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Cytotoxic Illudane Sesquiterpenes from the Fungus *Granulobasidium vellereum* (Ellis and Cragin) Jülich

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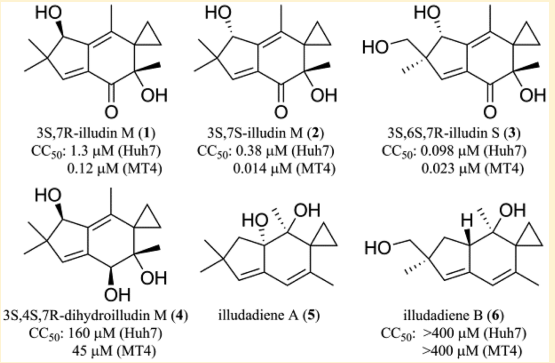
Supporting Information

ABSTRACT: Eight illudane sesquiterpenes were obtained from the wood-decomposing fungus *Granulobasidium vellereum* (Ellis and Cragin) Jülich; among them were the enantiomers of the known compounds illudin M (**1**) and dihydroilludin M (**4**) and the diastereomers of illudin M (**2**) and illudin S (**3**), as well as two previously undescribed illudanes (**5**, **6**). The cytotoxicity of compounds **1**−**4** and **6** was evaluated against two tumor cell lines (Huh7 and MT4), which showed that compounds **1**−**3** had potent cytotoxic activity, whereas compounds **4** and **6** had no or only moderate effects at concentrations up to 400 μM. Surprisingly, both compounds **2** and **3** were about 10 times more potent than **1**. When the chemical reactivity of **1** and **2** was tested, compound **2** was shown to have a substantially higher reaction rate when reacted both with 2 M HCl and with cysteine, indicating that the difference in cytotoxicity is probably due to chemical reactivity and not to enzymatic affinity.



Fungi from the phylum Basidiomycotina, one of the five subdivisions of Eumycota (true fungi), have been found to produce a large variety of secondary metabolites, of which many are of terpenoid origin. Most common among the terpenes are the sesquiterpenes, which in the basidiomycetes are mainly formed via the humulene protoilludane biosynthetic pathway.[1,2] Through rearrangements of the protoilludane carbon skeleton a number of different classes of compounds are formed. Some of the skeletal types have been described to possess interesting biological properties, of which the illudane skeletal type with its characteristic highly electrophilic spirocyclopropyl moiety has been of particular interest due to their high cytotoxicity.[2]

The naturally occurring illudanes illudin M and S were originally obtained from the poisonous Jack-o'-lantern mushroom *Omphalotus olearius* (prev. *Clitocybe illudens*).[3] They have, due to their extreme cytotoxicity, been the subject of numerous studies in order to determine their mechanism of action. They were found to be able to initiate DNA breakage in the cell through a two-step reaction initiated by a Michael-type addition of a thiol bionucleophile followed by a second nucleophilic attack on the spirocyclopropane group.[4−6] Unfortunately illudins M and S have turned out to be too toxic for clinical use against different cancer types. Instead the work has been focused on finding analogues with more beneficial therapeutic windows.[6,7] The most successful was an analogue of illudin S named irofulven,[8] which was subjected to phase III clinical trials against several types of cancer before being canceled in 2012 due to lack of efficacy.[9]

Granulobasiodium vellereum (Ellis & Cragin) Jülich is a saprotrophic wood decay fungus from which we have previously obtained a large variety of sesquiterpenoid metabolites,[10−12] of which two have shown potent cytotoxic activity.[12,13] This paper describes work to continue the identification and characterization of secondary metabolites from *G. vellereum* and to assess their cytotoxic effects.

RESULTS AND DISCUSSION

From the wood-decomposing fungus *G. vellereum* eight illudane sesquiterpenes were isolated through a combination of solid-phase extraction (SPE) and chromatographic techniques. Their respective structures were elucidated with spectroscopic techniques, and the cytotoxicity of compounds **1**−**4**, **6**, and 7,9-illudadiene-3,14-diol was evaluated against Huh7 and MT4 tumor cell lines.

