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Antineoplastic agents 582. Part 1: Isolation and structure of a cyclobutane-type sesquiterpene cancer cell growth inhibitor from *Coprinus cinereus* (Coprinaceae) ☆

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ABSTRACT

Bioassay-guided (murine P388 lymphocytic leukemia and human cancer cell lines) separation of an ethyl acetate extract prepared from the inky cap fungus *Coprinus cinereus* led to the isolation of three new sesquiterpenes, 7,7a-diepicoprinastatin 1 (**1**), 14-hydroxy-5-desoxy-2*S*,3*S*,9*R*-illudosin (**2**), and 4,5-dehydro-5-deoxyarmillol (**3**), together with the known armillol (**4**). The structure and relative configuration of **1** was determined by single-crystal X-ray diffraction experiments. The structures of compounds **2**, **3**, and **4** were each deduced by a combination of HRMS and 1D and 2D NMR techniques. Cyclobutane **2** led to modest inhibition of the murine P388 leukemia cell line.

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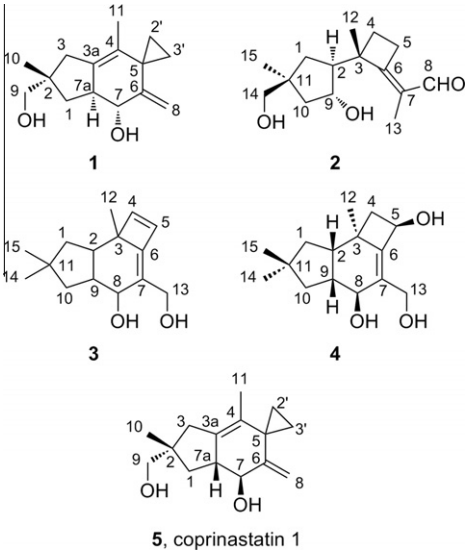
1. Introduction

Terrestrial fungi continue to be an increasing source of potentially useful substances with a wide range of biological activities. As it appears that less than 5% of fungal species have even been identified,[2] the prospect for making important new contributions to medical problems is certainly unconstrained. Recent examples of significant advances include the isolation and initial evaluations of terrestrial fungal species with cancer cell growth inhibitory properties (berkelic acid,[3] chrysosporide,[4] monocillin I[5]), and other fungal constituents with antifungal (pestafolide A[6]), antibacterial (scoparasin B[7]), and insecticidal (cycloaspeptide E[8]) activities. A review of endophytic fungal constituents known (230) to late 2005 has been prepared by Gunatilaka.[2]

In 2002, we began to focus on constituents of the leaf-litter inky cap fungus *Coprinus cinereus* (Schaeff) Gray, collected in the Shasta-Trinity National Forest (California), with respect to potential anticancer constituents. Interestingly, over 140 references to this fungus have appeared in just the past five years. Considerable interest in *C. cinereus* has developed because of its ease of culture and the commercial applications of its peroxidase and other enzymes. A brief summary of these applications, the few prior isolations of small molecules from this species, and our isolation and structural elucidation of a new cancer cell growth inhibitor designated coprinastatin 1 appear in our initial report on constituents of *C. cinereus*.[9]

In the previous contribution, we reported the isolation and structural elucidation of four new sesquiterpene components from *C. cinereus*, as well as a known oxazolinone component.[9] In a continuing examination of this fungus, the isolation and structure determination of three new sesquiterpene components, 7,7a-diepicoprinastatin 1 (**1**), 14-hydroxy-5-desoxyilludosin (**2**), and 4,5-dehydro-5-deoxyarmillol (**3**), respectively, will now be reported. In addition, the known sesquiterpene armillol (**4**) was identified. The anticancer and antimicrobial activities of these four sesquiterpenes were also evaluated.



