# Illudinic Acid, a Novel Illudane Sesquiterpene Antibiotic

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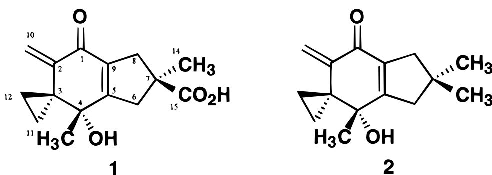
A novel illudane sesquiterpene antibiotic (**1**) with activity against methicillin-resistant *Staphylococcus aureus* (MRSA), has been isolated. Its MIC against MRSA was found to be 16 $\mu$g/mL. It also shows L-1210 cytotoxicity with an $IC_{50}$ of 10−15 $\mu$g/mL.

In the course of a natural products screening effort for new antibacterial agents, we have found a new member of the sesquiterpene illudin family for which we propose the name illudinic acid. There exists a number of naturally occuring illudins. The first reported examples are those of illudins S and M produced by *Omphalotus illudens*, the jack-O-lantern mushroom.[1,2]

The illudins are metabolically activated to unstable intermediates that cause irreparable DNA damage.[3] They have been investigated as anticancer agents, as it was found that the number of molecules necessary to kill a cell was several orders of magnitude less than that required of cisplatin[4] and that they exhibit some degree of selectivity in their antitumor activity.[5] Indeed, some analogues do show an improved therapeutic index.[6] For example, deoxyilludin M causes an increased life span of 24% when tested at 5 mpk (ip, 5 days) against murine leukemia P-388, while illudin S is ineffective.[7] Similarly, tumor growth was inhibited by dehydroilludin M by about 75% when tested on human myeloid leukemia xenographs established in mice. This efficacy was observed at a dose of 3 mpk (iv, 14 days), which was well tolerated by the animals.[8]

Similar compounds have been isolated from *O. illudens* and other basidiomycetes.[9,10] Illudins C−E have recently been reported.[11] We report our discovery of illudinic acid (**1**), a compound closely related to illudin C (**2**).




The fungus MF 6186 was isolated from a sample of leaf litter of oak (*Quercus pyrenaica* L.) collected in El Escorial, Madrid, Spain. It was isolated following a particle-filtration procedure[12] and was grown on several mycological media, some of them including cellulosic materials such as sterilized leaves and filter paper. It failed to sporulate under any of those conditions and thus could not be identified. It was cultivated on a corn meal/sucrose medium for 21 days, a time point that gave optimal antibiotic production.

A sample aliquot of the whole broth was characterized using a modified agarose−gel−electrophoresis bioautography procedure.[13] An agar overlay with a liquid surface inoculation of methicillin-resistant *Staphylococcus aureus* was used to characterize the charge and mobility of the antibiotic present. At pH 6, the antibiotic was found to be anionic. The whole broth was thus extracted with MeOH and adsorbed on an anion exchange resin. After elution with a high salt concentration, the eluate was desalted using a HP-20 resin. The desalted eluate was then fractionated using HPLC to afford a pure sample of a novel antibiotic, which we termed illudinic acid (**1**).

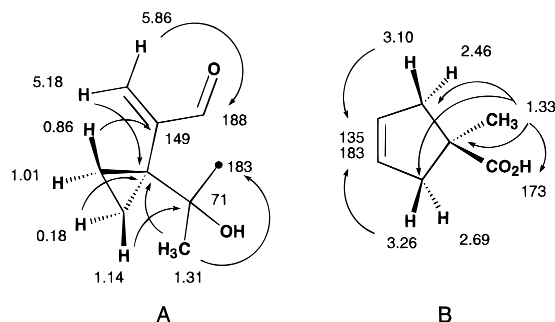



**Figure 1.** Partial structures A and B with selected HMBC correlations.

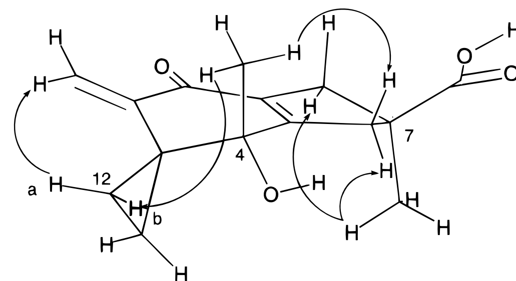



**Figure 2.** Selected NOE correlations for **1**.

The presence of a cyclopropane ring is immediately apparent from the $^{1}$H NMR of this new antibiotic. The only additional resonances that can be easily assigned are to an exocyclic methylene, two isolated methylene groups long-range coupled to each other, and two methyl groups on quaternary carbons. With the aid of HMBC and HMQC experiments, partial structures A and B (Figure 1) could be readily assembled. Combining fragments A and B afforded **1**, which possesses the illudane skeleton. A comparison with the assigned chemical shifts of **2**[10] confirmed our structural assignments. As shown in Figure 2, NOE experiments in both $CD_3OD$ and $C_5D_5N$ allowed for the determination of the

