Variation in Cytostatic Constituents of a Sponge-Derived *Gymnascella dankaliensis* by Manipulating the Carbon Source

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The *Halichondria* sponge-derived fungus, *Gymnacella dankaliensis*, was cultured in two different media conditions. A modified malt extract medium containing soluble starch instead of glucose resulted in two extremely unusual steroids, dankasterones A (**2**) and B (**3**), while four additional unusual steroids, gymnasterones A (**4**), B (**5**), C (**6**), and D (**7**), were isolated from the original malt extract medium. Their stereostructures have been established on the basis of spectroscopic analyses along with X-ray crystal structure analyses, modified Mosher's method, CD exciton method, and a chemical transformation. All the steroids except for **4** exhibited significant growth inhibition against the murine P388 cancer cell line. Dankasterone A (**2**) also exhibited potent growth inhibition against human cancer cell lines.

The challenge to broaden chemoprofiles of microorganisms that produce bioactive secondary metabolites is an important facet in natural products research. The OSMAC (one strain, many compounds) approach is one of the concise methods to optimize diversity of secondary metabolites by variation of culture conditions and mutagenesis.[1] Noteworthy examples of this concept include our discovery of three different classes of cytostatic metabolites produced by a *Penicillium* sp. separated from a marine alga. Saltwater culture of this *Penicillium* strain resulted in two different classes of alkaloids, communesins[2] and penochalasins,[3] whereas polyketide penostatins[4] were isolated independently from non-saltwater culture. In addition, there is an impressive article reported by Crews *et al.* in which an actin inhibitor, jasplakinolide, added to the culture medium activated biosynthesis of new chaetoglobosins isolated from a sponge-derived *Phomospis asparagi*.[5] Each of these discoveries has shed light on new modalities of fungal bioactive metabolites research.

Our focus has been on exploring anticancer lead compounds from microorganisms separated from marine environments, and important successes of this research encompass the finding of potent cytostatic polyketide-alkaloids, gymnastatins A (**1**) to H.[6] The gymnastatins were isolated from a sponge-derived *Gymnascella dankalisensis* by following our cell-based assay results.[7] Our attention on cytostatic substances from *G. dankaliensis* next shifted to several previously unexamined cytotoxic semipure fractions, which showed significant growth inhibition against the murine P388 lymphocytic leukemia cell line. Interestingly, those fractions appeared to contain no gymnastatins but did contain steroid-type compounds on the basis of the rationale discussed below. In addition, we have conducted an investigation of the metabolite variation pattern for this fungal strain by manipulating media components. The achievement of these themes was realized by the discovery of unusual steroids designated dankasterones A (**2**) and B (**3**) and gymnasterones A (**4**), B (**5**) C (**6**), and D (**7**). Gymnasterones were isolated as major steroidal components by the follow-up study of the remaining active fractions obtained from the malt–glucose–yeast media,[6] while dankasterones were mainly isolated from the MeOH mycelia extract obtained from a new media condition, in which one component of the original medium, glucose, was replaced by soluble starch. We report herein the isolation and stereostructure determination of the novel steroids **2**, **3**, **4**, **5**, **6**, and **7**, of which the stereostructure for **2** and partial stereostructures of **4** and **5** have been briefly reported in the preliminary forms.[8,9] The discussion below also includes cancer cell growth inhibitory properties of the steroids isolated in this study.

Results and Discussion

The fungal strain was cultured at 27 °C for 28 days in two kinds of culture media (types A and B). The media type A contained 1% malt extract, 1% soluble starch, and 0.05% peptone in artificial seawater adjusted to pH 7.5. The 1% soluble starch in the media type A was replaced by 1% glucose in media type B as reported previously.[6] The MeOH extracts of the mycelia grown in media types A and B were separated by bioassay (PS)-guided fractionation using Sephadex LH-20 followed by silica gel column chromatography to afford several cytostatic fractions, which were eluted with 0.5%–2% MeOH in CH_2Cl_2. We have already examined the most potent cytostatic fractions (1%–2% MeOH in CH_2Cl_2) that resulted in the isolation of gymnastatins. The compounds discussed below were isolated from the unexamined active fractions that were eluted with more nonpolar solvent (0.5%–1% MeOH in CH_2Cl_2) than those containing gymnastatins from the silica gel column chromatography. The ^{1}H NMR spectra of the active fractions showed many singlet and doublet methyls at δ_H 0.5–1.5, a hump from overlapped methylenes and methines at δ_H 1.0–2.5, and *trans* sp^2-methines at δ_H 5.0–5.5. On the basis of the observed proton resonances, the active fractions were expected to be rich in steroid-type compounds, which was confirmed by isolation of dankasterones A (**2**) and B (**3**) and gymnasterones A (**4**), B (**5**), C (**6**), and D (**7**) by final HPLC purification.

Detailed structure elucidation began with dankasterone A (**2**) isolated from media type A. The molecular formula of **2** was established as $C_{28}H_{40}O_3$ by HREIMS. The IR spectrum showed bands at 1695, 1682, and 1607 cm^{-1}, characteristic of unconjugated and conjugated ketones, and a double bond. A close inspection of the proton and carbon NMR spectra of **2** (Table 1) by DEPT and HSQC (^{1}H–^{13}C COSY) experiments revealed the following five functional groups: (1) six methyl groups including two tertiary methyls (C-18 and C-19) and four secondary methyls (C-21, C-26, C-27, C-28), (2) seven methylenes (C-1, C-2, C-7, C-11, C-12, C-15, and C-16), (3) one disubstituted (C-22 and C-23) and one

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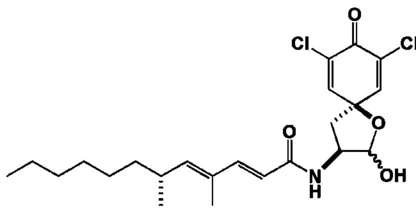
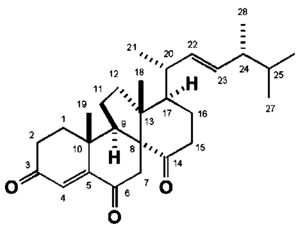
Table 1. ^{1}H and ^{13}C NMR Data of Dankasterones A (**2**) and B (**3**) in $CDCl_3$