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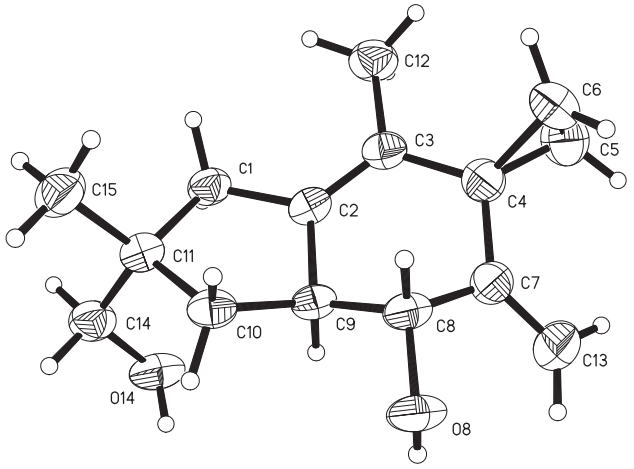

Figure 1. The X-ray crystal structure of one of the two possible enantiomers of the illudinoid, 7,7a-diepicoprinastatin 1 (**1**). The atomic thermal parameters are shown at 50% probability levels.

2. Results and discussion

7,7a-Diepicoprinastatin 1 (**1**) was isolated as colorless prisms from methanol and was assigned molecular formula $C_{15}H_{22}O_2$, based upon high-resolution APCI$^+$ mass spectroscopy experiments. On the basis of a comparison with those of coprinastatin 1 (**5**),[9] the ^{1}H and ^{13}C NMR spectra were consistent with structure **1**. The structure and relative configuration was confirmed by single-crystal X-ray diffraction analysis as depicted in Figure 1. However, these X-ray results did not allow convincing assignment of the absolute configuration, and thus Figure 1 represents one of the two possible enantiomeric forms of this illudinoid.

Terpene **2** was isolated as a colorless oil, and its molecular formula was assigned as $C_{15}H_{24}O_3$ by high-resolution FAB$^+$ mass spectroscopy ([M+1]$^+$ at *m/z* 253). Analyses of its ^{1}H NMR, ^{13}C NMR (APT) and HMBC spectra (Table 1) revealed 15 carbon signals that corresponded to three aliphatic methyl groups, five aliphatic methylenes (one oxygenated), three methines (two aliphatic with one oxygenated and one bearing an aldehyde group), and four quaternary carbons (one aliphatic, two olefinic, and one carbonyl). The ^{1}H–^{1}H COSY spectrum (Table 1) showed the correlations between H1 and H2, H2 and H9, H9 and H10, and H4 and H5, and thus the linkage of C1–C2–C9–C10 and C4–C5. The linkage of the other carbons in the molecule was easily deduced from the HMBC spectrum (Table 1). For example, the connections of C1 and C10 to quaternary carbon C11 were indicated by correlations from H1 and H10 to C11, while the C15 methyl group and the C14 hydroxymethyl group were also connected to C11, according to correlation peaks observed from H14 and H15 to C11. The presence of the cyclobutane ring (C3–C4–C5–C6–C3) was deduced from the strong correlations between both H4 and H5 and quaternary carbons C3 and C6. The correlations from H12 and H2 to C3 and from H4 and H12 to C2 determined the connection of C2 and C3. The C6–C7–C8 and C7–C13 segments were suggested by the correlations observed from H5 to C6 and C7 and from H13 to C6, C7, and C8. From these observations and from a comparison of the NMR data with those of 5-desoxyilludosin,[10] compound **2** was identified as the new secoprotoilludane sesquiterpene, 14-hydroxy-5-desoxyilludosin.

The relative configuration of the four chiral centers at C2, C3, C9, and C11 in compound **2** as drawn was deduced from its NOESY spectrum (Table 1). Strong NOEs were observed from H2 to H15, whereas no NOEs were observed from H2 to H9. This suggested that the 15-methyl group and H2 are on the same side (α' as drawn) of the five-carbon planar ring, while H9 is on the other (β as drawn), and so it was inferred that the relative stereochemistry of C2 was *S*, C9 was *R*, and C11 was *S*. The strong NOEs observed from H9 to H4α' suggested that both H4α' and the cyclopentane ring system are below (as drawn) the four-carbon planar ring, and the NOEs observed from H12 to H4β indicated that the 12-methyl is above (as drawn) the four-carbon planar ring. Thus, the relative configuration of C3 was assigned as *S*, and compound **2** is therefore identified as 14-hydroxy-5-desoxy-2*S*,3*S*,9*R*-illudosin or its 2*R*,3*R*,9*S*,11*R* enantiomer. The absolute configuration of **2** has yet to be determined conclusively.