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**Table 1.** NMR Assignments for **1** ($CD_3OD$, 25 °C, 400 MHz)

| carbon # | $^1H$ | mult. | $^{13}C$ |
|---|---|---|---|
| 1 | | | 188.14 |
| 2 | | | 149.80 |
| 3 | | | 34.21 |
| 4 | | | 70.87 |
| 5 | | | 183.85 |
| 6 a | 2.69 | dd (19.2, 1.2) | 45.89 |
| 6 b | 3.26 | dt (19.2, 2.0) | |
| 7 | | | 27.05 |
| 8 a | 2.46 | dd (16.0, 1.2) | 43.08 |
| 8 b | 3.10 | dt (16.4, 2.0) | |
| 9 | | | 135.32 |
| 10 a | 5.86 | d (1.2) | 116.89 |
| 10 b | 5.18 | d (1.2) | |
| 11 a | 0.18 | ddd (9.6, 6.4, 3.2) | 13.51 |
| 11 b | 1.14 | ddd (9.2, 5,6, 3,2) | |
| 12 a | 1.01 | dt (9.2, 5,2) | 5.33 |
| 12 b | 0.86 | ddd (10.0, 6.8, 5.2) | |
| 13 | 1.31 | s | 26.08 |
| 14 | 1.33 | s | 27.04 |
| 15 | | | 172.86 |

**Table 2.** Minimum Inhibitory Concentrations for Illudinic Acid

| organism | strain | MIC (μg/mL) |
|---|---|---|
| *Staphylococcus aureus* | MB108 | 1 |
| *MRSA* (COL) | MB5393 | 16 |
| *Escherichia coli* | MB1418 | 125 |
| *Erwinia atroseptica* | MB1159 | >125 |
| *Bacillus subtilis* | MB964 | 16 |
| *Proteus vulgaris* | MB838 | >125 |
| *Pseudomonas aeruginosa* | MB2824 | >125 |
| *Enteroccoccus faecium* | MB5572 | >125 |

**Table 3.** Cytotoxicity of Illudinic Acid

| assay (μg/mL) | illudinic acid | vancomycin | doxorubicin |
|---|---|---|---|
| L-1210 cytotoxicity | | | |
| MTT $IC_{50}$ | 15 | > 1000 | 0.2 |
| trypan blue $IC_{50}$ | 10 | > 1000 | < 0.1 |
| cell lysis | none | none | none |
| MIC | | | |
| *S.aureus* (COL) | 16 | 0.5 | 16 |

relative stereochemistry of **1**. As expected, the methyl group at C-7 shows NOEs to only one of the two hydrogens for each one of the two methylene groups at C-6 and C-8. The methyl group at C-4 shows NOEs to one of the cyclopropane hydrogens (H-12b) and to the hydrogen at C-6 on the opposite side of the methyl at C-7. This is only possible for the stereoisomer shown, having the two methyl groups on opposing faces. A full NMR assignment is shown in Table 1.

Illudinic acid was found to have good potency against *S. aureus* but very poor activity against other organisms, as shown in Table 2. In spite of its seemingly reactive chemical structure, illudinic acid was not found to be acutely cytotoxic. It demonstrates cytotoxicity in a mammalian cell culture system[14,15] that is similar to its MICs against Gram-positive organisms, as shown in Table 3. This is in contrast to vancomycin, an inhibitor of bacterial peptidoglycan synthesis, whose antibacterial MIC of 0.5 μg/mL against the COL strain of *S. aureus* is much lower than its cytotoxic $IC_{50}$'s, and doxorubicin (Adriamycin), an inhibitor of mammalian topoisomerase II, which is cytotoxic at levels much below its antibacterial MICs. Whereas the cytotoxicity of doxorubicin is probably mechanistically distinct from its antibacterial activity, the antibacterial and cytotoxic effects of illudinic acid may be due to the same mechanism. The mechanism of illudinic acid's cytotoxicity is not known, but it does not act by causing extensive cell lysis.