	2					3				
position	δ_H (mult, *J* in Hz)	δ_C (type)	^1H–^1H COSY	HMBC (C)	NOESY	δ_H (mult, *J* in Hz)	δ_C (type)	^1H–^1H COSY	HMBC (C)	NOESY
1α	2.08 (td, 13.2, 5.1)	38.9 (CH_2)	1β, 2α, 2β	3, 10, 19	1β, 2α, 9	1.54 (td, 13.2, 5.7)	32.7 (CH_2)	1β, 2α, 2β	2, 10, 19	1β, 2α
1β	2.03 (m)		1α, 2α, 2β	2, 3, 5, 10, 19	1α, 2α, 2β, 9, 11α, 19	1.31 (ddt, 13.2, 6.9, 2.5)		1α, 2α, 2β, 5	3, 5	1α, 2α, 2β, 9
2α	2.48 (dt, 17.6, 5.1)	34.3 (CH_2)	1α, 1β, 2β	1, 3, 10	1α, 2β	2.21 (ddt, 13.0, 5.7, 2.2)	36.8 (CH_2)	1α, 1β, 2β	10	1α, 1β, 2β
2β	2.53 (ddd, 17.6, 13.2, 6.0)		1α, 1β, 2α	1, 3, 10	1β, 2α, 19	2.29 (td, 13.0, 6.9)		1α, 1β, 2α	1, 3	1β, 2α, 4β, 19
3		199.1 (qC)					207.6 (qC)			
4	6.36 (s)	126.5 (CH)		2, 5, 6, 10		2.83 (dt, 16.2, 1.6)	35.8 (CH_2)	4β, 5	3, 5, 10	4β, 5
						2.19 (dd, 16.2, 6.2)		4α, 5	3, 5, 6, 10	2β, 4α, 5, 19
5		156.1 (qC)				2.89 (m)	50.0 (CH)	1β, 4α, 4β	1, 3, 4, 6, 10	4α, 4β, 7β, 11β, 19
6		200.0 (qC)					208.5 (qC)			
7α	2.66 (dd, 16.8, 1.3)	40.8 (CH_2)	7β, 9	5, 6, 9, 13, 14	7β, 16β, 18	2.95 (dd, 13.2, 1.8)	40.0 (CH_2)	7β, 9	5, 6, 8, 9, 14	7β, 18
7β	2.50 (d, 16.8)		7α	6, 8, 9, 13, 14	7α, 18	1.95 (d, 13.2)		7α	6, 8, 14	5, 7α, 18
8		62.2[a] (qC)					65.6[b] (qC)			
9	2.81 (td, 9.0, 1.3)	49.4 (CH)	7α, 11α, 11β	1, 7, 10, 11, 13, 14, 19	1α, 1β, 11α, 15, 17	3.05 (td, 9.6, 1.8)	53.2 (CH)	7α, 11α, 11β	5, 7, 8, 11	1β, 11α, 15α, 17, 19
10		36.0 (qC)					40.5 (qC)			
11α	2.02 (m)	25.1 (CH_2)	11β, 12α, 12β	13	1β, 11β, 12α, 17	2.31 (m)	25.5 (CH_2)	9, 11β, 12α, 12β		9, 11β
11β	1.85 (m)		11α, 12α, 12β	9	11α, 18, 19	2.10 (m)		9, 11α, 12α, 12β		5, 11α, 19
12α	1.77 (dt, 13.0, 7.2)	38.3 (CH_2)	11α, 11β, 12β	8, 13, 17, 18	11α, 12β, 17, 20	2.14 (m)	34.1 (CH_2)	11α, 11β, 12β	11, 13, 18	12β, 20
12β	1.71 (m)		11α, 11β, 12α	17	12α	1.65 (m)		11α, 11β, 12α	8, 9	12α, 18
13		54.0[a] (qC)					60.1[b] (qC)			
14		214.8 (qC)					214.7 (qC)			
15α	2.48 (2H, m)	37.9 (CH_2)	16α, 16β	16	16α, 16β, 17	2.78 (ddd, 13.0, 12.8, 5.9)	38.5 (CH_2)	15β, 16α, 16β	14, 16, 17	9, 15β, 17
15β						2.36 (ddd, 13.0, 4.3, 2.5)		15α, 16α, 16β	14, 17	15α, 16α, 16β
16α	1.90 (m)	23.2 (CH_2)	15, 16β, 17		15, 16β, 17, 21	1.99 (m)	27.2 (CH_2)	15α, 15β, 16β, 17	13, 17	15β, 16β, 18
16β	1.69 (m)		15, 16α, 17		7α, 15, 16α, 18	1.63 (m)		15α, 15β, 16α	13	15β, 16α
17	1.47 (dd, 13.2, 4.2)	49.3 (CH)	16α, 16β, 20		9, 11α, 12α, 15, 16α, 20, 21	1.95 (m)	45.4 (CH)	16α	13, 18, 21	9, 15α, 20, 21
18	0.98 (s)	17.1 (CH_3)		8, 12, 13, 17	7α, 7β, 12β, 16β, 20	0.75 (s)	15.2 (CH_3)		8, 12, 13, 17	7α, 7β, 12β, 20
19	1.26 (s)	24.0 (CH_3)		1, 5, 9, 10	1β, 2β, 11β	1.27 (s)	23.4 (CH_3)		1, 5, 9, 10	2β, 4β, 5, 11β
20	2.42 (m)	37.2 (CH)	17, 21	22, 23	12α, 17, 18, 21	2.42 (m)	36.9 (CH)	20, 21	16, 17, 21, 22, 23	12α, 17, 18, 21
21	1.09 (d, 6.8)	23.6 (CH_3)	20	17, 20, 22	16α, 17, 20	1.14 (d, 6.9)	24.1 (CH_3)	20	17, 20, 22	17, 20
22	5.25 (dd, 15.1, 6.8)	132.3 (CH)	20, 23	20	17, 18, 20, 21, 24, 25, 26, 28	5.22 (dd, 15.3, 6.9)	132.0 (CH)	20, 23	21	17, 18, 20, 21, 24, 25, 26, 28
23	5.29 (dd, 15.1, 6.8)	135.1 (CH)	22, 24	24	17, 18, 20, 21, 24, 25, 26, 28	5.25 (dd, 15.3, 6.8)	135.2 (CH)	22, 24	24	17, 18, 20, 21, 24, 25, 26, 28
24	1.88 (m)	43.2 (CH)	23, 25, 28	22, 23	22, 23, 25, 26, 27, 28	1.84 (m)	43.2 (CH)	23, 25, 28	22, 23, 25, 28	22, 23, 25, 26, 27, 28
25	1.47 (octet, 6.8)	33.0 (CH)	24, 26, 27	24	22, 23, 24, 26, 27, 28	1.45 (m)	33.0 (CH)	24, 26, 27	23, 24, 26, 27, 28	22, 23, 24, 26, 27, 28
26	0.81 (d, 6.8)	19.7 (CH_3)	25	24, 25, 27	22, 23, 24, 25, 27, 28	0.79 (d, 6.8)	19.7 (CH_3)	25	24, 25, 27	22, 23, 24, 25, 27, 28
27	0.84 (d, 6.8)	20.0 (CH_3)	25	24, 25, 26	22, 23, 24, 25, 26, 28	0.81 (d, 6.8)	20.0 (CH_3)	25	24, 25, 26	22, 23, 24, 25, 26, 28
28	0.91 (d, 6.8)	17.6 (CH_3)	24	23, 24, 25	22, 23, 24, 25, 26, 27	0.88 (d, 6.8)	17.5 (CH_3)	24	23, 24, 25	22, 23, 24, 25, 26, 27

[a,b] Assignments can be switched.



1



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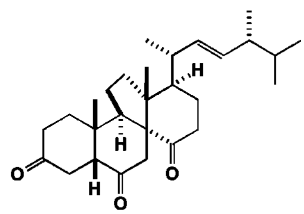

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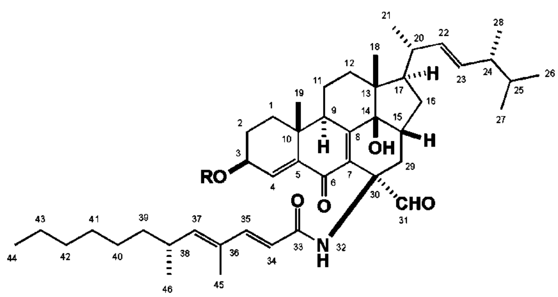
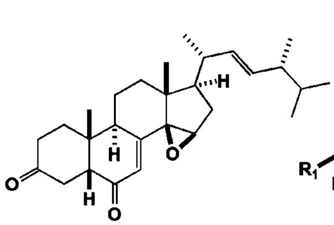
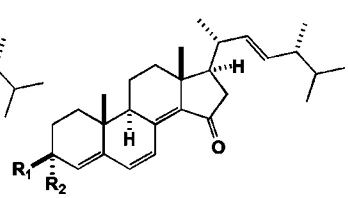


4: R=H
4a: R=*p*BrBz



5



6: R_1 = OH, R_2 = H
7: R_1, R_2 = O

trisubsituted (C-4 and C-5) double bond, (4) five sp^3-methines (C-9, C-17, C-20, C-24, and C-25), (5) two sp^3-quaternary carbons (C-10 and C-13), and three ketones including two unsaturated (C-3 and C-6) and one saturated ketone (C-14). The ^{1}H–^{1}H COSY analysis of **2** led to three spin systems (C-1–C-2, C-9–C-11–C-12, and C-15–C-17–C-20–C-28) as shown by boldfaced lines in Figure 1, which were supported by HMBC correlations. The geometry of the disubstituted double bond (C-22–C-23) in the side chain was deduced to be *trans* from the large coupling constant ($J_{22,23}$ = 15.1 Hz) of the olefinic protons. The connection of these spin systems and the remaining functional groups was determined on the basis of the key HMBC correlations summarized in Figure 1. Thus, the planar structure of **2** was assembled by integration of the data from each of former experiments.

The stereochemistry and conformation of **2** were established by detailed analysis of NOESY data and vicinal proton coupling constants (Table 1). The observation of NOEs from H-19 to H-1β and H-2β and the large coupling constant ($J_{1\alpha,2\beta}$ = 13.2 Hz) between H-1α and H-2β suggested that the A ring exists in a twist-chair conformation with H-2β and 10-CH_3 in a co-pseudoaxial arrangement. NOEs from H_3-18 to H-7α, from H-1α to H-9, and from H_3-19 to H-11β implied that the B ring exists in a twist-boat conformation with 10-CH_3 and H-7β in a co-pseudoaxial arrangement, and 10-CH_3 is arranged *trans* to H-9 in a pseudoaxial arrangement. In addition, NOEs from H_3-18 to H-16β, and from

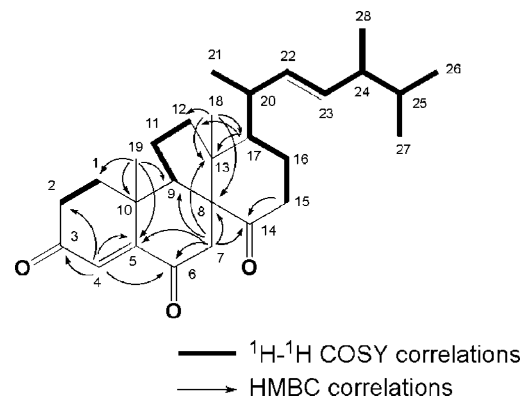


Figure 1. Selected ^{1}H–^{1}H COSY and HMBC correlations in **2**.

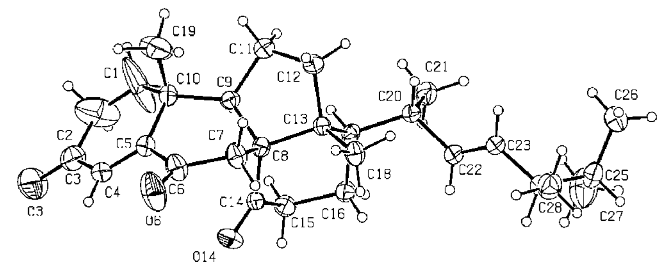


Figure 2. X-ray structure for **2**.

H-15α to H-17, implied that the D ring exists in a chair conformation with 13-CH_3 and H-16β in a co-axial arrangement, which are arranged *trans* to H-15α and H-17 in a co-axial arrangement. Although two protons of C-15 were observed as an overlapping signal, a proton showing an NOE to H-17 must be H-15α. An NOE between H-17 and H-9 indicated these protons to be on the same side. In order to determine the configuration of the 20- and 24-positions in the side chain, an X-ray crystal structure analysis was carried out for a single crystal of **2**.[10] The result obtained (Figure 2) allowed assignment of the configuration of the side chain and confirmation of the configuration of the other asymmetric centers.[11] It should be mentioned that the A ring of **2** exists in a planar conformation in crystalline state, while in a twist-chair conformation in solution state ($CDCl_3$) as mentioned above.

