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THE METHYL ESTER OF A NEW GIBBERELLIN, GA_{73}: THE PRINCIPAL ANTHERIDIOGEN IN LYGODIUM JAPONICUM

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Summary: The principal antheridiogen in the fern Lygodium japonicum was isolated and characterized as the methyl ester of a new gibberellin, GA_{73} (9,11-didehydro-GA_9). In L. japonicum, GA_{73} methyl ester exhibited high activity in inducing antheridial formation at 10^{-15} M and dark spore germination at 10^{-12} M and in inhibiting archegonial formation at 10^{-12} M.

It has been shown that fern prothallia produce substances which induce antheridial formation in fern gametophytes and which are called antheridiogens.[1,2,3] Antheridiogens in L. japonicum are unique because they not only induce antheridial formation but also inhibit archegonial formation in L. japonicum. Up to now, no information has been reported on active substances regulating formation of archegonia in ferns except in L. japonicum.[4]

In a previous paper,[5] we reported that gibberellin A_9 methyl ester (GA_9-Me: I in Fig. 1) was identified as an antheridiogen from the culture medium of L. japonicum prothallia. In L. japonicum, GA_9-Me was active in inducing antheridial formation at 10^{-10} M and inhibiting archegonial formation at 10^{-9} M. However, since the concentration of GA_9-Me in the culture medium was too low to explain the total activity from the culture medium, the possible presence of some other, more active substance (tentatively named Ly-I) was suggested. We report herein isolation and characterization of Ly-I.

Spores of L. japonicum (Thunb.) Sw. collected at The University of Tokyo, Botanic Gardens, Tokyo, were sowed and aseptically cultured for 3 weeks on 1/10 strength modified Murashige and Skoog's mineral salts solution solidified with 0.3% agar under continuous white light of 5 Wm^{-2} and then cultured on the same medium without agar for another 3 weeks under the same conditions. The prothallia were filtered off and the filtrate (12 liter) was fractionated with ethyl acetate to give a neutral ethyl acetate (NE) fraction. The entire above procedure was carried out as described previously.[5]

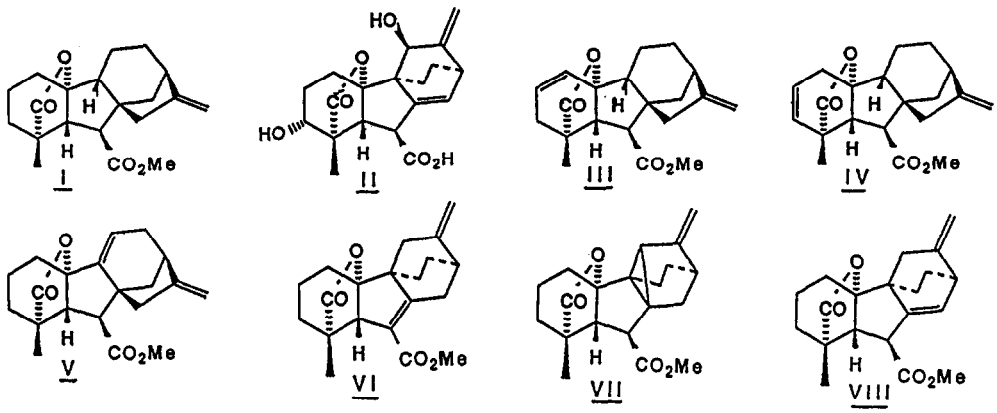

Fig. 1. Structures of antheridiogens and their relatated compounds.

The NE fraction was successively purified by Sep-Pak (ODS) treatment (eluted with methanol), silica gel short column (eluted with 10% ethyl acetate in n-hexane) and high performance liquid chromatography on Develosil-3 (eluted with 0.5% 2-propanol in n-hexane) and Nucleosil ODS-5 (eluted with 70% aqueous methanol) to give approximately 40 ng of Ly-I. Throughout all of the above purification procedure, Ly-I was monitored by the antheridium-inducing bioassay with dark-grown protonemata of L. japonicum.[5]

The results of capillary GC/MS of Ly-I indicated that Ly-I was a didehydro-GA_9-Me-like compound showing characteristic ions at m/z 328 (M^+), 297 (M^+-31), 284 (M^+-44), 269 (M^+-59) and 225 (M^+-103) in its mass spectrum. The prominent peak at m/z 225 was considered to be due to a hydrocarbon with the largest number of carbon atoms. The presence of the base peak at m/z 284 due to the loss of γ-lactone as CO_2 from the molecular ion suggested that Ly-I possesses a double bond (or a double bond equivalent functional group) in an α,β or β,γ relationship to the C-10 carbon.

Given the occurrence of antheridic acid II in the related species Anemia phyllitidis[6], and the co-occurrence of GA_9-Me in L. japonicum[5], the series of compounds III-VI were considered to be plausible candidates for the structure of Ly-I. The ent-9,15-cyclogibberellane structure VII was also considered to be a possibility in view of the recent discovery that the principal antheridiogen from A. mexicana is based on this skeleton.[7,8] The absence of a prominent M^+-28 peak in the mass spectrum of Ly-I allows structure VIII to be rejected, since it has been established that antheridane derivatives possessing a 8(14) olefinic bond undergo a facile retro Diels-Alder cleavage of the molecular ion.

