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Biosynthesis of GA_{73} Methyl Ester in *Lygodium* Ferns[1]

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Biosynthesis of GA_{73} methyl ester (GA_{73}-Me), the principal antheridiogen in *Lygodium* ferns, was investigated. From the methanol extract of prothallia of *Lygodium circinnatum*, GA_{25}, GA_{73}, GA_{73}-Me, GA_{88}-Me, and a few unknown GA_{73} derivatives were detected by GC-MS. Because the presence of GA_{25} suggests that GA_{24}, a direct precursor of GA_{25}, could also be present in *L. circinnatum* prothallia, we used feeding experiments to investigate the possibility that GA_{24} is a precursor of GA_{73}-Me. In *L. circinnatum* prothallia, $[^2H_2]GA_{24}$ was converted into $[^2H_2]GA_{73}$-Me and a trace amount of $[^2H_2]GA_9$-Me, whereas $[^2H_3]GA_9$ was converted into $[^2H_3]GA_9$-Me and $[^2H_3]$monohydroxy-GA_9-Me. Because GA_{73}-Me, GA_9-Me, and their monohydroxy derivatives had been identified by GC-MS from the culture medium of *L. circinnatum* prothallia, our results suggest that GA_{73}-Me is biosynthesized from GA_{24} via GA_{73}, and that neither GA_9 nor GA_9-Me is a precursor of GA_{73}-Me. Though the possibility had been suggested that GA_{73}-Me is biosynthesized from 9,15-cyclo-GA_9 (GA_{103}), $[^2H_2]GA_{103}$ was not converted into $[^2H_2]GA_{73}$-Me.

In Schizaeaceous ferns, antheridiogens have been reported to be GA-related compounds. Antheridic acid (Fig. 1) is a major antheridiogen in four *Anemia* species, i.e. *A. phyllitidis* (Nakanishi et al., 1971; Corey et al., 1986), *A. hirsuta* (Zanno et al., 1972), *A. rotundifolia*, and *A. flexuosa* (Yamane et al., 1987). In *A. mexicana*, 1β-hydroxy-9,15-cyclo-GA_9 (GA_{104}; Fig. 1) was isolated as a major antheridiogen (Nester et al., 1987; Furber et al., 1989; Oyama et al., 1996). The principal antheridiogen in three *Lygodium* ferns (*L. japonicum*, *L. circinnatum* and *L. flexuosum*) was found to be GA_{73}-Me (Fig. 1) (Yamane et al., 1988; Yamauchi et al., 1996). GA_9-Me (Fig. 1) was also identified in *L. japonicum* as an antheridiogen (Yamane et al., 1979). In *L. circinnatum*, GA_9-Me and monohydroxylated derivatives of GA_{73}-Me and GA_9-Me were detected as minor antheridiogens (Yamauchi et al., 1996).

The occurrence of three skeletal types as the principal antheridiogens in the Schizaeaceous ferns, i.e. antheridic acid (20-norantherid-16-ene derivative), GA_{104} (*ent*-9,15-cyclo-20-norgibberell-16-ene derivative), and GA_{73}-Me [*ent*-20-norgibberell-9(11),16-diene derivative], suggests that they might be related biosynthetically. The *ent*-9,15-cyclo-20-norgibberell-16-ene structure could be a precursor of either 20-norantherid-16-ene and/or *ent*-20-norgibberell-9(11),16-diene derivatives. Alternatively, the *ent*-20-norgibberell-16-ene structure could be a common precursor to each of the three classes of compounds. We reported previously that antheridic acid is biosynthesized from 9,15-cyclo-GA_9 (GA_{103}) via 3α-hydroxy-GA_{103} (GA_{107}) (Yamauchi et al., 1991; Oyama et al., 1996). However, almost no information has been obtained so far on the biosynthetic origin of GA_{73}-Me. The only information on the biosynthesis of antheridiogens in *Lygodium* ferns is that exogenously applied GA_9 was converted into GA_9-Me and then into 13-OH-GA_9-Me (GA_{20}-Me), 12β-OH-GA_9-Me (GA_{69}-Me), and 12α-OH-GA_9-Me (GA_{70}-Me) in *L. japonicum* prothallia (Yamane et al., 1979; Sato et al., 1985).

