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Four illudane sesquiterpenes from *Coprinopsis episcopalis*

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Abstract

Four new illudane derivatives with antibiotic and cytotoxic properties, illudins I (**1**), I$_2$ (**2**), J (**3**) and J$_2$ (**4**), have been isolated from the fungus *Coprinopsis episcopalis* (syn. *Coprinus episcopalis*). These sesquiterpenes are stereoisomers, and their relative structures have been determined taking into consideration 2D NMR data.

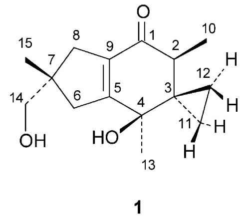
Keywords: *Coprinopsis episcopalis*; Basidiomycetes; Sesquiterpenes; Illudins

1. Introduction

Natural products from plants and microorganisms have proved to be a major source of important anticancer agents. Illudins are a group of sesquiterpene antibiotics that have been widely reported as antibacterial and antitumor agents (Mc Morris and Anchel, 1965). These low molecular weight natural products are cytotoxic to human tumor cells in vitro (Kelmer et al., 1990). Due to this bioactivity, illudin congeners have been the focus of considerable attention (Anchel et al., 1950; Mc Morris et al., 1999). Several species of Basidiomycetes have been reported in the literature to produce different classes of illudins and other related compounds (Arnone et al., 1991; Burguess et al., 1999; Cardillo et al., 1992). In the course of a program aimed at identifying bioactive substances from *Coprinopsis episcopalis* (syn. *Coprinus episcopalis*), four new illudin-type sesquiterpenes were isolated.

A total of 24.586 Ascomycetes and 718 Basidiomycetes were screened for the detection of new compounds with antibacterial activity against methicillin-resistant *Staphylococcus aureus*. Illudin-like compounds with antibacterial and cytotoxic activity were observed in ten strains, all of them belonging to the Basidiomycetes. All these strains were shown by PCR-based fingerprinting techniques to be genetically related but not identical. Nine of them were identified as *Coprinopsis episcopalis*. Another strain, MF6183, also identified as a *Coprinopsis* species, produces illudinic acid, an illudine previously discovered in the Merck, Sharp & Dohme Research Laboratories (Dufresne et al., 1997).

In this paper, we report on the full experimental evidence that led to the assignment of the relative structures of four new illudin sesquiterpenes, illudins I (**1**), I$_2$ (**2**), J (**3**) and J$_2$ (**4**), based mainly on data obtained by NMR spectroscopy, including 2D NMR experiments. These substances are produced by a basidiomycetous strain, which was identified as *Coprinopsis episcopalis*.



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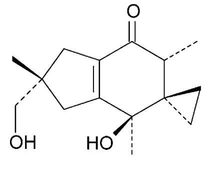

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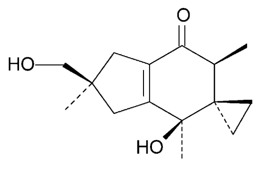

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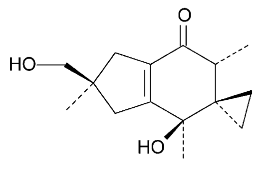

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2. Results and discussion

The structure **1** was assigned to illudin I on the basis of the following considerations: Its molecular formula was determined as $C_{15}H_{22}O_3$ by high resolution MS. The IR spectrum exhibited absorptions at 3450 and 1654 cm^{-1} due to the hydroxyls and to the unsaturated carbonyl group, respectively, whilst an absorbance in the UV spectrum at 250 nm (log ε 3.45) confirmed the conjugated system. In the ^1H NMR spectrum (Table 1) four high field multiplets of intensity one proton were observed at δ 0.40 and 0.69, assigned to H-12α and H-12β, respectively, and δ 0.51 and 0.55, to H-11α and H-11β, respectively. The corresponding carbons appear at δ 2.9 and 5.1. All these values are characteristic of the resonances of the cyclopropane moiety. Furthermore, the ^1H NMR spectrum showed signals of the hydroxymethylene group, two tertiary methyls, one secondary methyl at δ 0.91 (*d*, $J = 6.9$ Hz) and its vicinal proton at δ 2.69 (*q*, $J = 7.0$ Hz), and two isolated methylene groups, which resonate as two double doublets, the first at δ 2.45 and 2.69 and the second at δ 2.33 and 2.50. The carbon resonances bearing these hydrogens appear at δ 42.1 and 39.7, respectively. The complete assignment of the hydrogen and carbon signals (Tables 1–3) was realized on the basis of 2D NMR data (COSY, NOESY, HSQC and HMBC). Thus, for example, correlations in the HMBC spectrum were observed of the H-2 with C-1, C-3, C-4, C-9, C-10, C-11 and C-12, of the 10-methyl with C-1, C-2 and C-3, of the 14-hydroxymethylene with C-6, C-8 and C-15, and of the 15-methyl with C-6, C-8 and C-14.