On the basis of high-resolution APCI$^+$ mass spectroscopy and ^{1}H and ^{13}C (APT) NMR spectra, the molecular formula of sesquiterpene **3** (isolated as a colorless oil) was found to be $C_{15}H_{22}O_2$. Analyses of the ^{1}H, ^{13}C, and HMBC NMR spectra (Table 2) provided evidence that the 15 carbon signals for **3** corresponded to three aliphatic methyl groups, three aliphatic methylenes (one oxygenated), five methines (three aliphatic, including one oxygenated, and two olefinic), and four quaternary carbons (two aliphatic, two olefinic). The ^{1}H–^{1}H COSY spectrum (Table 2) established the correlations

Table 1
^{1}H and ^{13}C NMR assignments for sesquiterpene **2** in $CDCl_3$[a]

Position	δ ^{1}H	^{1}H–^{1}H COSY	NOESY	δ ^{13}C	HMBC[b]
1	1.45 (2H, d, 11.5)	H2	H14	37.85	H10α, H10β, H14, H15
2	2.32 (1H, dd, 11.5, 7.5)	H1, H9	H15, H12	55.57	H1, H4α, H4β, H9 (w), H10α, H10β, H12
3				50.63	H2, H4α, H4β, H5α, H5β, H9, H12
4α	2.05 (1H, td, 11.5, 6.5)	H4β, H5α, H5β	H9, H5α (w), H1	25.56	H2, H5α, H5β, H12
4β	1.76 (1H, td, 11.5, 6.5)	H4α, H5α, H5β	H5β		
5α	2.60 (1H, ddd, 10.0, 6.0, 1.5)	H4α, H4β, H5β	H4α (w)	25.72	H4α, H4β, H12
5β	2.75 (1H, ddd, 12.0, 6.0, 1.5)	H4α, H4β, H5α	H4β		
6				174.32	H2 (w), H4α, H4β, H5α, H5β, H13
7				131.26	H4α (w), H4β, H5α, H5β, H8, H13
8	9.81 (1H, s)			190.55	H13
9	3.99 (1H, q, 7.5)	H2, H10α, H10β	H4α	74.91	H2, H10α, H10β
10α	1.40 (1H, dd, 15.0, 7.5)	H9, H10β		46.32	H1, H14, H15
10β	2.00 (1H, dd, 15.0, 7.5)	H9, H10α	H9		
11				41.28	H1, H10α, H10β, H14, H15
12	1.56 (3H, s)		H2	28.46	H2, H4α, H4β
13	1.56 (3H, s)			9.82	
14	3.37 (2H, s)		H10β	71.44	H1, H10α, H10β, H15
15	1.10 (3H, s)		H2	24.98	H1, H10α, H10β, H14

[a] Measured at 500 MHz.
[b] w, weak.

Table 2
^{1}H and ^{13}C NMR assignments for sesquiterpene **3** in $CDCl_3$[a]

Position	δ ^{1}H	^{1}H–^{1}H COSY	δ ^{13}C	HMBC[b]
1α	1.28 (1H, dd, 11.5, 9.5)	H1β, H2	42.36	H10α, H10β, H14, H15
1β	1.40 (1H, ddd, 9.5, 8.5)	H1α, H2		
2	2.32 (1H, ddd, 11.5, 9.5, 8.5)	H1, H9	44.64	H12
3			52.37	H4, H5, H12
4	6.73 (1H, d, 2.5)	H5	152.30	H2, H12
5	6.48 (1H, d, 2.5)	H4	134.18	
6			147.97	H4 (w), H5, H8, H12, H13
7			125.60	H5, H8, H13
8	4.42 (1H, d, 10.0)	H9	75.11	H9 (w), H10α, H13
9	2.67 (1H, m)	H8, H10α, H10β	60.41	H1β, H10α, H10β
10aα	1.24 (1H, dd, 9.5, 7.0)	H9, H10β	47.91	H1α, H1β, H8, H14, H15
10β	1.72 (1H, dd, 12.5, 9.5)	H9, H10βα		
11			40.77	H1α, H1β, H10α, H10β, H14, H15
12	1.12 (3H, s)		21.12	H2
13	4.32 (2H, d, 10.0)		59.71	
14	1.08 (3H, s)		27.97	H1α, H1β, H10α, H10β, H15
15	0.92 (3H, s)		27.47	H1α, H1β, H10α, H10β, H14

[a] Measured at 500 MHz.
[b] w, weak.

between H1 and H2, H2 and H9, H8 and H9, and H9 and H10, and thus evidence for the C1–C2–C9–C10 and C8–C9 segments.

