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3-EPI-GA_{63}, ANTHERIDIOGEN IN *ANEMIA PHYLLITIDIS*

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Key Word Index—*Anemia phyllitidis*; Schizaeaceae; fern; prothallia; antheridiogen; antheridic acid; 3-epi-gibberellin A_{63}.

Abstract—3-Epi-Gibberellin A_{63} (3-epi-GA_{63}) was identified by full-scan GC-mass spectrometry of a purified extract from culture media of prothallia of the fern, *Anemia phyllitidis*. This is the third antheridiogen, following antheridic acid and 3α-hydroxy-9,15-cyclo-GA_9, in this species. 3-Epi-GA_{63} showed slightly less activity than antheridic acid in antheridial formation and dark spore germination assays.

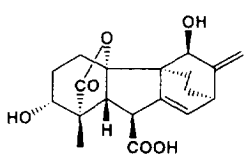
INTRODUCTION

Fern gametophytes secrete antheridium-inducing substances (antheridiogens) into culture media and induce antheridial formation. In *Anemia phyllitidis*, and three other *Anemia* species, antheridic acid (**1**) was identified as the principal antheridiogen [1–3]. 3α-Hydroxy-9,15-cyclo-GA_9 (**2**), a biosynthetic precursor of antheridic acid, was also identified as an antheridiogen in *A. phyllitidis* [4].

In the process of biosynthetic studies on antheridic acid that were reported in our previous paper [4], we had detected by GC-mass spectrometry a novel GA-like compound from a purified extract of the culture medium of prothallia of *A. phyllitidis* as a candidate for a third antheridiogen, although we had not noted the presence of this compound in the paper. In this paper, we describe the identification of this novel GA-like compound and its biological activities.

RESULTS AND DISCUSSION

The purified extract from culture medium of 45-day-old prothallia of *A. phyllitidis* was analysed by capillary GC-mass spectrometry after derivatization and a novel GA-like compound was detected, together with antheridic acid and 3α-hydroxy-9,15-cyclo-GA_9. The ratio of the GA-like compound/3α-hydroxy-9,15-cyclo-GA_9/antheridic acid was *ca* 1:1:10, based on comparison of the peak areas of total ions of the three compounds in the mass chromatogram. The methyl ester-trimethylsilyl ether (Me-TMSi) derivative of the GA-like compound showed a very similar mass spectrum to that of the Me-TMSi derivative of GA_{63} (**3**) [5], but their Kovats retention indices (KRIs) [6] were different from each other (Table 1). Because both the C-3α- and C-3β-epimers of 3-hydroxy-GAs show very similar mass spectra, and antheridic acid possesses a 3α-hydroxyl, we prepared 3-epi-GA_{63} (**4**) as a candidate for the new GA-like compound and carried out a direct comparison of the GA-like compound with synthetic 3-epi-GA_{63} by GC-mass spectrometry (Table 1). KRI and a mass spectrum of the Me-TMSi derivative of the GA-like compound were identical with those of the Me-TMSi derivative of 3-epi-GA_{63}. Thus, the GA-like compound was identified as 3-epi-GA_{63}. Since a 3β-hydroxy-GA, such as GA_{63}, epimerizes under basic conditions to give a mixture of 3β- and 3α-hydroxy-GAs in the ratio of 1:3 [7], 3-epi-GA_{63} might be



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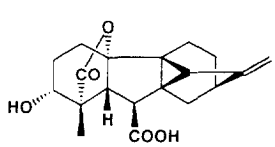

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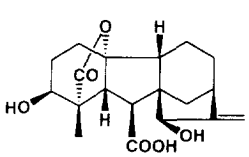

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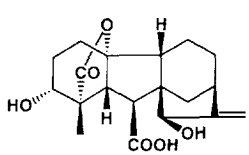

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Table 1. GC-MS data of the MeTMSi derivative of the GA-like compound from the culture medium of *A. phyllitidis* prothallia