Compound **1** had a molecular formula of $C_{15}H_{20}O_3$ according to HRESIMS analysis. The ^{1}H NMR data of compound **1** were identical to the literature data of illudin M,[4,14] but the specific rotation of compound **1** (96°) did not match that reported for illudin M (−284°).[3] This indicates that compound **1** might be an enantiomer of illudin M, and to

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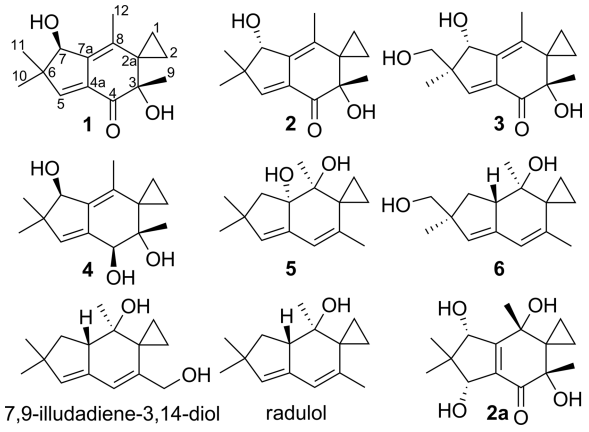

investigate this, the Mosher's test was performed on compound **1**.[15] From the shift differences between the S-MTPA and R-MTPA monoesters of **1** (Figure 1) it could be deduced that compound **1** had a 3*S*,7*R* configuration and indeed was the enantiomer of illudin M (3*R*,7*S*); compound **1** was named (3*S*,7*R*)-illudin M.

According to HRESIMS analysis, compound **2** had a molecular composition of $C_{15}H_{20}O_3$. The NMR data for compound **2** (Table 1) closely resembled those of illudin M and compound **1**. The HMBC spectrum showed that the bonding pattern of compound **2** was identical to that of compound **1**, but since the 1D NMR data were not identical, the structures must be diastereomeric. When stored over extended periods of time (−18 °C, >1 year), compound **2** decomposed and formed a variety of degradation products, of which the most abundant was the highly oxygenated compound **2a**. Compound **2a** had the same HMBC correlations as would be expected for illudin B and illudin H,[16,17] two illudane sesquiterpenes obtained from the fungi *Omphalotus olearius* and *Omphalotus nidiformis*, but the ^{1}H NMR data of **2a** did not match those reported for these compounds,[16,17] indicating that compound **2a**, illudin B, and illudin H are diastereomeric compounds. The relative configuration of compound **2a** could, unlike that of compound **2**, be determined from ROESY data (Figure 2), displaying diagnostic correlations between Me-9 and H-5 and from the latter to Me-11. H-7 had correlations to both Me-11 and Me-12, resulting in the relative configuration of compound **2a**. The relative configuration of compound **2** can also be deduced from these data if it is assumed that the configuration of C-3 and C-7 is retained in **2a** compared to compound **2** during the degradation process. To be able to determine the absolute configuration of compound **2**, the Mosher's test was performed,[15] which demonstrated the configuration to be 3*S*,7*S* for compound **2** (Figure 1). Compound **2** was consequently named (3*S*,7*S*)-illudin M.

The molecular formula of compound **3** was determined to be $C_{15}H_{20}O_4$ from HRESIMS analysis. The ^{1}H NMR data of compound **3** (Table 1) closely resembled but were not identical to those of illudin S,[18] which differs from illudin M only through the oxidation of methyl-11 in illudin M to a CH_2OH group in illudin S. The HMBC bonding pattern of **3** was also identical to what could be expected from illudin S, making it likely that they are diastereomers.

The relative configuration of C-6 and C-7, though not of C-3, could be deduced through diagnostic ROESY correlations between H-7 and H_2-11 (Figure 2). To be able to perform the Mosher's test on compound **3**, the primary hydroxy group CH_2OH was selectively acetylated, since it might otherwise react with the chiral Mosher's reagent [α-methoxy-(trifluoromethyl)phenylacetyl chloride] and interfere with the result of the test. The resulting acetylated product (**3a**) was then subjected to the Mosher's test,[15] from which the absolute configuration of C-6 and C-7 then could be deduced as 6*S*,7*R*. If compound **3** had a 3*R* configuration, it would be identical to illudin S, which according to NMR and polarimetric data it is not,[3,18] and consequently the absolute configuration of compound **3** must be 3*S*,6*S*,7*R*. Compound **3** was named (3*S*,6*S*,7*R*)-illudin S.