The HMBC spectrum (Table 2) led to the other carbon connections in **3**. From the correlations observed between C11 and H1, H10, H14, and H15, it was determined that C1, C10, C14, and C15 were all bonded to quaternary carbon C11 to form a five-carbon ring. Strong correlations between a methyl group (C12) signal that appeared at δ 1.12 as a singlet, showing that the methyl group was bonded to a quaternary carbon, and both C2 and the quaternary carbon at δ 52.37 (C3) led to the C2–C3–C12 segment. Four olefinic carbons at δ 125.6 (s), 147.97 (s), 134.18 (d), and 152.3 (d) implied the inclusion of two double bonds, and therefore, in order to satisfy the unsaturation number, the structure of **3** required another two rings besides the five-carbon ring already determined. The C13–C7–C6 and C7–C8 segments were suggested by the correlation of the hydroxymethyl group protons at δ 4.32 (H13) with C8 and with two olefinic quaternary carbons at δ 125.60 (s, C7) and 147.97 (s, C6). The correlations observed between H8 and the olefinic carbons (C6, C7) confirmed the linkage. The strong correlations observed from the 12-methyl protons to C6 (δ 147.97) indicated that C3 and C6 are bonded to form a six-carbon ring. Another two coupled olefinic carbon signals appeared at δ 134.18 (d) and 152.3 (d), with corresponding proton signals at δ 6.48 (1H, d, 2.0) and 6.73 (1H, d, 2.0), respectively. The small coupling constants between these two proton signals showed that they corresponded to two olefinic methines of a cyclobutene ring, thereby providing segment C3–C4–C5–C6 and fulfilling the unsaturation index. Apart from the signals corresponding to positions C4 and C5 of the cyclobutene ring, the NMR data were comparable to those of riparol C[11] and of armillol.[12] Therefore, the structure of **3** was established as 4,5-dehydro-5-deoxyarmillol, a new sesquiterpene. The relative configuration of **3** could not be determined because of the extremely limited supply (0.2 mg).

The colorless oily terpene **4** was found to have the molecular formula $C_{15}H_{24}O_3$ on the basis of EI mass spectroscopy and ^{1}H and ^{13}C NMR (APT) spectra. The structure was assigned as armillol by on 1D and 2D NMR analysis and comparison with corresponding data for the known compound.[12]

Substances **1**–**4** were evaluated for biological activity against the murine P388 lymphocytic leukemia cell line[13] and six human cancer cell lines.[14] Terpene **2** showed marginal P388 cancer cell inhibitory activity at ED_{50} 5.3 μg/ml, while **1**, **3**, **4** were considered inactive.

Compounds **1**, **2**, and **4** were available in sufficient quantity for antimicrobial evaluation using broth microdilution susceptibility assays.[15,16] At 64 μg/ml, these compounds did not inhibit the growth of *Stenotrophomonas maltophilia* ATCC 13637, *Micrococcus luteus* Presque Isle 456, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Enterobacter cloacae* ATCC 13047, *Enterococcus faecalis* ATCC 29212, *Candida albicans* ATCC 90028 or *Cryptococcus neoformans* ATCC 90112.

3. Experimental

3.1. General experimental procedures

All chromatographic solvents were redistilled. Sephadex LH-20 for partition column chromatography was obtained from Pharmacia Fine Chemicals AB. Melting points were measured on a Fisher Scientific electrothermal melting point apparatus and were uncorrected. Analytical HPLC was conducted with a Hewlett-Packard Model 1050 HPLC coupled with a Hewlett-Packard diode-array detector. Semipreparative HPLC was performed with a Waters 600 HPLC coupled to a Waters 2487 dual wavelength absorbance detector. The optical rotation measurements were recorded with a Perkin-Elmer 241 polarimeter. UV spectra were collected using a Perkin-Elmer Lambda 3B UV–vis spectrometer. NMR spectra were recorded with a Varian XL-400 or a Varian UNITY INOVA-500 spectrometer using tetramethylsilane (TMS) as an internal reference. A mixing time of 0.4 s was used for the NOESY experiment. High-resolution mass spectra were obtained with a JEOL LCMate magnetic sector instrument by APCI (positive ion mode) with a polyethylene glycol reference or FAB. EI mass spectra were recorded using a JEOL GCMate magnetic sector instrument. Single-crystal X-ray diffraction experiments were performed employing a Bruker 6000 X-ray diffractometer.

