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KM-01, A Brassinolide Inhibitor, Its Production, Isolation and Structure from Two Fungi *Drechslera avenae* and *Pycnoporus coccineus*

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Abstract—A new brassinolide inhibitor, named KM-01, was isolated from the culture filtrates of two fungal species, *Drechslera avenae* and *Pycnoporus coccineus*, and the structure with absolute stereochemistry was elucidated as the fatty acid ester of an eremophilane sesquiterpene, bipolaroxin, based on spectroscopic analysis, chemical degradation, and synthesis of the fatty acid.

Introduction

Brassinolide, the first steroidal plant growth regulator, has been isolated from rape pollen with the bioassay measuring elongation activity of the second internode of bean seedlings.[1] After the structure of brassinolide was announced by the USDA group,[2] we have shown that the rice lamina inclination bioassay, which was originally devised as an auxin bioassay, was an extremely sensitive method for bioassay of brassinolide and its 24-homo analogue.[3] By using this bioassay, the second natural brassinosteroid, castasterone, was then isolated from chestnut insect gall.[4] Since then, an extensive survey of naturally occurring brassinolide and its related brassinosteroids using this rice lamina inclination bioassay has been carried out by many researchers. Up to now, reports verifying the presence of brassinosteroid activity in various plant sources have been published. Among them, structures of thirty kinds of brassinosteroids have been chemically characterized[5] as well as their biological activities using various bioassay systems.[6–11] In parallel with these investigations from plant resources, attempts to find brassinosteroid-like substances from fungal metabolite were also conducted by several groups. We also commenced similar investigation among fungal metabolites, since we have succeeded previously in the isolation of natural abscisic acid, a plant hormone, from a fungus, *Botrytis cinerea*.[12]

In the course of our screening program to find new brassinosteroid-like substances from the agar cultured materials of fungi, we found that several fungal strains, including *Drechslera avenae* and *Pycnoporus coccineus*, caused severe inhibition against brassinolide-induced lamina inclination of rice seedling explants. We were interested in this unexpected inhibitory activity, because no brassinolide-inhibitor has hitherto been 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 two fungal species, *Drechslera avenae* and *Pycnoporus coccineus*, and its structure with absolute stereochemistry was determined as shown in Figure 3.

Results and Discussion

Production of KM-01

We screened fungal products cultured under blue light, because abscisic acid production by *B. cinerea* was extremely stimulated by blue-light irradiation during culture.[12] The crude extracts of *D. avenae* and *P. coccineus* showed

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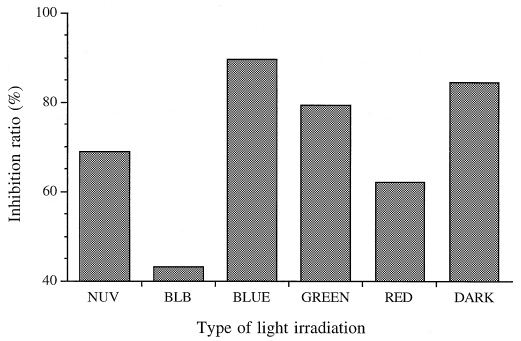


Figure 1. Production of KM-01 under various light irradiation.

brassinolide-inhibitory activities. Then, we examined this activity of *D. avenae* cultured under various wave length light. As shown in Figure 1, blue light was most effective for the production of the inhibitor. Unexpectedly, dark condition was similarly effective as blue light; however, the amount of inhibitor severely decreased by BLB irradiation. These light effects for production of the inhibitor were much different from those observed on the production of abscisic acid by *B. cinerea*.[13] The precise light effects on the inhibitor named KM-01 production and their mechanism are now under investigation.

Isolation of KM-01

In the previous procedure,[14] the yield of KM-01 was only 0.8 mg/L. Since we noticed that KM-01 was very unstable under acidic conditions, we changed the partition conditions from the acidic to basic. The acetone extract of mycelium on agar was partitioned between water (pH 12) and ether. The ether layer was concentrated in vacuo and then purified by silica gel column chromatography and HPLC monitored with bioassays as previously described.[14,15] The yield of KM-01 was 20.4 mg/L with this purification procedure.