The relative stereochemistry of illudin I (**1**) was determined by a study of its NOESY spectrum (Table 2). Thus, the 15-methyl (δ 1.14) showed strong NOEs with the protons at δ 2.45 (H-6β) and 2.33 (H-8β), whilst the 14-hydroxymethylene showed cross-peaks with the hydrogens at δ 2.69 (H-6α) and 2.50 (H-8α). The 6α-H also showed a nuclear Overhauser effect with the 13-methyl (δ 1.43) and this with H-2 (δ 2.69) and H-11β (δ 0.55).

The molecular formulas of illudins I_2 (**2**), J (**3**) and J_2 (**4**) were also determined as $C_{15}H_{22}O_3$ by HREIMS. A study of their spectral data showed them to have a carbon skeleton and functional groups similar to those of illudin I (**1**), and in consequence to be stereoisomers. The relative stereochemistry for these compounds was also assigned by interpretation of their NOESY data (Table 2).

The H-14 hydroxymethylene group in illudin I_2 (**2**) showed a NOE with H-6α δ 2.72), and this latter signal with the 13-methyl (δ 1.32), which indicated the same stereochemistry at C-4 and C-7 in both compounds, **1** and **2**. However, the stereochemistry at C-2 was different, because in illudin I_2 (**2**) a NOE was observed between the H-13 methyl and the H-10 methyl (δ 1.07), indicating α-stereochemistry for the latter.

In illudin J (**3**), as in **1**, a NOE was observed between the H-13 methyl and H-2, indicative of the same cyclohexene stereochemistry for these two compounds. Thus, these products must be stereoisomers at C-7, with β-stereochemistry for the hydroxymethylene group in illudin J (**3**). This was confirmed by the observation, also in the NOESY spectrum (Table 2), of cross-peaks corresponding to the interactions of H-14 with H-6α and H-8α, and of H-15 with H-6β and 8β.

With reference to illudin J_2 (**4**), as in the case of illudin I_2 (**2**), a NOE was observed between the H-13 methyl and the H-10 methyl, showing that both compounds have a cyclohexene ring with the same stereochemistry. In consequence, the structural difference between them must be the stereochemistry of the hydroxymethylene at C-7, which must be β in illudin J_2 **(4)**, as in illudin J (**3**), which was also confirmed by nuclear Overhauser effects observed of H-14 with H-6α and H-8α, and of H-15 with H-6β and H-8-β.

The cyclopentene stereochemistry assigned to compounds **1**–**4** was confirmed by analysis of the ^1H NMR

Table 1
^1H NMR data of compounds **1**–**4** (δ, J_{H-H} in Hz)

H	**1**	**2**	**3**	**4**
2	2.69, *q* (7.0)	2.40, *q* (7.4)	2.73, *q* (7.0)	2.32, *q* (7.2)
6α	2.69, *br d* (18.4)	2.72, *br d* (18.5)	2.31, *br d* (18.3)	2.32, *br d* (18.5)
6β	2.45 *br d* (18.4)	2.46, *br d* (18.5)	2.82, *br d* (18.3)	2.80, *br d* (18.5)
8α	2.50, *br d* (16.6)	2.53, *br d* (16.3)	2.27, *br d* (16.3)	2.30, *br d* (16.5)
8β	2.33, *br d* (16.6)	2.32, *br d* (16.3)	2.56, *br d* (16.3)	2.55, *br d* (16.5)
10	0.91, *d* (6.9)	1.07, *d* (7.4)	0.90, *d* (7.0)	1.09, *d* (7.4)
11α	0.51, *m*	0.43, *m*	0.51, *m*	0.44, *m*
11β	0.55, *m*	0.57, *m*	0.56, *m*	0.58, *m*
12α	0.40, *m*	0.40, *m*	0.39, *m*	0.36, *m*
12β	0.69, *m*	0.84, *m*	0.66, *m*	0.85, *m*
13	1.43, *s*	1.32, *s*	1.44, *s*	1.34, *s*
14	3.45 and 3.46, *s*	3.45 and 3.46, *s*	1.14, *s*	1.14, *s*
15	1.14, *s*	1.14, *s*	3.46 and 3.49, *d* (11)	3.45 and 3.49, *d* (11)