In the present work, we investigated the biosynthetic pathway of GA_{73}-Me in *L. circinnatum* by the analyses of the endogenous GAs and GA methyl esters in prothallia and by feeding experiments using possible precursor candidates.

MATERIALS AND METHODS

Analyses of Endogenous GAs and GA Methyl Esters in Fern Prothallia

Spores of *Lygodium circinnatum* were collected from sporangia-bearing pinnae harvested in the greenhouse at Ulm University, Ulm, Germany, and stored in a glass tube at 4°C until use. Spores of *L. circinnatum* were sterilized with 0.5% NaOCl and washed repeatedly with sterile water. They were then sown onto 0.5% agar-solidified medium (Mohr, 1956) and cultured under continuous white light (10 W m^{-2}) at 21°C. After about 5 weeks of culture, the prothallia were transferred individually to plastic Petri dishes (3.5 cm in diameter) containing 2 mL of the above medium and cultured further under the same conditions. After 10 weeks of culture, the prothallia were collected from the agar medium. The fresh weight of prothallia was 7.09 g.

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Abbreviations: AE, acidic ethyl acetate; DEA, diaminoethylpropyl; EtOAc, ethyl acetate; GA_n-Me, GA_n methyl ester; HOAc, acetic acid; MeOH, methanol; MSTFA, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide; NE, neutral ethyl acetate; ODS, octadecylsilane; Rt, retention time.

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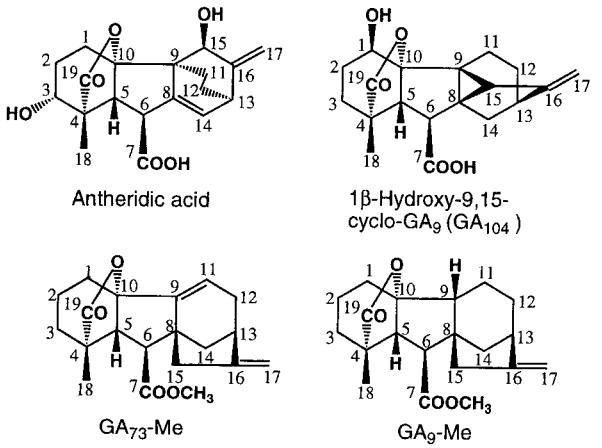

Figure 1. Chemical structures of antheridic acid, GA_{104}, GA_{73}-Me, and GA_9-Me. Numbering of the carbon skeletons is also shown.

Extraction and Fractionation

The prothallia were homogenized and extracted with MeOH. The MeOH extract was concentrated in vacuo, and the resultant aqueous residue was fractionated by the usual method (Toyomasu et al., 1992) to give an AE fraction and an NE fraction.

Purification of the AE Fraction

The AE fraction was dissolved in 1 mL of MeOH:H_2O (50:50, v/v) and passed through a Sep-Pak C_{18} cartridge (Waters, Milford, MA). The cartridge was eluted three times with 2 mL of MeOH:H_2O (80:20, v/v) and the combined eluate was evaporated to dryness in vacuo. The residue was dissolved in 1 mL of MeOH and loaded onto a column containing 0.1 g of Bondesil (DEA) (Varian Sample Preparation Products, Harbor City, CA). The column was eluted with 2 mL of MeOH and then with 2 mL of 0.5% HOAc in MeOH. The MeOH eluate was evaporated to dryness, dissolved in 1 mL of MeOH, and loaded onto another column containing 0.1 g of Bondesil (DEA). The column was eluted as described above. The eluates with 0.5% HOAc in MeOH from the first and second Bondesil (DEA) columns were combined and evaporated to dryness in vacuo. The residue was dissolved in 200 μL of MeOH:H_2O (30:70, v/v) and subjected to HPLC using a Senshu-Pak ODS 4253-D column (250 × 10 mm in diameter; Senshu Scientific, Tokyo, Japan). The solvents used were 1% HOAc in H_2O (A) and MeOH (B). A 28-min linear gradient (30–100% B) was applied 2 min after injection, at a flow rate of 3 mL min^{-1} and a column temperature of 40°C. The fractions were collected every 1 min. The GA-like activity of each fraction was examined using the dwarf rice (*Oryza sativa* L. cv Tan-ginbozu) micro-drop method modified by Nishijima and Katsura (1989), in which an aliquot equivalent to 0.1 g fresh weight was applied to each assay plant. The fractions showing GA-like activity were subjected to GC-MS analysis.

