Pergamon

Tetrahedron Letters, Vol. 35, No. 11, pp. 1731-1734, 1994
Elsevier Science Ltd
Printed in Great Britain
0040-4039/94 $6.00+0.00

0040-4039(94)E0124-G

A Brassinolide-Inhibitor KM-01, Its Isolation and Structure Elucidation from a Fungus *Drechslera avenae*

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Abstract: A first brassinolide-inhibitor named KM-01 was isolated from a fungus, *Drechslera avenae*, and its structure with absolute stereochemistry determined as the fatty acid ester of bipolaroxin.

Since the discovery of brassinolide, the first steroidal plant growth regulator, isolated from rape pollen,[1] twenty-eight naturally occurring brassinosteroids have been isolated from various plant species.[2-7] Although brassinosteroids were not firmly recognized as a new type of plant hormone, because of an ambiguity of their specific biological activity discriminated from other known plant hormones, its extraordinary low concentrations to show activity at 0.0005 ppm in rice lamina inclination bioassay and to stimulate at 0.00001 ppm hypocotyl growth of bean seedlings suggest that they must be a potent new plant hormone, hence, whose characteristic activity should be identified in the nearest future.

In the course of our screening program to find brassinolsteroid-like substances from fungi, we found that several fungal strains, including *D. avenae* caused severe inhibition against brassinolide-induced lamina inclination of rice seedling explants. We were interested in this unexpected inhibitory activity, because there is no brassinolide-inhibitor hitherto known, hence an active substance, once isolated, may be helpful to evaluate characteristic activity of brassinosteroids. We report here the isolation and structure elucidation of a brassinolide-inhibitor named KM-01 from *D. avenae* and its structure with absolute stereochemistry determined as shown in Fig.1. KM-01 is the first naturally occurring brassinosteroid-inhibitor as evaluated by our several bioassayed results.

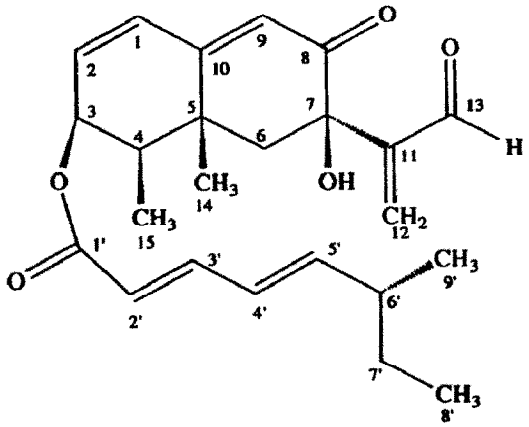


Fig. 1. The structure of KM-01

D. avenae NOY-0098[8] was cultured on a potato-lactose agar medium containing 2% orange powder under blue light irradiation at 25℃ for 10 days. The agar-cultured material was macerated with 70% aq. acetone and the extracted acetone solution was condensed below 40℃ to an aq. residue, which was extracted with diethyl

ether at pH 3. The active ether soluble extract was subjected to silica gel column chromatography with a stepwise elution of ethyl acetate in *n*-hexane. The active fraction eluted with the 30% solvent mixture was purified by reverse phase HPLC with Develosil ODS-5 column eluted with 60% aq. acetonitrile to give an active compound as a single peak. The yield was 0.8 mg / liter of the cultured agar medium.

KM-01 has the molecular formula of $C_{24}H_{30}O_5$ by HRMS (obsd. m/z 398.2098, calcd. 398.2093); UV (MeOH) λ_{max} 277 nm (ε 55,000); [α]$_D^{20}$ +472.8° (c 0.027, $CHCl_3$). The IR spectrum ($CHCl_3$) exhibited absorptions at 3,553, 1,720, 1,694, 1,662 and 1,636 cm^{-1}. The ^1H (270 MHz, $CDCl_3$; values in parenthesises) and ^{13}C (67.5 MHz, $CDCl_3$) NMR spectra and their extensive analysis using ^1H-^1H and ^{13}C-^1H COSY deduced nine partial structures of [A] to [I], as shown in Fig. 2.