3.2. Specimen collection and isolation of compounds

Coprinus cinereus was isolated from a plant sample collected (2002) by one of us (GRP) in the Shasta-Trinity US National Forest in California. The collection, isolation, taxonomic identification, fermentation, extraction, and initial separation of constituents are described in our earlier report.[9] The CH_2Cl_2 fraction (13.4 g) prepared from an ethyl acetate extract[9] of the *C. cinereus* fermentation broth was passed through a Sephadex LH-20 column, in CH_3OH, to give five fractions that inhibited P388 growth. One of these fractions was passed again through Sephadex LH-20 in hexane–CH_3OH–2-propanol, to give 15 cancer cell inhibitory fractions, of which fractions A, B, and C were further separated by HPLC to

give coprinastatin 1 and the other previously reported compounds.[9] Separation of fraction A on a semipreparative Luna 5μ C18 (2) column with a gradient mobile phase (20–60% CH_3CN in H_2O for 60 min, followed by 100% CH_3CN for 20 min) had yielded 13 P388-active fractions[9]; for this report, further HPLC was carried out on three of these fractions (4, 6, and 8). Fraction 4 was applied to a semipreparative Luna 5μ C18 (2) column, beginning with a gradient mobile phase (30–55% CH_3CN in H_2O for 80 min) and followed by 100% CH_3CN for 20 min, at a flow rate of 2.8 ml/min, to give **3** (0.4 mg) as a colorless oil. On the same column, fraction 6 was subjected to HPLC (10–42% CH_3CN in H_2O for 20 min, followed by 18–28% CH_3CN in H_2O for 20 min, at a flow rate of 2.8 ml/min) to yield **2** (3.4 mg).

Fraction 8 of the original 13 active fractions was analogously separated by HPLC using the same column and a gradient mobile phase (25–40% CH_3CN in H_2O for 54 min followed by 100% CH_3CN for 20 min, at a flow rate of 2.8 ml/min). Two fractions *a* and *b* were obtained, of which *a* was separated further by HPLC using the same column and a slightly modified gradient mobile phase (22–33% CH_3CN in H_2O for 50 min at a flow rate of 2.8 ml/min) to provide 34.6 mg of **4** (colorless oil). From a CH_3OH solution of fraction *b*, compound **1** (9.1 mg) crystallized in colorless prisms.

3.3. 7,7a-Diepicoprinastatin 1 (1)

Colorless prisms from CH_3OH: mp 146–147 °C (CH_3OH); $[\alpha]_D^{24}$ −31.2° (0.17, CH_3OH); UV (CH_3OH) λ_{max} (log ε) 202 (4.7) nm; ^1H NMR ($CDCl_3$, 500 MHz) δ 0.41 (1H, ddd, 9.0, 6.0, 3.0, H2′α), 0.92 (1H, ddd, 10.0, 6.0, 4.0, H3′α), 1.05 (1H, ddd, 12.0, 6.0, 3.0, H2′β), 1.13 (3H, s, H10), 1.19 (1H, ddd, 12.0, 6.0, 4.0, H3′β), 1.28 (1H, dd, 14.0, 11.0, H1α), 1.30 (3H, s, H11), 2.08 (1H, d, 17.0, H3α), 2.17 (1H, dd, 14.0, 7.0, H1β), 2.21 (1H, d, 17.0, H3β), 2.51 (1H, m, H7a), 3.34 (1H, d, 11.0, H9α), 3.43 (1H, d, 11.0, H9β), 4.07 (1H, d, 10.0, H7), 4.58 (1H, d, 2.0, H8α), 4.95 (1H, d, 2.0, H8β); ^{13}C NMR ($CDCl_3$, 500 MHz) δ (ppm) 9.99 (C3′), 12.80 (C11), 17.70 (C2′), 24.92 (C10), 26.23 (C5), 40.45 (C3), 41.09 (C1), 42.88 (C2), 49.01 (C7a), 69.77 (C9), 76.25 (C7), 98.94 (C8), 125.27 (C4), 134.49 (C3a), 154.32 (C6); HRAPCI (positive ion mode) *m/z* 235.1573 $[M+H]^+$ (Calcd for $C_{15}H_{23}O_2$: 235.1698).

