Trichomycins A and B: Antibacterial Triterpenes from the New Species *Tricholoma* sp. AU1

Simon P. B. Ovenden,[†] Jin Yu,[†] Jill Bernays,[†] Soo San Wan,[†] Larissa J. Christophidis,[†] Gian Sberna,[†] R. Murray Tait,[†] Howard G. Wildman,[†] Dominique Lebeller,[‡] Denis Platel,[‡] Tom W. May,[§] and Barbara M. Meurer-Grimes*,[†]

Cerylid Biosciences, 576 Swan Street Richmond, Victoria, 3121, Australia, Aventis Pharmaceuticals, Centre de Recherche de Paris, Etablissement de Romainville 102, Route de Noisy, F-93235 Romainville Cedex, Paris, France, and Royal Botanic Gardens Melbourne, Private Bag 2000, South Yarra, Victoria 3141, Australia

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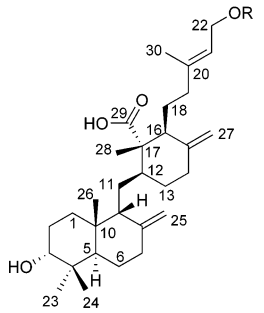
To new triterpenes, trichomycins A (**1**) and B (**2**), were purified from the new species *Tricholoma* sp. AU1 by activity-guided fractionation following their antibacterial activity. The two compounds were found to have a hitherto unreported triterpenoid skeleton. The structures and relative stereochemistry of **1** and **2** were determined through extensive 2D NMR spectroscopy, while the inhibitory activity of **1** and **2** against two Gram-positive and two Gram-negative bacteria and a mammalian cell line was determined.

Basidiomycetes of the genus *Tricholoma* occur ubiquitously, with several species highly valued due to their medicinal properties[1] or as culinary delicacies.[2] The genus is known to produce a multitude of different secondary metabolites derived from the polyketide,[3] fatty acid,[4] alkaloid,[5] and terpenoid[6] biosynthetic pathways.

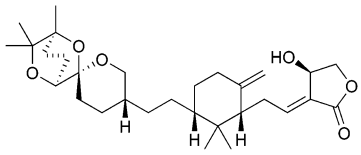
In the course of a high-throughput screening (HTS) campaign of a library of natural product extracts to identify novel antibacterial agents, the crude MeOH extract of a *Tricholoma* species (Tricholomataceae), collected in North West Victoria, Australia, exhibited notable antibacterial activity. The source organism was subsequently identified as a new species of the genus *Tricholoma* and is referred to as *Tricholoma* sp. AU1. Bioassay-guided fractionation of the crude MeOH extract led to the isolation of two new triterpenes, trichomycins A (**1**) and B (**2**). Reported below in detail is the structure elucidation, determination of relative stereochemistry, and biological profiles of **1** and **2**.

Trichomycin A (**1**) was isolated as a colorless oil and yielded a molecular ion in the positive HRESIMS spectrum at *m/z* 523.3389 ($[M + Na]^+$), indicative of the molecular formula $C_{31}H_{48}O_5$ (Δ +1.1 mmu) and equating to eight double-bond equivalents. Immediately identifiable from the NMR data for **1** (Table 1) were resonances consistent with one trisubstituted double bond [^{13}C: 144.6, 120.0 ppm; ^1H: δ 5.30], as well as two 1,1-disubstituted double bonds [^{13}C: 152.3, 114.6, 110.1, 108.6 ppm; ^1H: δ 4.91, 4.63 (×3)], a carboxy [^{13}C: 181.6 ppm; IR: 1720 cm^{-1}], and a formate carbonyl [^{13}C: 163.4 ppm; ^1H: δ 8.07; IR: 1699 cm^{-1}]. In the absence of any other sp or sp^2 carbons, the gross structure of **1** must be tricyclic. Also evident were resonances consistent with one olefinic [^{13}C: 16.8 ppm; ^1H: δ 1.69] and four tertiary methyls [^{13}C: 29.5, 23.5, 23.4, 11.4 ppm; ^1H: δ 0.92, 0.90, 0.83, 0.80], a hydroxy [^{13}C: 77.7 ppm; ^1H: δ 3.35], and an oxy methine [^{13}C: 61.9 ppm; ^1H: δ 4.65], as well as nine methylenes, five methines, and three quaternary carbons (Table 1).

