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Three New Metabolites of Hybrid Strain KO 0231, Derived from *Penicillium citreo-viride* IFO 6200 and 4692

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Abstract—Three new metabolites, anthrasteroids, have been isolated from the mycelia of a hybrid strain, KO 0231, prepared by cell fusion technique using *Penicillium citreo-viride* IFO 6200 and 4692. Their structures were determined based on their spectral data. Furthermore, these natural products were synthesized starting from ergosterol.

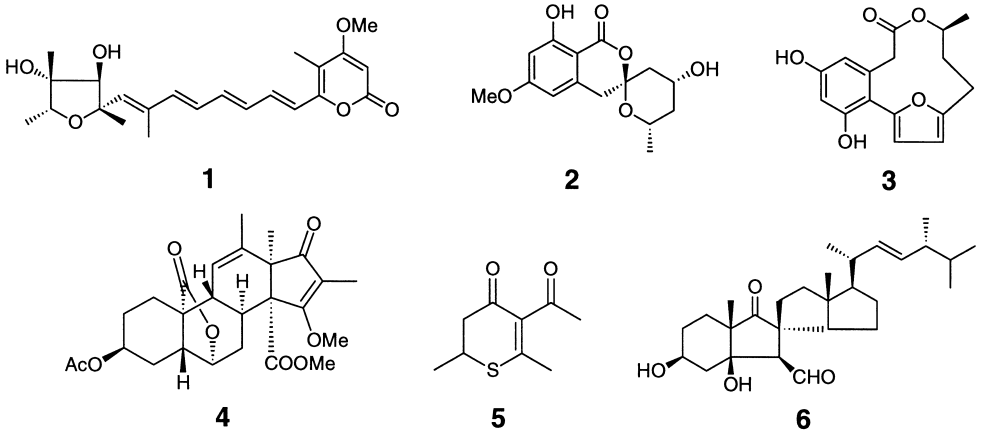
Introduction

In previous papers, a variety of bioactive metabolites with novel structures have been found in the mycelia of hybrid strains prepared by cell fusion of two different strains, *Penicillium citreo-viride* IFO 6200 and 4692. Of the two parent strains, the former mainly produced citreoviridin (**1**)[1,2] and related α-pyrones,[3,4] while the latter, *P. citreo-viride* IFO 4692, produced many phenolic compounds represented by citreoviranol (**2**).[5] Interestingly, however, the representative metabolites of hybrid strains have novel structures different from those of the two parents: citreofuran (**3**),[6] citreohybridone A (**4**),[7,8] citreothiopyrane A (**5**)[9] and citreospirosteroid (**6**)[10] are produced by the hybrid strains ME 0005, KO 0031, KO 0201 and KO 0011, respectively (Scheme 1). Therefore, the cell fusion technique is quite useful for finding new bioactive compounds. We describe herein another example using a hybrid strain, KO 0231, prepared from *P. citreo-viride* IFO 6200 and 4692.

Results and Discussion

According to the usual procedures previously reported, we were able to isolate three new metabolites (**I**, **II** and **III**) from the mycelium of a hybrid strain, KO 0231, newly prepared by cell fusion experiments using *P. citreo-viride* IFO 6200 and 4692 (Scheme 2). Their structures were determined based on the ^{1}H and ^{13}C NMR, HMQC and HMBC spectra with the aid of NOE experiments.

Citreoanthrasteroid A (**I**) as a colorless oil has a molecular



Scheme 1.

Keywords: hybrid strain; cell fusion; anthrasteroids.

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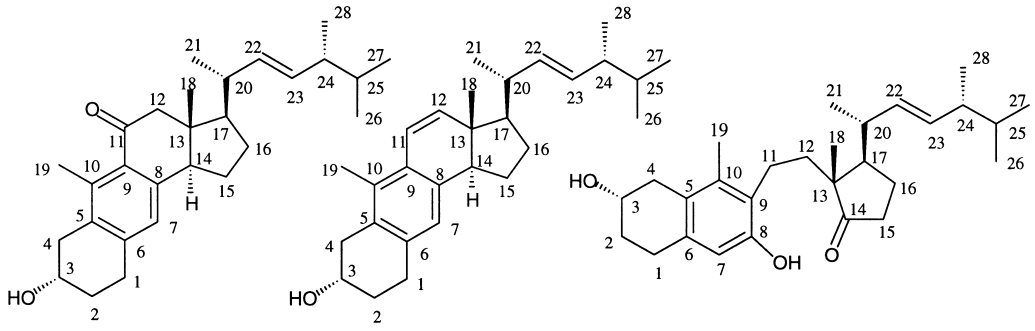


I **II** **III**

Scheme 2.

formula $C_{28}H_{40}O_2$ and one unsaturated CO and *sec*-OH group each (CO: IR 1667 cm^{-1} and ^{13}C NMR δ 201.2 ppm; OH: IR 3397 cm^{-1}, ^1H and ^{13}C NMR δ 4.19 and 67.4 ppm). The ^1H NMR spectrum indicates the presence of a disubstituted *trans*-olefinic double bond (δ 5.27 and 5.23 ppm), as shown in Table 1, where the ^1H and ^{13}C NMR spectral data were unambiguously assignable to each proton and carbon based on proton homonuclear decoupling and HMBC experiments. The partial structure (C-1–C-4) including the *sec*-OH group was determined by proton homonuclear decoupling experiments. The remaining moieties were also determined based on proton homonuclear decoupling and HMBC experiments [C-5–C-10 including aromatic ring with one methyl group C-19 (^1H and ^{13}C NMR δ 2.47 and 16.4 ppm) at C-10; C-11–C-17 with one methyl group C-18 (^1H and ^{13}C NMR δ 0.60 and 13.2 ppm) at C-13; C-20–C-28 with four methyls and the above-mentioned olefinic double bond], as shown in Fig. 1.

Citreoanthrasteroid B (**II**) as a colorless oil has a molecular formula $C_{28}H_{40}O$. The ^1H and ^{13}C NMR spectra of **II** are quite similar to those of citreoanthrasteroid A (**I**) except for the following points, as shown in Table 1. In the ^1H NMR spectrum, **II** has a *cis*-olefinic double bond [δ 6.48 ppm (1H, d, J=9.9 Hz) and δ 6.57 ppm (1H, d, J=9.9 Hz)], while there is the $-CO-CH_2-$ grouping [δ 201.2 ppm