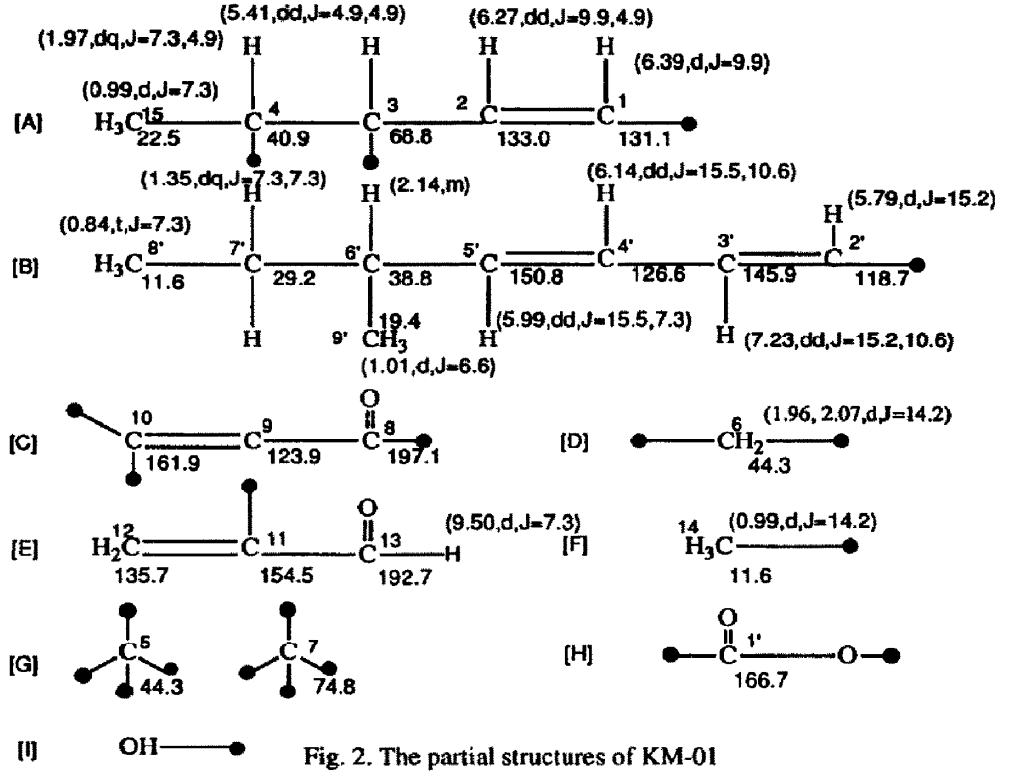


Fig. 2. The partial structures of KM-01

The nine partial structures were combined together by HMBC experiments, as conducted in Fig.3. Namely, the couplings were observed in the signals between a C-1' carbonyl (166.7) and H-2', H-3', H-3; between a C-5 quaternary carbon (36.2) and H-1, H-2, H-3, H-4, H_3-15, H_2-6, H-9, H_3-14; between a C-7 quaternary carbon (74.8) and H_2-6, H-9, H_2-12, H-13; between a C-8 carbonyl (197.1) and H_2-6, H-9; and between a C-10 carbon (161.9) and H-1, H-2, H_2-6, H-9, H_3-14, respectively. Thus, the plane structure of KM-01 was constructed reasonably.

The relative stereochemistry of KM-01 was determined based on ^1H-^1H coupling constants and NOESY experiments. The 2', 4'-*trans, trans*-conjugated dienoate system of the side chain was deduced from the large coupling constants of $J_{2',3'}$=15.2 Hz and $J_{4',5'}$=15.5 Hz. In the NOESY, the presence of a cross peak between H_3-14 and H_3-15 suggests that the two methyl groups are situated in a *cis*-relation. In addition, H-3 shows strong NOE with H-4, H_{eq}-6 with H_3-15, and also H_{ax}-6 (δ H 2.07) with H-4 and H_2-12, respectively, concluding that

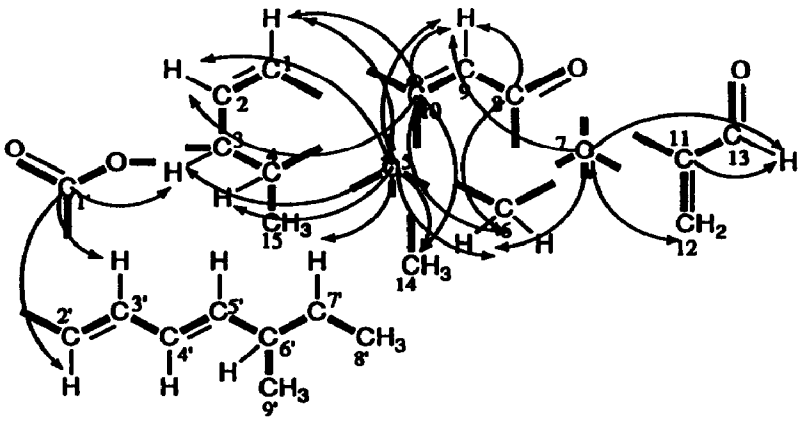


Fig. 3. HMBC analysis

all of these H-3, H-4, Hax-6 and H2-12 should be oriented on the same side of the ring moiety.

The proposed structure of the bicyclic ring system is an eremophilane type of sesquiterpene, which was found to accord with that of bipolaroxin that had been isolated from *bipolaris cynodontis* by Sugawara F. *et al.*[9] The relative stereochemistry of bipolaroxin was already determined by X-ray analysis, but its absolute stereochemistry remained unclear.