Structure analysis

KM-01 was obtained as a pale yellow oil, $[\alpha]_D^{20}$ +473 (*c* 0.027, $CHCl_3$). The UV spectrum (MeOH) showed a maximum absorption at 277 nm (ε 55000), and IR spectrum at 3553, 1694, 1662 and 1636 cm^{-1}. EI mass spectrum gave a molecular ion at *m/z* 398. The molecular formula $C_{24}H_{30}O_5$ was assigned by HREIMS; M^+ ion observed at 398.2098 (calcd. 398.2093). The molecular formula indicates the 10 double bond equivalents in the molecule. The ^1H and ^{13}C NMR spectra showed all of the protons and carbons in the molecule, and from these spectral data combined with DEPT analysis the presence of four methyls, three methylenes, three methins, two quaternaries, ten olefins, one each of ketone, aldehyde, ester and hydroxyl groups could be deduced. The definitive assignments of all of ^1H and ^{13}C NMR signals are summarized in Table 1.

The nine partial structures, (1)–(9) (shown in Figure 2) were deduced as follows. The partial structures (1) and (2), both of which had connective vicinal protons, were deduced from ^1H–^1H COSY and coupling constants. The methyl (3) and methylene (4) should be connected quaternary carbons. In (5) a conjugated ketonic carbon at 197.1 ppm was shown to be long range-coupled with C-9 proton of the trisubstituted double bond as ascertained by HMBC. The conjugated aldehyde carbon (192.7 ppm) in (6) was long-range coupled with C-12 terminal methylene protons. The ester in (7) should be a conjugated ester as shown by δ_c 166.7 ppm and ν 1662 cm^{-1}. The remaining two carbons were quaternary shown by DEPT experiments, and one free OH which disappeared with D_2O addition exists in the molecule. In order to combine the above nine partial structures together, we employed the HMBC technique, data from which are summarized in Table 1. Thus, the plane structure was constructed reasonably as shown in Figure 3.

The bicyclic ring moiety of the postulated structure for KM-01 was found to be the same as that of bipolaroxin which had been reported by Sugawara et al.[16] as a fungal metabolite from *Bipolaris cynodontis*. We tried to obtain bipolaroxin as a degradation product of KM-01 under various conditions; however, so far we have not succeeded.

Relative stereochemistry

Relative stereochemistry of KM-01 was clarified on the basis of NOESY and coupling constants data.

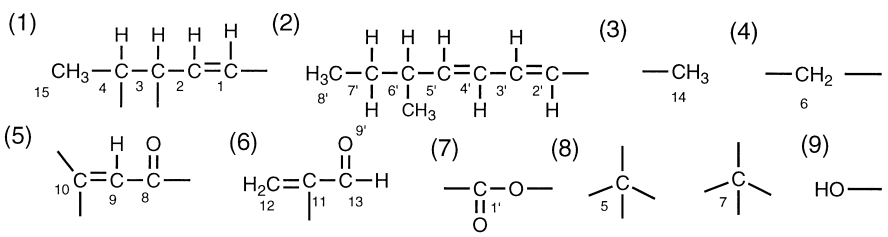


Figure 2. The partial structures of KM-01 (— implies quatanary carbons).

The C-2′–C-3′ and C-4′–C-5′ double bonds of the fatty acid moiety should be all *trans* from the values of $J_{2'3'} = 15.2$ and $J_{4'5'} = 15.5$ Hz. NOESY showed a cross peak between H_3-14 and H_3-15, indicating the two methyls to be situated in *cis*-relation. In addition, a strong NOE was observed on respective protons between H-3 and H-4, between H-4 and H_{ax}-6, and between H_{ax}-6 (δ_H 2.07) and H_2-12. These results indicated that all of H-3, H-4, H_{ax}-6, and H_2-12 should be oriented on the same side of the bicyclic ring moiety. The relative stereochemistry of the ring moiety deduced was the same as that of bipolaroxin and other eremophilane sesquiterpenoids.[17] The relative stereochemistry of bipolaroxin was determined by X-ray analysis, but its absolute stereochemistry remained unclear.