Although the fungus could not be precisely identified, the fact that illudins seem to be produced mainly by basidiomycetes suggested a taxonomic affiliation of the fungus at the level of subdivision (Basidiomycotina/Ascomycotina). The morphology of the colony and the microscopic appearance of the mycelium resembled basidiomycetes in pure culture. However, the absence of clamps (hyphal structures connecting contiguous cells), which are a diagnostic feature of this group, made it impossible to establish a taxonomic affiliation of this fungus based exclusively on morphological criteria. This made necessary the use of other techniques, not based on morphology. Recently, a primer has been designed that specifically recognizes a sequence of the internal transcribed spacer (ITS) region in the nuclear ribosomal repeat unit, which is conserved in basidiomycetes.[16] The polymerase chain reaction, using this oligonucleotide in combination with another fungus-specific primer, resulted in the unequivocal amplification of a DNA fragment of about 750 bp (data not shown). This result confirms the hypothesis that the producer fungus of illudinic acid belongs to the Basidiomycotina.

## Experimental Section

**General Experimental Procedures**. 3-4,5 Dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT), trypan blue, and the control drugs vancomycin and doxorubicin were obtained from Sigma. $^1H$- and $^{13}C$-NMR spectra were recorded at 25 °C on a Varian Unity 500 spectrometer. $^1H$ chemical shifts are shown in ppm relative to $Me_4Si$ at 0 ppm using the residual solvent peak at 3.30 ppm ($C\mathit{H}D_2OD$) as internal standard. $^{13}C$ chemical shifts are shown in ppm relative to $Me_4Si$ at 0 ppm using the residual solvent peak at 49.0 ppm ($\mathit{C}D_3OD$) as internal standard. IR spectra were obtained from a film on a ZnSe multiple internal reflectance crystal, using a Perkin-Elmer Model 1750 FTIR spectrometer. MS were recorded on a Finnigan MAT 90 mass spectrometer. Optical rotations were measured on a Perkin-Elmer Model 241 polarimeter.

**Minimum Inhibitory Concentrations**. Illudinic acid was two-fold serially diluted in DMSO at 20× final experimental concentrations, and 5 μL of these solutions were transferred to 45 μL LB medium in 96-well microtiter plates. Bacterial inocula were prepared by serially, diluting stationary phase cultures ($10^{-3}$ to $10^{-4}$) using LB medium.[17] A 50-μL sample of bacterial inocula was then added to each of the MIC wells.

**Cytotoxicity.**[14,15] Cytotoxicity determinations using MTT were performed using mouse L-1210 cells grown in Fisher's Medium (Difco) plus 10% heat-inactivated fetal bovine serum. The assays were performed in microtiter plates. Cell viability was also assessed using trypan blue exclusion; an equal aliquot of treated L-1210 cells and 0.4% trypan blue were mixed, and the total number of cells, as well as the proportion of viable (i.e., dye-excluding) cells, was determined by microscopic observation. In both MTT and trypan blue assays, the $IC_{50}$ indicates the concentration of compound that results in a viable cell number half the level of the control. The trypan blue assay was also used to determine the extent of lysis of L-1210 cells after a 24-h incubation.

**Culture Description**. The fungus was isolated from leaf litter of oak collected in El Escorial, Madrid, Spain. In agar culture, the fungus exhibits the following morphological characteristics (all observations were made after two weeks growing at 22 °C, 85% relative humidity, 12-h photoperiod). On yeast-malt extract agar (10 g malt extract, 2 g yeast extract, 20 g agar in 1 L distilled $H_2O$), colonies of 73−76 mm in diameter are formed. Aerial mycelium is appressed, subfelty, and constituted by intermingled hyphae radially disposed, with margin uneven, submerged, fimbriate. Colony color[18] was orange white, grayish orange at the center, sometimes with grayish orange dots densely grouped in the inner two-thirds of the colony. Reverse was yellowish white to orange white at the center, sometimes with grayish orange dots. Odors and exudates are absent. On potato dextrose agar (Difco), colonies of 55−59 mm in diameter are formed. Aerial mycelium was raised, cottony, felty at the center, with its margin even, submerged, fimbriate. Colony color was white to orange white, grayish orange at the center, white at the margin; reverse was yellowish white to pale yellow and orange white to pale orange at the center. On oatmeal agar (Difco), colonies of 75−80 mm are formed. Aerial mycelium is very sparse, white, appressed, and subfelty, with margin uneven, submerged, fimbriate. Colony is colorless. Odors and exudates are absent.