Table 1. ^1H and ^{13}C NMR data of anthrasteroids

C no.	Citreoanthrasteroid A (**I**)		Citreoanthrasteroid B (**II**)		Secocitreoanthrasteroid (**III**)	
	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H
1	27.8	2.80 (1H, m) 2.95 (1H, dt, *J*=17.6, 5.9 Hz)	28.0	2.85 (1H, m)	27.5	2.79 (2H, m)
2	30.4	1.77 (1H, m) 2.04 (1H, m)	31.3	1.72 (1H, m) 2.09 (1H, m)	31.2	1.72 (1H, m) 2.02 (1H, m)
3	67.4	4.19 (1H, m)	68.1	4.16 (1H, m)	68.2	4.10 (1H, m)
4	36.1	2.57 (1H, dd, *J*=6.8, 16.6 Hz) 3.04 (1H, dd, *J*=5.4, 16.6 Hz)	36.6	2.52 (1H, dd, *J*=7.7, 16.3 Hz) 3.04 (1H, dd, *J*=5.1, 16.3 Hz)	36.4	2.50 (1H, m) 2.95 (1H, dd, *J*=5.5, 16.1 Hz)
5	129.1		130.0		124.8	
6	140.7		135.1		136.2	
7	124.1	6.71 (1H, s)	123.4	6.64 (1H, s)	113.4	6.51 (1H, s)
8	142.7		137.1		152.3	
9	132.2		130.7		124.5	
10	139.8		131.7		135.3	
11	201.2		123.0	6.48 (1H, d, *J*=9.9 Hz)	22.2	2.75 (2H, m)
12	56.9	2.51 (1H, d, *J*=17.9 Hz) 2.87 (1H, d, *J*=17.9 Hz)	139.9	6.57 (1H, d, *J*=9.9 Hz)	36.1	1.72 (2H, m)
13	44.4		68.1		52.5	
14	50.7	2.83 (1H, m)	50.2	2.85 (1H, m)	226.2	
15	28.7	2.08 (2H, m)	29.6	1.60 (1H, m) 2.05 (1H, m)	36.9	2.10 (1H, m) 2.45 (1H, m)
16	24.0	1.95 (2H, m)	22.0	1.70 (1H, m) 1.90 (1H, m)	24.2	2.05 (1H, m) 1.50 (1H, m)
17	55.9	1.62 (1H, dq, *J*=11.7, 5.9 Hz)	51.7	1.65 (1H, m)	46.5	1.92 (1H, m)
18	13.2	0.60 (3H, s)	11.6	0.58 (3H, s)	17.6	0.93 (3H, s)
19	16.4	2.47 (3H, s)	14.6	2.18 (3H, s)	15.6	2.12 (3H, s)
20	40.4	2.00 (1H, m)	41.0	2.05 (1H, m)	39.2	2.22 (1H, m)
21	20.6	1.02 (3H, d, *J*=6.8 Hz)	20.9	1.13 (3H, d, *J*=6.8 Hz)	20.7	1.17 (3H, d, *J*=7.0 Hz)
22	132.6	5.21 (1H, dd, *J*=7.3, 15.2 Hz)	132.3	5.21 (1H, dd, *J*=5.9, 15.0 Hz)	133.6	5.33 (1H, dd, *J*=7.3, 15.2 Hz)
23	134.9	5.23 (1H, dd, *J*=8.4, 15.2 Hz)	135.1	5.24 (1H, dd, *J*=6.6, 15.0 Hz)	134.1	5.25 (1H, dd, *J*=8.4, 15.2 Hz)
24	42.8	1.86 (1H, q, *J*=6.8 Hz)	43.6	1.85 (1H, m)	42.9	1.91 (1H, m)
25	33.1	1.48 (1H, m)	33.2	1.50 (1H, m)	33.1	1.50 (1H, m)
26	19.6	0.81 (3H, d, *J*=6.8 Hz)	19.8	0.82 (3H, d, *J*=6.2 Hz)	19.7	0.83 (3H, d, *J*=6.2 Hz)
27	19.9	0.83 (3H, d, *J*=6.4 Hz)	20.1	0.84 (3H, d, *J*=6.6 Hz)	20. 0	0.85 (3H, d, *J*=6.2 Hz)
28	17.6	0.91 (3H, d, *J*=6.8 Hz)	17.7	0.92 (3H, d, *J*=7.0 Hz)	17.6	0.94 (3H, d, *J*=7.7 Hz)

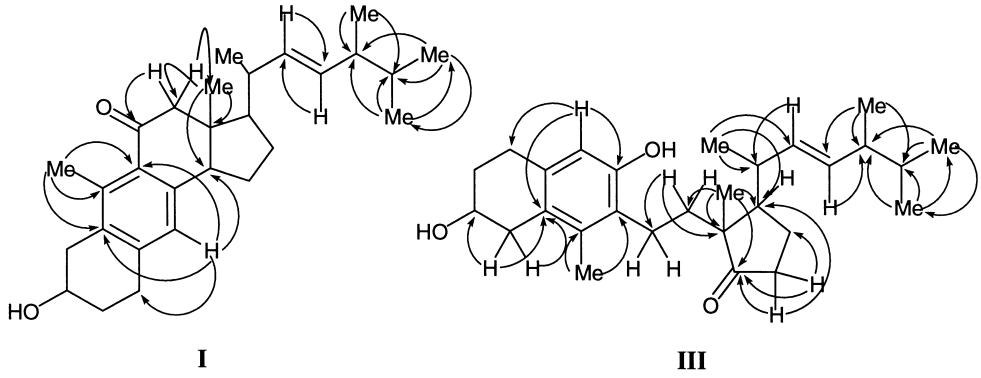
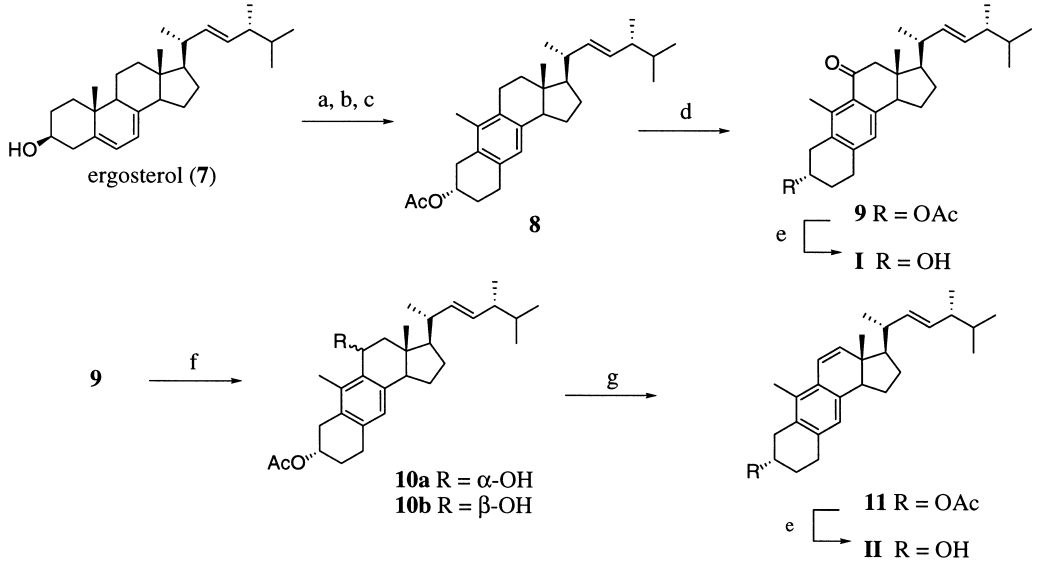


Figure 1. HMBC of anthrasteroids.