According to HRESIMS analysis, compound **4** had a molecular formula of $C_{15}H_{22}O_3$. The ^{1}H NMR data were identical to those described for dihydroilludin M,[14] another illudane sesquiterpene also originally isolated from *O. olearius*.[19] The specific rotation of compound **4** was +77° compared to −35° for dihydroilludin M,[19] indicating that compound **4** was the enantiomer of dihydroilludin M. To further verify this hypothesis, compound **4** was selectively oxidized at position C-4.[16] The ^{1}H NMR and specific rotation of the oxidized product were identical to those of compound **1** (the enantiomer of illudin M), and accordingly it was concluded compound **4** was the enantiomer of dihydroilludin M and named (3*S*,4*S*,7*R*)-dihydroilludin M.

The molecular composition of compound **5** was determined to be $C_{15}H_{22}O_2$ from HRESIMS analysis, resulting in an unsaturation index of five. Four sp^2 carbons were identified from the NMR data, indicating that **5** had a tricyclic structure. The NMR data also showed that the structure included four methyl groups, two quaternary carbons, two oxygen-linked tertiary carbons, and three CH_2 groups. From COSY and HSQC data a $-CH_2(1)-CH_2(2)-$ spin system was identified. HMBC showed that both Me-10 and Me-11 had correlations to C-6, C-7, and a protonated sp^2-carbon (C-5). Both H-5 and H_2-7 had correlations to an sp^2 carbon (C-4a) and an oxygen-linked tertiary carbon (C-7a), forming the cyclopentene moiety of the compound (Figure 3). C-7a also had a correlation to Me-12, which in turn correlated to C-8 and C-2a. The latter correlated also to both ends of the spin system, as well as to Me-9. Me-9 correlated to two sp^2 carbons (C-3 and C-4). Finally H-4 correlated to C-4a and C-5, resulting in the proposed structure of **5**, which was named illudadiene A. The relative configuration of **5** was determined through diagnostic ROESY correlations (in DMSO-d_6) between Me-12 and OH-7a and from the latter to H-7α. H-7β had a ROESY cross-peak to OH-8, giving the relative configuration of compound **5**. If it

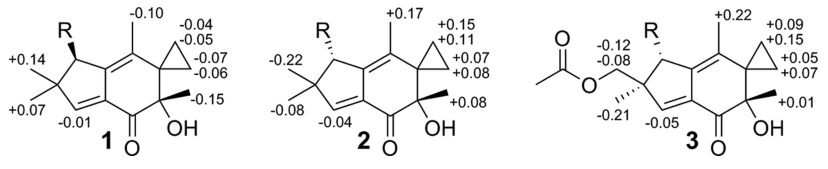

Figure 1. Chemical shift differences (in ppm) between the *S*-MTPA monoesters and the *R*-MTPA monoesters of **1**, **2**, and **3a** (pyridine-d_5).

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Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data (600 MHz ^{1}H NMR and 150 MHz ^{13}C NMR, MeOH-d_4) for (3*S*,7*S*)-Illudin M (**2**), (3*S*,6*S*,7*R*)-Illudin S (**3**), and Illudadienes A (**5**) and B (**6**)