Absolute stereochemistry

The absolute stereochemistry of KM-01 was determined by the CD method. In the CD spectrum (Figure 4), the large splitting type of Cotton effect at 280 and 255 nm in MeOH was clearly ascribed to the excited interaction of two chromophores between the dienone ring moiety and the diene ester side chain. The chiral exciton

Table 1. NMR assignments for KM-01 in $CDCl_3$

C no.		δ ^{1}H (multiplicy, *J* Hz)	δ ^{13}C	HMBC
1	CH=	6.39 (d, 9.9)	131.1	H-3, H-5, H-9
2	CH=	6.27 (dd, 9.9, 4.9)	133.0	H-3
3	CH(−O)	5.41 (dd, 4.9,4.9)	68.8	H-15, H-14, H-6, H-3, H-2, H-4, H-5
4	CH	1.97 (dq, 7.3,4.9)	40.9	H- 15, H-14, H-3, H-6, H-2
5	C		36.2	H-14, H-15, H-4, H-6, H-3, H-9, H-1
6	CH_2	ax 2.07 (d, 14.2)	44.3	H-14
		ex 1.96 (d, 14.2)		
7	C		74.8	H-6, H-9, H-12, H-13
8	C=O		197.1	H-6, H-9
9	CH=	5.98 (s)	123.9	H-1
10	C		161.9	H-14, H-6, H-2, H-1
11	C		154.5	H-6, H-12
12	CH_2=	6.82 (s) 6.29 (s)	135.7	H-12
13	CH=O	9.50 (S)	192.7	
14	CH_3	1.48 (s)	22.5	H-4
15	CH_3	1.02 (d, 7.3)	10.2	H-4, H-6
1′	C=O		166.7	H-3, H-2′, H-3′
2′	CH=	5.79 (d, 15.2)	118.7	H-4′
3′	CH=	7.23 (dd, 15.2, 10.6)	145.9	H-4′, H-5′
4′	CH=	6.14 (dd, 15.5, 10.6)	126.6	H-6′, H-2′, H-5′
5′	CH=	5.99 (dd, 15.5, 7.3)	150.8	H-9′, H-7′, H-6′, H4′, H-3′
6′	CH=	2.14 (m)	38.8	H-8′, H-9′, H-7′, H-6′, H-4′, H-3′
7′	CH_2	1.35 (dq, 7.3, 7.3)	29.2	H-8′, H-9′, H-6′, H-5′
8′	CH_3	0.84 (t, 7.3)	11.6	H-6′, H-7′
9′	CH_3	1.01 (d, 6.6)	19.4	H-7′, H-6′, H-5′
	OH	2.46 (s)		

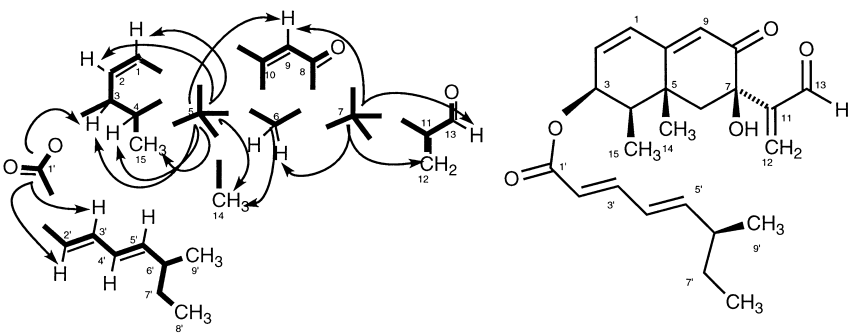


Figure 3. Carbon-proton connectivity by HMBC analysis (left) and the structures of KM-01 (right).

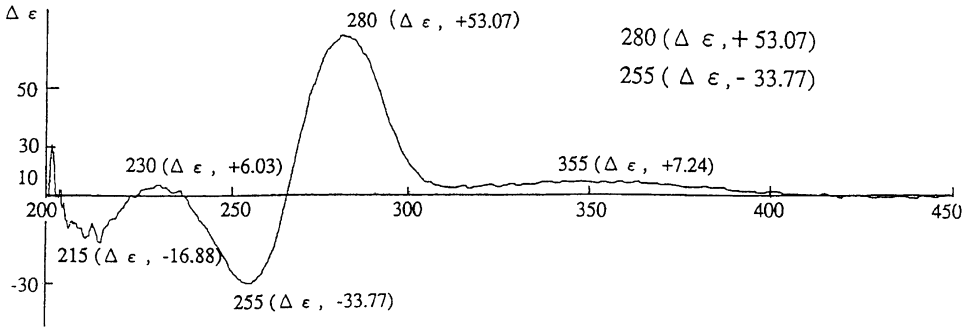


Figure 4. Splitting CD spectrum of KM-01.