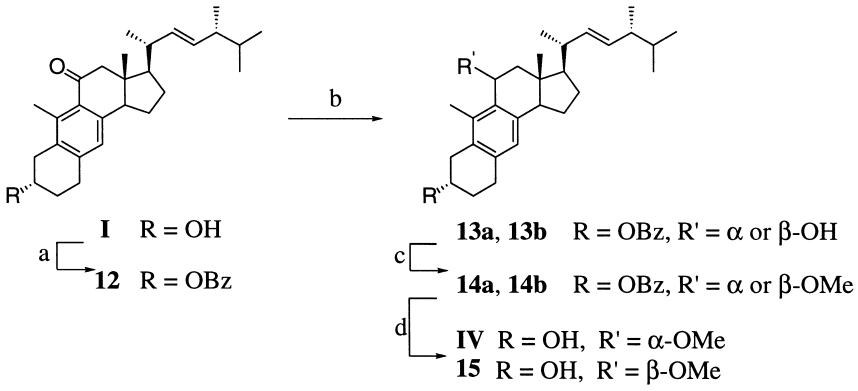
(CO); δ 2.51 ppm (1H, d, J=17.9 Hz), δ 2.87 ppm (1H, d, J=17.9 Hz) and δ 56.9 ppm (CH_2)] in **I**. Finally, the stereostructures of **I** and **II**, including the *sec*-OH group at the C-3-position, were unambiguously determined by the synthesis of citreoanthrasteroid A and B starting from ergosterol, as described later.

Secocitreoanthrasteroid (**III**) was obtained as a colorless oil and has a molecular formula $C_{28}H_{40}O_3$. The structure of **III** was determined based on the ^{1}H and ^{13}C NMR, HMQC and HMBC spectra with the aid of proton homonuclear decoupling experiments, as shown in Table 1 and Fig. 1. The presence of one CO group is suggested by the IR and ^{13}C NMR spectra (IR 1726 cm^{-1} and ^{13}C NMR δ 226.2 ppm). The ^{1}H NMR spectrum of **III** indicated the presence of a *trans*-olefinic double bond [δ 5.25 ppm (dd, J=8.4 and 15.2 Hz, H-23) and δ 5.33 ppm (dd, J=7.3 and 15.2 Hz, H-22)] included in the partial structure C-20–C-28, which is quite similar to those of citreoanthrasteroid A and B (**I** and **II**) in the corresponding ^{1}H and ^{13}C NMR spectral data. In the HMBC spectra, correlations between H-18 [δ 0.93 ppm (3H, s)] and both C-12–C-14 (δ 36.1, 52.5 and 226.2 ppm) and C-17 (δ 46.5 ppm) were observed. In addition, correlations between H-15 (δ 2.10 and 2.45 ppm, 2H) and C-14 (δ 226.2 ppm) were also observed. These results indicate that the methyl group (C-18) must be attached to the quaternary carbon (C-13) and the CO group (C-14) to both C-13 and C-15, respectively. The presence of a phenol ring is suggested by the IR and ^{13}C NMR spectral data of **III** (IR 3407 cm^{-1} and ^{13}C NMR δ 152.3 ppm). The HMBC spectrum indicated the correlations of H-7 (δ 6.51 ppm) with C-1 (δ 27.5 ppm), C-5 (δ 124.8 ppm) and C-8 (δ 152.3 ppm). In addition, H-19 (δ 2.12 ppm, 3H) was correlated with both C-9 (δ 124.5 ppm) and C-10 (δ 135.3 ppm). Therefore, the phenolic ring includes C-5–C-10. In the HMBC spectrum, correlations between H-4 (δ 2.50 and 2.95 ppm, 2H) and both C-3 (δ 68.2 ppm) and C-5 (δ 124.8 ppm) were observed, indicating that C-4 is connected to C-3 bearing the OH group (^{1}H and ^{13}C NMR δ 4.10 and 68.15 ppm) and C-5 of the phenolic ring. Finally, C-1 must be bonded to C-6 and C-11 to both C-9 and C-12.

The stereostructures of citreoanthrasteroid A and B were unambiguously determined by synthesis starting from ergosterol (**7**). The stereostructure of secocitreoanthrasteroid has not been determined yet, but must be cited as **III** based on the stereochemistry of **I** and **II**, which co-occur with secocitreoanthrasteroid.



Scheme 3. (a) Ac_2O, pyridine; (b) 4-phenyl-1,2,4-triazoline-3,5-dione, benzene; (c) BF_3–OEt_2, CH_2Cl_2, 87–95% in 3 steps; (d) PCC, Celite, benzene, 23%; (e) K_2CO_3, MeOH–THF 99%; (f) $NaBH_4$, EtOH then H_2O, 88%; (g) 1 M HCl aq., Et_2O–EtOH, 99%.

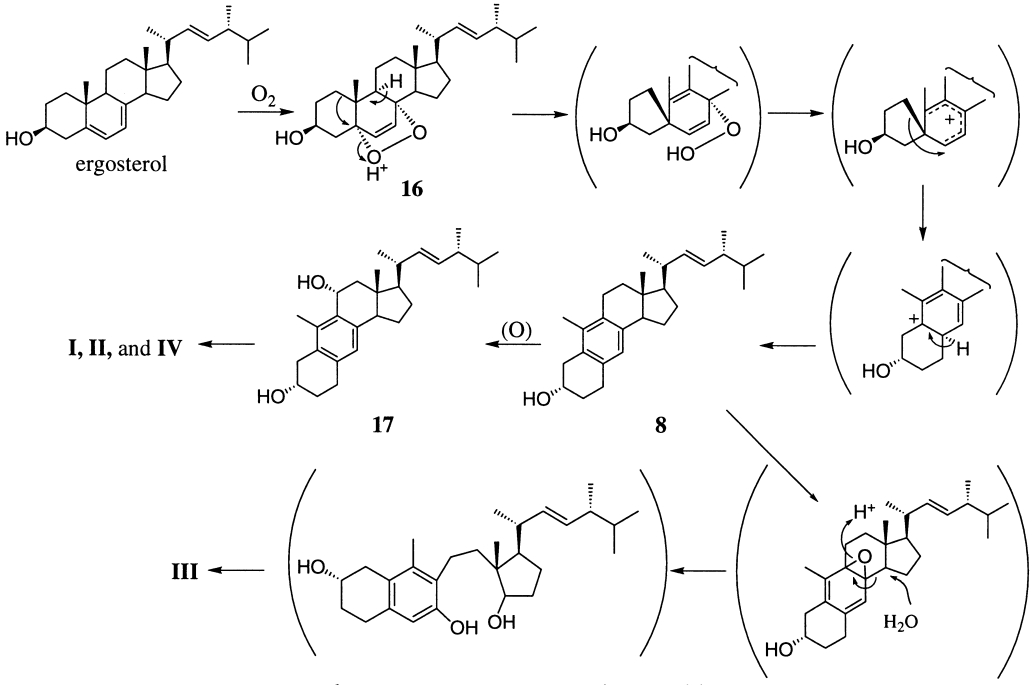


Scheme 4. (a) BzCl, pyridine, 95%; (b) $NaBH_4$, EtOH then H_2O, 99%; (c) MeI, NaH, THF, 95~96%; (d) K_2CO_3, MeOH–THF, 98~99%.

According to Bothworth's method,[11] 3-acetylergosterol was readily converted into the corresponding anthrasteroid (**8**), which was found in nature by Koshino et al.[12] Further oxidation of **8** with pyridinum chlorochromate (PCC) afforded ketone **9**, which was successfully hydrolyzed with K_2CO_3 in MeOH–THF to give rise to citreoanthrasteroid A (**I**). The synthetic compound is completely identical with an authentic sample of citreoanthrasteroid A in all respects of the spectral data, including optical rotation [synthetic sample: $[\alpha]_D^{24}=-9.8°$ (c=2.5 in $CHCl_3$); natural sample: $[\alpha]_D^{24}=-7.5°$ (c=1.0 in $CHCl_3$)] (Scheme 3).