Interpretation of the $^1H-^1H$ COSY and gHMBC data readily identified the three partial structures for **1** as shown in Figure 1. The observation of a gHMBC correlation from H-16 to C-19 allowed for the connection of partial structures A and B, while additional gHMBC correlations from H-12 to C-9 and C-11, and from H_b-11 to C-8, C-9, C-10, C-12, and C-13, allowed for the connection of partial structures B and C. This accounted for all atoms except for one exchangeable proton. The presence of a carboxylic acid accounted for the remaining proton and hence the gross structure of **1** as shown.



(**1**) R = CHO
(**2**) R = H



Saponaceolide C (**3**)

The relative stereochemistry of **1** was determined through 2D NOESY analysis and proton coupling constants. The observation of a NOESY correlation from H_2-22 to H_3-30 established an *E* configuration for $\Delta^{20,21}$, while NOESY correlations from H-3 to H_3-23 and H_3-24 suggested that H-3 was equatorial. Further NOESY correlations from H_3-24 to H_a-2, H_a-6, and H_3-26, and from H_3-26 to H_a-2, H_a-6, and H-9, placed H-9, H_3-24, and H_3-26 on the same face of the molecule. Similarly, a NOESY correlation from H-5 to H_3-23 placed H-5 on the opposite face of the molecule from H_3-26, suggesting an axial orientation for H-5 and hence a trans ring junction. These observations allowed for the determination of the relative stereochemistry of the labdane portion of **1** as shown in Figure 2. For the cyclohexane

* To whom correspondence should be addressed. Tel: +61 3 9208 4074. Fax: +61 3 9208 4126. E-mail: bmeurer-grimes@cerylid.com.au.
[†] Cerylid Biosciences.
[‡] Aventis Pharmaceuticals.
[§] Royal Botanic Gardens.

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Table 1. One- and Two-Dimensional NMR Data for Trichomycin A (**1**)

no.	^{13}C (δ, m)	^{1}H [δ, m, J(Hz)]	COSY	gHMBC ^{1}H to ^{13}C	NOESY
1	30.5 (t)	2.04 (m)	H_a-2		
2	27.2 (t)	1.93 (m)	H_2-1		H_3-24, H_3-26
		1.56 (m)	H-3		
3	77.7 (d)	3.35 (br s)	H_b-2	C-1, C-2, C-4, C-5, C-23, C-24	H_3-23, H_3-24
4	38.6 (s)				
5	41.1 (d)	1.64 (m)	H_b-6		H_3-23
6	24.9 (t)	1.54 (m)	H-7	C-8	H_3-24, H_3-26
		1.34 (ddd, 12.4, 5.6, 5.2)	H-5, H-7		
7	33.6 (t)	2.10 (m)	H_2-6	C-8, C-25	
8	152.3 (s)				
9	60.5 (d)	1.55 (m)	H_b-11	C-8, C-25	H_3-26
10	39.7 (s)				
11	30.9 (t)	1.64 (m)	H_b-11		H_3-28
		1.00 (dt, 14.2, 8.3)	H-9, H_a-11	C-8, C-9, C-10, C-12, C-13	
12	46.9 (d)	1.90 (m)	H_b-13	C-9, C-11, C-16, C-28	H_a-14
13	32.4 (t)	1.72 (m)	H_b-13		H_3-28
		1.12 (m)	H-12, H_a-13, H_a-14		
14	38.9 (t)	2.26 (m)	H_b-13, H_b-14	C-13, C-15, C-16, C-27	H-12, H-16
		1.94 (m)	H_a-14	C-13, C-15, C-16, C-27	
15	148.6 (s)				
16	50.7 (d)	2.37 (d, 10.8)	H_a-18	C-12, C-14, C-15, C-17, C-18, C-19, C-27, C-28, C-29	H_a-14
17	55.7 (s)				
18	27.4 (t)	1.64 (m)	H-16, H_a-19		
		1.18 (m)	H_a-19, H_b-19		
19	39.6 (t)	2.13 (m)	H_a-18, H_b-18		
		1.93 (m)	H_b-18		
20	144.6 (s)				
21	120.0 (d)	5.30 (t, 6.8)	H_3-20, H-22	C-19, C-22, C-30	
22	61.9 (t)	4.65 (d, 6.8)	H-21	C-20, C-31	H_3-30
23	29.5 (q)	0.92 (s)		C-3, C-4, C-5, C-24	H-5, H-3
24	23.5 (q)	0.80 (s)		C-3, C-4, C-5, C-23	H_a-2, H-3, H_a-6, H_3-26
25	110.1 (t)	4.63 (br s)		C-8, C-9	
26	23.4 (q)	0.90 (s)		C-1, C-5, C-9, C-10	H_a-2, H_a-6, H-9, H_3-24
27	108.6 (t)	4.91 (br s)	H_b-27	C-14, C-15, C-16	
		4.63 (br s)	H_a-27	C-14, C-15, C-16	
28	11.4 (q)	0.83 (s)		C-12, C-16, C-17, C-29	H_2-11, H_a-13
29	181.6 (s)				
30	16.8 (q)	1.69 (s)	H-21	C-19, C-20, C-21	H_2-22
31	163.4 (d)	8.07 (s)		C-22	