The next compound to be characterized was dankasterone B (**3**), possessing a molecular formula ($C_{28}H_{42}O_3$) that contained two more protons than dankasterone A (**2**) as deduced from HREIMS. The general features of the ^{1}H and ^{13}C NMR spectra closely resembled those of **2** except that the signals for the trisubstituted double bond in **2** were replaced by those of an sp^3-methylene (C-4) and an sp^3-methine (C-5) in **3** and the C-3 signal in **3** appeared shifted downfield by 8.5 ppm relative to **2**. This evidence led to the planar structure of **3**, which was supported by the detailed analysis of ^{1}H–^{1}H COSY and HMBC correlations (Table 1). The A and B rings were determined to be in a chair conformation on the basis of NOEs from H_3-19 to H-2β, H-4β, and H-5, from H-2β to H-4β, from H-5 to H-7β and H-11β, and from H_3-18 to H-7α and H-7β and the large and small values of coupling constants between H-1α and H-2β ($J_{1\alpha,2\beta}$ = 13.2 Hz) and H_2-4 and H-5 ($J_{4\alpha,5}$ = 1.6 Hz and $J_{4\beta,5}$ = 6.2 Hz). These NMR data further implied that the A/B ring conjunction is *cis*. The conformation of the D ring and stereochemistry of the side chain were determined to be same as those of **2** on the basis of NOEs and agreements of the ^{1}H and ^{13}C NMR data of **3** with those of **2** (Table 1). Thus the stereostructure of dankasterone B was determined as **3**.

Attention shifted next to the characterization of compounds isolated from media type B. Gymnasterone A (**4**) had a molecular formula of $C_{45}H_{67}NO_5$ established from HREIMS. The IR spectrum showed bands at 3339, 1729, 1658, and 1613 cm^{-1}, characteristic

Table 2. ^{1}H and ^{13}C NMR Data of Gymnasterone A (**4**) in $CDCl_3$

position	δ_H (mult, *J* in Hz)	δ_C (type)	^{1}H–^{1}H COSY	HMBC (C)	NOESY
1α	1.52 (m)	34.5 (CH_2)	1*β*, 2α, 2*β*	2, 3, 9, 19	1*β*, 3, 9
1*β*	1.90 (m)		1α, 2α, 2*β*, 3	2, 3, 5, 10, 19	1α, 19
2α	2.10 (m)	28.0 (CH_2)	1α, 1*β*, 2*β*, 3	3, 4, 10	2*β*, 3
2*β*	1.54 (m)		1α, 1*β*, 2α, 3	3, 10	2α, 19
3	4.34 (ddd, 8.0, 6.0, 2.0)	67.7 (CH)	1*β*, 2α, 2*β*, 4		1α, 2α, 4
4	6.72 (t, 2.0)	137.3 (CH)	3	2, 5, 6, 10	3
5		142.3 (qC)			
6		186.8 (qC)			
7		129.4 (qC)			
8		162.3 (qC)			
9	2.45 (dd, 13.0, 3.8)	48.4 (CH)	11α, 11*β*	1, 5, 7, 8, 10, 11, 12, 19	1α, 11α, 11*β*, 12α, 17
10		38.6 (qC)			
11α	1.50 (m)	17.5 (CH_2)	9, 11*β*, 12α, 12*β*	8, 9, 12	9, 11*β*
11*β*	2.14 (m)		9, 11α, 12α, 12*β*	8, 9, 12	9, 11α, 19
12α	1.69 (m)	36.7 (CH_2)	11α, 11*β*, 12*β*	9, 11, 13, 14, 17, 18	9, 17
12*β*	1.76 (m)		11α, 11*β*, 12α	9, 11, 13, 18	18
13		44.3 (qC)			
14		77.0 (qC)			
15	2.48 (m)	38.7 (CH)	16α, 16*β*, 29α, 29*β*	30	16*β*, 18, 29*β*, 14-OH
16α	1.10 (m)	33.6 (CH_2)	15, 16*β*, 17	13, 15, 17, 29	16*β*, 31
16*β*	1.59 (m)		15, 16α, 17	14, 15, 17, 20	15, 16α
17	1.26 (m)	51.8 (CH)	16α, 16*β*, 20	12, 13, 16, 20, 21, 22	9, 12α, 21
18	1.14 (s)	18.7 (CH_3)		12, 13, 14, 17	12*β*, 15, 20, 14-OH
19	1.05 (s)	19.1 (CH_3)		1, 5, 9, 10	1*β*, 2*β*, 11*β*
20	2.13 (m)	40.3 (CH)	17, 21, 22	17, 21, 22, 23	18, 21, 23
21	1.03 (d, 6.8)	21.7 (CH_3)	20	17, 20, 22	17, 20
22	5.06 (dd, 15.1, 8.7)	134.4 (CH)	20, 23	17, 20, 21, 23, 24	24, 28
23	5.24 (dd, 15.1, 8.0)	133.6 (CH)	22, 24	20, 22, 24, 25, 28	20, 25, 26, 27
24	1.83 (m)	42.8 (CH)	28	22, 23, 25, 26, 27, 28	22, 25, 26, 27, 28
25	1.46 (octet, 6.8)	33.2 (CH)	26, 27	23, 24, 26, 27, 28	23, 24, 26, 27, 28
26	0.80 (d, 6.8)	19.9 (CH_3)	25	24, 25, 27	23, 24, 25, 27, 28
27	0.82 (d, 6.8)	19.6 (CH_3)	25	24, 25, 26	23, 24, 25, 26, 28
28	0.89 (d, 6.8)	17.6 (CH_3)	24	23, 24, 25	22, 24, 25, 26, 27
29α	1.74 (dd, 15.0, 3.2)	30.1 (CH_2)	15, 29*β*	7, 14, 15, 16, 30, 31	29*β*, 31
29*β*	2.52 (dd, 15.0, 6.0)		15, 29α	14, 15, 16, 30, 31	15, 29α, 14-OH
30		59.3 (qC)			
31	9.18 (s)	194.1 (CH)		30	16α, 29α, 32
32	7.12 (s)			7, 30, 31, 33	31
33		165.5 (qC)			
34	5.72 (d, 15.1)	117.8 (CH)	35	33, 35, 36	45
35	7.11 (d, 15.1)	146.8 (CH)	34	33, 34, 36, 37, 45	37
36		130.8 (C)			
37	5.60 (d, 9.6)	148.1 (CH)	38	35, 38, 39, 45, 46	35, 39a, 46
38	2.48 (m)	33.0 (CH)	37, 46		39a, 39b, 45, 46
39a	1.21 (m)	37.3 (CH_2)		46	37, 38, 39b, 46
39b	1.33 (m)			37, 38, 40, 46	38, 39a, 46
40	1.20 (m)	27.4 (CH_2)		38, 41	
41	1.22 (m)	29.4 (CH_2)		42	
42	1.26 (m)	31.8 (CH_2)		40, 41, 43, 44	44
43	1.22 (m)	22.6 (CH_2)		41	44
44	0.87 (t, 6.8)	14.1 (CH_3)	43	42, 43	42, 43
45	1.72 (s)	12.5 (CH_3)		35, 36, 37	34, 38
46	0.95 (d, 6.8)	20.5 (CH_3)	38	37, 38, 39	37, 38, 39a, 39b
3-OH	nd[a]				
14-OH	6.19 (s)			13, 14	15, 18, 29*β*

[a] Not detected.

of a hydroxy group and/or an amine, an aldehyde, conjugated amide and ketone, and a double bond. A close inspection of the ^{1}H and ^{13}C NMR spectra (Table 2) of **4** by DEPT and HSQC (^{1}H–^{13}C COSY) experiments revealed the presence of the following functional groups: nine methyl groups including one vinylic methyl, one primary methyl, five secondary methyls, and two tertiary methyls, 11 methylenes, eight sp^3-methines including one hydroxyl methine, four quaternary sp^3-carbons including one oxygenated carbon, two disubstituted, two trisubstituted, and one tetrasubstituted double bonds, one secondary amide, one aldehyde, and one ketone in a cross-conjugated dienone system [δ_C 186.8 (C-6)].[12] The ^{1}H–^{1}H COSY analysis of **4** led to six substructures as shown by boldfaced lines in Figure 3, which were supported by HMBC correlations. The connection of the substructure and the remaining functional groups was determined on the basis of HMBC correlations shown in Figure 3. The connection of C-6 and C-7 was deduced from the evidence that C-6 is a ketone in a cross-conjugated cyclohexadienone system. This evidence led to the planar structure of **4**.