Purification of the NE Fraction

The NE fraction was dissolved in MeOH:H_2O (80:20, v/v) and partitioned three times against *n*-hexane. The combined aqueous MeOH phase was evaporated to dryness in vacuo. The residue was dissolved in 2 mL of MeOH:H_2O (40:60, v/v) and passed through a Sep-Pak C_{18} cartridge. The cartridge was washed twice with 2 mL of MeOH:H_2O (40:60, v/v) and eluted three times with 2 mL of MeOH. The combined MeOH eluate was evaporated to dryness in vacuo. The residue was dissolved in 200 μL of MeOH:H_2O (50:50, v/v) and subjected to HPLC using a Senshu-Pak ODS 3151-N column (150 × 8 mm in diameter). The solvents used were H_2O (A) and MeOH (B). A 30-min linear gradient (50–100% B) was applied 2 min after injection at a flow rate of 2 mL min^{-1} and a column temperature of 40°C. The fractions were collected every 1 min.

Antheridial formation activity of each fraction was assayed with *L. japonicum* protonemata, which are highly responsive to GA methyl esters. Spores of *L. japonicum* were aseptically inoculated on the surface of 1 mL of a 0.5% agar solidified 1/10 strength Murashige and Skoog's mineral salt medium (Murashige and Skoog, 1962) containing test samples (1/2000 aliquot of each fraction mL^{-1}) in a well of a 24-well plastic microplate. After incubation in darkness at 25°C for 5 d, spores were irradiated with red light for 24 h and incubated further for 7 d in darkness. The protonemata thus obtained were observed under a microscope to score antheridia formation. Fractions showing antheridial formation activity were subjected to GC-MS analysis.

GC-MS Analysis

A JEOL DX-303 GC-MS system was used, fitted with a fused silica chemically bonded capillary column DB-1 (15 m × 0.258 mm in diameter; J&W Scientific, Folsom, CA). Samples for the analysis of GAs were methylated with ethereal diazomethane and then trimethylsilylated with MSTFA at 80°C for 30 min. Samples for the analysis of GA methyl esters were trimethylsilylated with MSTFA at 80°C for 30 min. A 1-μL aliquot of each sample in MSTFA was injected onto the column at 120°C in a splitless mode. After 2 min of isothermal hold at 120°C, the column temperature was programmed at 16°C min^{-1} to 216°C with a 5-min isothermal hold at 216°C and subsequently at 8°C min^{-1} to 280°C. The pressure of the He carrier gas was 64 kPa.

Feeding Experiments

The preparation of [17-$^2H_2$]GA_{24} and of [17-$^2H_2$]GA_{103} was described by Nakayama et al. (1991) and Furber et al. (1990), respectively. [2,2,6-$^2H_3$]GA_9 was prepared according to the method of Nakayama et al. (1989) with modifications. GA_4-Me (40 mg) was refluxed with MeONa in MeOD (0.2 M, 3 mL) for 3 h. The solution was acidified with D_2SO_4 and diluted with water. After evaporating the MeOH, the solution was extracted with EtOAc. The EtOAc phase was dried over anhydrous Na_2SO_4 and evaporated. The resultant residue was again refluxed with

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Table I. *Endogenous GAs and their methyl esters identified by full-scan GC-MS in L. circinnatum prothallia*
X2 and X3 are monohydroxy-GA_{73}-Me-like compounds.