coupling theory predicts that the sign of the first CD band originates from the spatial absolute relationship of two chromophores.[18] The positive sign of the first Cotton effect indicates that two chromophores should be twisted in clockwise sense.[19] The conformational energy of KM-01 was calculated by use of the QUANTA/CHARMm molecular modeling program,[14] and the most stable conformation obtained showed the diene ester side chain to be oriented perpendicularly upward from the ring moiety. The most stable conformation was also calculated by the AMBER* force field (in water as a solvent) in the molecular modeling package MacroModel 6.0[20] (Figure 5) and found to be similar to that obtained by the QUANTA/CHARMm. The *S* configuration at C-3 of KM-01 was confirmed by applying the modified Mosher method.[21] On basic methanolysis KM-01 gave unstable enol **1** which, without purification, was converted to stable quinoxaline **2** by treatment with *o*-phenylenediamine (Scheme 1). Basic methanolysis of quinoxaline provided secondary alcohol **3**, which was esterified to give (*R*)- and (*S*)-MTPA esters (**4a,b**). ^{1}H NMR signals of the two MTPA esters were assigned and $\Delta\delta$ values were calculated. Satisfactory results were obtained as shown in Scheme 1 except for the abnormal $\Delta\delta$ value at H_3-14. However, this negative value could be due to the axial orientation of the H_3-14. For example, the most stable conformation of the (*S*)-MTPA ester, which was calculated by the AMBER* force field (in $CHCl_3$ as a solvent), showed that the H_3-14 was located in the upper side of the MTPA plane as shown in Figure 6.

The absolute stereochemistry of the ring system was thus elucidated as C-3 (*S*), C-4 (*R*), C-5 (*R*) and C-7 (*R*).

Determination of C-6′ stereochemistry

C-6′ stereochemistry of the side chain was determined first by obtaining the fatty acid moiety as its methyl ester by alkaline hydrolysis followed by methylation with diazomethane. Next, a possible enantiomeric ester of the natural acid, methyl (2*E*,4*E*,6*S*)-6-methyl-2,4-octadienoate, was synthesized (Figure 7). (*S*)-(−)-2-Methylbutanol was subjected to Swern oxidation to give (*S*)-(+)-2-methylbutanal. The aldehyde was then reacted, without isolation, with methyl (*E*)-4-triphenylphoranylidene-2-butenoate which was prepared from methyl 4-bromo-2-butenoate via (3-carbomethoxy-2-propen-1-yl)triphenylphosphonium bromide, affording the desired (6*S*)-dienoate.

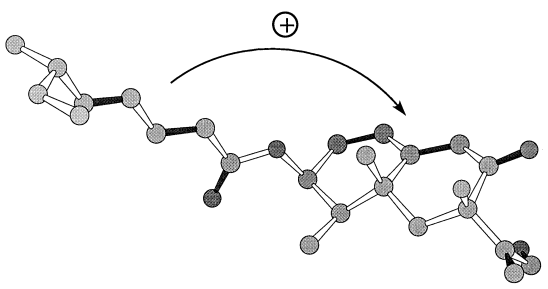


Figure 5. The most stable conformer of KM-01 calculated by the AMBER* force field.