The stereochemistry of **4** was deduced from analysis of the NOESY data and vicinal proton coupling constants (Figure 4). The observation of NOEs from H_3-19 to H-2*β* and from H-1α to H-3 indicated that the A ring exists in a twist-chair conformation with 10-CH_3 and H-2*β*, and H-1α and H-3, respectively, in co-pseudoaxial arrangements, implying 3-OH to be *cis* to 10-CH_3. In a NOESY experiment in $CDCl_3$, an NOE between H-1α and H-3 could not be distinguished from an NOE between H-2*β* and H-3 because the signals of H-1α (δ_H 1.52, m) and H-2*β* (δ_H 1.54, m) appeared almost overlapped. Therefore, the configuration of 3-OH was previously reported as α, based on the coupling constant between H_2-2 and H-3.[9] When the NOESY for **4** was measured in

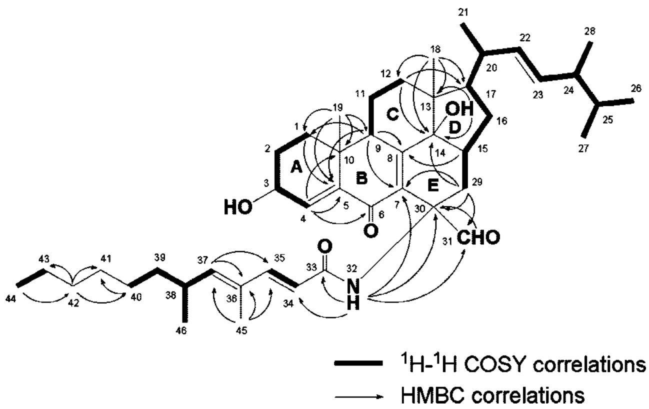


Figure 3. Selected ^{1}H–^{1}H COSY and HMBC correlations in **4**.

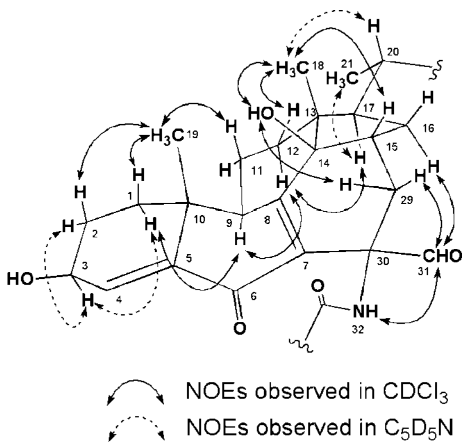


Figure 4. Key NOE correlations for **4**.

pyridine-d_5, the signals of H-1α (δ_H 1.49) and H-2β (δ_H 1.76) appeared separately, and an NOE between H-1α and H-3 was observed (Table S1), implying that the configuration of 3-OH must be revised as β. In addition, an NOE between H-1α and H-9 indicated that 10-CH_3 is arranged *trans* to H-9 in a pseudoaxial arrangement. NOEs from H_3-18 to H-12β, H-15, and 14-OH and from H-9 to H-12α and H-17 and the large coupling constant ($J_{9,11\beta}$ = 13.0 Hz) between H-9 and H-11β indicated that the C ring exists in a twist-boat conformation with 13-CH_3 and 14-OH in a *cis* orientation and H-9 is oriented *trans* to 13-CH_3 and on the same side as H-17. In addition, the observation of NOEs from H-31 to H-16α and from H-29β to 14-OH suggested that the E ring exists in a twist-chair conformation with the formyl group in a pseudoaxial arrangement, which is oriented *trans* to 14-OH in a pseudoaxial arrangement. The geometry of the Δ^{22}-double bond in the side chain (C-20–C-28) was deduced as *trans* from the large coupling constant ($J_{22,23}$ = 15.1 Hz) of the olefinic protons. The geometry of the diene (Δ^{34}- and Δ^{36}-double bonds) in the conjugated amide moiety (N-32–C-46) was deduced as *trans-s-trans* in three ways: (1) the large coupling constant between H-34 and H-35 ($J_{34,35}$ = 15.1 Hz), (2) a chemical shift [δ_C 12.5 (C-45)] of the ^{13}C NMR signal of a vinylic methyl,[13] and (3) NOEs from H-34 to H_3-45 and from H-35 to H-37.

The configuration of the chiral centers (C-20 and C-24) in the side chain of gymnasterone A (**4**) was determined by comparison of the ^{1}H and ^{13}C NMR data of the side chain of **4** with those of gymnasterone D (**6**), of which the configuration was determined by the X-ray analysis as described below. The absolute configuration of the chiral center (C-38) of **4** was assumed by agreement of the ^{1}H and ^{13}C NMR data of the conjugated amide moiety of **4** with those of gymnastatin A (**1**), previously reported,[6b] and by a consideration of the co-occurrence of **1**. The absolute stereochemistry of the steroidal part of **4** was supported by application of the CD exciton chirality method to the *p*-bromobenzoate **4a**. The CD spectrum of **4a** showed a typical split by two excitons. The negative first Cotton effect at 266 nm ($\Delta\varepsilon$ −24.6) and a second positive Cotton effect at 240 nm ($\Delta\varepsilon$ +16.9) indicated that the conjugated amide moiety at C-30 and the *p*-bromobenzoyl group at C-3 are twisted in a counterclockwise direction, implying that the configuration of the chiral centers at C-3 and C-30 is 3*S* and 30*R*.[14] The above-summarized evidence allowed assignment of the absolute stereostructure of **4**.

The second new compound from media type B was gymnasterone B (**5**), having a molecular formula of $C_{28}H_{40}O_3$ established by HREIMS. The ^{1}H and ^{13}C NMR spectra of **5** closely resembled those of **4** except that the Δ^4-olefin, the methylene (C-29), and quaternary sp^3-carbon (C-30) of the E ring, the formyl group (C-31), and the conjugated amide moiety (N-32–C-46) in **4** were missing from **5** and two hydroxyl groups in **4** were replaced by a ketone (C-3) and an epoxide (C-14 and C-15, $J_{CH(15)}$ = 183 Hz) in **5**. The planar structure of **5** thus deduced from the 1D NMR spectral analysis was confirmed by analysis of ^{1}H–^{1}H COSY and HMBC correlations (Table 3). The stereochemistry of **5** was established by the NOESY data (Figure 5). The observation of NOEs from H-19 to H-1α, H-1β, and H-5, from H-5 to H-1β, and from H-2α to H-4α and the large coupling constant ($J_{4\alpha,5}$ = 13.5 Hz) between H-5 and H-4α implied that the A ring exists in a chair conformation with 10-CH_3 and H-5 in respective equatorial and axial, consequently, *cis* arrangements. In addition, NOEs from H-9 to H-2α and H-4α indicated H-9 to be arranged *trans* to 10-CH_3 because H-2α and H-4α are *trans* to 10-CH_3, as deduced from the above-mentioned NOEs. NOEs from H-9 to H-12α and H-15, from H_3-18 to H-12β, and from H-12α to H-17 implied that the C ring exists in a twist-chair conformation with H-9 and H-12α in a co-pseudoaxial arrangement, 13-CH_3 with a pseudoaxial arrangement is oriented *trans* to H-9 and *cis* to the C-14–O bond of the epoxide, and H-15 is on the same side as H-9. The geometry of the Δ^{22}-double bond in the side chain was deduced as *trans* from the large coupling constant ($J_{22,23}$ = 15.5 Hz) of the olefinic protons. An attempt to deduce the configuration of the chiral centers (C-20 and C-24) in the side chain of **5** from NMR spectral analysis including NOESY was unsuccessful. However, their configurations were assumed to be the same as for the co-metabolites, gymnasterones A (**4**), C (**6**), and D (**7**).

The structure of gymnasterone C (**6**)[15] was assumed to have a different chromophore from those of **2**–**5** on the basis of strong UV absorbance at 335 and 385 nm. Its molecular formula was assigned as $C_{28}H_{40}O_3$ by HREIMS, which contained one oxygen atom less than that of **5**. The IR spectrum showed bands at 3388, 1697, and 1597 cm^{-1}, characteristic of a hydroxy group, a conjugated ketone, and a double bond. The inspection of the ^{1}H and ^{13}C NMR spectra (Table 4) of **6** revealed that the signals of the α,β-unsaturated ketone system of the B ring and the epoxide in **5** were missing in **6** and the signals of a hydroxymethine (C-3) and disubstituted and tetrasubstituted double bonds (C-6, C-7, C-8, and C-14) appeared additionally in **6**. The position of these functional groups was deduced from analysis of ^{1}H–^{1}H COSY and HMBC correlations (Table 4), leading to planar structure **6** for gymnasterone C.

The observation of NOEs from H-1α to H-3 and from H_3-19 to H-2β and the large coupling constants between H-1α and H-2β ($J_{1\alpha,2\beta}$ = 13.1 Hz) and H-3 and H-2β ($J_{2\beta,3}$ = 9.8 Hz) suggested that the A ring exists in a twist-chair conformation with 10-CH_3 and H-3 in a *trans* dipseudoaxial arrangement and consequently with the 3-hydroxy group in a pseudoequatorial arrangement. NOEs from H-9 to H-1α and H-12α, from H-11β to H_3-19 and H_3-18, and from H-12α to H-17 implied that the C ring exists in a twist-chair conformation with 13-CH_3 and H-9 in a *trans* dipseudoaxial arrangement, and both 10-CH_3 and 13-CH_3 are oriented *cis* to H-9 and H-17. The geometry of the Δ^{22}-double bond in the side chain