Rt on ODS-HPLC	Derivative	Kovats Retention Index	Principal Ions and Relative Intensity (% Base Peak)	Identity
min				
GAs				
18–20	MeTMSi[a]	2520	416(M^+; 39), 401(11), 385(13), 372(100), 357(26), 313(51), 141(10)	Free acid of X2
22–26	Me[b]	2329	328(M^+; 5), 297(17), 284(100), 269(16), 241(17), 225(77), 209(12), 183(23)	GA_{73}
26–29	Me	2439	404(M^+; 2), 372(33), 344(2), 312(75), 284(100), 253(8), 225(43)	GA_{25}
GA Mes				
12–13	TMSi[c]	2521	416(M^+; 40), 401(8), 385(6), 372(100), 357(15), 313(72), 223(5)	X2
13–15	TMSi	2520	416(M^+; 10), 372(100), 357(12), 313(17), 282(13), 267(18), 223(19), 129(20)	X3
18–19	TMSi	2495	416(M^+; 28), 360(44), 326(22), 287(77), 282(100), 259(28), 223(46), 129(37)	GA_{88}-Me
27–32	—[d]	2326	328(M^+; 1), 297(11), 284(100), 269(13), 241(24), 225(81), 209(12), 183(48)	GA_{73}-Me

[a] MeTMSi, Methylester trimethylsilyl ester. [b] Me, Methyl ester. [c] TMSi, Trimethylsilyl ether. [d] —, Not derivatized.

MeONa in MeOD under the above conditions. The reaction mixture was acidified, extracted with EtOAc, and evaporated as described above. Purification of the residual gum by silica gel TLC (*n*-hexane-EtOAc-HOAc; 28:12:1, V/V/V) gave [2,2,6-$^2H_3$]3-*epi*-GA_4-Me (14.9 mg). [2,2,6-$^2H_3$]3-*Epi*-GA_4-Me (14.9 mg) was dissolved in dry pyridine (1 mL) with $POCl_3$ (40 μL), and the mixture was heated in a sealed tube at 100°C for 3 h. The reaction mixture was diluted with acidic water and extracted with EtOAc at pH 3. The EtOAc phase was washed with 5% aqueous $NaHCO_3$, dried over anhydrous Na_2SO_4 and evaporated. The residue was dissolved in dry benzene (8 mL), to which *n*-Bu_3SnH (110 μL) and α,α-azobisisobutyronitrile (small quantity) were added. The solution was refluxed under an N_2 stream for 1 h and then diluted with EtOAc, washed with water, and evaporated. The residual gum was subjected to preparative Si gel TLC (*n*-hexane-EtOAc-HOAc; 28:12:1, V/V/V) to give [2,2,6-$^2H_3$]GA_9-Me (12 mg), which was demethylated with *n*-PrSLi by the method of Bartlett and Johnson (1970). The crude product was purified by preparative Si gel TLC with the above solvent system to afford [2,2,6-$^2H_3$]GA_9 (8.8 mg).

Culture of Prothallia

About 30 mg of spores of *L. circinnatum* were aseptically inoculated and cultured for 3 weeks on 100 Petri dishes (3 cm in diameter) containing 6 mL of 1/10 strength Murashige and Skoog's mineral salts medium containing uniconazole-p (1 μg mL^{-1}) solidified with 0.5% agar, at 25°C under continuous white light (5 W m^{-2}).

Procedure of Feeding Experiments

Ten micrograms of [$^2H_2$]GA_{103}, [$^2H_3$]GA_9, or [$^2H_2$]GA_{24} in MeOH (5 μL) was aseptically added to a 50-mL conical

Table II. *Metabolities identified by full-scan GC-MS in feeding experiment of [$^2H_2$]GA_{103} in L. circinnatum prothallia*