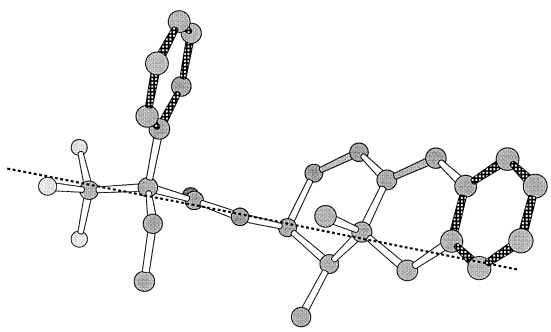
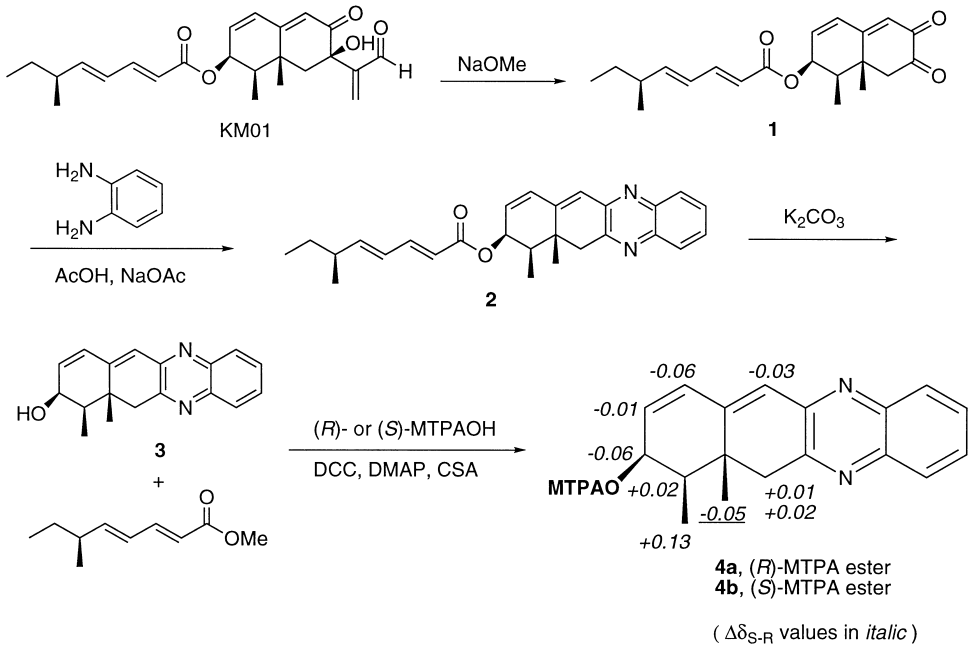


Figure 6. The lowest energy conformer of an analogue of (*S*)-MTPA ester **4b**. The dotted line indicates the MTPA plane. The quinoxaline moity of the (*S*)-MTPA-ester **4b** is replaced by benzene in the analogue.



Scheme 1.

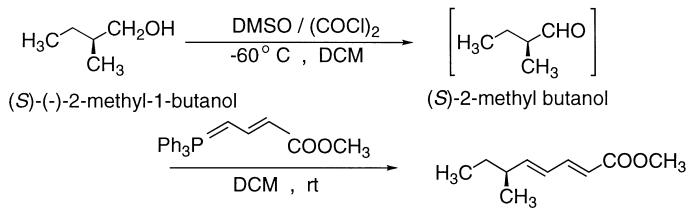


Figure 7. Synthesis of methyl (2*E*,4*E*,6*S*)-6-methyl-2,4-octadienoate.

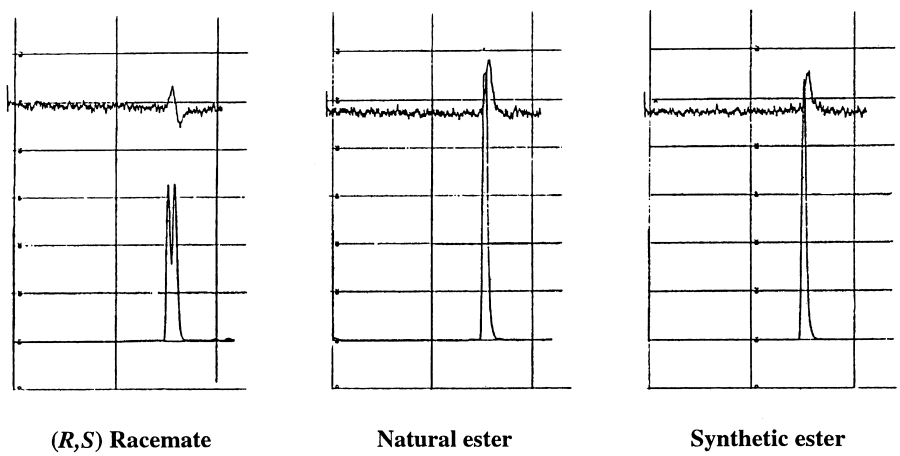


Figure 8. HPLC Chromatogram and polarimetric diagram for the resolution of racemate, natural and synthetic ester on a CHIRALCEL OD column.

Comparative identification of the natural ester with the synthetic product was conducted with HPLC equipped with a polarimeter. When racemates were subjected to HPLC on a column of CHIRALCEL OD by using *n*-hexane and 2-propanol (90:20) as an eluant, the resolution was attained as shown in Figure 8. The retention time of the natural compound, monitored with λ_{max} 254 nm, was well coincident with that of the synthetic compound, and the former's upward signal, monitored polarimetrically at 365 nm, was the same as that of the synthetic compound. Thus it is concluded that the C-6′ of the fatty acid side chain has the *S* stereochemistry and the absolute stereochemistry of KM-01 was established as shown in Figure 3.