Table 3. ^{1}H and ^{13}C NMR Data of Gymnasterone B (**5**) in $CDCl_3$

position	δ_H (mult, *J* in Hz)	δ_C (mult)	^{1}H–^{1}H COSY	HMBC (C)	NOESY
1α	2.14 (ddd, 14.3, 6.2, 2.5)	34.8 (CH_2)	1*β*, 2α, 2*β*	2, 3, 5, 10, 19	1*β*, 11, 19
1*β*	1.61 (td, 14.3, 4.9)		1α, 2α, 2*β*	2, 3, 5, 9, 10	1α, 2*β*, 5, 19
2α	2.53 (ddd, 15.5, 14.3, 6.2)	36.9 (CH_2)	1α, 1*β*, 2*β*	1, 3	2*β*, 4α, 9
2*β*	2.37 (ddd, 15.5, 4.9, 2.5)		1α, 1*β*, 2α	3	1*β*, 2α
3		207.9 (qC)			
4α	2.28 (dd, 15.0, 13.5)	39.6 (CH_2)	4*β*, 5	3, 5, 6, 10	2α, 9
4*β*	2.33 (dd, 15.0, 5.2)		4α, 5	5, 6, 10	
5	2.43 (dd, 13.5, 5.2)	56.1 (CH)	4α, 4*β*, 7	6, 7, 9, 10, 19	1*β*, 19
6		198.2 (qC)			
7	6.06 (d, 2.6)	119.8 (CH)	5	5, 9, 14	15
8		158.6 (qC)			
9	2.93 (ddd, 7.8, 6.8, 2.6)	39.0 (CH)	11	7, 8, 10, 11, 19	2α, 4α, 11, 12α, 15
10		37.4 (qC)			
11	1.81 (2H, m)	20.5 (CH_2)	9, 12α, 12*β*	8, 9	1α, 9, 19
12α	1.68 m	39.0 (CH_2)	11, 12*β*	11, 13, 17, 18	9, 12*β*, 17
12*β*	1.84 m		11, 12α	10, 11, 13, 14, 18	12α, 18
13		45.6 (qC)			
14		71.9 (qC)			
15	3.18 (d, 1.5)	69.0 (CH)	16α	8, 16, 17	7, 9, 12α, 16α, 16*β*
16α	2.03 (ddd, 15.3, 10.0, 1.5)	29.5 (CH_2)	15, 16*β*, 17	13, 17, 20	15, 16*β*, 17, 21
16*β*	2.09 (dd, 15.3, 3.4)		16α, 17	13, 14, 15, 17, 20	15, 16α, 17, 21
17	1.72 m	53.2 (CH)	16α, 16*β*, 20	12, 13, 14, 15, 16, 21	12α, 16α, 16*β*, 18, 20
18	1.13 s	15.8 (CH_3)		12, 13, 14, 17	11, 12*β*, 20, 21
19	1.08 s	22.7 (CH_3)		1, 5, 9, 10	1α, 1*β*, 5, 11
20	2.33 m	38.3 (CH)	17, 21, 22	13, 16, 17, 21, 22, 23	17, 21, 22, 23, 24
21	0.95 (d, 6.8)	23.0 (CH_3)	20	17, 20, 22	17, 18, 20, 22, 23
22	5.28 (dd, 15.5, 8.0)	133.5 (CH)	20, 23	17, 20, 21, 23, 24	18, 20, 21, 23, 24, 26, 27
23	5.20 (dd, 15.5, 8.0)	133.3 (CH)	22, 24	20, 22, 24, 25, 28	18, 20, 21, 22, 24, 26, 27
24	1.92 (m)	43.1 (CH)	28	22, 23, 25, 26, 27, 28	20, 21, 22, 23, 26, 27, 28
25	1.49 (octet, 6.9)	33.1 (CH)	26, 27	23, 24, 26, 27, 28	26, 27, 28
26	0.84 (d, 6.9)	19.7 (CH_3)	25	24, 25, 27	24, 25, 27
27	0.86 (d, 6.9)	20.0 (CH_3)	25	24, 25, 26	24, 25, 26
28	0.96 (d, 6.9)	17.7 (CH_3)	24	23, 24, 25	24, 25, 26, 27

was deduced as *trans* from the large coupling constant ($J_{22,23}$ = 15.8 Hz) of the olefinic protons. Also, the configuration of the chiral center (C-20 and C-24) in the side chain of gymnasterone C (**6**) was determined by agreements of the ^{1}H and ^{13}C NMR data of the side chain of **6** with those of gymnasterone D (**7**), the stereostructure of which was determined by X-ray structure analysis described below. The absolute configuration of **6** was supported by application of the modified Mosher method.[16] The ^{1}H chemical-shift difference between (*R*)- and (*S*)-MTPA esters (**6a** and **6b**) prepared by the standard method shown in Figure 6 suggested the *S* configuration for the asymmetric center at C-3 and, consequently, confirmed the 9*R*, 10*R*, 13*R*, 17*R*, 20*R*, and 24*R* configurations for the other asymmetric centers of gymnasterone C (**6**).

Closely related to **6** was gymnasterone D (**7**),[15] which was assigned a molecular formula with two proton atoms less than that of **6**. The IR spectrum showed bands at 1689, 1687, and 1597 cm^{-1}, characteristic of two conjugated ketones and a double bond. The general features of the ^{1}H and ^{13}C NMR spectra of **7** closely resembled those of **6** except that the signal for the 3-hydroxymethine in **6** was replaced by a conjugated ketone (C-3). This planar structure of **7** was supported by analysis of ^{1}H–^{1}H COSY and gHMBC correlations (Table 4). The NOESY of **7** exhibited correlation patterns similar to **6**, suggesting that the configuration and ring conformation of **7** are the same as those of **6** except for C-3, C-20, and C-24 (Table 4). The configuration of the 20- and 24-positions in the side chain was determined by an X-ray crystal structure analysis for a single crystal of **7**.[17] In the asymmetric unit of the crystal, compound **7** turned out to exist as two independent molecules (Orteps A and B) possessing the same stereostrucure (Figure 7). The X-ray analysis allowed confirmation of the configuration of the other asymmetric centers and the conformation deduced from NOESY data. In addition, compound **7** was derived by oxidation of **6**, supporting the absolute stereochemistry of gymnasterone D (**7**).

The cancer cell growth inhibitory properties of the isolated steroids were examined using the murine P388 lymphocytic leukemia cell line[7] and a disease-oriented panel of 39 human cancer cell lines (HCC panel) in the Japanese Foundation for Cancer

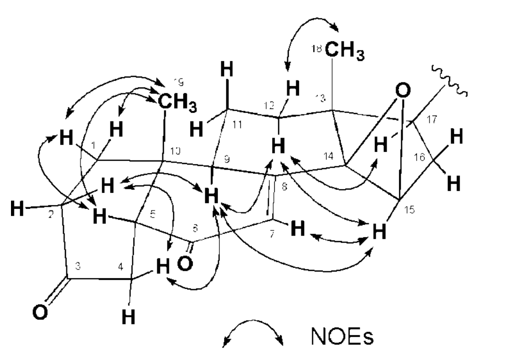
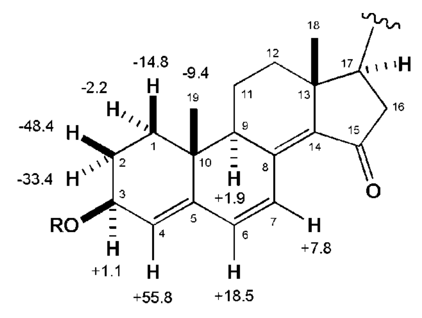


Figure 5. Key NOE correlations for **5**.



6a: R = (*R*)-MTPA
6b: R = (*S*)-MTPA

Figure 6. Proton chemical shift differences ($\Delta\delta = \delta_S - \delta_R$) between the (*R*)- and (*S*)-MTPA esters **6a** and **6b**. $\Delta\delta$ values are expressed in Hz (500 MHz).

Table 4. ^{1}H and ^{13}C NMR Data of Gymnasterones C (**6**) and D (**7**) in $CDCl_3$