Table 2
NOESY data of **1**–**4**

H	**1**	**2**	**3**	**4**
2	H-10, H-13	H-10, H-11α	H-10, H-13	H-10, H-11α
6α	H-6β, H-13, H-14	H-6β, H-13, H-14	H-6β, H-13, H-14	H-6β, H-13, H-14
6β	H-6α, H-15	H-6α, H-15	H-6α, H-15	H-6α, H-15
8α	H-8β, H-14	H-8β, H-14	H-8β, H-14	H-8β, H-14
8β	H-2, H-8α, H-15	H-8α, H-15	H-8α, H-15	H-8α, H-15
10	H-2, H-11α, H-12α	H-2, H-11α, H-13	H-2, H-11α, H-12α	H-2, H-11α, H-13
11α	H-10, H-11β, H-12α	H-10, H-11β, H-12α	H-10, H-11β, H-12α	H-10, H-11β, H-12α
11β	H-12β, H-11α, H-13	H-12β, H-11α, H-13	H-12β, H-11α, H-13	H-12β, H-11α, H-13
12α	H-10, H-11α, H-12β	H-2, H-11α, H-12β	H-10, H-11α, H-12β	H-2, H-11α, H-12β
12β	H-11β, H-12α	H-11β, H-12α	H-11β, H-12α	H-11β, H-12α
13	H-2, H-6α, H-11β	H-6α, H-10, H-11β	H-2, H-6α, H-11β	H-6α, H-10, H-11β
14	H-6α, H-8α, H-15	H-6α, H-8α, H-15	H-6α, H-8α, H-15	H-6α, H-8α, H-15
15	H-6β, H-8β, H-14	H-6β, H-8β, H-14	H-6β, H-8β, H-14	H-6β, H-8β, H-14

Table 3
^{13}C NMR data of **1, 2** and **4**

C	**1**	**2**	**4**
1	198.9	199.0	200.0
2	44.9	47.8	48.2
3	30.0	30.8	30.0
4	70.5	70.1	70.5
5	164.8	163.8	164.0
6	42.1	42.4	42.4
7	42.3	42.7	42.4
8	39.7	39.7	39.7
9	135.0	135.0	136.0
10	9.5	13.7	14.0
11	5.1	5.5	5.5
12	2.9	9.2	9.5
13	24.5	24.6	24.8
14	24.6	24.9	70.5
15	70.7	70.5	24.6

data (Table 1). Thus, in illudins I (**1**) and I_2 (**2**), which have the same stereochemistry at C-7, the protons on C-6 have similar chemical shifts (δ 2.69 and 2.45 in **1,** 2.72 and 2.46 in **2**, α and β respectively), as is also true of the two hydrogens on C-8 (α and β) (δ 2.50 and 2.33 in **1,** 2.53 and 2.32 in **2**). These values were different from the resonances observed in illudins J (**3**) and J_2 (**4**) for H-6α and H-6β (δ 2.31 and 2.82 in **3**, 2.32 and 2.80 in **4**) or of the 8-H (α and β) (δ 2.27 and 2.56 in **3**, 2.30 and 2.55 in **4**). Moreover, this also indicated that the chemical shifts of the respective hydrogens in **3** and **4** were very similar, because in both the C-7 stereochemistry is the same, opposite to that of **1** and **2**.

The assignment of the H-6 and H-8 resonances from the 2D NMR data was confirmed by the observation, showed in Table 1, that coupling constants involving H-6α and H-6β are similar in the four stereoisomers, ~18 Hz. This is also true of 8α and 8β where coupling constant was ~16 Hz.

The stereochemistry assigned to the cyclohexene ring in the four sesquiterpenes was also confirmed by study of the 1D NMR data. Thus, compounds with the same cyclohexene stereochemistry as **1** and **3**, or **2** and **4**, exhibit similar chemical shifts. The 10- and 13-methyls appear, in the ^{1}H NMR spectra, at δ 0.91 and 1.43 in **1**, and 0.90 and 1.44 in **3**, respectively, whilst they resonate at δ 1.07 and 1.32 in **2**, and 1.09 and 1.34 in **4**, respectively. A similar behaviour can be observed with the cyclopropyl hydrogens (H-11 and H-12, α and β) (Table 1) and the carbon resonances of C-2 and C-10 (Table 3). Thus, in **2** and **4**, C-2 appear at δ 47.8 and 48.2, and C-10 at 13.7 and 14.0, respectively, whilst in **1**, with a different stereochemistry at this ring, the same carbons, C-2 and C-10, resonate at δ 44.9 and 9.5, respectively. The effect of the change in stereochemistry at C-2 is also clear in the chemical shifts of C-12, δ 9.2 and 9.5 in **2** and **4**, respectively, and δ 2.9 in **1**. Unfortunately, the ^{13}C NMR spectrum of **3** could not be obtained, due to the small amount isolated.