	2		3		5		6	
pos.	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)
1	9.1, CH_2	1.08, ddd (9.9, 6.2, 4.0)	9.0, CH_2	1.08, ddd (9.9, 6.0, 3.8)	12.3, CH_2	0.95, m	9.7, CH_2	1.00, ddd (9.7, 5.8, 3.8)
		0.46, ddd (9.6, 6.8, 4.0)		0.46, ddd (9.5, 6.7, 3.8)		0.80, m		0.46, ddd (9.6, 6.7, 3.8)
2	5.9, CH_2	0.99, ddd (9.6, 6.2, 4.6)	5.9, CH_2	0.99, ddd (9.5, 6.0, 4.7)	5.7, CH_2	0.89, m	6.5, CH_2	0.90, ddd (9.6, 5.8, 4.5)
		0.78, ddd (9.9, 6.8, 4.6)		0.78, ddd (9.9, 6.7, 4.7)		0.81, m		0.80, ddd (9.7, 6.7, 4.5)
2a	33.5, C		33.5, C		29.4, C		33.8, C	
3	77.8, C		77.8, C		139.9, C		143.2, C	
4	201.8, C		201.6, C		119.4, CH	6.06, d (1.3)	120.9, CH	5.99, d (1.6)
4a	139.9, C		138.5, C		139.7, C		143.9, C	
5	146.7, CH	6.43, s	143.0, CH	6.39, s	137.7, CH	5.39, s	129.4, CH	5.15, d (2.6)
6	49.9, C		56.3, C		44.5, C		51.4, C	
7	79.8, CH	4.43, s	75.5, CH	4.68, s	47.7, CH_2	2.09, d (13.1)	36.2, CH_2	1.95, dd (13.1, 7.9)
						1.60, d (13.1)		1.45, dd (13.1, 9.2)
7a	136.2, C		140.4, C		87.6, C		54.3, CH	2.99, ddd (9.2, 7.8, 2.6)
8	135.1, C		134.8, C		75.7, C		72.8, C	
9	24.9, CH_3	1.27, s	24.9, CH_3	1.29, s	19.4, CH_3	1.53, d (1.3)	19.5, CH_3	1.52, d (1.6)
10	28.4, CH_3	1.11, s	16.2, CH_3	1.11, s	30.3, CH_3	1.24, s	24.9, CH_3	1.11, s
11	21.0, CH_3	1.13, s	69.8, CH_2	3.44, d (10.8)	29.8, CH_3	1.11, s	70.5, CH_2	3.34, m
				3.38, d (10.8)				
12	14.3, CH_3	1.71, s	14.3, CH_3	1.71, s	16.8, CH_3	0.94, s	19.7, CH_3	1.00, s

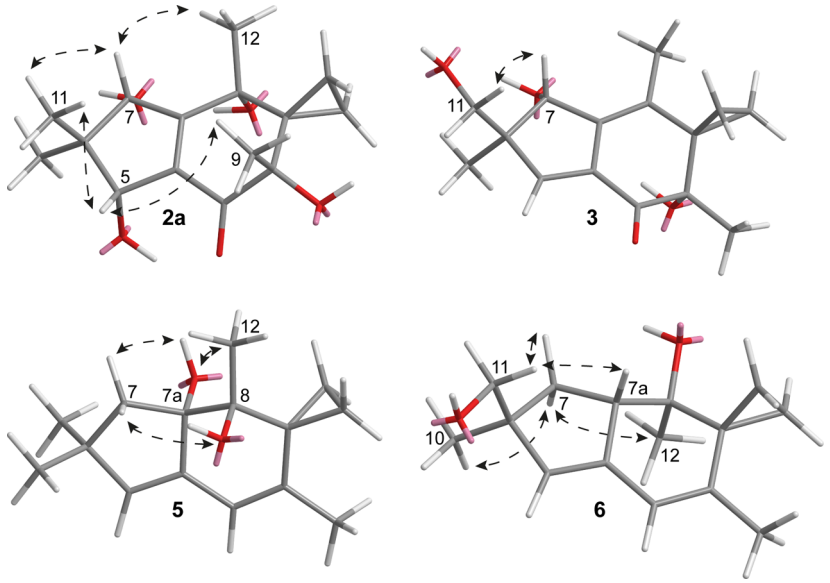

Figure 2. Molecular models of compounds **2a**, **3**, **5**, and **6** obtained with MM2 energy minimization displaying diagnostic ROESY correlations used for structure determination. ROESY data for **2a** and **3** were obtained in MeOH-d_4, for compound **5** in DMSO-d_6, and for compound **6** in acetone-d_6.

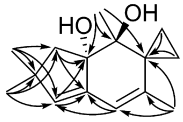

Figure 3. Diagnostic HMBC correlations of compound **5** (MeOH-d_4).

assumed that compound **5** is produced by the same enzymatic machinery as 7,9-illudadiene-3,14-diol and radulol, they are likely to share the configuration on C-8, resulting in a 7aS,8S configuration of **5**.

HRESIMS data showed that compound **6** had the molecular formula $C_{15}H_{22}O_2$, as with compound **5**. The ^{1}H and ^{13}C NMR data of compound **6** were also similar to those of compound **5** (Table 1), and 2D NMR data showed that the only difference between the two compounds was that methyl-11 was oxidized to an CH_2OH in compound **6** and that C-8 was oxidized in compound **5**. Consequently compound **6** was named illudadiene B. The relative configuration of **6** was deduced through ROESY correlations from H-7α to Me-12 and Me-10 (Figure 2). H_2-11 correlated to H-7β and H-7a, giving the relative configuration of compound **6**. If it is again assumed that 7,9-illudadiene-3,14-diol, radulol, and compounds **5** and **6** share the configuration on C-8, the absolute configuration of compound **6** would be 5*S*,7a*S*,8*R*.