position	**6** δ_H (mult, J in Hz)	**6** δ_C (type)	**6** ^{1}H–^{1}H COSY	**6** HMBC (C)	**6** NOESY	**7** δ_H (mult, J in Hz)	**7** δ_C (type)	**7** HMBC (C)	**7** NOESY
1α	1.52 (td, 13.1, 3.0)	33.5 (CH_2)	1β, 2β	2, 3, 9, 10, 19	1β, 2α, 2β, 3, 9	1.89 (td, 13.7, 6.0)	34.0 (CH_2)	2, 3, 9, 10, 19	1β, 2α, 9
1β	1.74 (dt, 13.1, 3.4)		1α, 2α, 2β	2, 3, 5, 10, 19	1α, 2α, 2β, 11β, 19	2.06 (ddd, 13.7, 5.0, 2.5)		2, 3, 5, 10, 19	1α, 2β, 11α, 19
2α	2.10 (m)	28.3 (CH_2)	1β, 2α, 3	4	1α, 1β, 2β, 3	2.49 (dddd, 17.9, 6.0, 2.5, 0.7)	33.9 (CH_2)	1, 3, 10	1α
2β	1.61 (m)		1α, 1β, 3	1, 3	2α, 19	2.55 (ddd, 17.9, 13.7, 5.0)		1, 3, 10	1β, 19
3	4.34 (ddd, 9.8, 6.9, 2.7)	68.0 (CH)	2α, 2β, 4	4, 5	1α, 2α		199.0 (qC)		
4	5.60 (br s)	129.3 (CH)	3	2, 6, 10	6	5.83 (d, 0.7)	125.1 (CH)	2, 6, 10	6
5		144.0 (qC)					161.9 (qC)		
6	6.17 (d, 9.8)	133.4 (CH)	7	4, 5, 6, 8, 10	4, 7	6.31 (d, 9.8)	131.3 (CH)	4, 5, 8, 10	4, 7
7	7.37 (d, 9.8)	124.2 (CH)	6	5, 8, 9, 14	6	7.69 (d, 9.8)	131.5 (CH)	4, 5, 8, 10	6
8		141.5 (qC)					139.0 (qC)		
9	2.23 (m)	46.3 (CH)	11α	1, 5, 7, 10, 19	1α, 11α, 12α	2.36 (dd, 11.2, 6.2)	45.2 (CH)	1, 5, 8, 10, 11, 14, 19	1α, 11α, 12α
10		36.7 (qC)					37.6 (qC)		
11α	1.67 (m)	18.8 (CH_2)	9, 11β, 12α, 12β	8, 9, 13	9, 11β, 12α, 12β	1.76 (m)	18.7 (CH_2)	8, 9, 13	1β, 9, 11β, 12α, 12β, 19
11β	1.58 (m)		11α, 12α, 12β	9, 12, 13	1β, 11α, 12α, 12β, 18, 19	1.64 (m)		10, 12	11α, 18, 19
12α	1.41 (td, 13.0, 3.2)	36.2 (CH_2)	11α, 11β, 12β, 18	9, 11, 13, 17, 18	9, 11α, 11β, 12β, 17	1.44 (td, 13.2, 3.2)	35.6 (CH_2)	9, 11, 13, 17	9, 11α, 12β, 17
12β	2.18 (dt, 13.0, 3.2)		11α, 11β, 12α	9, 11, 13, 14, 18	11α, 11β, 12α, 21	2.24 (dt, 13.2, 3.4)		9, 11, 13, 14, 17, 18	11α, 12α, 17, 18, 21
13		42.4 (qC)					42.6 (qC)		
14		141.5 (qC)					145.3 (qC)		
15		206.8 (qC)					206.5 (qC)		
16α	2.29 (dd, 19.0, 8.0)	42.8 (CH_2)	16β, 17	13, 14, 15, 17	16β, 17	2.33 (dd, 19.2, 8.0)	42.5 (CH_2)	13, 14, 15, 17	16β, 17
16β	2.11 (dd, 19.0, 12.2)		16α, 17	15, 17, 20	16α, 18	2.14 (dd, 19.2, 12.1)		15, 17, 20	16α, 18
17	1.62 (m)	51.2 (CH)	16α, 16β, 20	12, 13, 19, 18, 20, 21, 22	12α, 16α, 21, 22, 23	1.66 (m)	51.0 (CH)	12, 16, 18, 20, 21, 22	12α, 12β, 16α, 21
18	1.06 (s)	20.4 (CH_3)	12α	12, 13, 14, 17	11β, 16β, 20	1.09 (s)	20.0 (CH_3)	12, 13, 14, 17	11β, 12β, 16β
19	0.97 (s)	18.4 (CH_3)		1, 5, 9, 10	1β, 2β, 11β	1.07 (s)	17.0 (CH_3)	1, 5, 9, 10	1β, 2β, 11α, 11β
20	2.22 (m)	39.4 (CH)	17, 21, 22	16, 17, 21, 22, 23	18, 21, 23	2.24 (m)	39.4 (CH)	22, 23	21, 22, 23
21	1.11 (d, 6.6)	21.3 (CH_3)	20	17, 20, 22	17, 12β, 20, 22, 23	1.13 (d, 6.6)	21.3 (CH_3)	17, 20	12β, 17, 20, 22, 23
22	5.17 (dd, 15.8, 8.8)	134.1 (CH)	20, 23	17, 20, 21, 23, 24	17, 20, 21, 23, 24, 28	5.18 (ddd, 15.3, 8.7, 0.7)	133.8 (CH)	17, 20, 21, 23, 24	17, 20, 21, 23, 24, 26, 27, 28
23	5.28 (dd, 15.8, 8.0)	133.5 (CH)	22, 24	20, 22, 24, 25, 28	17, 20, 21, 22, 24, 25, 26, 27	5.31 (dd, 15.3, 8.0)	133.8 (CH)	20, 22, 24, 25, 28	17, 20, 21, 22, 24, 26, 27, 28
24	1.87 (m)	42.9 (CH)	23, 28	22, 23, 25, 26, 27, 28	22, 23, 25, 26, 27, 28	1.86 (m)	42.9 (CH)	22, 23, 25, 26, 27, 28	25, 26, 27, 28
25	1.47 (octet, 6.8)	33.0 (CH)	26, 27	23, 24, 26, 27, 28	23, 24, 26, 27, 28	1.47 (octet, 6.8)	33.0 (CH)	23, 24, 26, 27, 28	24, 26, 27, 28
26	0.82 (d, 6.8)	19.7 (CH_3)	25	24, 25, 27	23, 24, 25, 27, 28	0.82 (d, 6.8)	19.7 (CH_3)	24, 25, 27	24, 25, 27, 28
27	0.84 (d, 6.8)	20.0 (CH_3)	25	24, 25, 26	23, 24, 25, 26, 28	0.84 (d, 6.6)	20.0 (CH_3)	24, 25, 26	24, 25, 26, 28
28	0.92 (d, 6.8)	17.7 (CH_3)	24	23, 24, 25	22, 23, 24, 25, 26, 27	0.92 (d, 6.8)	17.7 (CH_3)	22, 23, 24	22, 23, 24, 25, 26, 27
3-OH	nd[a]								

[a] Not detected.

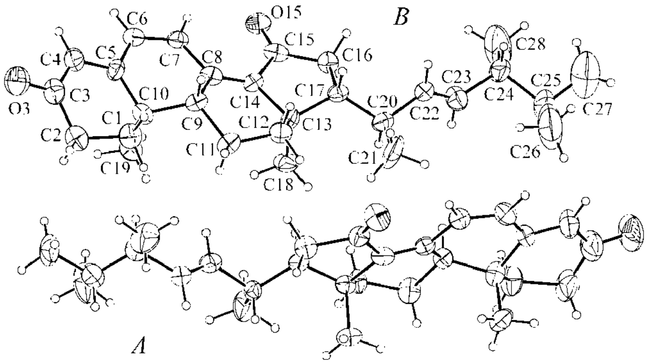


Figure 7. X-ray structure for **7**.

Research.[18] All the steroids (**2**, **3**, **5**, **6**, and **7**) except gymnasterone A (**4**) (10.1 μg/mL) exhibited significant and marginal growth inhibition against the murine P388 cell line with ED_{50} values of 2.2, 2.8, 1.6, 0.9, and 2.5 μg/mL, respectively. This result suggested that a conjugated ketone system plays an important role for enhancement of the cancer cell growth inhibition in compounds **2**, **5**, and **6**. A decrease of the cancer cell growth inhibitory activity in compound **4** is most likely due to steric hindrance of the conjugated amide moiety (N-32–C-46) to the conjugated ketone.

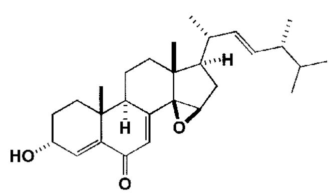
Dankasterone A (**2**) and gymnasterones A (**4**) and B (**5**) were also evaluated against the HCC panel (Table S2). Compound **2** showed appreciable growth inhibition against human cancer cell lines (MG-MID –5.41), whereas growth inhibition of the two other compounds (**4** and **5**) was moderate. The delta and range values of **2** were 0.35 and 0.97, respectively (effective value; delta >0.5 and range >1.0), indicating that selective inhibitory activity of this compound is not appreciable. On the other hand, evaluation of the pattern of differential inhibition using the COMPARE program[18] suggested the possibility that the mode of action for **2** might be different from that shown by any other anticancer drug developed to date.

Conclusions

Steroids separated from marine invertebrates have been known to possess unusual structures with potent biological activity such as the cortistatins[19] and/or rare functional groups as in the haplosamates.[20] Despite the number of marine-derived steroids that have been found to date,[21] marine-derived fungi appear to be an infertile source of novel bioactive steroids.[22] It is likely that marine-derived fungi and terrestrial fungi share the same steroid biosynthetic pathways. In addition, the difficulty of finding novel steroids may also be due to a lack of the structural diversity of fungal steroids represented by ergosterol.

Interestingly, dankasterones and gymnasterones isolated in this study from *Gymnascella dankaliensis* were all structurally unusual, and they were the first examples of cytostatic steroids from sponge-derived fungi. Dankasterones A (**2**) and B (**3**) were unprecedented steroids possessing a 13(14→8)*abeo*-8-ergostane skeleton from nature. Only one exception has been found by a photochemical reaction of the insect molting hormone, 20α-hydroxyecdysone.[23] This extremely rare skeleton occurs most likely on the basis of the 1,2-migration of the C-13–C14 bond to the C-8 position. On the other hand, all the gymnasterones isolated from media type B were also structurally unique stereoids. First, gymnasterone A (**4**) represents an especially interesting structure because it consists of an unprecedented steroid alkaloid with an additional ring and a side chain derived from gymnastatin. In addition, the structure of gymnasterones B (**5**) was also rare in terms of having an epoxide on the D ring. Recently, Li's group has reported the total synthesis of gymnasterone B.[24] Surprisingly, the product of their synthesis, while stated to be gymnasterone B, is different than the structure we assigned for compound **5**.[9] The target of their synthesis, structure **8**, is similar to a related ergostanoid, gymnasterol (**9**), recently isolated from *G. dankaliensis* by Hayakawa *et al.*[25] A final remark on the structural characteristics of the unusual 4,6,8(14)-conjugated triene system of gymnasterones C (**6**) and D (**7**) is that terrestrial fungi and mushrooms are known to produce this type of steroid.[26,27] However, compounds **6** and **7** are the first examples of the conjugated triene ergostanoids from marine-derived fungi. Interestingly, this type of steroid has also been isolated from a marine sponge, *Dysidea herbacea*.[28]

The independent isolation of dankasterones and gymnasterones as major steroidal components from two different carbon sources demonstrates an exciting new approach for obtaining novel bioactive secondary metabolites from one fungal strain. The structure diversity and chemical profile of *G. dankaliensis* were clearly different between the original malt extract and the modified carbon source media conditions. We have continued this project by looking for new gymnastatin analogues from modified malt extract media conditions, and the results of this investigation will be described in the future.



