministry of Education and Science and the Spanish Research Council (CSIC) for a 'Formacion de Personal Investigador' fellowship.

REFERENCES

1. Hanson, J. R., Sarah, F. Y., Fraga, B. M. and Hernández, M. G. (1979) *Phytochemistry* **18**, 1875.
2. Beale, M. H., Bearder, J. R., Down, G. H., Hutchison, M., MacMillan, J. and Phinney, B. O. (1982) *Phytochemistry* **21**, 1279.
3. Hedden, P. and Graebe, J. E. (1981) *Phytochemistry* **20**, 1011.
4. Pinar, M., Rodriguez, B. and Alemany, A. (1978) *Phytochemistry* **17**, 1978.
5. Fraga, B. M., Guillermo, R. and Hanson, J. R. (1990) *J. Chem. Res.* 99.
6. Dennis, D. T., Upper, C. D. and West, C. A. (1965) *Plant. Physiol.* **40**, 948.
7. Cross, B. E. and Myers, P. L. (1969) *Phytochemistry* **8**, 79.
8. Bateson, J. H. and Cross, B. E. (1972) *J. Chem. Soc., Perkin Trans I* 1117.
9. Fraga, B. M., González, A. G., González, P., Hanson, J. R. and Hernández, M. G. (1983) *Phytochemistry* **22**, 691.
10. Hanson, J. R., Savona, G. and Siverns, M. (1974) *J. Chem. Soc., Perkin Trans. I* 2001.
11. Hanson, J. R., Hawker, J. and White, A. F. (1973) *J. Chem. Soc., Perkin Trans I* 1892.