Experimental

General methods

^{1}H (270 MHz) and ^{13}C (67.5 MHz) NMR spectra were recorded on a JEOL JNM GSX-270 (270 MHz), a Bruker ARX400 (400 MHz) or a Bruker AMX600 (600 MHz) NMR spectrometer. Electron impact mass spectra (EIMS) were obtained on a JEOL JMS-DX 705L mass spectrometer. High-resolution EIMS was performed on a JEOL Mstation JMS-700. UV absorption spectra were measured with Hitachi 330 spectrophotometer. Infrared absorption spectra were obtained with a JASCO FT-IR 7000S infrared spectrometer. Optical rotations were determined with a JASCO DIP-371 digital polarimeter. The CD spectra were obtained on a JASCO DP-500N spectropolarimeter. The Macromodel V6.0 calculations were performed on a Silicon Graphics O2 R5000SG computer.

Fungal culture

Two fungal species, *Drechslera avenae* and *Pycnoporus coccineus*, were kindly gifted by the late Dr. N. Nishihara, National Grassland Research Institute, and their morphological identification was conducted by Dr. M. Ito, Shin Nippon Chem Ind., Anjo City, Japan.

D. avenae and *P. coccineus* were cultured with a potato-lactose agar medium that was prepared by dissolving lactose (20 g) and agar (20 g) in 1 L of a potato extract (200 g) solution. The medium (30 mL) in a Petri dish (9 cm in diameter) was inoculated at four points with the fungus, and incubated at 26 °C for 10 days under blue light using Toshiba FL20S B19 lamp combined with an acrylite No. 302 filter (λ_{max} 457 ± 20 nm, 1.5 W/m^2). For the production of the inhibitor under various light irradiation, the following lights were used with the same energy (1.5 W/m^2): near UV, Toshiba FL20S E lamp (320 ± 20 nm); black light blue (BLB), with Toshiba FL20S BLB lamp (352 ± 20 nm); green, Toshiba FL20S G with Fuji Film BPB 55 filter (547 ± 10 nm); red, FL20S Re66 with Acrylite No. 102 filter (663 ± 20 nm). Each cultured material under the various light conditions was extracted with acetone, concentrated and partitioned with ether at pH 2. The ether extracts were bioassayed using modified *Raphanus* test with brassinolide as described in the previous paper.[14]

Enol 1. To a stirred solution of KM-01 (2.1 mg) in MeOH (0.9 mL) at 0 °C was added a 1 M solution of NaOMe in MeOH (0.1 mL). The mixture was stirred at ambient temperature for 30 min. The mixture was diluted with ether (20 mL) and water (20 mL). The organic layer was separated and the aqueous layer extracted with ether (2×20 mL). The combined organic extracts were washed with saturated NaCl, dried on Na_2SO_4, and concentrated to give **1** (2.2 mg) as a crude oil: ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.87 (t, *J* = 7.4 Hz, 3 H), 1.04 (d, *J* = 6.8 Hz, 3 H), 1.18 (d, *J* = 6.8 Hz, 3 H), 1.26 (s, 3 H), 1.30 (dq, *J* = 6.8 and 6.8 Hz, 2 H), 2.08 (m, 1 H), 2.18 (m, 1 H), 5.55 (dd, *J* = 5.4 and 4.4 Hz, 1 H), 5.82 (d, 15.3 Hz, 1 H), 6.05 (dd, *J* = 15.2 and 7.7 Hz, 1 H), 6.17 (dd, *J* = 15.2 and 10.7 Hz, 1 H), 6.19 (dd, *J* = 9.6 and 5.4 Hz, 1 H), 6.20 (s, 1 H), 6.29 (s, 1 H), 6.33 (s, 1 H), 6.45 (d, *J* = 9.6 Hz, 1 H), and 7.24 (dd, *J* = 15.3 and 10.7 Hz, 1 H); EIMS *m*/*z* 342 (M^+), 188, 160, 137 (base), and 109.