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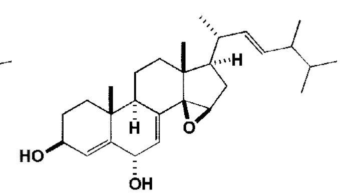

9

Experimental Section

General Experimental Procedures. Optical rotations were obtained on a JASCO ORD/UV-5 spectropolarimeter. CD spectra were recorded on a JASCO J-500A spectrometer. UV spectra were recorded on a Shimadzu spectrophotometer and IR spectra on a Perkin-Elmer FT-IR spectrometer 1720X. 1D and 2D NMR spectra were recorded at 27 °C on a Varian UNITY INOVA-500 spectrometer, operating at 500 and 125.7 MHz for ^{1}H and ^{13}C, respectively, with TMS as an internal reference. High-resolution and low-resolution EIMS were obtained using a Hitachi M-4000H mass spectrometer. Liquid chromatography over silica gel (mesh 230–400) was performed in medium pressure using a metering pump. HPLC was run on a Waters ALC-200 instrument equipped with a differential refractometer (R401) and Shim-pack PREP-ODS (250 mm × 20 mm i.d.). Analytical TLC was performed on precoated Merck aluminum sheets (DC-Alufolien Kieselgel 60 F254, 0.2 mm) with the solvent CH_2Cl_2–MeOH (19:1), and compounds were viewed under UV lamp and sprayed with 10% H_2SO_4 followed by heating.

Biological Materials. The fungal strain (OUPS-N134) was initially isolated from the sponge *Halichondria japonica*, collected in Osaka Bay, Japan, in April 1994. The fungal culture was submitted for identification to the Institute for Fermentation, Osaka, Japan, and identified as *Gymnascella dankaliensis* (Castellani) Currah on the basis of the analysis of its fruiting body.

Culture Conditions and Extraction. The fungal strain was grown in two kinds of stationary liquid media (types A and B). Media type A was composed of 1% malt extract, 1% soluble starch, and 0.05% peptone in artificial seawater adjusted to pH 7.5 for 28 days at 27 °C. Media type B was composed of 1% malt extract, 1% glucose, and 0.05% peptone in artificial seawater adjusted to pH 7.5 for 28 days at 27 °C. The culture was filtered under suction, and the mycelium collected was extracted three times with MeOH. The combined extracts were evaporated *in vacuo* to give crude extracts EA1 (31.0 g; 40 L of medium A) and EA2 (76.6 g; 100 L of media A2) and EB (11.0g; 90 L of medium B).

Isolation of Pure Compounds. The CH_2Cl_2–MeOH (1:1) soluble portion of EA1 was passed through Sephadex LH-20, using CH_2Cl_2–MeOH (1:1) as the eluent. The second fraction (F1; 14.4 g), in which the activity was concentrated, was chromatographed on a Si gel column with an *n*-hexane–CH_2Cl_2–MeOH gradient as the eluent to give an active fraction (F2; 685.0 mg) obtained from MeOH–CH_2Cl_2 (1:99). The Si gel fraction F2 was purified by RP HPLC using acetone–H_2O (9:1) to afford **2** (10.7 mg). The CH_2Cl_2–MeOH (1:1) soluble portion of EA2 was passed through Sephadex LH-20, using CH_2Cl_2–MeOH (1:1) as the eluent. The second fraction (F3; 61.1 g), in which the activity was concentrated, was chromatographed on a Si gel column with an *n*-hexane–CH_2Cl_2–MeOH gradient as the eluent to give an active fraction (F4; 3.9g) obtained from MeOH–CH_2Cl_2 (1:99). The active fraction F4 was repeatedly chromatographed on a Si gel column with a CH_2Cl_2–MeOH gradient as the eluent to afford an active fraction (F5; 101 mg) obtained from MeOH–CH_2Cl_2 (1:199). The Si gel fraction F5 was purified by HPLC using acetone–H_2O (17:3) twice to afford **3** (1.6 mg). The CH_2Cl_2–MeOH (1:1) soluble portion of EB was passed through Sephadex LH-20, using CH_2Cl_2–MeOH (1:1) as the eluent. The second fraction (F6; 7.0 g), in which the activity was concentrated, was chromatographed on a Si gel column with an *n*-hexane–CH_2Cl_2–MeOH gradient as the eluent to give an active fraction (F7; 559 mg). The active fraction F7 was separated by RP HPLC using acetone to afford three fractions, F8 (15.9 mg), F9 (48.3 mg), and F10 (20.4 mg). The HPLC fractions F8, F9, and F10 were purified by RP HPLC using acetone–H_2O (9:1) separately to afford **5** (12.0 mg) from F8, **6** (16.9 mg) and **7** (11.2 mg) from F9, and **4** (14.9 mg) from F10, respectively.

Dankasterone A (2): colorless prisms (MeOH); mp 133–134 °C; $[\alpha]^{27}_D$ +57.8 (*c* 0.7, $CHCl_3$); IR (film) ν_{max} 1695, 1682, 1607 cm^{-1}; UV (EtOH) λ_{max} (log ε) 254 nm (4.02); ^{1}H and ^{13}C NMR data, see Table 1; HREIMS *m/z* 424.2988 [M]$^+$ (calcd for $C_{28}H_{40}O_3$ 424.2976).

Crystal data for 2: $C_{28}H_{40}O_3$, $M = 424.60$, orthorhombic, $P2_12_12_1$, $a = 12.667(3)$ Å, $b = 23.829(5)$ Å, $c = 8.134(4)$ Å, $V = 2455.4(14)$ Å^3, $Z = 4$, $d_x = 1.149$ g cm^{-3}, $F(000) = 928$, μ(Cu Kα) = 0.563 mm^{-1}. Data collection was performed on a Rigaku AFC5R using graphite-monochromated radiation ($\lambda = 1.5418$ Å); 5117 reflections were collected until $\theta_{max} = 70.14°$, of which 3467 reflections were observed [$I > 2\sigma(I)$]. The crystal structure was solved by direct methods using SHELXS-86.[29] The structure was refined by full-matrix least-squares methods on F^2 using SHELXL-93.[30] In the structure refinements, non-hydrogen atoms were calculated on the geometrically ideal positions by a "riding" method and were included in the calculation of structure factors with isotropic temperature factors. In the final stage, $R = 0.0625$, $Rw = 0.1504$, and $S = 1.036$ were obtained. CCDC: 182/1288.

Dankasterone B (3): colorless powder; mp 182–183 °C; $[\alpha]^{22}_D$ +38.4 (*c* 0.2, $CHCl_3$); IR (film) ν_{max} 1719 cm^{-1}; UV (EtOH) λ_{max} (log ε) 269 nm (2.57); ^{1}H and ^{13}C NMR data, see Table 1; HREIMS *m/z* 426.3139 [M]$^+$ (calcd for $C_{28}H_{42}O_3$ 426.3123).

Gymnasterone A (4): pale yellow oil; $[\alpha]^{20}_D$ –110.7 (*c* 1.4, $CHCl_3$); IR (film) ν_{max} 3397, 3339, 1729, 1658, 1623 cm^{-1}; UV (EtOH) λ_{max} (log ε) 270 nm (4.58); ^{1}H and ^{13}C NMR data, see Table 2; HREIMS *m/z* 701.5028 [M]$^+$ (calcd for $C_{45}H_{67}NO_5$ 701.5016).

Formation of *p*-Bromobenzoate from 4. To a solution of **4** (0.9 mg) in pyridine (0.5 mL) was added *p*-bromobenzoyl chloride (0.5 mg). The reaction mixture was stirred at room temperature for 12 h. The residue obtained by evaporation under reduced pressure was purified by RP HPLC using isocratic conditions of MeOH–H_2O (19:1) as the eluent to afford **4a** (0.7 mg) as a colorless oil.

***p*-Bromobenzoate (4a):** EIMS *m/z* 883 [M]$^+$ (0.6%); UV (EtOH) λ_{max} (log ε) 251 (4.35), 268 nm (4.33); CD (EtOH) λ_{max} ($\Delta\varepsilon$) 220 (+2.1), 240 (+16.9), 252 (0), 266 (−24.6), 313 nm (0); ^{1}H NMR δ ppm ($CDCl_3$) 9.19 (1H, s, H-31), 7.89 (2H, d, $J = 8.0$ Hz, Ar-H), 7.56 (2H, d, $J = 8.0$ Hz, Ar-H), 7.13 (1H, d, $J = 15.3$ Hz, H-35), 7.12 (1H, s, H-32), 6.74 (1H, br. s, H-4), 6.22 (1H, br. s, 14-OH), 5.72 (1H, d, $J = 15.3$ Hz, H-34), 5.65 (1H, m, H-3), 5.61 (1H, d, $J = 9.8$ Hz, H-37), 5.26 (1H, dd, $J = 15.3$, 8.0 Hz, H-23), 5.08 (1H, dd, $J = 15.3$, 8.0 Hz, H-22), 2.45–2.55 (4H, m, H-9, H-15, H-29β, H-38), 2.24 (1H, m, H-2α), 2.13–2.20 (2H, m, H-11β, H-20), 2.00 (1H, m, H-1β), 1.84 (1H, m, H-24), 1.73 (3H, d, $J = 0.7$ Hz, H_3-45), 1.52–1.80 (7H, m, H-1α, H-2β, H-11α, H-12α, H-12β, H-16β, H-29α), 1.46 (1H, octet, $J = 6.8$ Hz, H-25), 1.21–1.37 (11H, m, H-17, H_2-39, H_2-40, H_2-41, H_2-42, H_2-43), 1.16 (3H, s, H_3-18), 1.11 (3H, s, H_3-19), 1.05 (3H, d, $J = 6.8$ Hz, H_3-21), 0.95 (3H, d, $J = 6.8$ Hz, H_3-46), 0.90 (3H, d, $J = 6.8$ Hz, H_3-28), 0.87 (3H, t, $J = 6.6$ Hz, H_3-44), 0.83 (3H, d, $J = 6.8$ Hz, H_3-27), 0.81 (3H, d, $J = 6.8$ Hz, H_3-26).