Furthermore, **9** was reduced with $NaBH_4$ in MeOH to give a mixture of two epimers, **10a** and **10b**, which were directly treated with 1 M HCl to yield **11**. Finally, **11** was hydrolyzed with K_2CO_3 in MeOH–THF to give rise to citreoanthrasteroid B (**II**), whose spectral data were compatible with those of an authentic sample, including optical rotation [synthetic sample: $[\alpha]_D^{24}=+33.6°$ (c=1.0 in $CHCl_3$); natural sample: $[\alpha]_D^{24}=+22.7°$ (c=0.05 in $CHCl_3$)].

In a previous paper[10] we reported on the isolation and structure determination of citreoanthrasteroid (**IV**), which was found in the culture filtrate of a hybrid strain, KO 0011, prepared by cell fusion technique using *P. citreo-viride* IFO 6200 and 4692. This metabolite was successfully synthesized from citreoanthrsteroid A (**I**), as shown in Scheme 4. Citreoanthrasteroid A (**I**) was treated with benzoyl chloride in pyridine to give **12**, which was reduced with $NaBH_4$ in EtOH to afford a mixture of two diastereomers (**13a** and **13b**). This mixture was successfully separated by flash column chromatography. Of these two diastereomers, whose stereochemistry was determined by NOE experiments, the α-isomer was treated with MeI and NaH to afford methyl ether **14**. Finally, **14** was hydrolyzed with K_2CO_3 in MeOH–THF to give rise to citreoanthrasteroid



Scheme 5. A plausible biosynthetic pathway of citreoanthrasteroids.

(**IV**). The synthetic compound was completely identical with an authentic sample of citreoanthrasteroid in all respects of the spectral data (Scheme 4).

From a biogenetic point of view, in the light of Scheme 3 (**7** to **8**), ergosterol (**7**) will be oxygenated to give peroxide **16**, which is subjected to double 1,2-shifts to afford anthrasteroid (**8**), as shown in Scheme 5. Then, enzymatic oxidation of **8** will afford **17**, which is dehydrated or methylated to give **II** or **IV**, respectively. Further oxidation of **17** gives rise to **I**.

In the present study, cell fusion technique using two different strains has been proved to be a useful method to find new bioactive compounds

Materials and Methods

General procedures

^{1}H NMR (400 MHz) and ^{13}C NMR spectra (100 MHz) were recorded on a Jeol GX-400 or JNM-α400 spectrometer in $CDCl_3$ as solvent, unless otherwise stated. IR spectra were measured as a film on a JASCO FT/IR-410 spectrometer. HREIMS were taken on a HITACHI M-80B GC-MS spectrometer. Optical rotations were determined on a JASCO DIP-360 polarimeter. All reactions were carried out under Ar atmosphere and monitored by TLC and reaction products were purified by using silica gel column chromatography (Fuji Silysia BW-300).

Cell fusion

Sliced potato (400 g) was immersed in boiling water (2 L) for 20 min and filtered. Each strain, *P. citreo-viride* IFO 6200 and 4692, was grown on PSA medium (5% sucrose and 5% agar in the above filtrate) for 4 days. Spores were separated from the mycelial debris by filtration through nylon mesh and incubated on malt extract broth (3% malt extract and 0.5% peptone in deionized water) for 12 h, respectively. The germinal hypha were independently separated from the malt extract broth by centrifugation (700 rpm, 10 min), washed twice by centrifugation (700 rpm, 10 min) with 0.1 M phosphate buffer (stabilized with 0.6 M NaCl) and then treated with a mixed solution [pectolyase Y23 (Seishin Pharm. Co. Ltd) (10 mg), cellulase (Sigma C-0901) (5.0 mg), sulfatase (Sigma S-9751) (1.10 mL) and zymolyase (Seikagaku Co. 20000 unit) in 10 mL of 0.1 M phosphate buffer (stabilized with 0.6 M NaCl) at 30°C for 3 h. Each protoplast was separated from the mycelial debris by filtration through a glass filter (Shibata P250) and then washed by centrifugation (700 rpm, 5 min) with 0.1 M phosphate buffer solution (stabilized with 0.6 M NaCl). The two different protoplasts in a high pH Ca^{2+} solution [(0.1 M $CaCl_2{\cdot}2H_2O$ and 0.8 M mannitol in deionized water)/(0.01 M glycine in deionized water (pH 10.5 with 2 M NaOH aq.))=1:1] were mixed (each protoplast: ca. 5×10^5–10^6 mL^{-1}). A mixed solution of the two protoplasts (100 mL) was put on a glass plate and suspended in a mixed solution of 50% polyethylene glycol 6000, 3.6% glucose, 0.5% $CaCl_2{\cdot}2H_2O$ and 0.1% KH_2PO_4 (100 mL) for 20 min. The suspension was diluted with a high pH Ca^{2+} solution (100 mL) for 20 min and then further diluted overnight. A mixed solution of a high pH Ca^{2+} solution and PS medium (5% sucrose in the potato extract) (9:1) (100 mL) was added to the above mixture and allowed to stand at room temperature for 10 h. Then, this mixture was also treated with a mixed solution of a high pH Ca^{2+} solution and PS medium (5% sucrose in the potato extract) (3:1) (100 mL) overnight, and then 20 mL of the mixture was suspended in 2 mL of PSA medium at 40°C, sprayed over PSA medium plates and then incubated for 7 days. From some hybrid strains, a hopeful strain KO 0231 was selected.

Isolation of citreoanthrasteroid A (I), citreoanthrasteroid B (II) and secocitreoanthrasteroid (III)

Polished rice (200 g) in deionized water (500 mL) was successively entered into an Erlenmeyer flask (3 L), cooked using an autoclave (121°C, 20 min at 2 atm), and then inoculated with a suspension of the above hybrid strain KO 0231 in sterilized water and incubated stationary at room temperature for 61 days, giving a yellow rice, which was extracted with a lot of acetone. The acetone solution was concentrated under reduced pressure to give a yellow oil, which was partitioned between EtOAc and water. The EtOAc extract (32 g) was chromatographed on silica gel using a gradient solvent of EtOAc–hexane (50–100%) to afford 6 fractions (A–F). Fraction C eluted with EtOAc (100%) gave a brown oil, which was further separated by repeated preparative TLC [EtOAc–hexane (1:4)] and then EtOAc–hexane (1:4)] to afford a colorless oil including **I** and **II**. This oil was further separated by preparative TLC [$CHCl_3$–MeOH (30:1)] to two fractions. The more polar fraction was further separated by preparative TLC [EtOAc–hexane (1:2)] to afford **I** as a colorless oil (4.8 mg). The remaining fraction was subjected to HPLC [ODS-UG-5 Develosil ϕ10×250 mm, flow 6.0 mL/min, Rt. 9.3 min] using MeOH to afford **II** as a colorless oil (0.9 mg).

Fraction D eluted with EtOAc (100%) gave a brown oil, which was further separated by repeated preparative TLC [EtOAc–benzene (1:1) and then EtOAc–hexane (1:1)] to afford secocitreoanthrasteroid (**III**) as a colorless oil (4.1 mg).