The absolute stereochemistry of KM-01 was determined by the CD spectrum (Fig. 4). The large splitting type of Cotton effect at 280 nm ($\Delta\ \varepsilon$,+53.07) and 255 nm ($\Delta\ \varepsilon$,-33.77) in MeOH was clearly ascribed to a chromophore interaction between the dienone ring system and the diene ester side chain. The positive sign of the first Cotton effect indicates that the two chromophors should be twisted in clockwise sense. Fig.5 showed the most stable conformation of KM-01 drawn by calculating of the energy using the QUANTA. The absolute stereochemistry of the ring system was thus elucidated as shown in Fig.1. Chiral centers were therefore C-3 (*S*), C-4 (*R*), C-5 (*R*) and C-7 (*R*).

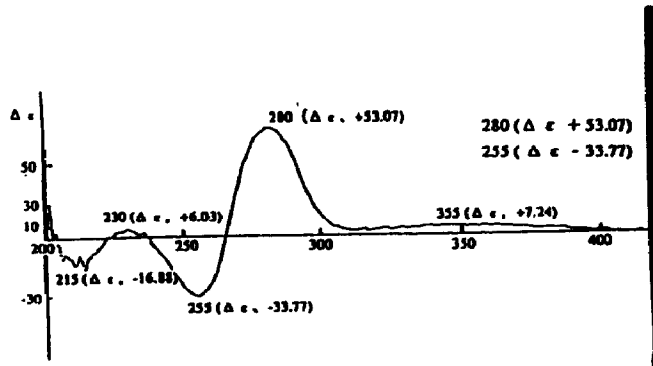
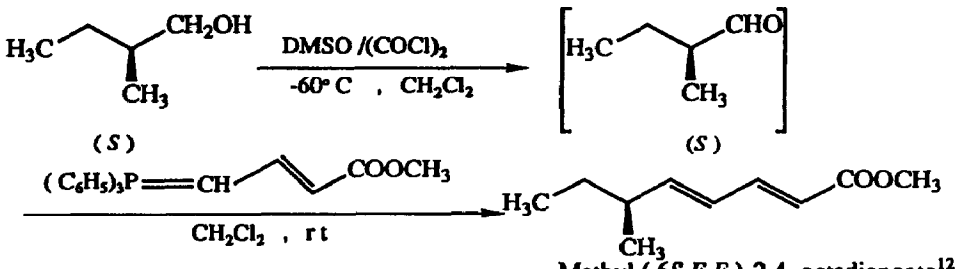


Fig. 4. Splitting CD spectrum of KM-01

Fig. 5. The most stable conformation of KM-01

The remaining problem was a C-6' stereochemistry of the side chain, being clarified by hydrolysis of KM-01 to isolate the acid moiety that was then synthesized. First, KM-01 was hydrolyzed in 2N HCl aq. MeOH for 1hr, the free acid liberated being methylated (diazomethane), and the methyl ester purified by HPLC. Next, methyl (6*S*, *E*, *E*)-2, 4, octadienoate was synthesized by that (*S*)-(-)-2-methylbutanol was subjected to Swern

oxidation[10] to give (*S*)-(+)-2- methylbutanal, which was reacted, without isolation, with methyl 4-triphenylphosphoranylidene-2*E*-butenoate[11] to the desired (6*S*)-dienoate. The major reaction product was the *trans,trans*-dienoate, whose optical rotation was $[\alpha]_{365}^{20}$ + 204.3°



Methyl (6*S*,*E*,*E*)-2,4, octadienoate[12]

Comparative identification of the natural ester with the synthetic product was conducted with HPLC equipped with polarimeter. The retention time and peak height relative to the positive optical value of the natural compound was well coincident with those of the synthetic one,[13] concluding that the C-6' has the *S* stereochemistry. The absolute stereochemistry of KM-01 was thus established as shown in Fig.1.

KM-01 inhibited brassinolide activity in raphanus test[14] and rice lamina inclination test.[15-16] Namely, KM-01 at a 1 μ g/ml concentration showed 90% inhibition in hypocotyl elongation of radish seedlings induced by addition of 0.01 ppm of brassinolide, and the bending angle (148°) between sheath and leaf of rice lamina explants induced by 0.01 ppm of brassinolide was completely cancelled by 0.1 μ g/ml of KM-01.

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12. $[\alpha]_D^{20}$ + 52.8° (c 0.301, CHCl3), $[\alpha]_{365}^{20}$ +204.3° ; ^{1}H NMR (270MHz, CDCl3) δ =0.86 (3H, t, J=7.4), 1.02 (3H, d, J=6.7), 1.37 (2H, dq, J=7.1,7.1), 2.16 (1H, m), 3.73 (3H, s), 5.80 (1H, d, J=15.2), 6.01 (1H, dd, J=15.2, 7.4), 6.14 (1H, dd, J=15.2,10.6), 7.27 (1H, dd, J=15.2,10.6).

13. Column: CHIRALCEL OD (Daicel) (4.6 x 250 mm) ; Eluent: *n*-hexane/ 2-propanol (90:10) ; Flow rate: 0.5 ml/min ; UV-detector: 254 nm ; Polarimeter detector: 365 nm ; retention time: 12.5 min.

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(*Received in Japan* 15 *November* 1993)