Metabolite	Derivative	Kovats Retention Index	Principal Ions and Relative Intensity
			%
Authentic GA_{103}-Me	—[a]	2232	328(M^+; 85), 284(78), 269(37), 255(20), 225(100), 209(20), 196(22)
[$^2H_2$]GA_{103}-Me	—	2231	330(M^+; 95), 286(62), 271(36), 257(17), 227(100), 211(11), 198(13)
Authentic 11α-OH-9,15-cyclo-GA_9-Me	TMSi[b]	2403	416(M^+; 5), 384(10), 356(12), 280(50), 266(100), 241(25), 223(50), 195(22)
[$^2H_2$]11α-OH-9,15-cyclo-GA_9-Me	TMSi	2401	418(M^+; 8), 386(6), 357(9), 282(49), 268(100), 241(31), 225(64), 197(14)
Authentic GA_{108}-Me	TMSi	2413	416(M^+; 10), 384(10), 356(12), 282(45), 280(52), 266(100), 223(50)
[$^2H_2$]GA_{108}-Me	TMSi	2412	418(M^+; 8), 386(8), 358(9), 284(37), 282(53), 268(100), 225(54)
Authentic 12α-OH-9,15-cyclo-GA_9-Me	TMSi	2458	416(M^+; 100), 326(27), 313(40), 267(12), 258(25), 240(28), 223(45), 196(25)
[$^2H_2$]12α-OH-9,15-cyclo-GA_9-Me	TMSi	2456	418(M^+; 100), 328(32), 315(45), 269(18), 256(28), 242(48), 225(58), 198(25)
Authentic 2α-OH-9,15-cyclo-GA_9-Me	TMSi	2471	416(M^+; 28), 372(85), 357(26), 341(78), 313(25), 282(35), 223(100), 197(32)
[$^2H_2$]2α-OH-9,15-cyclo-GA_9-Me	TMSi	2466	418(M^+; 39), 374(90), 359(29), 343(100), 315(23), 284(40), 225(86), 199(29)
[$^2H_2$]Monohydroxy-9,15-cyclo-GA_9-Me-like compound	TMSi	2486	418(M^+; 100), 343(43), 328(40), 315(62), 256(38), 242(44), 225(85), 198(52)

[a] —, Not derivatized. [b] TMSi, Trimethylsilyl ether.

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Table III. *Metabolites identified by full-scan GC-MS in feeding experiment of $[^2H_3]GA_9$ in L. circinnatum prothallia*

Compound	Derivative	Kovats Retention Index	Principal Ions and Relative Intensity
			%
$[^2H_3]GA_9$-Me	—[a]	2318	333(M^+; 8), 301(100), 289(16), 273(85), 246(43), 229(51), 220(25)
Unknown compound	TMSi[b]	2493	421(M^+; 49), 406(33), 361(22), 331(62), 298(74), 286(50), 271(100), 225(71)
$[^2H_3]GA_{84}$-Me	TMSi	2468	421(M^+; 71), 361(34), 345(29), 331(77), 286(97), 266(100), 226(63)
$[^2H_3]GA_{20}$-Me	TMSi	2478	421(M^+; 100), 406(20), 376(47), 303(14), 235(11), 207(27), 193(8)
$[^2H_3]GA_{70}$-Me	TMSi	2540	421(M^+; 35), 361(33), 331(53), 313(31), 298(66), 285(76), 271(100), 226(66)
Unknown compound	TMSi	2588	421(M^+; 100), 406(12), 376(21), 295(14), 272(10), 226(16), 207(25), 183(15)

[a] —, Not derivatized. [b] TMSi, Trimethylsilyl ether.

flask containing 10 mL of the same medium as above without agar. To the conical flask, 0.2 g fresh weight of 3-week-old prothallia was transferred and incubated at 25°C under continuous white light (5 W m^{-2}). After 10 d the medium was separated from the prothallia by filtration. The filtrate was adjusted to pH 3 with 6 M HCl and extracted 3 times with EtOAc. The combined EtOAc phase was dried over anhydrous Na_2SO_4 and evaporated to dryness in vacuo. The residue was dissolved in 1 mL of MeOH:H_2O (40:60, v/v) and passed through a Sep-Pak C_{18} cartridge. The cartridge was washed twice with 2 mL of MeOH:H_2O (40:60, v/v) and eluted three times with MeOH. The combined MeOH eluate was evaporated to dryness in vacuo. The residue was trimethylsilylated with MSTFA at 80°C for 30 min and analyzed by GC-MS as described above.

RESULTS AND DISCUSSION

Endogenous GAs and GA Methyl Esters in Prothallia of *L. circinnatum*

To obtain information on the biosynthetic intermediates leading to GA_{73}-Me, we analyzed the endogenous GAs and GA methyl esters in prothallia of *L. circinnatum*.