I. $[\alpha]_D^{24}=-7.5°$ (c=1.0, in $CHCl_3$); IR: 3397, 1667, 1594, 1459, 1381, 1233 cm^{-1}; HREIMS: Found m/z 408.3026, $C_{28}H_{40}O_2$ requires m/z 408.3026. ^{1}H and ^{13}C NMR spectral data are cited in Table 1.

II. $[\alpha]_D^{24}=+22.7°$ (c=0.05, in $CHCl_3$); IR: 3348 cm^{-1}; HREIMS: Found m/z 392.3056, $C_{28}H_{40}O$ requires m/z 392.3076. ^{1}H and ^{13}C NMR spectral data are cited in Table 1.

III. $[\alpha]_D^{24=}+16.9°$ (c=0.5, in EtOH); IR: 3407, 1726 cm^{-1}; HREIMS: Found m/z 426.3153, $C_{28}H_{40}O_2$ requires m/z 426.3132. ^{1}H and ^{13}C NMR spectral data are cited in Table 1.

Synthesis of 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11-one-3α-acetate (9). A solution of 1(10→6)abeo-ergosta-5,7,9,22-tetraen-3α-acetate (**8**) (1.04 g, 2.4 mmol) including

PCC (9.56 g, 44.3 mmol), and celite (25 g) in benzene (200 mL) was stirred for 30 h under refluxing, and then cooled and filtered. The filtrate was separated by column chromatography [Si gel, EtOAc–hexane (1:10)] to give 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11-one-3α-acetate (**9**) (251 mg, 23%) as a colorless oil: $[\alpha]_D^{25}=-18.1$ (c=1.90 in $CHCl_3$); IR: 1738, 1670, and 1595 cm^{-1}; ^1H NMR δ/ppm 6.71 (1H, s), 5.28–5.14 (3H, m), 3.02 (1H, dd, J=5.4, 17.1 Hz), 2.93–2.83 (4H, m), 2.70 (1H, dd, J=6.4, 17.1 Hz), 2.53 (1H, d, J=7.6 Hz), 2.45 (3H, s), 2.07–1.83 (6H, m), 2.03 (3H, s), 1.63 (1H, dq, J=6.4, 12.2 Hz), 1.55–1.43 (3H, m), 1.03 (3H, d, J=6.4 Hz), 0.91 (3H, d, J=6.8 Hz), 0.83 (3H, d, J=6.8 Hz), 0.81 (3H, d, J=6.8 Hz), 061 (3H, s); ^{13}C NMR δ/ppm 201.2, 170.8, 142.8, 140.5, 139.6, 134.9, 132.7, 131.7, 129.2, 124.2, 68.9, 56.9, 55.9, 50.7, 44.4, 42.9, 40.5, 33.2, 32.5, 28.8, 27.4, 26.9, 24.1, 21.5, 20.7, 20.1, 19.8, 17.7, 16.5, 13.4; HREIMS: Found m/z 450.3161, $C_{30}H_{42}O_3$ requires m/z 450.3131.

Synthesis of citreoanthrasteroid A (I). A solution of 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11-one-3α-acetate (**9**) (92 mg, 0.21 mmol) and K_2CO_3 (1.5 g, 1.1 mmol) in a mixed solvent of MeOH (10 mL) and THF (3 mL) was stirred at room temperature for 30 min, and filtered through Celite. The filtrate was poured into water and extracted with EtOAc (3×10 mL). The combined extracts were dried over Na_2SO_4 and concentrated to give a crude product, which was then separated by column chromatography [Si gel, EtOAc–hexane (1:4)] to afford citreoanthrasteroid A (**I**) as a colorless oil (84 mg, 99%): $[\alpha]_D^{24}=-9.8°$ (c=2.5, in $CHCl_3$).

Synthesis of 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11-hydroxy-3α-acetate (10a and 10b). A solution of 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11-one-3α-acetate (**9**) (133 mg, 0.30 mol) and $NaBH_4$ (66.7 mg, 1.7 mmol) in EtOH (10 ml) was stirred for 5 min at 0°C, and then poured into water (50 mL) and allowed to stand at room temperature for 1 h. The reaction mixture was extracted with EtOAc (3×25 ml). The combined extracts were washed with saturated NaCl aq. (20 mL), dried over Na_2SO_4, and concentrated to give a crude oil, which was then separated by column chromatography [Si gel, EtOAc–hexane (1:5)] to afford a diastereomeric mixture (120 mg, 88%) as a colorless oil. This mixture was separated by flash column chromatography [Si gel, acetone–hexane (1:10)] to afford 1(10→6)abeo-ergost-5,7,9,22-tetraen-11α-hydroxy-3α-acetate (**10a**) as a colorless oil (43.8 mg): $[\alpha]_D^{26}=-28.0°$ (c=1.00 in $CHCl_3$); IR: 3276 and 1738 cm^{-1}; ^1H NMR (400 MHz, C_6D_6) δ/ppm 6.69 (1H, s), 5.37–5.30 (3H, m), 5.05 (1H, t, J=7.6 Hz), 2.87–2.76 (3H, m), 2.66–2.54 (3H, m), 2.17 (3H, s), 2.01–1.25 (11H, m), 1.69 (3H, s), 1.11 (3H, d, J=6.4 Hz), 1.01 (3H, d, J=6.8 Hz), 0.92 (3H, d, J=6.8 Hz), 0.91 (3H, d, J=6.8 Hz), 0.46 (3H, s); ^{13}C NMR (100 MHz, C_6D_6) δ/ppm 169.8, 138.3, 136.2, 136.0, 134.8, 134.5, 132.4, 130.7, 123.9, 70.4, 67.3, 56.0, 51.3, 49.8, 45.4, 43.5, 41.2, 33.7, 33.2, 29.6, 27.8, 27.3, 24.8, 21.1, 20.5, 20.1, 18.2, 15.6, 13.0; HREIMS: Found m/z 452.2354, $C_{30}H_{44}O_3$ requires m/z 452.2388.

1(10→6)abeo-ergosta-5,7,9,22-tetraen-11β-hydroxy-3α-acetate (**10b**) (38.9 mg) was also obtained as a colorless oil: $[\alpha]_D^{26}=+1.80°$ (c=2.10 in $CHCl_3$). IR: 3415 and 1738 cm^{-1}; ^1H NMR (400 MHz, C_6D_6) δ/ppm 6.70 (1H, s), 5.32–5.21 (2H, m), 4.84 (1H, d, J=5.2 Hz), 2.89 (1H, dd, J=5.2, 16.8 Hz), 2.74 (1H, dt, J=5.6, 16.4 Hz), 2.63–2.57 (2H, m), 2.45–2.42 (2H, m), 2.13 (3H, s), 2.13–2.09 (1H, m), 2.02–1.78 (4H, m), 1.72 (3H, s), 1.71–1.64 (2H, m), 1.55–1.47 (3H, m), 1.35–1.20 (1H, m), 1.17 (3H, d, J=6.4 Hz), 1.02 (3H, d, J=6.8 Hz), 0.92 (3H, d, J=6.4 Hz), 0.91 (3H, d, J=6.8 Hz), 0.86 (3H, s); ^{13}C NMR (100 MHz, C_6D_6) δ/ppm 169.7, 137.1, 136.8, 136.1, 134.8, 134.4, 132.4, 130.9, 124.0, 70.8, 66.8, 55.6, 53.2, 48.4, 43.5, 41.2, 40.9, 33.7, 33.4, 29.6, 28.2, 27.8, 24.7, 21.4, 21.2, 20.4, 20.2, 18.2, 15.9, 13.6; HREIMS: Found m/z 452.2390, $C_{30}H_{44}O_3$ requires m/z 452.2388.

