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3-EPI-GA₆₃, ANTHERIDIOGEN IN ANEMIA PHYLLITIDIS

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Abstract—3-Epi-Gibberellin A_{63} (3-epi-GA₆₃) 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-GA₉, in this species. 3-Epi-GA₆₃ 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-9,15cyclo-GA₉ (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-dayold 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-GA₉. The ratio of the GA-like compound/ 3α -hydroxy-9,15-cyclo-GA₉/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 GA₆₃ (3) [5], but their Kovats retention indices (KRIs) [6] were different from each other (Table 1). Because both the C-3 α - and C-3 β -epimers of 3hydroxy-GAs show very similar mass spectra, and antheridic acid possesses a 3a-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-GA₆₃ 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-GA₆₃. Thus, the GA-like compound was identified as 3-epi- GA_{63} . Since a 3 β -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-GA₆₃ might be



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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	2749	506 ([M] ⁺ ; 100), 491 (15), 446 (21), 416 (14),	3-epi-GA ₆₃	
the GA-like compound		369 (17), 287 (51), 156 (20), 129 (29)		

Table 2. Activity of antheridic acid, 3-epi-GA₆₃ and GA₆₃ in inducing antheridial formation in A. phyllitidis

Sample	Antheridial formation (% ± SE) Concentration (M)							
	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-GA₆₃ and GA₆₃ in inducing dark spore germination in A. phyllitidis

Sample	Dark spore germination (% ± SE) Concentration (M)							
	0	10 ⁻¹⁰	10 ⁻⁹	10 ⁻⁸	10 ⁻⁷	10 ⁻⁶		
Antheridic acid 3-epi-GA ₆₃ GA ₆₃	1.3 ± 0.2	$2.5 \pm 0.3 \\ 2.6 \pm 0.3 \\ 1.2 \pm 0.1$	$43.9 \pm 2.1 \\ 20.2 \pm 3.1 \\ 5.7 \pm 0.6$	$62.7 \pm 0.5 \\38.6 \pm 3.2 \\22.0 \pm 1.8$	93.3 ± 1.0 82.8 ± 2.0 44.5 ± 2.0	97.1 ± 0.4 96.0 ± 0.7 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 cooccurrence 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-GA₆₃ and GA₆₃ 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-GA₆₃ was slightly less active than antheridic acid, but slightly more active than GA₆₃ 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 3 α -hydroxy group. These results indicate that the introduction of a 3 α -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-GA₆₃, it was shown that 9,15-cyclo-GA₉, a precursor of 3α -hydroxy-9,15-cyclo-GA₉ and antheridic acid, was not metabolized into 3-epi-GA₆₃ in *A. phyllitidis* prothallia (data not shown). Investigations into the biosynthetic origin of 3epi-GA₆₃ 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 $25 \pm 1^{\circ}$. 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 \times 0.258 mm i.d., 0.25 mm thick stationary phase, J&W Scientific). Each sample was methylated with CH₂N₂-Et₂O 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-GA₆₃ methyl ester. The methodology is based on that reported in refs [5, 11]. $3-Epi-GA_4$ methyl ester (ent-3\beta,10\beta-dihydroxy-20-norgibberell-16ene-7,19-dioic acid 7-methyl ester 19,10-lactone). A solution of Li t-butoxide was formed by the addition of nbutyllithium (1.6 M, 2 ml) to dry t-butyl alcohol (4.5 ml) dissolved in dry THF (15 ml) with stirring at $\,-\,10^\circ$ under a N₂ atmosphere. To this soln was added GA₄ 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 NH₄Cl soln (20 ml). The aq. phase was back extracted with EtOAc $(2 \times 20 \text{ ml})$. 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 v cm⁻¹: 3500, 1770, 1730. ¹H NMR (300 MHz, CDCl₃): δ 1.18 (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, M)1H, H-3), 3.72 (s, 3H, OMe), 4.84 (br s, 1H, H-17), 4.97 (br s, 1H, H'-17). ¹³C NMR (75 MHz, CDCl₃): 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-GA₄ methyl ester 3-acetate (ent-3 β -acetoxy-10 β hydroxy-20-norgibberell-16-ene-7,19-dioic acid 7-methyl ester 19,10-lactone). To a sample of 3-epi-GA₄ Me ester (170 mg, 0.49 mmol) dissolved in dry CH₂Cl₂ (12 ml) stirring under a N2 atmosphere was added Et3N (1.2 ml) and DMAP (50 mg). The soln was cooled to 0° , Ac₂O (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 $(3 \times 10 \text{ ml})$ and then with brine $(1 \times 10 \text{ ml})$. The aq. phase was extracted with EtOAc $(2 \times 10 \text{ ml})$, the combined organic layers dried (Na₂SO₄), 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 - 3.78°. IR: 1770, (EtOAc-hexane, 1:2). $[\alpha]_{\mathbf{D}}$ 1730 cm⁻¹. ¹H NMR (300 MHz, CDCl₃): δ 1.06 (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). ¹³C NMR (75 MHz, CDCl₃): *δ*12.6 (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]⁺, C22H28O6: 388.1886: found 388.1887. Ent-3β-acetoxy-10B,15B-dihydroxy-20-norgibberell-16-ene-7,19-dioic acid 7-methyl ester 19,10-lactone. To a soln of 3-epi-GA₄ Me ester 3-acetate (115 mg, 0.294 mmol) dissolved in dry CH₂Cl₂ (2.5 ml) was added, SeO₂ (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 \times 10 ml) and H₂O (1 \times 10 ml). The combined aq. phases were extracted with EtOAc $(2 \times 10 \text{ ml})$. The combined organic phases were washed with NaHCO₃ soln (1 \times 10 ml), dried (Na₂SO₄), 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-3ß-acetoxy-10ß-hydroxy-15-oxo-20-norgibberell-16-ene-7,19-dioic acid 7-methyl ester 19,10-lactone. The 15ahydroxy compound was dissolved in dry CH₂Cl₂ (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 CH₂Cl₂ (20 ml) and satd. NaHCO₃ 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 NaHCO₃ soln (4×10 ml), then with brine (1×15 ml), dried (Na₂SO₄), 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. ¹H NMR (300 MHz, CDCl₃): δ 1.12 (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). ¹³C NMR (75 MHz, CDCl₃): 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]⁺, C₂₂H₂₆O₇: 402.1679, found