GAs

The AE fraction from the MeOH extract of the prothallia was subjected to ODS-HPLC after prepurification using a Sep-Pak C_{18} cartridge and a Bondesil (DEA) column. The fractions from ODS-HPLC were assayed using the dwarf rice micro-drop method to give GA-active fractions, which were analyzed by full-scan GC-MS after derivatization. As shown in Table I, GA_{73}, GA_{25}, and the free acid of X2 (X2 is a monohydroxy-GA_{73}-Me-like compound that was detected from the culture medium of prothallia of *L. circinnatum* [Yamauchi et al., 1996]) were identified by their Kovats retention index and full-scan mass spectra of their Me or Me-trimethylsilyl ether derivatives. Because the presence of GA_{25} suggests that GA_{24}, a direct precursor of GA_{25}, could be present in *L. circinnatum* prothallia, GA_{24} may be one of the precursor candidates of GA_{73}-Me.

GA Methyl Esters

The NE fraction was purified by solvent fractionation using *n*-hexane and MeOH:H_2O (80:20, v/v), and the aqueous MeOH fraction was subjected to ODS-HPLC after prepurification using a Sep-Pak C_{18} cartridge. The fractions from the ODS-HPLC were subjected to the antheridial formation assay with *L. japonicum* protonemata. The bioactive fractions were analyzed by full-scan GC-MS after derivatization. GA_{73}-Me, GA_{88}-Me, X2, and X3 (another monohydroxy-GA_{73}-Me-like compound) were identified by full-scan GC-MS (Table I). Of these GA methyl esters, the major component was GA_{73}-Me (approximately 0.5 $\mu g\ g^{-1}$ fresh weight); GA_{73} was the level of GA_{73}-Me. The levels of other GAs were the same as that of GA_{73} or less. Co-occurrence of GA_{73}-Me and GA_{73} in the prothallia suggests that GA_{73}-Me is biosynthesized from GA_{73}.

Feeding Experiments in Prothallia of *L. circinnatum*

To investigate the biosynthetic pathway of GA_{73}-Me in *L. circinnatum*, the precursor candidates, $[^2H_2]GA_{103}$, $[^2H_3]GA_9$, and $[^2H_2]GA_{24}$, were fed individually to 3-week-old *L. circinnatum* prothallia and the prothallia were cultured an additional 10 d. In these feeding experiments, the prothallia cultured in the presence of 1 $\mu g\ mL^{-1}$ of uniconazole-P, an inhibitor of GA biosynthesis (Izumi et al., 1985), were used to reduce the levels of native antheridiogens and to promote the metabolism of the feeding sub-

Table IV. *Metabolites identified by full-scan GC-MS in feeding experiment of $[^2H_2]GA_{24}$ in L. circinnatum prothallia*

Compound	Derivative	Kovats Retention Index	Principal Ions and Relative Intensity
			%
$[^2H_2]GA_{73}$-Me	—[a]	2328	330(M^+; 4), 299(8), 286(100), 271(19), 241(15), 227(94), 211(8)
$[^2H_2]GA_9$-Me	—	2312	332(M^+; 14), 300(70), 290(46), 272(100), 243(37), 228(93), 217(42)

[a] —, Not derivatized.

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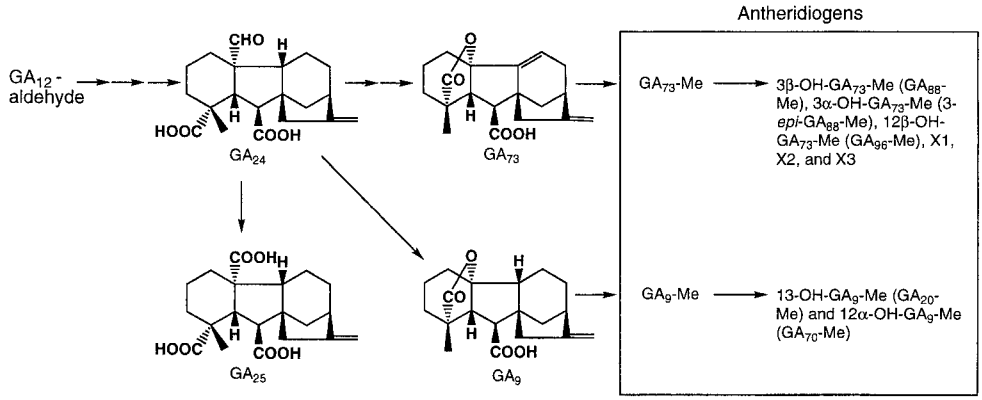