Radulol and 7,9-illudadiene-3,14-diol were identified by comparison of NMR, MS, and polarimetric data with literature values.[13,20,21] 7,9-Illudadiene-3,14-diol has previously been isolated from the fungi *Agrocybe aegerita* and *Russula*

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Table 2. Cytotoxicity of Compounds 1−6 and 7,9-Illudadiene-3,14-diol against the Two Tumor Cell Lines Huh7 and MT4 (Mean Values, Samples in Duplicate)

	CC_{50} (μM)							
cell line	1	2	3	4	5	6	7,9-illudadiene-3,14-diol	INX-189[a]
Huh7	1.3	0.38	0.098	160	n.t.[b]	>400	240	3
MT4	0.12	0.014	0.023	45	n.t.	>400	110	3

[a]Positive control.[22] [b]Not tested.

delica,[20,21] whereas radulol has been obtained from the fungus *Radulomyces confluens*.[13]

Compounds **2** and **3** were found to have very potent cytotoxic effects against the two tested tumor cell lines Huh7 and MT4 with CC_{50} values (the concentration that results in 50% cell viability compared to untreated control cells) of 0.38 μM (Huh7) and 0.014 μM (MT4) for compound **2** and 0.098 μM (Huh7) and 0.023 μM (MT4) for compound **3**, respectively (Table 2). Surprisingly, compound **1** was found to be substantially less cytotoxic than its diastereomer **2**, with CC_{50} values of 1.3 μM (Huh7) and 0.12 μM (MT4), whereas compounds **4**, **6**, and 7,9- illudadiene-3,14-diol, as expected, due to the lack of possibility to facilitate Michael-type reactions, had no or moderate activity at concentrations up to 400 μM (Table 2).

To test if the difference in cytotoxicity between compounds **1** and **2** could be explained with chemical reactivity, the compounds were subjected to diluted aqueous HCl and then in a second experiment to cysteine in aqueous solution, using experimental conditions similar to previous studies on illudin M.[4,5] Both compounds **1** and **2** reacted with 2 M HCl in the same way as described for illudin M, with the chloride acting as a nucleophile on the cyclopropyl moiety as the rate-determining step followed by a Michael-type addition of water, resulting in two isomeric phenolic products.[4] The reaction rate of compound **1** ($k = 4.1 \times 10^{-3}$ min^{-1}, $t_{1/2}$ = 170 min) was similar to that described for illudin M ($k = 4.7 \times 10^{-3}$ min^{-1}, $t_{1/2}$ = 147 min),[4] whereas compound **2** ($k = 12 \times 10^{-3}$ min^{-1}, $t_{1/2}$ = 38 min) reacted noticeably faster. The reaction of illudin M with cysteine and other thiol nucleophiles has been shown to be highly pH dependent, with a maximum rate at pH 5−6 for the reaction between illudin M and cysteine. Therefore, the reactions between cysteine and compounds **1** and **2** were carried out in a phosphate buffer at pH 5.4. Compound **1** reacted in a similar though not identical way to that described for illudin M, forming a 2:1 mixture of two isomeric reaction products (**1a** and **1b**, respectively) with a $t_{1/2}$ of 27 min (Figure 3).[5] Compound **2** reacted much faster with a $t_{1/2}$ of 5.6 min and formed mainly one of the two expected reaction products (20:1 ratio) (Figure 4, compound **2b**). Together this indicates that the cis configuration of OH-3 and OH-7 as observed for compounds **2** and **3** compared to the trans configuration in compound **1** and illudin M is likely responsible for the increased reactivity observed for compound **2** and subsequently for the increase in cytotoxicity.

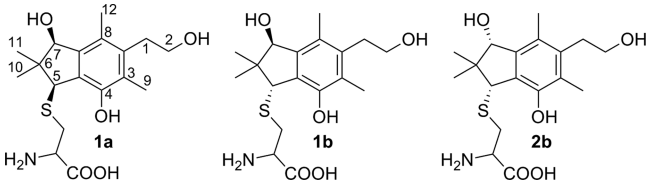

Figure 4. Reaction products **1a**, **1b**, and **2b**.