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 3acetate (ent-3 β -acetoxy-10 β ,15 α -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 C₆H₆ (2 ml)

402.1678.

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 Et₂O (30 ml). The organic phase was washed with H_2O (1 × 10 ml), satd NaHCO₃ soln $(1 \times 10 \text{ ml})$ and, finally, with brine (1 \times 10 ml). The organic phase was dried (Na₂SO₄), filtered and the solvent removed in vacuo. Purification by silica gel CC (hexane-EtOAc, 2:1) yielded GA₆₃ Me ester 3acetate (58.4 mg, 83%). Rf 0.61 (EtOAc-hexane, 1:1). IR v cm⁻¹: 3500, 1770, 1730. ¹H NMR (300 MHz, CDCl₃): 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). ¹³C NMR (75 MHz, CDCl₃): 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-GA₆₃ 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-GA₆₃ Me ester 3-acetate (55 mg, 0.136 mmol) dissolved in MeOH (6 ml) was added freshly activated Zn (195 mg), followed by ZnCl₂ 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 $H_2O(10 \text{ ml})$ and EtOAc (30 ml). $H_3PO_4(10\%, 2 \text{ ml})$ 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 $(2 \times 15 \text{ ml})$, dried (Na_2SO_4) , filtered and the solvent removed in vacuo. Purification on silica gel (hexane-EtOAc, $3:1 \rightarrow 1:1$) yielded 3-epi-GA₆₃ 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). $[\alpha]_D - 8.49^\circ$. IR ν cm⁻¹: 1765, 1720. ¹H NMR (300 MHz, CDCl₃): δ1.18 (s, 3H, 4-Me), 1.20-2.80 (m, 9H), 2.0-2.3 (m, 3H), 2.42 (dd, J₁ = 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). ¹³C NMR (75 MHz, CDCl₃): 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-GA₆₃ 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 NaH₂PO₄ and the product extracted into EtOAc-*n*-BuOH (4:1). The EtOAc soln was washed with brine (2 ×), dried (Na₂SO₄) and reduced to dryness. The residue was chromatographed on silica gel, eluting with hexane-EtOAc-CH₂Cl₂-MeOH-HOAc (15:30:10:2:2) to afford a mixt. of 3-epi-GA₆₃ and GA₆₃, (total 6 mg). The mixt. was subjected to prep. silica gel TLC (EtOAc-hexane-HOAc, 10:1:1) to give 3-epi-GA₆₃ (2.4 mg; R_f 0.35) and GA₆₃ (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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