Sample	KRI	Principal ions and relative intensity (% base peak)	Identity
GA_{63}-MeTMSi	2691	506 ($[M]^+$; 100), 491 (34), 446 (31), 416 (19), 287 (25), 197 (35), 156 (28), 129 (8)	—
3-epi-GA_{63}-MeTMSi	2749	506 ($[M]^+$; 100), 491 (11), 446 (21), 416 (12), 369 (17), 287 (30), 156 (18), 129 (12)	—
MeTMSi derivative of the GA-like compound	2749	506 ($[M]^+$; 100), 491 (15), 446 (21), 416 (14), 369 (17), 287 (51), 156 (20), 129 (29)	3-epi-GA_{63}

Table 2. Activity of antheridic acid, 3-epi-GA_{63} and GA_{63} in inducing antheridial formation in *A. phyllitidis*

	Antheridial formation (% ± SE) Concentration (M)					
Sample	0	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}
Antheridic acid	0 ± 0	0 ± 0	49.6 ± 2.8	95.3 ± 1.0	99.1 ± 0.5	98.4 ± 0.8
3-epi-GA_{63}		0 ± 0	17.4 ± 3.0	87.3 ± 4.5	94.0 ± 1.3	97.8 ± 0.9
GA_{63}		0 ± 0	0 ± 0	77.4 ± 2.2	97.8 ± 0.8	98.3 ± 0.7

Each value represents the mean of results from three replicates with the standard error.

Table 3. Activity of antheridic acid, 3-*epi*-GA_{63} and GA_{63} in inducing dark spore germination in *A. phyllitidis*

	Dark spore germination (% ± SE) Concentration (M)					
Sample	0	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}
Antheridic acid	1.3 ± 0.2	2.5 ± 0.3	43.9 ± 2.1	62.7 ± 0.5	93.3 ± 1.0	97.1 ± 0.4
3-epi-GA_{63}		2.6 ± 0.3	20.2 ± 3.1	38.6 ± 3.2	82.8 ± 2.0	96.0 ± 0.7
GA_{63}		1.2 ± 0.1	5.7 ± 0.6	22.0 ± 1.8	44.5 ± 2.0	87.9 ± 1.3

Each value represents the mean of results from three replicates with the standard error.

an artefact from GA_{63} as a consequence of the purification procedure. However, in our experiment the co-occurrence of 3-epi-GA_{63} and GA_{63} was not observed. Thus, 3-epi-GA_{63} is considered to be native and to be the third antheridiogen in *A. phyllitidis*.

Activities of antheridic acid, 3-epi-GA_{63} and GA_{63} in the induction of antheridial formation and dark spore germination in *A. phyllitidis* were tested. The results of these assays are shown in Tables 2 and 3. Antheridic acid was active down to 10^{-9} M in antheridial formation and dark spore germination. 3-Epi-GA_{63} was slightly less active than antheridic acid, but slightly more active than GA_{63} in both assays. The activity of 3-epi-antheridic acid was weaker than that of antheridic acid in both assays [8] and all three antheridiogens indentified in *A. phyllitidis* possess a 3α-hydroxy group. These results indicate that the introduction of a 3α-hydroxyl is important for the high biological activity and secretability into the medium of antheridiogens in *A. phyllitidis*.

With regard to biosynthesis of 3-epi-GA_{63}, it was shown that 9,15-cyclo-GA_9, a precursor of 3α-hydroxy-9,15-cyclo-GA_9 and antheridic acid, was not metabolized into 3-epi-GA_{63} in *A. phyllitidis* prothallia (data not shown). Investigations into the biosynthetic origin of 3-epi-GA_{63} are now under way.

EXPERIMENTAL

Preparation of conditioned medium and purification. Prothallia of *A. phyllitidis* (0.5 g fr. wt, 35 days old) growth on 0.3% agar solidified medium [9] were aseptically transferred into a 50 ml conical flask containing 7 ml of the liquid medium and cultured under continuous white light at 25 ± 1°. After 10 days, the conditioned medium from 10 flasks (total 70 ml) was sepd from the prothallia by filtration. The filtrate was extracted with EtOAc at pH 3 and the extract was purified using

Sepralyte (diethylaminopropyl, Analytichem) in the same manner as described previously [4].