Gymnasterone B (5): colorless powder; mp 197–199 °C; $[\alpha]^{22}_D$ −76.3 (*c* 0.763, $CHCl_3$); IR (film) ν_{max} 1719, 1657 cm^{-1}; UV (EtOH) λ_{max} (log ε) 255 nm (4.13); ^{1}H and ^{13}C NMR data, see Table 3; HREIMS *m/z* 424.2975 [M]$^+$ (calcd for $C_{28}H_{40}O_3$, 424.2976).

Gymnasterone C (6): pale yellow needles (MeOH); mp 197–199 °C; $[\alpha]^{22}_D$ +224.0 (*c* 0.25, $CHCl_3$); IR (film) ν_{max} 3388, 1697, 1597 cm^{-1}; UV (EtOH) λ_{max} (log ε) 254 (3.62 sh), 336 (4.22), 354 (4.08 sh) nm; ^{1}H and ^{13}C NMR data, see Table 4; HREIMS *m/z* 408.3032 [M]$^+$ (calcd for $C_{28}H_{40}O_2$ 408.3026).

Formation of the (*R*)- and (*S*)-MTPA Esters (6a and 6b) from 6. (*R*)-MTPA (14.2 mg), dicyclohexylcarbodiimide (14.7 mg), and 4-(dimethylamino)pyridine (8.4 mg) were added to a CH_2Cl_2 solution (0.5 mL) of **6** (3.0 mg), and the reaction mixture was stirred for 3 h at rt. The solvent was evaporated under reduced pressure. The residue was purified by a Si gel column chromatography using *n*-hexane–EtOAc (3:1) and a RP HPLC using MeOH–H_2O (4:1) to afford ester **6a** (3.4 mg). The same reaction with **6** (2.9 mg) using (*S*)-MTPA (13.6 mg) gave ester **6b** (3.7 mg).

(*R*)-MTPA ester (6a): colorless oil; EIMS *m/z* 624 [M]$^+$ (0.2); ^{1}H NMR δ ppm ($CDCl_3$) 7.55 (2H, m, Ar-H), 7.42 (3H, m, Ar-H), 7.40 (1H, d, $J = 9.8$ Hz, H-7), 6.12 (1H, d, $J = 9.8$ Hz, H-6), 5.67 (1H, ddd, $J = 9.6$, 6.5, 2.4 Hz, H-3), 5.44 (1H, br s, H-4), 5.28 (1H, dd, $J = 15.2$, 8.0 Hz, H-23), 5.17 (1H, dd, $J = 15.2$, 8.6 Hz, H-22), 3.58 (3H, s, OCH_3), 2.29 (1H, dd, $J = 19.2$, 7.8 Hz, H-16α), 2.22 (1H, m, 2α), 2.21 (1H, m, H-9), 2.21 (1H, m, H-20), 2.18 (1H, dt, $J = 13.3$, 3.0 Hz, H-12β), 2.10 (1H, dd, $J = 19.2$, 12.2 Hz, H-16β), 1.86 (1H, m, H-24), 1.83 (1H, m, H-2β), 1.81 (1H, m, H-1β), 1.68 (1H, m, H-11α), 1.62 (1H, m, H-17), 1.58 (1H, m, H-1α), 1.57 (1H, m, H-11β), 1.47 (1H, octet, $J = 6.8$ Hz, H-25), 1.42 (1H, td, $J = 13.3$, 3.5 Hz, H-11β), 1.11 (3H, d, $J = 6.9$ Hz, H_3-21), 1.05 (3H, s, H_3-18), 0.96 (3H, s, H_3-19), 0.92 (3H, d, $J = 6.8$ Hz, H_3-28), 0.84 (3H, d, $J = 6.8$ Hz, H_3-27),0.82 (3H, d, $J = 6.8$ Hz, H_3-26).

(*S*)-MTPA ester (6b): colorless oil; EIMS *m/z* 624 [M]$^+$ (0.2); ^{1}H NMR ^{1}H NMR δ ppm ($CDCl_3$) 7.55 (2H, m, Ar-H), 7.42 (3H, m, Ar-H), 7.41 (1H, d, $J = 9.9$ Hz, H-7), 6.16 (1H, d, $J = 9.9$ Hz, H-6), 5.67 (1H, ddd, $J = 9.6$, 6.8, 2.5 Hz, H-3), 5.55 (1H, br s, H-4), 5.28 (1H, dd, $J = 15.3$, 8.0 Hz, H-23), 5.17 (1H, dd, $J = 15.3$, 8.7 Hz, H-22), 3.58 (3H, s, OCH_3), 2.31 (1H, dd, $J = 19.3$, 7.8 Hz, H-16α), 2.24 (1H, m, H-9), 2.23 (1H, m, H-20), 2.19 (1H, m, H-12β), 2.18 (1H, m, H-2α), 2.10 (1H, dd, $J = 19.3$, 12.4 Hz, H-16β), 1.87 (1H, m, H-24), 1.78 (1H, m, H-1β), 1.71 (1H, m, H-2β), 1.67 (1H, m, H-11α), 1.63 (1H, m, H-17), 1.58 (1H, m, H-11β), 1.57 (1H, m, H-1α), 1.47 (1H, octet, $J = 6.8$ Hz, H-25), 1.42 (1H, td, $J = 13.3$, 3.2 Hz, H-11β), 1.11 (3H, d, $J = 6.6$ Hz, H_3-21), 1.05 (3H, s, H_3-18), 0.94 (3H, s, H_3-19), 0.92 (3H, d, $J = 6.8$ Hz, H_3-28), 0.84 (3H, d, $J = 6.8$ Hz, H_3-27), 0.82 (3H, d, $J = 6.8$ Hz, H_3-26).

Dess-Martin Oxidation of 6. To a solution of Dess-Martin reagent (9.6 mg) in dry CH_2Cl_2 (1.0 mL) was added **5** (5.0 mg) in CH_2Cl_2 (1.0 mL), and the mixture was stirred at rt for 6 h. After diluting with ether, the reaction mixture was treated with saturated $Na_2S_2O_3$–saturated $NaHCO_3$ (1:1) twice and washed with H_2O and brine. The organic layer was evaporated in a vacuum under reduced pressure to afford a residue. The residue was purified by RP HPLC using isocratic MeOH–H_2O (19:1) as the eluent to afford **7** (0.7 mg) as a colorless oil. All the spectral data including optical rotation were identical with those of **7** obtained from the fungal extract.

Gymnasterone D (7): colorless needles (MeOH); mp 166–168 °C; $[\alpha]^{22}_D$ +473.7 (*c* 0.88, $CHCl_3$); IR (film) ν_{max} 1689, 1674, 1601 cm^{-1}; UV (EtOH) λ_{max} (log ε) 258 (4.36 sh), 336 (4.51), 356 (4.36 sh) nm; ^{1}H and ^{13}C NMR data, see Table 4; HREIMS *m/z* 406.2877 [M]$^+$ (calcd for $C_{28}H_{38}O_3$ 406.2870).

Crystal data for 7: $C_{28}H_{38}O_2$, $M = 424.60$, orthorhombic, $P2_12_12_1$, $a = 22.038(6)$ Å, $b = 34.524(10)$ Å, $c = 6.446(2)$ Å, $V = 4904.1(24)$ Å^3, $Z = 8$, $d_x = 1.101$ g cm^{-3}, $F(000) = 1776$, μ(Cu Kα) = 0.514 mm^{-1}. Data collection was performed on a Rigaku AFC5R using graphite-monochromated radiation (λ=1.5418 Å); 4194 reflections were collected until $\theta_{max} = 60.04°$, of which 2296 reflections were observed [$I > 2\sigma(I)$]. The crystal structure was solved by direct methods using SHELXS-86.[29] The structure was refined by full-matrix least-squares methods on F^2 using SHELXL-93.[30] In the structure refinements, non-hydrogen atoms were calculated on the geometrically ideal positions by a "riding" method and were included in the calculation of structure factors with isotropic temperature factors. In the final stage, $R = 0.1012$, $Rw = 0.2300$, and $S = 1.284$ were obtained. CCDC: 635811.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of **2**, **3**, **4**, **5**, **6**, and **7**, the crystal data of **2** and **7**, and the HCC panel data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) Crystallographic data for dankasterone A (**2**) have been deposited at the Cambridge Crystallographic Data Center. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk or http://www.ccdc.cam.ac.uk/].
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