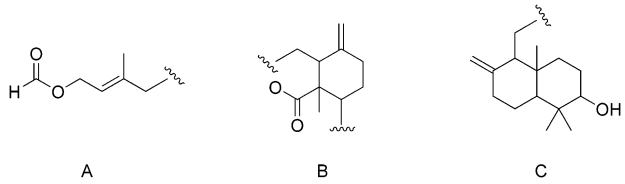

Figure 1. Generated partial structures for trichomycin A (**1**).

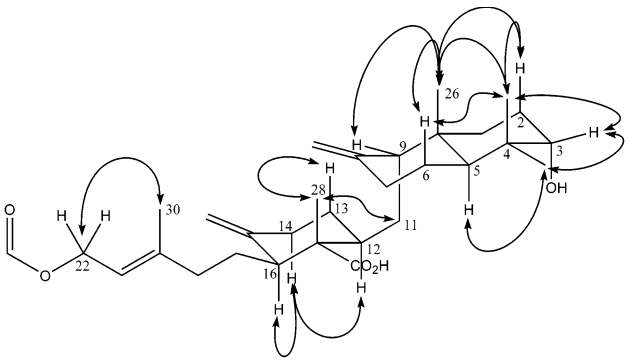

Figure 2. Observed NOESY correlation for trichomycin A (**1**). For clarity only protons where NOESY correlations were observed are shown.

moiety of **1**, the observation of NOESY correlations from H_a-14 to H-12 and H-16 place them on the same face of the molecule. In addition, the large coupling constant for H-16 (J = 10.8 Hz) suggested that these protons were all axial (Figure 2). Finally, NOESY correlations from H_3-28 to H_a-13 and H_2-11 were suggestive of H_3-28 and H_a-13 axial, and H_2-11 equatorial (Figure 2). Hence the relative stereochemistry of **1** is proposed as shown. The absolute stereochemistry of **1** remains unassigned at this time.

Trichomycin B (**2**) was isolated and gave a molecular ion at *m/z* 471.3472 in the negative HRESIMS ([M − H]$^-$) spectrum, indicative of the molecular formula $C_{30}H_{48}O_4$ (Δ +0.2 mmu), 28 amu less than the molecular ion measured for trichomycin A (**1**) and equating to seven double-bond equivalents. Analysis of the NMR data for **2** showed that it was remarkably similar to that for **1**, with the exception of the ^{13}C NMR data of C-20 [**2** (139.4 ppm) vs **1** (144.6 ppm)] and C-21 [**2** (124.5 ppm) vs **1** (120.0 ppm)], the ^{13}C and ^{1}H NMR data for C-22 [**2** (^{13}C: 59.0 ppm; ^{1}H: δ 4.05) vs **1** (^{13}C: 61.9 ppm; ^{1}H: δ 4.65)], and the absence of a formate proton at ~δ 8.00. Further analysis of the ^{1}H−^{1}H COSY and gHMBC showed that indeed the only difference between **2** and **1** was the absence of the formate group. Hence the gross structure for **2** is as shown. The proposed relative stereochemistry of **2** has been assigned the same as **1** on the basis of ^{13}C chemical shifts. Interestingly, when **2** was first purified, it had a molecular weight 28 amu higher than **1**, as well as an additional formate resonance in the ^{1}H NMR spectrum. It appears that over time, compound **2** has undergone a di-deformylation. It is possible that the formate substitution observed in **1** and initially present in **2** are artifacts of the isolation process, as formic acid was used as a solvent modifier. The pos-

Table 2. Antibacterial and Cytotoxic Activities for Trichomycins A (**1**) and B (**2**) against *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *S. pneumoniae*, and THP-1 Cells (IC_{50}'s in μM)

	K. pneumoniae	*P. aeruginosa*	*S. aureus*	*S. pneumoniae*	THP-1
1	>500	>500	12	5	>1000
2	>473	>473	12	6	640

sibility of artifact formation could not be addressed any further, because no further dried fungal material was available to repeat the isolation under acid-free conditions.

