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GIBBERELLINS A_{45} AND A_{61} FROM GAMETOPHYTE CULTURE EXTRACTS OF *ANEMIA MEXICANA*

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Key Word Index—*Anemia mexicana*; Anemiaceae; fern; gibberellins; antheridiogens.

Abstract—Two gibberellins, GA_{45} and GA_{61}, were identified for the first time from gametophyte culture media extracts of *Anemia mexicana* along with the previously characterized primary antheridiogen, 1β-hydroxy-9,15-cyclo-GA_9 (GA_{104}). © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Although antheridiogens are substances which hasten the formation of antheridia in fern gametophytes, and may also substitute for the light requirement for spore germination in *Anemia* and *Lygodium* sp., very little is known about their structure. An antheridiogen from *Anemia phyllitidis* was initially characterized as the GA-like compound **1** [1], but its structure was later revised to the epimer, antheridic acid **2** [2], which has also been identified in *Anemia hirsuta* [3], *Anemia rotundifolia* and *Anemia flexuosa* [4]. Two additional antheridiogens were also characterized in *A. phyllitidis* as 3α-hydroxy-9,15-cyclo-GA_9 (GA_{107}, **3**) [5] and 3-*epi*-GA_{63} (**5**) [6]), and several other antheridiogens and GAs were recently identified from its gametophytes and sporophytes [7].

Additionally, primary antheridiogen of *Anemia mexicana* Klotzsch was identified as 1β-hydroxy-9,15-cyclo-GA_9 (**4**) [8] and designated GA_{104}. Comparisons of spectral data of synthetic preparatives with the native antheridiogen aided in its structural determination. A second antheridiogen was detected but not structurally identified [9]. The present paper describes the isolation and identification of two new GAs produced by gametophytes of *A. mexicana* using capillary GC-MS, in addition to the known primary antheridiogen, GA_{104}.

RESULTS AND DISCUSSION

Gametophyte aq. culture media of *Anemia mexicana* was purified by EtOAc extraction followed by QAE-Sephadex chromatography. Biologically active fractions (spore germination in darkness) after chromatography were purified further by HPLC. Biological activity of HPLC fractions revealed a broad band of activity from 20 to 30 min. GC-MS analysis of bioactive HPLC fractions indicated the presence of the previously characterized primary antheridiogen (**4**) and at least four other GA or GA-like compounds. GA_{45} (**6**) and GA_{61} (**7**) were eluted from the HPLC column at 24–28 min and identified by comparisons of mass spectra of their methyl ester and methyl ester, TMSi derivatives (Table 1) [10]. GA_{104} was eluted from the HPLC column at 20–24 min and was also identified by comparison of its mass spectra of methyl ester and methyl ester, TMSi derivatives [9]. Two other unknown compounds with GA-like mass spectra were also present in the 20–24 min HPLC fractions, and their methyl ester derivatives had molecular ions of *m/z* 362 indicating the presence of two hydroxyl groups. The chemical structures of these two unknowns are presently under investigation.

This is the first report of GA_{45} or GA_{61} from a pteridophyte gametophyte culture media extract and is of considerable interest since their hydroxylation sites correspond to those in GA_{104} and *epi*-GA_{63}, with the latter being previously identified in gametophytes of *A. phyllitidis* [6]. In our studies, antheridic acid was not detected in any of the GC-MS samples analyzed from HPLC fractions between 16 and 32 min, although it was isolated from several other *Anemia* species. It may be that *A. mexicana* does not synthesize antheridic acid (**2**) since *A. mexicana* and *A. phyllitidis* are in separate subgenera. In *Anemia phyllitidis*, GA_9, GA_{24}, GA_{25}, 3-*epi*-GA_{63} and antheridic acid were identified while GA_4, GA_9, GA_{15}, GA_{19}, GA_{20} and GA_{24} have been isolated from sporophyte tissue [7]. In *Lygodium japonicum*, two antheridiogens were characterized as GA_9-methyl ester [11] and GA_{73}-methyl ester [12]. These two GA-methyl esters, tog-

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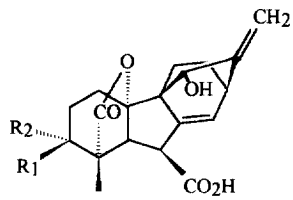
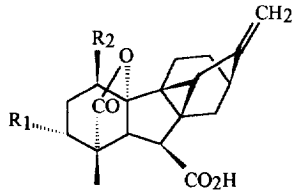
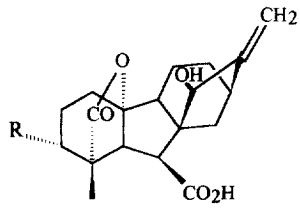


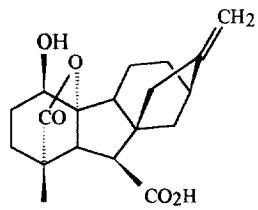
1. *A. phyllitidis* antheridiogen: R_1 = OH, R_2 = H
2. Antheridic acid: R_1 = H, R_2 = OH



3. GA_{107}: R_1 = OH, R_2 = H
4. GA_{104}: R_1 = H, R_2 = OH



5. 3-*epi*-GA_{63}: R = OH
6. GA_{45}: R = H



7. GA_{61}

ether with four other GA-methyl esters (GA_{20}, GA_{70}, GA_{88}, 3-*epi*-GA_{88}), were also identified in *Lygodium circinnatum* and *Lygodium flexuosum* [13]. GAs have been isolated from young sporophyte fronds of three tree ferns including ten GAs from *Cyathea australis* [14], 17 GAs from *Cibotium glaucum*, and seven GAs from *Dicksonia antarctica* [15], but neither GA_{45} nor GA_{61} were described as present in tree ferns.