Synthesis of 1(10→6)abeo-ergosta-5,7,9,11,22-hexaen-3α-acetate (11). A solution of 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11-hydroxy-3α-acetate (**10**) (52.4 mg, 0.12 mol) in Et_2O (3 mL) was poured into a mixed solution of 1 M HCl aq. (2 mL) and EtOH (3 mL) and then stirred at room temperature for 5 h. The reaction mixture was poured into saturated aqueous solution $NaHCO_3$ (5 mL) and then extracted with EtOAc (3×20 ml) at 0°C. The combined extracts were dried over Na_2SO_4 and concentrated to give a crude oil, which was then purified by column chromatography [Si gel, EtOAc–hexane (1:4)] to afford 1(10→6)abeo-ergosta-5,7,9,11,22-hexaen-3α-acetate (**11**) (49.9 mg, 99%) as a colorless oil: $[\alpha]_D^{25}=+35.3°$ (c=1.00 in $CHCl_3$); IR: 1738 cm^{-1}; ^1H NMR δ/ppm 6.55 (1H, s), 6.47 (1H, d, J=10.0 Hz), 6.39 (1H, d, J=10.0 Hz), 5.20–5.09 (3H, m), 2.93 (1H, dd, J=5.4, 16.6 Hz), 2.83–2.72 (3H, m), 2.55 (1H, dd, J=6.8, 17.1 Hz), 2.07 (3H, s), 1.95 (3H, s), 1.95–1.75 (6H, m), 1.59 (1H, q, J=9.3 Hz), 1.52 (1H, dd, J=5.9, 12.2 Hz), 1.41–1.35 (2H, m), 1.04 (3H, d, J=6.3 Hz), 0.83 (3H, d, J=6.8 Hz), 0.75 (3H, d, J=6.8 Hz), 0.73 (3H, d, J=6.3 Hz), 0.49 (3H, s); ^{13}C NMR δ/ppm 170.9, 140.4, 140.1, 137.3, 135.2, 134.0, 132.5, 131.8, 130.9, 129.6, 123.6, 123.1, 70.6, 51.8, 50.3, 43.6, 42.9, 41.0, 33.2, 32.8, 29.6, 27.5, 27.3, 22.0, 20.9, 20.1, 19.8, 17.8, 14.5, 11.6; HREIMS: Found m/z 434.3161, $C_{30}H_{42}O_3$ requires m/z 434.3181.

Synthesis of citreoanthrasteroid B (II). A solution of 1(10→6)abeo-ergosta-5,7,9,11,22-hexaen-3α-acetate (**11**) (49.9 mg, 0.11 mmol) and K_2CO_3 (0.50 g, 3.6 mmol) in a mixed solvent of MeOH (5 mL) and THF (1 mL) was stirred at room temperature for 6 h, and then filtered through Celite. The filtrate was poured into water and extracted with EtOAc (3×10 mL). The combined extracts were dried over Na_2SO_4 and concentrated to give a crude product, which was then separated by column chromatography [Si gel, EtOAc–hexane (1:4)] to give citreoanthrasteroid B (**II**) (44.6 mg, 99%) as a colorless oil: $[\alpha]_D^{24}=+33.6°$ (c=1.0 in $CHCl_3$). The spectral data of the synthetic compound were completely identical with those of an authentic sample.

Synthesis of 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11-one-3α-benzoate (12). A solution of citreoanthrasteroid A (**I**) (252 mg 0.62 mmol) and benzoyl chloride (236 mg, 1.7 mmol) in pyridine (10 mL) was stirred at 0°C for 5 h. The reaction mixture was poured into saturated aqueous solution $NaHCO_3$ (10 mL). The mixture was extracted with EtOAc (3×30 mL). The combined extracts were dried over Na_2SO_4 and concentrated to give a crude product,

which was then separated by column chromatography [Si gel, EtOAc–hexane (1:8)] to afford 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11-one-3α-benzoate (302 mg, 95%) as a colorless oil: $[\alpha]_D^{25}=+11.8°$ (c=2.50 in $CHCl_3$); IR: 1717, 1670 and 1596 cm^{-1}; ^1H NMR δ/ppm 8.00 (2H, dd, J=1.5, 7.8 Hz), 7.53 (1H, tt, J=1.5, 7.8 Hz), 7.40 (2H, t, J=7.8 Hz), 6.75 (1H, s), 5.53 (1H, quintet, J=5.9 Hz), 5.25 (1H, dd, J=7.3, 15.1 Hz), 5.18 (1H, dd, J=8.3, 15.1 Hz), 3.14 (1H, dd, J=5.4, 16.6 Hz), 3.04 (1H, dt, J=6.8, 17.6 Hz), 2.94–2.84 (4H, m), 2.54 (1H, d, J=18.1 Hz), 2.48 (3H, s), 2.12–2.03 (4H, m), 2.00–1.91 (1H, m), 1.87 (1H, q, J=6.8 Hz), 1.65 (1H, dq, J=5.4, 6.4 Hz), 1.55–1.44 (3H, m), 1.01 (3H, d, J=6.4 Hz), 0.92 (3H, d, J=6.8 Hz), 0.84 (3H, d, J=6.8 Hz), 0.82 (3H, d, J=6.3 Hz), 0.63 (3H, s,); ^{13}C NMR δ/ppm 201.0, 165.9, 142.6, 140.4, 139.5, 134.7, 132.8, 132.5, 131.6, 130.3, 129.5, 129.1, 128.2, 124.1, 70.3, 56.8, 55.9, 50.7, 44.4, 42.9, 40.4, 33.1, 32.6, 28.8, 27.4, 27.0, 24.0, 20.7, 20.0, 19.8, 17.7, 16.5, 13.4; HREIMS: Found m/z 512.3266, $C_{30}H_{42}O_3$ requires m/z 512.3245.