There have been 12 reports[6−17] in the literature concerning the isolation of terpenes from species of *Tricholoma*, four of which describe the isolation of steroids,[6,8−10] and a further four describing the isolation of diterpenes.[7,11−13] Furthermore, an additional four publications concern the unusual oxygenated triterpenes saponaceolides A (**3**), B, C, and D, which were reported from *T. saponaceum*[14−16] and *T. terreum*.[17] These compounds show some resemblance to trichomycins A (**1**) and B (**2**) by sharing similar partial structures A and B (Figure 2). To the best of our knowledge, this is the first report of ring-opened triterpenes from a *Tricholoma* species. Furthermore, to the best of our knowledge, the triterpene skeleton as exemplified by **1** and **2** has not previously been reported.

The antibacterial activity for **1** and **2** was determined against two Gram-positive and two Gram-negative bacterial strains. In addition, cytotoxicity and selective antibacterial activity was assessed by exposure to a human leukemic cell line (THP-1). The results are summarized in Table 2. Both **1** and **2** were found to be selective against Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus pneumoniae*), with no activity at the highest concentration detected against Gram-negative bacteria (*Klebsiella pneumoniae* and *Pseudomonas aeruginosa*). Very weak activity at the highest concentration was noted for **2** against the mammalian cell line (THP-1), indicating a selectivity index toward the antibacterial activity of almost 100-fold.

Experimental Section

General Experimental Procedures. SPE was performed using Varian Megabond Elute C18 SPE cartridges (10 g, 50 μm). HPLC was performed on either a Waters Delta Prep 4000 chromatography system equipped with a Waters 2487 dual wavelength UV detector, a Waters prep LC system controller and a Waters fraction collector, or a system equipped with a Waters 600 controller, a Waters 996 photodiode array detector, a Waters 717 plus autosampler, and a Waters fraction collector II. All data generated from these chromatographic systems were collected using Waters Millenium[32] data collection package.

All NMR spectra were collected on a Varian Unity Inova 400 MHz spectrometer in the solvents indicated, with spectra referenced to residual ^{1}H in the deuterated NMR solvents.

Optical rotations were performed on a Jasco Dip-1000 digital polarimeter, while infrared spectra were acquired on a Bio-Rad FTS-165 Fourier transform infrared spectrometer.

Low-resolution mass spectral data were collected on a ThermoFinnigan LCQ ion trap mass spectrometer, with an ESI probe. High-resolution mass measurements were collected on a Bruker BioApex FT mass spectrometer.

Fungal Material. Fruiting bodies of *Tricholoma* sp. AU1 (Tricholomataceae) were collected in a river flood plain near the Wimmera River at the border of the Little Desert National Park, 2.1 km south of Horseshoe Bend camping ground (36°30.86′ S, 142°01.58′ E), in *Eucalyptus microcarpa* woodland, Victoria, in June 1998. Description: Fruit-body robust, lamellate, growing on soil. Pileus large, to 9 cm diameter, white with slight yellow stain. Stipe central, white, without annulus. Spores 5−6 × 3−4 μm, broadly ellipsoid, hyaline, smooth, inamyloid. Pileipellis composed of repent, cylindrical, hyaline hyphae, looser at surface. Clamp connections absent. All characteristics are typical for *Tricholoma*, but do not match those of known Australian species. A voucher specimen (MEL2049115) was deposited with the National Herbarium of Victoria, Australia.