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotation of the compounds was measured with PerkinElmer 341 or 343 polarimeters (λ 589 nm, path length 10.0 cm, 20 °C). The UV absorption maxima were obtained with a Hitachi U-2001 spectrophotometer, whereas a PerkinElmer Lambda 2 UV/vis spectrophotometer was used to monitor the decrease of the absorption bands at 318 nm. A Bruker Avance III 600 MHz NMR spectrometer (5 mm QXI probe, 5 mm CryoProbe, or 5 mm SmartProbe) or a Bruker DRX400 NMR spectrometer (5 mm QNP probe) was used to obtain the NMR data, with the chemical shifts reported relative to the residual solvent signal of MeOH-d_4 (δ_H 3.31; δ_C 49.00). HRESIMS data were obtained with a Bruker maXis Impact ESI UHR Q-TOF with Na formate as calibrant (positive mode). Preparative HPLC was performed on a Gilson 305/306 system, with a Gilson 118 UV/vis detector (254 nm). Energy minimization modeling of the compounds was performed employing the MM2 force field using ChemBio3D Ultra version 11.0 (CambridgeSoft Corp.).

Fungus and Cultivation. Isolation and identification of a fungal culture of *G. vellereum* strain olrim243 is described in a previous study.[10] In the present study, supernatants of *G. vellereum* were produced by growing cultures in 500 mL Erlenmeyer flasks each containing 250 mL of liquid Hagem medium.[23] Five agar plugs 0.5 × 0.5 cm in size with established fungal mycelia from an actively growing colony were aseptically inoculated in each flask and incubated on a rotary shaker at 120 rpm at room temperature (ca. 21 °C) for average periods of 4 weeks. After cultivation, supernatants were filtered to obtain cell-free samples.

Extraction and Isolation. Cell-free filtrates of *G. vellereum* were extracted through SPE columns [C18 (EC); 50 mL filtrate/per 1 g packing material; International Sorbent Technology, Hengoed, UK] using aqueous 95% MeCN as eluent. The extracts were dried in a vacuum centrifuge and redissolved in aqueous 40% MeCN before being fractionated on a preparative HPLC system (Reprosil-Pur ODS-3, C_{18}, 5 μm, 100 × 20 mm and guard column 30 × 20 mm, Dr Maisch GmbH, Ammerbuch, Germany) using a linear gradient (10−95% aqueous MeCN in 10 min, followed by a hold at 95% MeCN for 10 min, 10 mL/min).

Radulol was obtained pure from the first chromatography step, whereas the other compounds had to be purified further as described below. Fractions containing crude compounds **1**, **2**, **4**−**6**, and 7,9-illudadiene-3,14-diol were individually pooled and rechromatographed using preparative HPLC (column as above, 13.2 mL/min) and at isocratic conditions with either 30% aqueous MeCN for compounds **1**, **2**, **5**, **6**, and 7,9-illudadiene-3,14-diol or 20% aqueous MeCN for compound **4**. Crude compound **3** was rechromatographed using a linear gradient with 7.5% to 40% aqueous MeCN in 30 min (column as above, 13.2 mL/min). The maximum yields obtained for the compounds were 11.7 (**1**), 15.0 (**2**), 1.9 (**3**), 48.5 (**4**), 0.3 (**5**), 0.4 (**6**), 1.0 (7,9-illudadiene-3,14-diol), and 0.4 (radulol) mg/L filtrate.

*(3S,7R)-Illudin M (**1**):* light yellow, amorphous solid; $[\alpha]_D$ 96 (*c* 0.1 in methanol); HRESIMS *m/z* 249.1489 $(M + H)^+$ (calcd for $C_{15}H_{21}O_3$, 249.1485).

*(3S,7S)-Illudin M (**2**):* light yellow, amorphous solid; $[\alpha]_D$ 59 (*c* 0.2 in methanol); UV λ_{max} (MeOH) nm (log ε) 224, 243, 307 (4.29, 4.25,

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3.71); NMR data, see Table 1; HRESIMS m/z 249.1482 $(M + H)^+$ (calcd for $C_{15}H_{21}O_3$, 249.1485).