3.4. X-ray crystal structure determination of 7,7a-diepicoprinastatin 1 (1)

A thin, colorless, plate-shaped crystal (~0.58 × 0.09 × 0.13 mm), grown from a CH_3OH solution, was mounted on the tip of a glass fiber. Cell parameter measurements and data collection were performed at 123 ± 2 K on a Bruker SMART 6000 diffractometer. Final cell constants were calculated from a set of 3428 reflections from the actual data collection. Frames of data were collected in the θ range of 5.09–69.35° ($-10 \leqslant h \leqslant 10$, $-8 \leqslant k \leqslant 7$, $-12 \leqslant l \leqslant 12$) using 0.396° steps in ω such that a comprehensive coverage of the sphere of reflections was performed. After data collection, an empirical absorption correction was applied with the program SADABS.[17] Subsequent statistical analyses of the complete reflection set using the XPREP[18] program indicated the monoclinic space group $P2_1$.

3.5. Crystal data

$C_{15}H_{22}O_2$, $a = 8.9133(3)$, $b = 7.3964(2)$, $c = 10.5093(3)$ Å, $\beta = 102.9040(10)$, $V = 675.34(3)$ Å^3, $\lambda = (Cu\ K\alpha) = 1.54178$ A, $\mu = 0.584$ mm^{-1}, $\rho_c = 1.152$ g cm^{-3} for $Z = 2$ and fw = 234.33, $F(0\,0\,0) = 256$. A total of 4893 reflections were collected, of which 1981 were unique ($R_{int} = 0.0354$), and considered observed ($I_o > 2\sigma(I_o)$). These were used in the subsequent structure solution and refinement with SHELXTL-Version 5.1.[18] All non-hydrogen atoms for **1** were located using the default settings of that program. Hydrogen atoms were placed in calculated positions, assigned thermal parameters equal to either 1.2 or 1.5 (depending upon chemical type) of the *U*iso value of the atom to which they were attached, then both coordinates and thermal values were forced to ride that atom during final cycles of refinement. All non-hydrogen atoms were refined anisotropically in a full-matrix least-squares refinement process. The final standard residual R_1 value for the model shown in Figure 1 converged to 0.0428 (for observed data) and 0.0444 (for all data). The corresponding Sheldrick *R* values were wR_2 of 0.1173 and 0.1191, respectively, and the GOF = 1.070 for all data. The difference Fourier map showed small residual electron density; the largest difference peak and hole being +0.257 and −0.270 e/Å^3, respectively. Final bond distances and angles were all within acceptable limits. The structure depicted in Figure 1 represents only one of the two possible enantiomeric structures.[19] Deduction of the absolute stereochemistry of **1** by X-ray methods failed. Thus, refinement of the Flack absolute structure parameter,[20] which relies upon small anomalous dispersion differences displayed by the two enantiomers, yielded inconclusive results; for example, refinement of said parameter resulted in a value of 0.7, with an esd of 3.

3.6. 14-Hydroxy-5-desoxy-2*S*,3*S*,9*R*-illudosin (2)

Colorless oil: $[\alpha]_D^{26}$ +71.8° (0.17, CH_3OH); UV λ_{max} (CH_3OH) 247 (log ε 4.1) nm; ^1H and ^{13}C NMR (see Table 1); HRFAB (positive ion mode) *m/z* 253.1793 $[M+H]^+$ (Calcd for $C_{15}H_{25}O_3$: 253.1804).

3.7. 4,5-Dehydro-5-deoxyarmillol (3)

Colorless oil: $[\alpha]_D^{24}$ −177° (0.013, CH_3OH); UV λ_{max} (CH_3OH) 209 (log ε 4.3) nm; ^1H and ^{13}C NMR (see Table 2); HRAPCI (positive ion mode) *m/z* 235.1638 $[M+H]^+$ (Calcd for $C_{15}H_{23}O_2$: 235.1698).

3.8. Cancer cell line bioassay procedures

The National Cancer Institute's standard sulforhodamine B assay was used to assess inhibition of human cancer cell growth as previously described.[13,14] The P388 lymphocytic leukemia cell line results were obtained as described previously.[14]

Acknowledgments

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19. Crystallographic data (excluding structure factors) for structure **1** have been deposited with the Cambridge Crystallographic Data Center as supplementary publication number CCDC 772476. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 (0)1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).
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