Extraction and Isolation. Dried and ground fungal material (10 g) was extracted twice with MeOH (500 mL) over 36 h. The combined MeOH was concentrated in vacuo and subjected to C18 SPE (10% gradient elution from 20% MeOH/H_2O to 80% MeOH/H_2O, and a flush with 100% MeOH), generating eight fractions, with activity localized in the 80% MeOH/H_2O fraction. This fraction was further purified on C18 preparative HPLC [16 mL/min, gradient elution from 3:7 (MeCN/H_2O + 0.1% formic acid) to 9:1 (MeCN/H_2O + 0.1% formic acid) over 25 min through a Varian C18 250 × 50 mm, 5 μm, preparative HPLC axial compression column] and C18 semipreparative HPLC [4 mL/min, gradient elution from 1:1 (MeCN/H_2O + 0.1% formic acid) to MeCN (+0.1% formic acid) over 18 min through a Waters C18 Xterra 100 × 7.8 mm, 5 μm, HPLC column] to yield trichomycins A (**1**) (5.4 mg, 0.054%) and B (**2**) (0.4 mg, 0.004%) as the compounds responsible for the activity of the extract.

Trichomycin A (1): colorless oil; $[\alpha]_D$ 16.6° (*c* 0.27, $CHCl_3$); IR (film) ν_{max} 3406, 2937, 2879, 1720, 1699 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD), see Table 1; ^{13}C NMR data (100 MHz, CD_3OD), see Table 1; HRESIMS *m/z* 523.3389 (calcd for $C_{31}H_{48}O_5Na$, 523.3400).

Trichomycin B (2): colorless oil; $[\alpha]_D$ 69.5° (*c* 0.04, $CHCl_3$); IR (film) ν_{max} 3406, 2931, 2874, 1720 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 5.29 (1H, t, J = 6.6 Hz, H-21), 4.90 (1H, br s, H_a-27), 4.64 (1H, br s, H_b-25), 4.63 (1H, br s, H_a-25), 4.60 (1H, br s, H_b-27), 4.05 (2H, d, J = 6.6 Hz, H_2-22), 3.34 (1H, br s, H-3), 2.37 (1H, d, J = 10.5 Hz, H-16), 2.25 (1H, d, J = 12.1 Hz, H_a-19), 2.15 (2H, m, H_2-7), 2.13 (1H, m, H_a-14), 2.04 (2H, m, H_2-1), 1.94 (1H, m, H_b-19), 1.93 (1H, m, H_a-2), 1.90 (1H, m, H_b-14), 1.88 (1H, m, H-12), 1.75 (1H, m, H_a-13), 1.66 (1H, m, H_a-11), 1.65 (1H, m, H-5), 1.64 (1H, m, H_a-18), 1.63 (3H, s, H_3-30), 1.56 (2H, m, H_b-2, H_a-6), 1.55 (1H, m, H-9), 1.25 (1H, m, H_b-6), 1.13 (2H, m, H_b-13, H_b-18), 1.00 (1H, m, H_b-11), 0.92 (3H, s, H_3-23), 0.90 (3H, s, H_3-26), 0.82 (3H, s, H_3-28), 0.80 (3H, s, H_3-24); ^{13}C NMR (100 MHz, CD_3OD) δ 181.1 (s, C-29), 151.4 (s, C-8), 147.9 (s, C-15), 139.4 (s, C-20), 124.5 (d, C-21), 109.4 (t, C-25), 107.9 (t, C-27), 76.9 (d, C-3), 59.7 (d, C-9), 59.0 (t, C-22), 55.1 (s, C-17), 50.2 (d, C-16), 46.4 (d, C-12), 41.4 (s, C-10), 40.0 (d, C-5), 39.1 (s, C-4), 38.6 (t, C-14), 37.8 (t, C-19), 32.8 (t, C-7), 31.6 (t, C-13), 29.8 (t, C-11), 29.6 (t, C-1), 28.8 (t, C-2), 28.6 (q, C-23), 26.9 (t, C-18), 26.5 (t, C-6), 22.5 (q, C-24), 22.4 (q, C-26), 15.9 (q, C-30), 10.8 (q, C-28); HRESIMS *m/z* 471.3472 (calcd for $C_{30}H_{47}O_4$, 471.3474).