*(3S,6S,7R)-Illudin S (**3**):* colorless oil; $[\alpha]_D$ 34 (c 0.2 in methanol); UV λ_{max} (MeOH) nm (log ε) 232, 240, 213 (4.05, 4.03, 3.46); NMR data, see Table 1; HRESIMS m/z 265.1429 $(M + H)^+$ (calcd for $C_{15}H_{21}O_4$, 265.1434).

*(3S,4S,7R)-Dihydroilludin M (**4**):* white, amorphous solid; $[\alpha]_D$ 77 (c 0.3 in methanol); UV λ_{max} (MeOH) nm (log ε) 204, 251 (3.68, 3.68); NMR data, see Table 1; HRESIMS m/z 273.1460 $(M + Na)^+$ (calcd for $C_{15}H_{22}NaO_3$, 273.1461).

*Illudadiene A (**5**):* white, amorphous solid. $[\alpha]_D$ −6 (c 0.1 in methanol); UV λ_{max} (MeOH) nm (log ε) 204, 248 (3.84, 4.08); NMR data, see Table 1; HRESIMS m/z 257.1511 $(M + Na)^+$ (calcd for $C_{15}H_{22}NaO_2$, 257.1512).

*Illudadiene B (**6**):* white, amorphous solid; $[\alpha]_D$ 59 (c 0.06 in methanol); UV λ_{max} (MeOH) nm (log ε) 204, 250 (3.86, 4.40); NMR data, see Table 1; HRESIMS m/z 257.1512 $(M + Na)^+$ (calcd for $C_{15}H_{22}NaO_2$, 257.1512).

Reaction of Compounds 1 and 2 with Cysteine. To a solution of **1** (1.2 mg, 4.7 μmol) in 5 mL of acetate buffer pH 5.4 (50 mM) was added L-cysteine (5.7 mg, 47 mmol) at room temperature. The reaction was monitored both spectrophotometrically at 318 nm and through LC-HRESIMS analysis (aqueous 10−90% MeCN, 10 min and then a hold at 90% for 10 min; Reprosil-Pur ODS-3, C_{18}, 3.5 μm, 125 × 4.6 mm, Dr Maisch GmbH, Ammerbuch, Germany) during a time period of 3 h. The reaction mixture was left overnight and was then extracted by SPE (C18 (EC), 300 mg). The SPE column was eluted with MeCN (1 mL), and the resulting crude product was purified by preparative HPLC (aqueous 10−90% MeCN, 10 min and then a hold at 90% MeCN for 10 min; column as above, 10.0 mL/min), resulting in the isolation of two isomeric reaction products (**1a** and **1b**) in a 2:1 ratio. Compound **1a**: NMR data, see Table S3; HRESIMS m/z 370.1688 $(M + H)^+$ (calcd for $C_{18}H_{28}NO_5S$, 370.1683). Compound **1b**: NMR data, see Table S3; HRESIMS m/z 370.1687 $(M + H)^+$ (calcd for $C_{18}H_{28}NO_5S$, 370.1683). Compound **2** was subjected to the same reaction procedure as compound **1**, with 2.0 mg (8.1 μmol) of compound **2** and 9.8 mg (81 μmol) of L-cysteine, resulting in one reaction product (**2b**). Compound **2b**: NMR data, see Table S3; HRESIMS m/z 370.1685 $(M + H)^+$ (calcd for $C_{18}H_{28}NO_5S$, 370.1683).

Reaction of Compounds 1 and 2 with HCl. Compound **1** (0.64 mg, 2.6 μmol) was added to 10 mL of 2 M HCl at room temperature. The reaction was monitored both spectrophotometrically at 318 nm and through LC-HRESIMS analysis (aqueous 10−90% MeCN, 10 min and then a hold at 90% for 10 min; Reprosil-Pur ODS-3, C_{18}, 3.5 μm, 125 × 4.6 mm, Dr Maisch GmbH, Ammerbuch, Germany). The reaction mixture was left overnight before being neutralized with NaOH and passed through a SPE-column (C18 (EC), 1 g). After elution of the SPE column with MeCN (1 mL) the resulting crude product was purified by preparative reversed-phase HPLC (aqueous 10−90% MeCN, 10 min and then a hold at 90% MeCN for 10 min; column as above, 10.0 mL/min), resulting in two isomeric products (**1c** and **1d**). Compound **1c**: NMR data, see Table S3; HRESIMS m/z 307.1071 $(M + Na)^+$ (calcd for $C_{15}H_{21}ClNaO_3$, 307.1071). Compound **1d**: NMR data, see Table S3; HRESIMS m/z 307.1068 $(M + Na)^+$ (calcd for $C_{15}H_{21}ClNaO_3$, 307.1071). Compound **2** was subjected to the same reaction procedure as compound **1**, with 1.8 mg (7.0 μmol) of compound **2**, resulting in the formation of two isomeric reaction products (**2c** and **2d**). Compound **2c**: NMR data, see Table S3; HRESIMS m/z 307.1075 $(M + Na)^+$ (calcd for $C_{15}H_{21}ClNaO_3$, 307.1071). Compound **2d**: NMR data, see Table S3; HRESIMS m/z 3071073 $(M + Na)^+$ (calcd for $C_{15}H_{21}ClNaO_3$, 307.1071).