With little prospect of obtaining sufficient material to obtain further structural information on Ly-I, the decision was taken to synthesize the series of candidate structures III-VII, details of which will be published elsewhere. Direct comparison of Ly-I with synthesized compounds III-VII by capillary GC/MS (Table 1) and bioassay[9] gave an unambiguous identification of Ly-I as V (9,11-didehydro-GA_9-Me). Since compound V was a new GA-Me, a new GA-number, GA_{73}, was allocated to the corresponding free acid. In L. japonicum, the synthesized GA_{73}-Me exhibited high activity in inducing antheridial formation in dark-grown protonemata at 10^{-15} M and dark spore germination at 10^{-12} M and in inhibiting archegonial

Table 1. Direct comparison of Ly-I with synthetic compounds III-VII by capillary GC/MS. The GC/MS was carried out with a JEOL DX 303 (ionization voltage, 70 eV). Samples were dissolved in methanol and injected onto a fused silica capillary column DB-1 (J & W Scientific Inc., CA; 0.258 mm diameter and 15 m length; 0.25 μm thick stationary phase) at 120°C in the splitless mode. After 2 min isothermal hold at 120°C, the column temperature was programmed at 16°C/min to 280°C with a 10 min isothermal hold at the end of the programm. The pressure of the helium carrier gas was 0.65 kg/cm^2. The column was directly led to the ion source.

Sample	Retention time	Principal ions and relative abundance (% base peak)
Ly-I	9'28"	328 (M^+, 6), 297 (8), 284 (100), 269 (13), 241 (17), 225 (79) and 183 (34)
Compound III	9'20"	328 (M^+, 27), 296 (62), 284 (100), 268 (42), 241 (18), 224 (86) and 181 (41)
Compound IV	9'20"	328 (M^+, trace), 297 (8), 284 (53), 269 (7), 252 (10), 224 (100) and 181 (30)
Compound V	9'28"	328 (M^+, 4), 297 (7), 284 (100), 269 (13), 241 (12), 225 (67) and 183 (22)
Compound VI	10'08"	328 (M^+, 16), 300 (22), 297 (14), 284 (100), 269 (18), 225 (43) and 183 (30)
Compound VII	9'05"	328 (M^+, 77), 297 (2), 296 (3), 284 (68), 269 (35), 225 (100) and 181 (27)

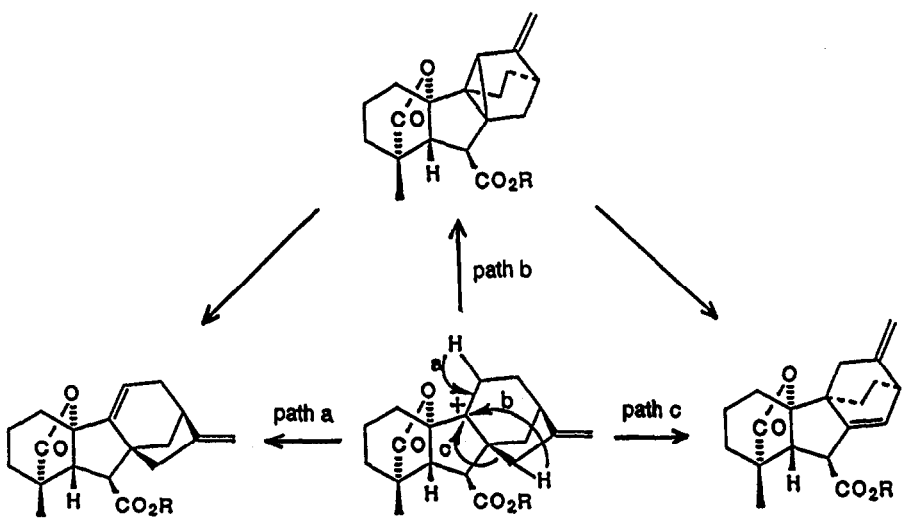

Fig. 2. Speculations on antheridiogen biosynthesis.

formation in light-grown prothallia at 10^{-12} M. The natural Ly-I exhibited similar activities to the synthesized GA_{73}-Me in the three bioassays. Thus, it is regarded as conclusive that GA_{73}-Me is the principal antheridiogen in L. japonicum.

The occurrence of three skeletal types, i.e. II, V and VII in the Schizaeaceae family of ferns suggests a close biosynthetic relationship. The ent-9,15-cyclogibberellane structure could be a precursor of either antheridane or ent-9,11-didehydrogibberellane derivatives, or possibly both. Alternatively, a C-9 cationic intermediate could be a common precursor to each of the three classes of compounds (Fig. 2). A study of the various biosynthetic relationships is now under way.

The physiological role of Ly-I as the principal antheridiogen in L. japonicum is considered to be as follows. As described previously,[10] in rapidly-grown L. japonicum prothallia archegonial formation is initiated, and Ly-I is secreted into the medium. Then by the effect of Ly-I, archegonial formation in juvenile prothallia is inhibited and antheridial formation is induced. Germination of dormant spores is also induced by Ly-I, resulting in increase of population of juvenile prothallia around the rapidly-grown prothallia. Thus the role of Ly-I in the formation of sexual organs in L. japonicum evidently is to minimize intragametophytic selfing, which would increase genetic risk, and to favor cross fertilization.

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