Synthesis of 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11-hydroxy-3α-benzoate (13a and 13b). A solution of 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11-one-3α-benzoate (**12**) (302 mg, 0.59 mol) and $NaBH_4$ (126 mg, 3.3 mmol) in EtOH (20 mL) was stirred at 0°C for 5 min. The reaction mixture was poured into water (60 mL) and allowed to stand at room temperature for 1 h. The mixture was extracted with EtOAc (3×25 mL). The combined extracts were washed with saturated NaCl aq. (20 mL) and then dried over Na_2SO_4 and concentrated to give a crude oil, which was separated by column chromatography [Si gel, EtOAc–hexane (1:5)] to give 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11-hydroxy-3α-benzoate (302 mg, 99%) as a diastereomeric mixture. This mixture was successfully separated by flash column chromatography [Si gel, acetone–hexane (1:10)] to give 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11α-hydroxy-3α-benzoate (**13a**) (119 mg) as a colorless oil: $[\alpha]_D^{26}=-20.3°$ (c=1.19 in $CHCl_3$); IR: 3281, 1717 cm^{-1}; ^1H NMR (400 MHz, C_6D_6): δ/ppm 8.22 (2H, dd, J=1.5, 7.3 Hz), 7.13 (1H, t, J=7.3 Hz), 7.05 (2H, t, J=7.3 Hz), 6.77 (1H, s), 5.58 (1H, m), 5.34 (1H, dd, J=8.3, 15.1 Hz), 5.32 (1H, dd, J=7.8, 15.1 Hz), 5.03 (1H, dd, J=7.7, 7.8 Hz), 2.93–2.89 (3H, m), 2.80 (1H, dd, J=5.9, 7.1 Hz), 2.68–2.60 (2H, m), 2.23 (3H, s), 2.07–1.95 (5H, m), 1.87 (1H, m), 1.69–1.40 (7H, m), 1.17 (3H, d, J=6.4 Hz), 1.07 (3H, d, J=6.8 Hz), 0.978 (3H, d, J=6.8 Hz), 0.982 (3H, d, J=6.3 Hz), 0.53 (3H, s); ^{13}C NMR (100 MHz, C_6D_6): δ/ppm 165.9, 138.3, 136.4, 136.0, 134.8, 134.5, 132.8, 132.4, 131.3, 130.6, 129.9, 128.5, 123.9, 71.1, 67.2, 56.0, 51.4, 49.8, 45.4, 43.5, 41.2, 33.6, 33.2, 29.6, 27.8, 27.2, 24.8, 21.4, 20.5, 20.2, 18.2, 15.6, 12.9; HREIMS: Found m/z 514.3488, $C_{35}H_{46}O_3$ requires m/z 514.3444. 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11β-hydroxy-3α-benzoate (**13b**) (115 mg) was also obtained as a colorless oil: $[\alpha]_D^{25}=+15.4°$ (c=2.00 in $CHCl_3$); IR: 3422 and 1717 cm^{-1}; ^1H NMR (400 MHz, C_6D_6): δ/ppm 8.24 (2H, dd, J=1.5, 6.8 Hz), 7.15 (1H, dt, J=1.5, 6.8 Hz), 7.08 (2H, d, J=6.8 Hz), 6.79 (1H, s), 5.56 (1H, m), 5.35 (1H, dd, J=7.3, 15.1 Hz), 5.29 (1H, dd, J=6.8, 15.1 Hz), 4.90 (1H, br.s), 2.99 (1H, dd, J=4.9, 16.6 Hz), 3.88 (1H, dt, J=5.9, 16.6 Hz), 2.78 (1H, dd, J=6.3, 16.6 Hz), 2.69 (1H, dt, J=6.3, 17.6 Hz), 2.50 (1H, d, J=13.2 Hz), 2.51–2.48 (1H, m), 2.18 (3H, s), 2.06–1.94 (5H, m), 1.74 (1H, dd, J=5.4, 13.7 Hz), 1.64 (1H, dd, J=8.3, 13.7 Hz), 1.61–1.53 (2H, m), 1.29–1.26 (2H, m), 1.22 (3H, d, J=6.8 Hz), 1.07 (3H, d, J=6.8 Hz), 0.978 (3H, d, J=6.8 Hz), 0.982 (3H, d, J=6.8 Hz), 0.92 (3H, s); ^{13}C NMR (100 MHz, C_6D_6): δ/ppm 165.8, 137.1, 136.9, 136.1, 134.8, 134.4, 132.8, 132.4, 131.4, 130.8, 129.9, 128.5, 124.1, 71.4, 66.8, 55.6, 53.2, 48.4, 43.5, 41.2, 40.9, 33.6, 33.3, 29.6, 28.1, 27.6, 24.7, 21.5, 20.5, 20.2, 18.2, 16.0, 13.6; HREIMS: Found m/z 514.3425, $C_{35}H_{46}O_3$ requires m/z 514.3444.

Synthesis of 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11α-methoxy-3α-benzoate (14a). A solution of 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11α-hydroxy-3α-benzoate (95 mg, 0.18 mmol) and MeI (1.2 mL, 12.7 mmol) in THF (10 mL) was stirred at −78°C under Ar. Then, NaH (24.5 mg (60%), 0.61 mmol) was added to the solution at −78°C and stirred at the same temperature for 10 min under Ar. The reaction solution was poured into saturated NH_4Cl aq. (10 mL) at 0°C. The mixture was extracted with EtOAc (3×30 mL). The combined extracts were dried over Na_2SO_4 and then concentrated to give a crude oil, which was separated by column chromatography [Si gel, EtOAc–benzene (20:1)] to afford 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11α-methoxy-3α-benzoate (**14a**) (93 mg, 95%) as a colorless oil: $[\alpha]_D^{25}=-1.2°$ (c=2.21 in $CHCl_3$); IR: 1715 and 1603 cm^{-1}; ^1H NMR (400 MHz, C_6D_6): δ/ppm 8.16 (2H, dd, J=1.6, 7.2 Hz), 7.09 (1H, dt, J=1.6, 7.2 Hz), 7.01 (2H, t, J=7.2 Hz), 6.75 (1H, s), 5.58 (1H, m), 5.30 (1H, dd, J=7.2, 15.2 Hz), 5.23 (1H, dd, J=7.6, 15.2 Hz), 4.74 (1H, dd, J=6.0, 8.0 Hz), 3.10 (3H, s), 3.06–2.97 (2H, m), 2.82–2.77 (2H, m), 2.65(1H, m), 2.43 (1H, dd, J=7.6, 13.2 Hz), 2.15 (3H, s), 2.07–2.04 (1H, m), 1.97– 1.83 (6H, m), 1.64–1.55 (1H, m), 1.51 (1H, q, J=6.8 Hz), 1.44–1.39 (2H, m), 1.13 (3H, d, J=6.8 Hz), 1.03 (3H, d, J=6.8 Hz), 0.94 (3H, d, J=6.8 Hz), 0.93 (3H, d, J=6,0 Hz), 0.50 (3H, s); ^{13}C NMR (100 MHz, C_6D_6): δ/ppm 165.8, 139.4, 136.6, 136.0, 134.9, 132.7, 132.4, 132.0, 131.4, 130.2, 129.9, 128.5, 123.8, 75.2, 71.2, 56.2, 53.8, 50.6, 45.3, 44.9, 43.5, 41.2, 33.6, 33.3, 29.7, 27.5, 24.4, 21.4, 20.4, 20.2, 18.2, 15.3, 14.0; HREIMS: Found m/z 528.3593, $C_{36}H_{48}O_3$ requires m/z 528.3600.