EXPERIMENTAL

Plant material

Spores of *Anemia mexicana* were collected from Uvalde County, Texas, U.S.A. and stored in glass vials at 4 C. A voucher specimen is deposited in the Warner Herbarium at Sam Houston State University.

Spore sowing and antheridiogen production

Spores were sown onto the surface of sterile plastic petri plates (100 × 15 mm) and gametophytes were grown for three months as previously described [9]. Eighteen plates were placed in an aluminum pan and the pan was placed in a closed plastic bag which had a strip hole made and covered with Micropore tape. Pans were placed under fluorescent light (approximately 150 lumens ft^2) with 16 h light, 8 h darkness at 24 C.

Table 1. GAs identified by GC-MS from gametophyte culture extracts of *A. mexicana* with comparisons to published spectra of GA_{104}, GA_{45}, and GA_{61}

HPLC fraction	Compound	Derivative	Principal ions and relative intensities
20–22 min	GA_{104}	Me	344 (M^+; 25), 312 (7), 285 (17), 282 (19), 273 (7), 239 (11), 223 (100)
		MeTMSi	416 (M^+; 56), 384 (13), 357 (35), 345 (17), 285 (50), 282 (63), 259 (37), 239 (25), 223 (100)
26–28 min	GA_{45}	Me	346 (M^+; 2), 314 (100), 284 (13), 269 (12), 241 (20), 225 (23)
		MeTMSi	418 (M^+; 100), 403 (20), 358 (44), 284 (24), 269 (29), 241 (20), 225 (43)
	GA_{61}	Me	346 (M^+; 5), 314 (49), 286 (100), 258 (19), 241 (15), 225 (47)
		MeTMSi	418 (M^+; 7), 403 (7), 386 (14), 358 (31), 296 (100), 268 (14), 241 (17), 225 (41)
	GA_{104}*	Me	344 (M^+; 44), 312 (5), 285 (19), 282 (23), 273 (6), 239 (9), 223 (100)
		MeTMSi	416 (M^+; 83), 384 (16), 357 (38), 345 (28), 285 (62), 282 (72), 259 (55), 239 (33), 223 (100)
	GA_{45}†	Me	346 (M^+; 6), 314 (100), 284 (14), 269 (12), 241 (20), 225 (16)
		MeTMSi	418 (M^+; 100), 403 (15), 358 (36), 284 (19), 269 (19), 241 (10), 225 (19)
	GA_{61}†	Me	346 (M^+; 6), 314 (33), 286 (100), 258 (22), 241 (16), 225 (46)
		MeTMSi	418 (M^+; 12), 403 (12), 386 (15), 358 (50), 296 (100), 268 (19), 241 (17), 225 (33)

* Relative intensity data obtained from Ref. [9].
† Relative intensity data obtained from Ref. [10].

Isolation

After three months, aq. gametophyte culture media was obtained, adjusted to pH 2.5 with 2 M HCl and extracted ×3 with EtOAc. The EtOAc fraction was extracted ×3 with 5% sodium bicarbonate adjusted to pH 3.0 with 2 M HCl and extracted ×3 with EtOAc. Dried fractions were stored at −20° C. Initial purification was carried out on QAE Sephadex A-25 (Diethyl[2-hydroxypropyl]aminoethyl Sephadex, Sigma Chemical Company) with 1 M to 2 M HOAc as the eluant. All fractions were dried and stored at −20° C until bioassayed.

Bioassays

Chromatographic fractions were bioassayed for location of fractions with activity for spore germination in darkness. Fractions were dissolved in a known volume of EtOH and an aliquot was removed and added to liquid culture media buffered with 25 mM MES (2-[N-Morpholino]ethanesulfonic acid) pH 6. Bioassays were prepared in 24-well sterile plastic plates in duplicate. Spores were added to the bioassay fractions and replicate plates were placed in both the light and in darkness. After 10 days, bioassays were observed for spore germination in darkness using an inverted microscope.

HPLC

Fractions with biological activity after ion-exchange chromatography were pooled, and passed through a Sep-pak column (Millipore Corporation). The sample was fractionated by HPLC with a Spherisorb C-18 column 250 × 4.6 mm with detection at 205 nm. A gradient of 20–100% MeOH and water (with 50 ml MeOH and 50 μl HOAc added/l) over 40 min was used with a flow rate of 1 ml/min. Fractions were evaporated to dryness with a Labconco Centrivap Evaporation System at 50° C, stored dry in silylated glass vials at −20° C and bioassayed.

Combined GC-MS

HPLC fractions with biological activity were analyzed by GC-MS. Methyl-ester derivatives were prepared by treating samples dissolved in MeOH with CH_2N_2 in ethyl ether. Methyl-ester trimethylsilyl (TMSi)-ether derivatives were prepared by treating methyl-ester derivatives with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) in closed vials at 70° C for 30 min. Samples were analysed with a Hewlett-Packard 5890 GC-MS with a DB-5 WCOT fused silica capillary column (J&W Scientific). Injector temperature was 220° C. Compounds were eluted with a temperature gradient of 50° C to 200° C at 30° C min^{-1}, to 300° C at 10° C min^{-1}, holding at 300° C for 4 min.

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