Quinoxaline 2. *o*-Phenylenediamine (54 mg) was dissolved in 2 M AcOH (0.25 mL) by gentle heating, and 4 M NaOAc (0.125 mL) was added. To the mixture at 60 °C was added a solution of the above crude enol **1** (2.2 mg) in MeOH (0.75 mL), and the mixture was stirred at 60 °C for 30 min. The mixture was diluted with water (2 mL) and extracted with hexane:EtOAc (4:1) (3×3 mL). The combined organic extracts were washed with 2 M AcOH (1 mL) and concentrated. The residual oil was chromatographed on silica gel (hexane:EtOAc (5:1)) to give **2** (1.7 mg, 77% yield from KM-01) as a yellow oil: $[\alpha]_D^{23}$ + 770 (*c* 0.14, $CHCl_3$); UV (MeOH) λ_{max} 265 (ε 34100), 278 (30000, sh), 348 (18200, sh), 361 (22900), and 377 (19400) nm. IR ($CHCl_3$) 1701, 1640, 1617, 1261, 1141, 1003, and 911 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.85 (t, *J* = 7.4 Hz, 3 H), 1.01 (d, *J* = 6.8 Hz, 3 H), 1.18 (d, *J* = 6.8 Hz, 3 H), 1.18 (s, 3 H), 1.36 (dq, *J* = 6.8 and 6.8 Hz, 2 H), 2.16 (m, 1 H), 2.22 (dq, *J* = 4.4 and 6.8 Hz, 1 H), 3.00 (d, *J* = 15.8 Hz, 1 H), 3.34 (d, *J* = 15.8 Hz, 1 H), 5.54 (dd, *J* = 5.4 and 4.4 Hz, 1 H), 5.81 (d, 15.3 Hz, 1 H), 6.00 (dd, *J* = 15.2 and 7.7 Hz, 1 H), 6.14 (dd, *J* = 15.2 and 10.7 Hz, 1 H), 6.21 (dd, *J* = 9.6 and 5.4 Hz, 1 H), 6.53 (d, *J* = 9.6 Hz, 1 H), 6.67 (s, 1 H), 7.24 (dd, *J* = 15.3 and 10.7 Hz, 1 H), 7.67 (m, 2 H), and 7.98 (m, 2 H); EIMS *m*/*z* 414 (M^+, base), 278, 262, 246, 231, 219, 137, and 109; HREIMS calcd. for $C_{27}H_{30}N_2O_2$ 414.2307 (M), found 414.2321.

Alcohol 3. To a solution of **2** (1.7 mg) in MeOH (1 mL) was added K_2CO_3 (13.8 mg), and the mixture was stirred at room temperature for 3.5 h and then at 50 °C for 3 h. The reaction mixture was diluted with water (4 mL) and extracted with dichloromethane (3×4 mL). The combined organic layers were washed with water (1 mL) and concentrated. The residual oil was chromatographed on silica gel (hexane:EtOAc (4:1, 3:2, and then 1:1)) to give alcohol (1.0 mg, 88%) as yellow crystals and methyl (2*E*, 4*E*, 6*S*)-6-methyl-2,4-octadienoate (0.5 mg, 73%) as an oil.

3: $[\alpha]_D^{22}$ +370 (*c* 0.057, $CHCl_3$); UV (MeOH) λ_{max} 287 (ε 15400), 348 (15600, sh), 362 (20000), and 378 (17300) nm. IR ($CHCl_3$) 3610, 1594, 1494, 1400, 1373, 1332, 1243, 1179, 1134, 1103, 1008, 968, and 884 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ 1.15 (s, 3 H), 1.29 (d, J=7.2 Hz, 3 H), 2.01 (dq, J=4.2 and 7.2 Hz, 1 H), 2.97 (d, J=15.8 Hz, 1 H), 3.33 (d, J=15.8 Hz, 1 H), 4.30 (br d, 1 H), 6.24 (dd, J=9.6 and 5.4 Hz, 1 H), 6.47 (d, J=9.6 Hz, 1 H), 6.65 (s, 1 H), 7.66 (m, 2 H), and 7.97 (m, 2 H); EIMS *m*/*z* 278 (M^+), 276, 260, 245 (base), 230, and 218; HREIMS calcd for $C_{18}H_{18}N_2O$ 278.1419 (M), found 278.1438. Methyl (2*E*, 4*E*, 6*S*)-6-methyl-2,4-octadienoate: $[\alpha]_D^{22}$ +40 (*c* 0.03, $CHCl_3$); ^1H NMR data were identical to those for the synthetic sample described below.