Formation of Degradation Product 2a. Pure compound **2** was stored in −18 °C for a prolonged period of time (>1 year) and was then rechromatographed by preparative HPLC at isocratic conditions with 30% aqueous MeCN (column as above, 13.2 mL/min), yielding a 5:2 mixture of two different decomposition products, of which the most abundant was compound **2a**. NMR data, see Table S1; HRESIMS m/z 305.1362 $(M + Na)^+$ (calcd for $C_{15}H_{22}NaO_5$, 305.1359).

Selective Acetylation of Compound 3. Acetic anhydride (2.0 μL, 21 μmol) was added to a stirred solution of **3** (3.8 mg, 14 μmol) in pyridine (500 μL) at 0 °C and was left stirring for 24 h. The solvent was then evaporated, after which the crude product was redissolved in 50% aqueous MeCN and purified with preparative HPLC (column as above; 10−90% aqueous MeCN in 10 min, followed by a hold at 90% $MeCN_{aq}$ in 10 min, 10 mL/min). The product was pooled and dried, yielding 0.94 mg (19%) of the desired product (**3a**).

Oxidation of Compound 4. To a stirred solution of **4** (27 mg, 0.11 mmol) in 10 mL of dichloromethane were added 0.04 mL of pyridine (0.49 mmol) and 20 mg (0.50 mmol) of NBS at room temperature. All the starting material had been consumed after 3 h according to TLC analysis (silica gel 60 F_{254}, mobile phase; 1:4 EtOAc−hexane), after which 2 mL of 2-propanol was added to the mixture. The solvent was then evaporated, resulting in a yellow oil, which was dissolved in 50% aqueous MeCN and purified on preparative HPLC (isocratic conditions, aqueous 22.5% MeCN; same column as above, 13.2 mL/min), yielding compound **1**.

Formation of MTPA Esters of Compounds 1, 2, and 3a. The (*S*)-MTPA esters were formed by treating compounds **1**, **2**, and **3a** (0.8−2.9 μmol) with (*R*)-(−)-MTPA-Cl (12- to 24-fold excess) in 500 μL of pyridine-d_5 for 72 h at room temperature. Analogously, the (*R*)-MTPA esters were formed by treating compounds **1**, **2**, and **3a** with (*S*)-(+)-MTPA-Cl (6- to 24-fold excess). All MTPA esters were analyzed by NMR spectroscopy without further purification.

Cytotoxicity Assay. MT4 (T-cell line, a kind gift from Prof. Yamamoto, Yamaguchi University, Japan) and Huh7 (hepatocarcinoma cell line, ReBlikon GmbH, Germany) cell lines were passaged into 96-well microplates (2×10^4 cells/well) followed by the addition of the test substances the next day, in duplicate samples. The number of viable cells was assessed after 6 days by using a soluble formazan (XTT) assay.[24] The compound INX-189 was used as positive control.[22]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00500.

^1H and ^{13}C NMR spectra for compounds **1**−**6** and ^1H NMR spectra for compounds **1a**−**d**, **2a**−**d**, **3a**, and the (*S*/*R*)-MTPA esters of **1**, **2**, and **3a**, along with tabulated ^1H NMR data for **1a**−**d**, **2a**−**d**, **3a**, **5**, **6**, and the (*S*/*R*)-MTPA esters of **1**, **2**, and **3a** (PDF)

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Notes

The authors declare no competing financial interest.

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