Figure 2. Hypothetical biosynthetic pathways of native antheridiogens in prothallia of *L. circinnatum*. X1, X2, and X3 are monohydroxy-GA_{73}-Me-like compounds (Yamauchi et al., 1996). Because the free acid of X2 was detected from the MeOH extract of prothallia of *L. circinnatum*, it remains to be clarified whether X2 is biosynthesized from GA_{73}-Me or from GA_{73} via the free acid of X2.

strates (Nakayama et al., 1991). In a preliminary experiment, we confirmed that the production of native antheridiogens in *L. circinnatum* prothallia cultured in the presence of 1 μg mL^{-1} of uniconazole-P was negligible, whereas the growth of prothallia was not affected (data not shown). The metabolites from each feed were identified by full-scan GC-MS after derivatization. In the feed of $[^2H_2]GA_{103}$, this substrate was converted into $[^2H_2]GA_{103}$-Me and several monohydroxylated derivatives, namely, 2α-, 11α-, 11β-, and 12α-hydroxy-GA_{103}-Me, plus one unknown derivative (Table II; Pour, 1994; M. Pour, P. Kraft-Klaunzer, L.N. Mander, B. Twichin, N. Oyama, N. Murofushi, H. Yamane, and T. Yamauchi, unpublished data). Conversion of $[^2H_2]GA_{103}$ into $[^2H_2]GA_{73}$-Me was not observed in this feed. In the feed of $[^2H_3]GA_9$, as shown in Table III, $[^2H_3]GA_9$-Me, $[^2H_3]$11β-OH-GA_9-Me (GA_{84}-Me), $[^2H_3]$ 12α-OH-GA_9-Me (GA_{70}-Me), $[^2H_3]$13-OH-GA_9-Me (GA_{20}-Me), and two unknown $[^2H_3]$monohydroxy-GA_9-Me-like compounds were detected. This suggests that GA_9 was converted into GA_9-Me, and then monohydroxylated at C-11β, C-12α, C-13 or the other positions, although GA_{84}-Me and the unknown $[^2H_3]$monohydroxy-GA_9-Me-like compounds were not detected as native antheridiogens in *L. circinnatum* (Yamauchi et al., 1996). The metabolism of GA_9 in *L. circinnatum* was similar to that reported in prothallia of *L. japonicum* (Yamane et al., 1979; Sato et al., 1985). On the other hand, in the feed of $[^2H_2]GA_{24}$, $[^2H_2]GA_{73}$-Me and a trace amount of $[^2H_2]GA_9$-Me were identified as metabolites (Table IV). Thus, it was clearly indicated that GA_{24} is a precursor of both GA_{73}-Me and GA_9-Me, whereas neither GA_9 nor GA_{103} is likely to be a precursor of GA_{73}-Me. In the feed of $[^2H_2]GA_{24}$, neither $[^2H_2]GA_{24}$-Me nor $[^2H_2]$monohydroxy-GA_{24}-Me-like compounds were detected in the culture medium. These results, together with the presence of GA_{25} in the free acid form in the prothallia, suggest that the C-7 carboxyl group is methylated after conversion of GA_{24} into GA_{73} or GA_9. On the basis of our current knowledge of GA biosynthesis, qualitative analysis of GAs and GA methyl esters in the prothallia, the feeding experiments described in this paper, and our previous studies (Yamauchi et al., 1996), the hypothetical biosynthetic pathways from GA_{12}-aldehyde to the native antheridiogens in *L. circinnatum* are presented in Figure 2.

In conclusion, this paper presents evidence that GA_{73}-Me is biosynthesized from GA_{24} via GA_{73}. Because GA_9 was not incorporated into GA_{73} or GA_{73}-Me, it is possible that the 9(11) double bond is introduced before the loss of C-20. Experiments to test this hypothesis will be conducted.

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