Antibacterial Assays. Strains of *K. pneumoniae, P. aeruginosa, S. aureus*, and *S. pneumoniae* were initially grown on Mueller-Hinton agar, then subcultured into Mueller-Hinton broth supplemented with $CaCl_2$/$MgCl_2$ and grown at 37 °C for 18 h. Serial dilutions were made (*K. pneumoniae* and *P. aeruginosa* diluted to 10^{-6} cfu/mL, *S. aureus* to 10^{-8} cfu/mL, and *S. pneumoniae* to 10^{-4} cfu/mL) and all broths left to equilibrate for 30 min at 37 °C. Extracts were added to a 96-well microtiter plate, and 200 μL of each culture was dispensed into each well. Negative control wells contained the respective bacterial strain without inhibitors, while positive control wells contained bacterial strains with 50 μg/mL of streptomycin. The plates were incubated and shaken at 37 °C and 60% humidity for 18 h, after which optical density at 650 nm was measured. Test wells showing little or no growth/turbidity were indicative of antibacterial activity.

Mammalian Cytotoxicity Assay. Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 5 units/mL penicillin, 5 mg/mL streptomycin, and 10 μM 2-mercaptoethanol at 37 °C, 5% CO_2. The assay was conducted in a 96-well microtiter plate at a cell density of 2.5 × 10^4 cells per well, in a total volume of 200 μL. Natural product samples in each well were tested at 250 μg/mL final concentration. Positive control wells contained 10 μM camp-

tothecin, while negative control wells contained 0.1% DMSO. Plates were incubated for 72 h at 37 °C, 5% CO_2, prior to the addition of WST-1 reagent (20 μL) and further incubated for 1−2 h. Absorbance at 450 nm was measured and activity compared to positive controls.

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References and Notes

(1) Francia, C.; Rapior, S.; Courtecuisse, R.; Siroux, Y. Y., *Int. J. Med. Mushrooms* **1999**, *1*, 169−172.
(2) Hawksworth, D. L.; Kirk, P. M.; Sutton, B. C.; Pegler, D. N. *Dictionay of the Fungi*, 8th ed.; CAB International: Wallingford, UK, 1995.
(3) Klostermeyer, D.; Knops, L.; Sindlinger, T.; Polborn, K.; Steglich, W. *Eur. J. Org. Chem.* **2000**, 603−609.
(4) Dagne, E.; Asmellash, S.; Abate, D. *J. Nat. Prod.* **1994**, *57*, 390−392.
(5) Pand, Z.; Sterner, O. *J. Nat. Prod.* **1994**, *57*, 852−857.
(6) Ohnuma, N.; Amemiya, K.; Kakuda, R.; Yaoita, Y.; Machida, K.; Kikuchi, M. *Chem. Pharm. Bull.* **2000**, *48*, 749−751.
(7) Tsukamoto, S.; Macabalang, A. D.; Nakatani, K.; Obara, Y.; Nakahata, N.; Ohta, T. *J. Nat. Prod.* **2003**, *66*, 1578−1581.
(8) Yaoita, Y.; Matsuki, K.; Iljima, T.; Nakano, S.; Kakuda, R.; Machida, K.; Kikuchi, M. *Chem. Pharm. Bull.* **2001**, *49*, 589−594.
(9) Regerat, F.; Pourrat, H. *Ann. Pharm. Fr.* **1976**, *34*, 323−328.
(10) Nozoe, S.; Takahashi, A.; Kusano, G.; Itai, A.; Iitaka, Y. *Chem. Lett.* **1982**, *10*, 1679−1680.
(11) Knops, L.; Nieger, M.; Steffan, B.; Steglich, W. *Liebigs Ann. Chem.* **1995**, 77−80.
(12) Benevelli, F.; Carugo, O.; Invernizzi, A. G.; Vidari, G. *Tetrahedron Lett.* **1995**, *36*, 3035−3038.
(13) Invernizzi, A. G.; Vidari, G.; Vita-Finzi, P. *Tetrahedron Lett.* **1995**, *36*, 1905−1908.
(14) De Bernardi, M.; Garlaschelli, L.; Gattla, G.; Vidari, G.; Vita-Finzi, P. *Tetrahedron* **1988**, *44*, 235−240.
(15) De Bernardi, M.; Garlaschelli, L.; Toma, L.; Vidari, G.; Vita-Finzi, P. *Tetrahedron* **1991**, *47*, 7109−7116.
(16) Yoshikawa K.; Kuroboshi, M.; Arihara, S.; Mirua, N.; Tujimura, N.; Sakamoto, K. *Chem. Pharm. Bull.* **2002**, *50*, 1603−1606.
(17) Geroci, G.; Piattelli, M.; Tringali, C. *Magn. Reson. Chem.* **1991**, *29*, 603−606.

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