The mycelium is composed of undifferentiated thin-walled hyphae, is branched and septate, is 3−6.5 $\mu$m in diameter, is mostly hyaline in $H_2O$ and KOH, and sometimes contains a faint yellow cytoplasmic pigment. Short irregular branches are often present, as well as irregular terminal and intercalary swellings of the hyphae, which can reach up to 15 $\mu$m across. Clamps and propagative structures were not observed.

**Fermentation**. The fungus MF 6186 was deposited in the Merck Microbial Resources Culture Collection. It was grown at 25 °C, 220 rpm for 5−6 days on a seed medium that contained corn steep liquor (5 g/L), tomato paste (40 g/L), oat flour (10 g/L), glucose (10 g/L), and the following trace elements: $FeSO_4 \cdot 7\ H_2O$ (10 $\mu$g/L), $MnSO_4 \cdot 4\ H_2O$ (10 $\mu$g/L, $CuCl_2 \cdot 2\ H_2O$ (0.025 $\mu$g/L), $CaCl_2$ (0.1 $\mu$g/L), $H_3BO_3$ (0.056 $\mu$g/L), $(NH_4)_6MoO_2 \cdot 4\ H_2O$ (0.019 $\mu$g/L), and $ZnSO_4 \cdot 7\ H_2O$ (0.2 $\mu$g/L). The seed was used to inoculate production medium, which contained sucrose (80 g/L), corn meal (50 g/L), and yeast extract (1 g/L). Cultures were grown for 21 days at 25 °C, in 250-mL Erlenmeyer flasks containing 50 mL of media, on a rotary shaker (220 rpm).

**Isolation**. MeOH (500 mL) was added to the whole broth (500 mL), and this solution (pH 7.7) was adsorbed on a bed of Dowex-1 (Cl; 50 mL) with a flow rate of 2.5 mL/min. The column was washed with $H_2O$ (100 mL) and eluted with 3% $NH_4Cl$ in 90% MeOH−$H_2O$, collecting 25-mL fractions. Eluate fractions 2−5 were combined and diluted with $H_2O$ (150 mL). This solution was adsorbed on a column of HP-20 resin (20 mL) with a flow rate of 2 mL/min. The column was washed with 30% MeOH−H2O, and eluted with MeOH (100 mL). The MeOH eluate was concentrated to dryness under reduced pressure to afford 139 mg of an oily residue. This residue was dissolved in 4 mL of 30% $CH_3CN$−$H_2O$ and chromatographed on RP-HPLC (Zorbax RX-C8, 5 $\mu$m, 25 × 250 mm, 10 mL/min) collecting fractions at 0.5 min intervals. Fractions 25−27 were dried to afford 1.6 mg of illudinic acid (**1**) as an oil: $[\alpha]^{25}{}_D$ −29° (*c* 0.45, MeOH); UV (MeOH) $\lambda$ max 207, 228 (sh), 282 nm; IR (film on ZnSe) $\nu$ max 3415, 2971, 1708, 1657, 1605, 1468, 1392, 1276, 1222, 1130, 934 $cm^{-1}$; EIMS (70 eV) *m/z* found 262.1162 ($C_{15}H_{18}O_4$, calcd 262.1205, 66), 247.1055 ($C_{14}H_{15}O_4$, 82, M − $CH_3$), 234.0984 ($C_{13}H_{14}O_4$, 29, M − $C_2H_4$), 219.0998 ($C_{13}H_{15}O_3$, 34, M − $CH_3$ − CO), 217.1191 ($C_{14}H_{17}O_2$, 40, M − $CO_2H$), 201.0918 ($C_{13}H_{13}O_2$, 95, M − $CH_3$ − CO − $H_2O$), 189.0968 ($C_{12}H_{13}O_2$, 100, M − $CO_2H$ − $C_2H_4$), 173.0907 ($C_{12}H_{13}O$, 42, M − $CH_3$ − CO − $HCO_2H$).

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