This is the first time that illudane sesquiterpenes hydroxylated at C-14 or C-15 have been isolated from the same fungus. However, protoilludane sesquiterpenes functionalized at these carbons have been found in *Armillaria mellea* (Arnone et al., 1988). Illudins I (**1**), I_2 (**2**) and J_2 (**4**) were moderately active against *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*, and also exhibited cytotoxic properties.

3. Experimental

3.1. General

Optical rotations were determined at room temperature using a Perkin-Elmer 241 polarimeter. IR and UV spectra were taken on a Perkin Elmer 1600 FT and Jasco V560 spectrometer, respectively. NMR spectra were measured on a Bruker AMX-2 500 MHz spectrometer with pulsed field gradient, using the solvent as internal standard. Exact mass measurements and EIMS were recorded on a Micromass Autospect instrument at 70 eV. HPLC was carried out on a 1100 HP Agilent ChemStation and a Beckman System Gold 168. Sephadex LH-20 (Pharmacia) and silica gel (Merck 7749, 15111 and 5554) were used for thin-layer (TLC), centrifugal thin-layer and column chromatography (CC). Compounds were visualized on TLC plates with a 25% H_2SO_4 solution.

3.2. Fungal isolation

The illudin-producing strains of this study were collected in the following places in Spain and Norway: MF6183 was isolated from leaf litter under *Quercus pyrenaica* (El Escorial, Madrid), F-043615 and F-050152 from twigs of *Q. ilex* (El Pardo, Madrid), F-043616 from the bark of a *Pinus* species (Fuenfría Valley, Madrid) and F-043898 from twigs of an undetermined bush (Puerto de Canencia, Madrid). Other isolates were recovered from fruit bodies of different Basidiomycetes: F-036104 and F-065192 from *Coltricia perennis* (San Rafael, Segovia, and Sierra de Aracena, Huelva, respectively), F-064720 from *Omphalina pyxidata* (Río Pelagallinas, Guadalajara) and F-062769 and F-061559 from *Hypoderma setigerum* and *Tylospora asterospora*, respectively (Akershus, Norway).

The isolation was made following standard procedures (Worrall, 1991; Peláez et al., 1998). All the strains isolated were maintained as PDA slants (Difco) until used for fermentation studies, and preserved as frozen agar plugs in glycerol 10% at −80 °C in the CIBE culture collection.

3.3. Fungal fermentations

The inocula for the fermentation studies were prepared as follows: The upper third of the agar slants from the cultures was removed and transferred aseptically into unbaffled 250 ml Erlenmeyer flasks, containing 50 ml of the seed medium (Bills et al., 1996). The seed flasks were incubated at 25 °C for 3–4 days with shaking (220 rpm). Aliquot parts (2 ml) of the resulting cultures were inoculated in flasks containing a production medium of sucrose (80 g l^{-1}), yellow corn meal (50 g) and yeast extract (Difco, 1 g l^{-1}). The production flasks were incubated at 25 °C for 21 days with similar shaking.

Flasks containing a brown rice medium (Jayasuriya et al., 1995) were used for the production of basidiomata from the cultures that produce illudins. These were inoculated from seed flasks, obtained as described above, and incubated statically at 25 °C for up to 4 months.

3.4. Extraction and isolation

Nine regrowths of *Coprinopsis episcopalis* (1 l each) were extracted with methyl ethyl ketone (MEK) (1400 ml each). Extraction of the fermentations was performed by liquid/liquid partition with MEK stirring at room temperature for 1 h. Analytical diode-array HPLC gradient characterization of samples was performed with a Zorbax Rx-C8 4.6 mm×250 mm column at 210 nm of UV detection. A 10–100% gradient of acetonitrile in water with a flow gradient of 0.9–1.2 ml/min was programmed at 20 °C. During the 22 min of each analysis trifluoracetic acid (0.01% TFA) was added for buffering the samples. The extracts were grouped according to the similarities of their chromatograms into crudes A (3.32 g), B (3.37 g), C (7.23 g), D (5.94 g), and E (11.1 g).