GC-MS. A JEOL DX-303 GC-MS system (ionization voltage, 70 eV) was used, fitted with a fused silica capillary column DB-1 (15 m × 0.258 mm i.d., 0.25 mm thick stationary phase, J&W Scientific). Each sample was methylated with CH_2N_2–Et_2O and trimethylsilylated with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) at 80° for 30 min, then injected on to the column at 120° in the splitless mode. After 2 min at 120°, the column temp. was prog. at 16° min^{-1} to 216° with a 5 min hold at 216° and subsequently at 8° min^{-1} to 280°. The pressure of the He carrier gas was 64 kPa. KRIs were determined by the method of ref. [10].

Preparation of 3-epi-GA_{63} methyl ester. The methodology is based on that reported in refs [5, 11]. *3-Epi-GA_4-methyl ester (ent-3β,10β-dihydroxy-20-norgibberell-16-ene-7,19-dioic acid 7-methyl ester 19,10-lactone).* A solution of Li *t*-butoxide was formed by the addition of *n*-butyllithium (1.6 M, 2 ml) to dry *t*-butyl alcohol (4.5 ml) dissolved in dry THF (15 ml) with stirring at −10° under a N_2 atmosphere. To this soln was added GA_4 Me ester (180 mg, 0.52 mmol) dissolved in dry THF (2 ml). The soln was left to warm slowly and then heated at 30° for 36 hr. After this time, the solvent was removed and the residue taken up in EtOAc (50 ml) and washed with satd NH_4Cl soln (20 ml). The aq. phase was back extracted with EtOAc (2 × 20 ml). The combined organic phases were dried (Na_2SO_4), filtered and the solvent removed *in vacuo* to yield a semi-crystalline yellow solid (170 mg). This material was used without further purification. R_f 0.16 (EtOAc–hexane, 1:1). IR ν cm^{-1}: 3500, 1770, 1730. ^{1}H NMR (300 MHz, $CDCl_3$): δ1.18 (*s*, 3H, 4Me), 1.20–2.30 (*m*, 14H), 2.55 (*d*, *J* = 10.4 Hz, 1H, H-5), 2.62 (*br t*, 1H, H-13), 2.77 (*d*, *J* = 10.4 Hz, 1H, H-6), 3.70 (*m*, 1H, H-3), 3.72 (*s*, 3H, OMe), 4.84 (*br s*, 1H, H-17), 4.97 (*br s*, 1H, H′-17). ^{13}C NMR (75 MHz, $CDCl_3$): δ12.7 (C-18), 16.0 (C-11), 29.2 (C-1), 30.0 (C-2), 31.2 (C-12), 36.7 (C-14), 38.6 (C-13), 44.4 (C-15), 51.3 (C-9), 52.0 (OMe), 52.3 (C-8), 53.2 (C-6), 54.3 (C-4), 56.5 (C-5), 72.7 (C-3), 92.9 (C-10), 107.3 (C-17), 156.4 (C-16), 173.0 (C-7), 177.6 (C-19). EIMS *m/z* (rel. int.): 346 $[M]^+$ (1%), 286 (10), 183 (5), 169 (10), 155 (13), 143 (20), 129 (25), 115 (25), 105 (35), 91 (100). *3-Epi-GA_4 methyl ester 3-acetate (ent-3β-acetoxy-10β-hydroxy-20-norgibberell-16-ene-7,19-dioic acid 7-methyl ester 19,10-lactone).* To a sample of 3-epi-GA_4 Me ester (170 mg, 0.49 mmol) dissolved in dry CH_2Cl_2 (12 ml) stirring under a N_2 atmosphere was added Et_3N (1.2 ml) and DMAP (50 mg). The soln was cooled to 0°, Ac_2O (0.6 ml) added and the soln left to warm to room temp. After 12 hr, the solvent was removed *in vacuo*, the residue taken up in EtOAc (50 ml), the soln washed with 20% sodium dihydrogen phosphate (3 × 10 ml) and then with brine (1 × 10 ml). The aq. phase was extracted with EtOAc (2 × 10 ml), the combined organic layers dried (Na_2SO_4), filtered and the solvent removed *in vacuo*. Purification by silica gel CC (hexane–EtOAc, 3:1) afforded a crystalline solid (152 mg, 79%). R_f: 0.56 (EtOAc–hexane, 1:2). $[\alpha]_D$ −3.78°. IR: 1770, 1730 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ1.06 (*s*, 3H, 4Me), 1.20–2.30 (*m*, 14H), 2.09 (*s*, 3H, OAc), 2.63 (*t*, 1H, H-13), 2.65 (*d*, *J* = 10.4 Hz, 1H, H-5), 2.76 (*d*, *J* = 10.4 Hz, 1H, H-6), 3.71 (*s*, 3H, OMe), 4.85 (*br s*, 1H, H-17), 4.90 (*br s*, 1H, H-3), 4.97 (*br s*, 1H, H′-17). ^{13}C NMR (75 MHz, $CDCl_3$): δ12.6 (C-18), 16.0 (C-11), 20.8 (OAc), 25.7 (C-1), 29.6 (C-2), 31.2 (C-12), 36.7 (C-14), 38.6 (C-13), 44.4 (C-15), 51.3 (C-9), 52.0 (OMe), 52.2 (C-8), 52.2 (C-4), 53.2 (C-6), 56.5 (C-5), 73.0 (C-3), 92.3 (C-10), 107.5 (C-17), 156.3 (C-16), 170.3 (OAc), 172.6 (C-7), 176.3 (C-19). EIMS *m/z* (rel. int.): 388 $[M]^+$ (1%), 356 (20), 328 (20), 284 (18), 225 (20), 129 (20), 105 (30), 85 (45). HRMS (EI) *m/z*: calcd for $[M]^+$, $C_{22}H_{28}O_6$: 388.1886: found 388.1887. *Ent-3β-acetoxy-10β,15β-dihydroxy-20-norgibberell-16-ene-7,19-dioic acid 7-methyl ester 19,10-lactone.* To a soln of 3-epi-GA_4 Me ester 3-acetate (115 mg, 0.294 mmol) dissolved in dry CH_2Cl_2 (2.5 ml) was added, SeO_2 (100 mg, 0.889 mmol, 3eq.), followed by one drop of *t*-butylhydroperoxide soln. The mixt. was sonicated for 3 hr, then the soln diluted with EtOAc (30 ml) and washed with dilute. HCl (1 × 10 ml) and H_2O (1 × 10 ml). The combined aq. phases were extracted with EtOAc (2 × 10 ml). The combined organic phases were washed with $NaHCO_3$ soln (1 × 10 ml), dried (Na_2SO_4), filtered and the solvent removed *in vacuo*. The yellow residue was used immediately in the next step. R_f 0.23 (EtOAc–hexane, 1:2). *Ent-3β-acetoxy-10β-hydroxy-15-oxo-20-norgibberell-16-ene-7,19-dioic acid 7-methyl ester 19,10-lactone.* The 15α-hydroxy compound was dissolved in dry CH_2Cl_2 (5 ml) and Dess–Martin reagent [12] (100 mg, 0.588 mmol, 3 eq.) was added. After 10 min, the reaction appeared as a cloudy milky-white soln. The reaction was diluted with CH_2Cl_2 (20 ml) and satd. $NaHCO_3$ soln containing 7% $Na_2S_2O_4$ (20 ml) was added. The soln was left stirring for 20 min or until the cloudiness had dissipated. The layers were sepd and the organic phase was washed with satd $NaHCO_3$ soln (4 × 10 ml), then with brine (1 × 15 ml), dried (Na_2SO_4), filtered and the solvent removed *in vacuo*. Purification by silica gel CC (hexane–EtOAc, 2:1) yielded the desired enone (100 mg, 84%) as a white foam. R_f 0.32 (EtOAc–hexane, 1:2). IR: 1770, 1735 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$): δ1.12 (*s*, 3H, 4Me), 1.20–2.35 (*m*, 14H), 2.09 (*s*, 3H, OAc), 2.66 (*d*, *J* = 10.3 Hz, 1H, H-5), 2.79 (*d*, *J* = 10.3 Hz, 1H, H-6), 3.04 (*m*, 1H, H-13), 3.62 (*s*, 3H, OMe), 4.97 (*m*, 1H, H-3), 5.37 (*s*, 1H, H-17), 5.93 (*s*, 1H, H′-17). ^{13}C NMR (75 MHz, $CDCl_3$): δ12.9 (C-18), 16.5 (C-11), 20.8 (OAc), 25.5 (C-1), 28.8 (C-14), 29.1 (C-2), 30.9 (C-12), 35.5 (C-13), 48.9 (C-9), 50.0 (C-6), 51.9 (OMe), 52.9 (C-4), 55.5 (C-5), 60.02 (C-8), 72.9 (C-3), 92.2 (C-10), 117.2 (C-17), 151.0 (C-16), 170.2 (OAc), 171.4 (C-7), 176.2 (C-19), 205.0 (C-15). EIMS *m/z* (rel. int.): 402 $[M]^+$ (30%), 371 (15), 360 (18), 342 (20), 310 (25), 282 (25), 264 (23), 238 (82), 211 (22), 183 (40), 155 (30), 143 (30), 129 (40), 117 (30), 105 (50), 91 (95), 82 (90), 55 (100). HRMS (EI) *m/z*: calcd for $[M]^+$, $C_{22}H_{26}O_7$: 402.1679, found 402.1678.