Synthesis of 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11β-methoxy-3α-benzoate (14b). A solution of 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11β-hydroxy-3α-benzoate (87 mg, 0.18 mmol) and MeI (1.2 mL, 12.7 mmol) in THF (10 mL) was stirred at −78°C under Ar. Then, NaH (24.5 mg (60%), 0.61 mmol) was added to the solution at −78°C and stirred at same temperature for 10 min under Ar. The mixture was poured into saturated NH_4Cl aq. (10 mL) at 0°C. The reaction mixture was extracted with EtOAc (3×30 mL). The combined extracts were dried over Na_2SO_4 and concentrated to give a crude oil, which was then separated by column chromatography [Si gel, EtOAc–benzene (20:1)] to give 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11β-methoxy-3α-benzoate (**14b**) (86 mg, 96%) as a colorless oil: $[\alpha]_D^{25}=-0.4°$ (c=2.75 in $CHCl_3$); IR: 1718 and 1602 cm^{-1}; ^1H NMR (400 MHz, C_6D_6): δ/ppm 8.17 (2H, dd, J=0.8, 8.4 Hz), 7.09 (1H, dt, J=0.8, 8.4 Hz), 7.02 (2H, t, J=8.4 Hz), 6.76 (1H, s), 5.49 (1H, m), 5.32 (1H, dd, J=4.4, 15.3 Hz), 5.27 (1H, dd, J=5.2, 15.3 Hz), 4.19 (1H, d, J=4.8 Hz), 3.19 (3H, s), 2.93 (1H, dd, J=5.4, 17.1 Hz), 2.88–2.78 (2H, m), 2.65 (1H, d, J=13.7 Hz), 2.67–2.59

(1H, m), 2.54 (1H, dd, *J*=7.8, 12.2 Hz), 2.15 (1H, dt, *J*=6.8, 10.3 Hz), 2.07 (3H, s), 2.07–2.00 (1H, m), 1.98–1.85 (4H, m), 1.64 (1H, dq, *J*=5.7, 6.8 Hz), 1.55–1.47 (2H, m), 1.39 (1H, dd, *J*=5.4, 14.2 Hz), 1.29 (1H, q, *J*=7.8 Hz), 1.23 (3H, d, *J*=6.8 Hz), 1.03 (3H, d, *J*=6.8 Hz), 0.94 (3H, s), 0.93 (6H, d, *J*=7.8 Hz); ^{13}C NMR (100 MHz, C_6D_6): δ/ppm 165.7, 137.6, 137.3, 136.1, 134.9, 133.1, 132.7, 132.4, 131.5, 130.4, 129.9, 128.5, 123.9, 76.4, 71.3, 55.6, 55.5, 53.3, 43.5, 41.2, 40.6, 33.6, 33.4, 29.8, 28.0, 27.5, 24.8, 21.5, 20.4, 20.2, 18.2, 15.9, 12.9; HREIMS: Found *m/z* 528.3592, $C_{36}H_{48}O_3$ requires *m/z* 528.3600.

Synthesis of 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11α-methoxy-3α-ol (IV). A solution of 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11α-methoxy-3α-benzoate (**14a**) (95 mg, 0.18 mmol) and K_2CO_3 (1.0 g, 0.73 mmol) in a mixed solvent of MeOH (5 mL) and THF (1.5 mL) was stirred at room temperature for 30 min and then filtered through Celite. The filtrate was poured into water and extracted with EtOAc (3×10 mL). The combined extracts were dried over Na_2SO_4 and concentrated to give a crude product, which was then separated by column chromatography [Si gel, EtOAc–hexane (1:4)] to give citreoanthrasteroid (**IV**)[10] (75 mg, 98%) as a colorless oil: $[\alpha]_D^{25}=-18°$ (*c*=1.35 in $CHCl_3$); IR: 3386 and 1604 cm^{-1}; ^{1}H NMR (400 MHz, C_6D_6), δ/ppm 6.74 (1H, s), 5.28 (1H, dd, *J*=5.4, 15.6 Hz), 5.23 (1H, dd, *J*=5.9, 15.6 Hz), 4.77 (1H, dd, *J*=7.3, 7.8 Hz), 3.81 (1H, m), 3.12 (3H, s), 3.05 (1H, dd, *J*=6.3, 10.7 Hz), 2.86–2.77 (2H, m), 2.61 (1H, dt, *J*=6.8, 8.3 Hz), 2.45– 2.37 (2H, m), 2.22 (3H, s), 2.03 (1H, m), 1.96–1.88 (4H, m), 1.72 (1H, m), 1.60–1.46 (3H, m), 1.43–1.40 (2H, m), 1.13 (3H, d, *J*=7.2 Hz), 1.01 (3H, d, *J*=7.2 Hz), 0.92 (3H, d, *J*=6.8 Hz), 0.471 (3H, s); ^{13}C NMR (100 MHz, C_6D_6): δ/ppm 139.2, 136.4, 136.0, 135.3, 132.4, 131.7, 131.2, 123.8, 75.2, 67.8, 56.2, 53.8, 50.6, 45.3, 44.9, 43.5, 41.2, 37.1, 33.6, 31.6, 29.7, 28.2, 24.5, 21.3, 20.4, 20.1, 18.2, 15.4, 14.0; HREIMS: Found *m/z* 424.3305, $C_{29}H_{44}O_2$ requires *m/z* 424.3339.

Synthesis of 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11β-methoxy-3α-ol. (15). A solution of 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11β-methoxy-3α-benzoate (**14b**) (82 mg, 0.16 mmol) and K_2CO_3 (1.0 g, 0.73 mmol) in a mixed solvent of MeOH (5 mL) and THF (1.5 mL) was stirred for 30 min, and then filtered through Celite. The filtrate was poured into water and extracted with EtOAc (3×10 mL). The combined extracts were dried over Na_2SO_4 and concentrated to give a crude product, which was then separated by column chromatography [Si gel, EtOAc–hexane (1:4)] to afford 1(10→6)abeo-ergosta-5,7,9,22-tetraen-11β-methoxy-3α-ol (**15**) (66 mg, 99%) as a colorless oil: $[\alpha]_D^{25}=+13.3°$ (*c*=1.00 in $CHCl_3$) IR: 3399 and 1602 cm^{-1}; ^{1}H NMR (400 MHz, C_6D_6): δ/ppm 6.74 (1H, s), 5.31 (1H, dd, *J*=2.9, 15.1 Hz), 5.27 (1H, dd, *J*=3.4, 15.1 Hz), 4.20 (1H, d, *J*=4.9 Hz), 3.61 (1H, m), 3.20 (3H, s), 2.82 (1H, dd, *J*=5.4, 16.6 Hz), 2.77 (1H, dt, *J*=4.9, 17.6 Hz), 2.64 (1H, d, *J*=14.2 Hz), 2.62–2.58 (1H, m), 2.53 (1H, dd, *J*=7.3, 11.2 Hz), 2.45 (1H, dd, *J*=8.3, 16.1 Hz), 2.13 (3H, s), 2.13 (1H, m), 2.03–1.91 (3H, m), 1.75 (1H, m), 1.64–1.47 (4H, m), 1.39 (1H, dd, *J*=4.9, 14.2 Hz), 1.28 (1H, dq, *J*=3.4, 10.7 Hz), 1.23 (3H, d, *J*=6.3 Hz), 1.02 (3H, d, *J*=6.8 Hz), 0.93 (6H, d, *J*=7.8 Hz), 0.91 (3H, s); ^{13}C NMR (100 MHz, C_6D_6): δ/ppm 137.5, 137.1, 136.1, 135.3, 132.9, 132.4, 131.7, 123.8, 76.5, 67.9, 55.6, 53.3, 43.5, 41.20, 41.18, 40.6, 37.4, 33.6, 32.1, 29.8, 28.8, 24.8, 21.5, 20.4, 20.2, 18.2, 16.2, 12.9; HREIMS: Found *m/z* 424.3371, $C_{29}H_{44}O_2$ requires *m/z* 424.3339.

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