MTPA esters 4a, b. A mixture of alcohol (0.4 mg), (*R*)-α-methoxy-α-(trifluoromethyl)phenylacetic acid ((*R*)-MTPA acid) (2.9 mg), 4-dimetylaminopyridine (1.2 mg), 10-camphorsulfonic acid (1.1 mg), and 1,3-dicyclohexylcarbodiimide (3.5 mg) in anhydrous dichloromethane (0.5 mL) was stirred at room temperature for 4 h. The reaction mixture was diluted with hexane (0.5 mL) and the resulting precipitates were removed by filtration and washed with hexane. The combined filtrate and washings were concentrated and chromatographed on silica gel (hexane:EtOAc (2:1) to give (*R*)-MTPA ester (0.5 mg) as an oil. (*S*)-MTPA ester was obtained in the same manner. ^1H NMR (600 MHz) spectra of MTPA esters were measured in $CDCl_3$.

Hydrolysis of KM-01 and methylation. KM-01 (4.9 mg, 0.012 mmol) dissolved in 0.1N-KOH (1 mL) and MeOH (1 mL) was stirred for 30 min at room temperature. The reaction was monitored by TLC until the sample disappeared. MeOH was removed. The aqueous solution was extracted with ethyl acetate (3×3 mL), acidified with HCl (0.1N) and extracted with diethyl ether (3×3 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated in vacuo. The residue was further purified by preparative TLC, showing R_f value of 0.23 in the petroleum ether-ethyl ether-acetic acid (80:20:1 (v/v)) system. This fatty acid was methylesterified with diazomethane. After the purification with HPLC, the yield was 0.52 mg.

Methyl (*E*)-triphenylphophoranylidene-2-butenoate.[22] To a solution of triphenylphosphine (2.6 g, 10 mmol) in dry benzene (10 mL), methyl 4-bromo-2-butenoate (1.8 g, 10 mmol) was added by slowly dropping with vigorous stirring at room temperature and the mixture was stirred overnight. The white solid was collected by filtration, washed with 100 ml of dry benzene, and dried in vacuum oven at 60–80 °C. The yield of phosphonium salt (mp 179–180 °C) was 4.06 g. The phosphonium salt (4.06 g) was dissolved in cold water (200 mL), and 2%-NaOH (20 mL) was dropped cautiously. The mixture was stirred overnight at room temperature and the precipitate was filtered by suction. The precipitate was washed with excess ice-cooled water, dried below 20 °C under vacuum. The yield was 2.58 g, mp 175–179 °C.

Methyl (2*E*,4*E*,6*S*)-6-methyl-2,4-octadienoate. DMSO was added to the stirred oxalyl chloride (11 mmol) dissolved in 25 mL dichloromethane at −50 °C to −60 °C. The reaction mixture was stirred for 2 min and (*S*)-2-methylbuthanol was added within 5 min, stirring was continued for an additional 15 min. Triethylamine (7.0 mL, 50 mmol) was added and the reaction mixture was stirred for 5 min and then allowed to warm to room temperature. Water (50 mL) was then added, the organic layer was separated, and the aqueous layer was reextracted with additional dichrolomethane (50 mL). The organic layers were combined, washed with saturated NaCl solution (100 mL), and dried over anhydrous magnesium sulfate. To the concentrated organic layer (25 mL), methyl (*E*)-triphenylphophoranylidene-2-butenoate (1.96 g, 5.5 mmol) was added rapidly and the reaction mixture was allowed to stand overnight at room temperature. The mixture was poured into water and the aqueous phase extracted with ether. The ethereal fraction was washed with saturated aqueous NaCl and concentrated to yield a viscous oil which was chromatographed over silica gel eluted with the solvent system of *n*-hexane-ethylacetate. The yield was 182 mg (1.12 mmol).

$[\alpha]_D^{20}$ +52.8° (*c* 0.3, $CHCl_3$), $[\alpha]_{365}^{20}$ +204.3; ^1H NMR (270 MHz, $CDCl_3$), δ 0.86 (3H, t, J=7.4 Hz), 1.02 (3H, d, J=6.7 Hz), 1.37 (2H, dq, J=7.1, 7.1 Hz), 2.16 (1H, m), 3.73 (3H, s), 5.80 (1H, d, J=15.2 Hz), 6.01 (1H, dd, J=15.2, 7.4 Hz), 6.14 (1H, dd, J=15.2, 10.6 Hz), 7.27 (1H, dd, J=15.2, 10.6 Hz).

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