Each one of these extracts was chromatographed on a silica column (60 H, hexane–EtOAc 100:0–0:100 and EtOAc–MeOH 100:0–0:100), and the active fractions f-1 (2.46 g, hexane–EtOAc, 9:11–5:15) and f-2 (3.17 g, 100% EtOAc) were used for subsequent isolation procedures.

Fractions f-1 and f-2 were chromatographed on a Si gel vacuum liquid chromatography column (VLC) using a hexane–EtOAc gradient, Sephadex LH-20 column (200×80 mm) with hexane–CH_2Cl_2–MeOH (3:1:1) as eluent, Si gel column chromatography and Si gel centrifugal thin-layer chromatography using an hexane–EtOAc gradient were applied. The active fractions were purified by preparative normal-phase HPLC using a 250×20 mm Si gel column (Gasukuro Kogyo Inertsil ODS-2, 5 μm particle size). An isocratic system of hexane–EtOAc (40:60), and a flow rate of 8 ml/min yielded illudin I (**1**) (4.5 mg), illudin J (**3**) (0.8 mg), illudin I_2 (**2**) (4.9 mg) and illudin J_2 (**4**) (3.8 mg).

3.4.1. Illudin I (1)

Oil, $[\alpha]_D$ −41 (*c* 0.09, MeOH); UV (EtOH) λ_{max} (log ε) 250 (3.45) nm; IR ($CHCl_3$): ν_{max} 3450, 1654, 1455, 1379, 1040, 755 cm^{-1}; EIMS *m/z* (rel. int.): 250 $[M]^+$ (6), 232 (10), 222, (10), 219 (10), 207 (100), 201 (17), 189 (24), 173 (13), 159 (12), 145 (11), 131 (13), 119 (15), 105 (23), 91 (24), 77 (17), 69 (12), 55 (17); HREIMS: $[M]^+$ at *m/z* 250.1542. $C_{15}H_{22}O_3$ requires 250.1568; ^1H and ^{13}C NMR data in Tables 1 and 3, respectively.

3.4.2. Illudin I_2 (2)

Oil, $[\alpha]_D$ +22.5 (*c* 0.08, MeOH); IR ($CHCl_3$): ν_{max} 3018, 1659, 1427, 1215, 1036, 756 cm^{-1}; EIMS *m/z* (rel. int.): 250 $[M]^+$ (8), 235 (17), 232 (18), 222, (30), 219 (24), 208 (62), 207 (100), 205 (24), 201 (29), 189 (33), 173 (24), 159 (21), 145 (21), 131 (23), 119 (26), 105 (40), 91 (43), 77 (35), 67 (20), 55 (34); HREIMS: $[M]^+$ at *m/z* 250.1565. $C_{15}H_{22}O_3$ requires 250.1568; ^1H and ^{13}C NMR data in Tables 1 and 3, respectively.

3.4.3. Illudin J (3)

Oil, EIMS *m/z* (rel. int.): 250 $[M]^+$ (4), 232 (25), 222 (3), 219 (28), 201 (49), 199 (24), 189 (11), 187 (21), 173 (21), 171 (17), 160 (10), 159 (26), 145 (33), 143 (31), 131

(33); HREIMS m/z $[M]^+$ 250.1483. $C_{15}H_{22}O_3$ requires 250.1568; ^1H and ^{13}C NMR data in Tables 1 and 3, respectively.

3.4.4. *Illudin J_2 (4)*

Oil, $[\alpha]_D$ −26 (c 0.034, MeOH); UV (EtOH): λ_{max} (log ε) 250 (4.62) nm; IR ($CHCl_3$): ν_{max} 3400, 1659, 1430, 1039, 755 cm^{-1}; EIMS m/z (rel. int.): 250 $[M]^+$ (2), 232 (94), 222, (13), 219 (16), 207 (31), 205 (14), 201 (100), 199 (38), 189 (17), 187 (17), 173 (30), 171 (18), 161 (14), 160 (23), 159 (21), 145 (21), 143 (29), 131 (24), 119 (18), 105 (29), 91 (40), 77 (30), 67 (13), 55 (21); HREIMS: $[M]^+$ at m/z 250.1599. $C_{15}H_{22}O_3$ requires 250.1568; ^1H and ^{13}C NMR data in Tables 1 and 3, respectively.

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