Microanalysis: $C_{22}H_{26}O_7 \cdot H_2O$ required C 62.85, H 6.71; found C 62.50, H 6.74%. *3-Epi-GA_{63} methyl ester 3-acetate (ent-3β-acetoxy-10β,15α-dihydroxy-20-norgibberell-16-ene-7,19-dioic acid 7-methyl ester 19,10-lactone).* To the enone (70 mg, 0.174 mmol) dissolved in C_6H_6 (2 ml)

was added HOAc (1 ml) and freshly activated Zn (60 mg). The reaction was then sonicated for 1 hr. The reaction mixt. was worked-up by filtering through a small pad of Celite and washed through with Et_2O (30 ml). The organic phase was washed with H_2O (1 × 10 ml), satd $NaHCO_3$ soln (1 × 10 ml) and, finally, with brine (1 × 10 ml). The organic phase was dried (Na_2SO_4), filtered and the solvent removed *in vacuo*. Purification by silica gel CC (hexane–EtOAc, 2:1) yielded GA_{63} Me ester 3-acetate (58.4 mg, 83%). R_f 0.61 (EtOAc–hexane, 1:1). IR ν cm^{-1}: 3500, 1770, 1730. ^{1}H NMR (300 MHz, $CDCl_3$): δ0.85 (*m*, 1H, H-11), 1.06 (*s*, 3H, 4Me), 1.20–2.25 (*m*, 17H), 2.08 (*s*, 3H, OAc), 2.47 (*dd*, J_1 = 12.73 Hz, J_2 = 5.95 Hz, 1H, H-9), 2.58 (*d*, J = 10.8 Hz, 1H, H-5), 2.62 (*t*, J = 8 Hz, 1H, H-13), 2.79 (*m*, J = 10.8 Hz, 1H, H-6), 3.76 (*s*, 3H, OMe), 3.93 (*br s*, 3H, H-15), 4.91 (*dd*, J_1 = 10.6 Hz, J_2 = 5.8 Hz, 1H, H-3), 5.10 (*br s*, 1H, H-17), 5.11 (*br s*, 1H, H′-17). ^{13}C NMR (75 MHz, $CDCl_3$): δ12.5 (C-18), 15.6 (C-11), 20.9 (OAc), 25.7 (C-1), 29.1 (C-2), 31.2 (C-12), 31.9 (C-14), 36.6 (C-13), 43.0 (C-9), 51.1 (C-6), 52.4 (C-4), 52.6 (OMe), 55.6 (C-8), 58.1 (C-5), 72.9 (C-3), 77.7 (C-15), 92.9 (C-10), 109.2 (C-17), 157.0 (C-16), 170.4 (OAc), 174.6 (C-7), 176.2 (C-19). EIMS *m/z* (rel. int.): 404 [M]$^+$ (1%), 240 (10), 183 (10), 157 (15), 149 (10), 143 (15), 129 (20), 105 (25), 91 (50), 79 (30), 77 (30), 69 (30), 67 (30), 57 (55), 55 (100). HRMS (EI) *m/z*: calcd for [M − 32]$^+$, $C_{21}H_{24}O_6$: 372.1573; found 372.1575. *3-Epi-GA$_{63}$ methyl ester* (*ent-3β,10β,15α-trihydroxy-20-norgibberell-16-ene-7,19-dioic acid 7-methyl ester* 19,10-*lactone*). To a soln of 3-epi-GA_{63} Me ester 3-acetate (55 mg, 0.136 mmol) dissolved in MeOH (6 ml) was added freshly activated Zn (195 mg), followed by $ZnCl_2$ in MeOH (1 M, 1.5 ml). The soln was left to reflux for 4 hr, until TLC indicated that the reaction had finished. The reaction mixt. was diluted with H_2O (10 ml) and EtOAc (30 ml). H_3PO_4 (10%, 2 ml) was added to digest all of the Zn. The layers were sepd and the aq. phase extracted with EtOAc (4 × 10 ml). The combined organic phases were washed with brine (2 × 15 ml), dried (Na_2SO_4), filtered and the solvent removed *in vacuo*. Purification on silica gel (hexane–EtOAc, 3:1 → 1:1) yielded 3-epi-GA_{63} Me ester as crystals (31.5 mg, 64%), with a further (14 mg, 28%) of slightly impure material. R_f 0.20 (EtOAc–hexane, 1:1). $[\alpha]_D$ − 8.49°. IR ν cm^{-1}: 1765, 1720. ^{1}H NMR (300 MHz, $CDCl_3$): δ1.18 (*s*, 3H, 4-Me), 1.20–2.80 (*m*, 9H), 2.0–2.3 (*m*, 3H), 2.42 (*dd*, J_1 = 12.8 Hz, J_2 = 6.0 Hz, 1H, H-9), 2.46 (*d*, J = 10.8 Hz, 1H, H-5), 2.62 (*t*, J = 5.9 Hz, 1H, H-13), 2.81 (*d*, J = 10.8 Hz, 1H, H-6), 3.66 (*dd*, J_1 = 10.9 Hz, J_2 = 6.0 Hz, 1H, H-3), 3.77 (*s*, 3H, OMe), 3.93 (*t*, J = 2.5 Hz, 1H, H-15), 5.09 (*br s*, 1H, H-17), 5.11 (*br s*, 1H, H′-17). ^{13}C NMR (75 MHz, $CDCl_3$): δ12.7 (C-18), 15.6 (C-11), 29.5 (C-1), 29.6 (C-2), 31.3 (C-12), 32.0 (C-14), 36.7 (C-13), 43.0 (C-9), 51.3 (C-6), 52.6 (OMe), 54.5 (C-4), 55.8 (C-8), 58.2 (C-5), 72.7 (C-3), 77.8 (C-15), 93.4 (C-10), 109.2 (C-17), 157.1 (C-16), 175.0 (C-7), 177.5 (C-19). EIMS *m/z* (rel. int.): 362 [M]$^+$ (1%), 330 (20), 149 (50), 143 (50), 129 (18), 115 (17), 105 (20), 95 (20), 91 (50), 79 (35), 77 (35), 69 (40), 57 (58), 55 (100). CIMS *m/z*: 380 [M + 18]$^+$ (55%), 363 [M + 1]$^+$, 347 (50), 345 (100), 332 (50), 330 (85), 313 (45), 303 (35), 284 (25). HRMS (EI) *m/z*: calcd for [M]$^+$, $C_{20}H_{26}O_6$: 362.1729; found 362.1730.

Chemicals for bioassays. A soln of 3-epi-GA_{63} Me ester (10 mg) in MeOH (3 ml) was treated with solid NaOH (80 mg) and the soln stirred for 24 hr. The sample was then concd under red. pres., the pH adjusted to 4 by addition of NaH_2PO_4 and the product extracted into EtOAc–*n*-BuOH (4:1). The EtOAc soln was washed with brine (2 ×), dried (Na_2SO_4) and reduced to dryness. The residue was chromatographed on silica gel, eluting with hexane–EtOAc–CH_2Cl_2–MeOH–HOAc (15:30:10:2:2) to afford a mixt. of 3-epi-GA_{63} and GA_{63}, (total 6 mg). The mixt. was subjected to prep. silica gel TLC (EtOAc–hexane–HOAc, 10:1:1) to give 3-epi-GA_{63} (2.4 mg; R_f 0.35) and GA_{63} (1.7 mg; R_f 0.50). The synthesis of antheridic acid was reported previously [13].

Bioassays. Effects of test compounds on antheridial formation in protonemata and on dark spore germination were determined by methods described previously [4].

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