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Lucidenic Acid O and Lactone, New Terpene Inhibitors of Eukaryotic DNA Polymerases from a Basidiomycete, *Ganoderma lucidum*

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Abstract—Terpenoids, **1**, **2** and **3**, which selectively inhibit eukaryotic DNA polymerase activities, were isolated from the fruiting body of a basidiomycete, *Ganoderma lucidum*, and their structures were determined by spectroscopic analyses. New terpenes, lucidenic acid O (**1**) and lucidenic lactone (**2**), prevented not only the activities of calf DNA polymerase α and rat DNA polymerase β, but also these of human immunodeficiency virus type 1 reverse transcriptase. Cerevisterol (**3**), which was reported to be a cytotoxic steroid, inhibited only the activity of DNA polymerase α. Although these compounds did not influence the activities of prokaryotic DNA polymerases and other DNA metabolic enzymes such as T7 RNA polymerase and deoxyribonuclease I.

Introduction

We have long been interested in the integrity of the genome of eukaryotes and its relation to the cell differentiation. DNA replication, recombination and repair in eukaryotes are key systems to maintain these processes.[1] The DNA polymerases have important roles in these processes. In this regard, we have concentrated our effort to investigate eukaryotic DNA polymerases associated with these processes.[2–12]

Multiple DNA polymerases have been identified in eukaryotes,[13] and recent investigations have revealed that eukaryotic cells contain at least six types (α, β, γ, δ, ε and ζ) of DNA polymerase.[13,14] DNA polymerase α and δ are required for nuclear DNA replication, whereas DNA polymerase γ participates in mitochondrial DNA replication.[13] DNA polymerase β and ε have been implicated to be involved in DNA repair, but there are also reports suggesting that these enzymes are related to recombination and DNA replication, respectively.[1,13,14] DNA polymerase ζ may be also involved in repair.[14] However, not all functions of eukaryotic DNA polymerases have been fully elucidated. Selective inhibitors of DNA polymerases are useful tools for distinguishing DNA polymerases and clarifying the biological functions cited above. We have found several DNA polymerase inhibitors; fatty acids,[15,16] bredenin 5′-monophosphate,[17,18] triterpenoids,[19,20] ergosterol peroxide,[21] incisterols,[22] cerebrosides,[23] carboxyflavins[24] and sulfate containing glycolipids.[25–27] These compounds are still not sufficient for completely investigating the processes, and therefore, we have continued screening. We recently found another novel terpenes from a mushroom, *Ganoderma lucidum*, selectively inhibiting the DNA polymerase activity. In this paper, we would like to report the isolation and structural determination of these novel terpenes.

The basidiomycete, *G. lucidum*, is known as a medicinal mushroom used in traditional Chinese medicine, as the so-called '*reishi*' or '*mannentake*' mushroom. Dried '*reishi*' powder was used for cancer chemotherapy in the Imperial Court of ancient China. The fruiting bodies of the basidomycete produced three terpenoids, which were found to inhibit the activities of mammalian DNA polymerases in vitro. Two of the compounds, lucidenic acid O (**1**) and lucidenic lactone (**2**), are new terpenes and purified by silica gel column chromatography. In

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addition, a steroid (**3**) was isolated and identified as cerevisterol (ref. 28, and references therein) based on its physicochemical and spectroscopic data. Cerevisterol (**3**) was previously reported as a cytotoxic agent in 1988.[28]

Results and Discussion

Extraction and purification of terpenoid compounds from the fruiting caps of the basidiomycete, *G. lucidum*

The fruiting caps (150 g) of the mushroom *G. lucidum* were homogenized in a Waring blender and extracted with acetone (5 L) for 3 days. Evaporation of the solvent yielded 4 g of a brown waxy material. The extract was partitioned between EtOAc (1 L) and water (1 L) adjusted to pH 7 and the organic layer evaporated. The fraction (30 mL/fraction) was subjected to silica gel column chromatography (Wakogel C-200, 200 mesh, 5.0×50 cm) and then eluted with EtOAc:MeOH:H_2O (v/v/v 50:1:0.1). Two active fractions which inhibited DNA polymerase activity, i.e. minimum inhibitory concentration was less than 1 mg/mL, (0.6 g of fraction No. 54-60 and 0.2 g of fraction No. 105-134, respectively) were obtained. The latter active fraction (No. 105-134) was purified through a second silica gel column chromatography (Wakogel C-300, 300 mesh, 2.0×30 cm) using IPA:MeOH:H_2O (v/v/v 5:1:0.1) and lucidenic acid O (**1**) was collected (5.8 mg). The first active fraction (No. 54-60) was purified through an additional silica gel column chromatography (Wakogel C-300, 300 mesh, 2.0×30 cm) using $CHCl_3$:MeOH:H_2O (v/v/v 5:1:0.1), and lucidenic lactone (**2**) and cerevisterol (**3**) were collected (5.2 mg of the latter active fraction, No. 49-58, and 16.4 mg of the first active fraction, No. 35-41, respectively).

Structure determination of terpenoid compounds from the fruiting caps of the basidiomycete, *G. lucidum*

The empirical formula of lucidenic acid O (**1**) was determined with HR-FABMS (high resolution fast atom bombardment mass spectrum) to be $C_{27}H_{38}O_7$. The ^{1}H NMR spectrum revealed one exomethylene group (C=CH_2) at δ 4.96 (1H, br.s) and 4.95 (1H, br.s), three secondary hydroxyl groups (-C*H*–OH) at 4.81 (1H, dd), 4.62 (1H, dd) and 3.66 (1H, dd), and one primary alcohol (-CH_2–OH) at 3.52 (1H, d) and 3.31 (1H, d), and four methyls (-CH_3) at 0.72 (3H, s), 0.79 (3H, s), 1.27 (s, 3H) and 1.31 (3H, s). ^{13}C NMR implied one exomethylene at δ 148.35 (*C*=) and 111.34 (=*C*H_2), one α,β-unsaturated carbonyl (*C*=*C*–CO) at 161.32, 142.89 and 202.05, one carboxylic acid (-*C*OOH) at 177.40, and four methyl groups (-CH_3) at 20.18, 20.13, 12.91 and 19.37. In the HMBC spectrum, the primary alcohol protons (H-24) at 3.52 and 3.31 showed cross peaks with 72.41 (C-3), 43.40 (C-4), 42.67 (C-5), 12.91 (C-23), while H-3 proton at 3.60 had cross peaks with 66.26 (C-24), 43.30 (C-4) and 12.91 (C-23). These correlation suggested that the primary alcohol should be C-24. The olefinic protons (H-19) at 4.96 and 4.96 had cross peaks at 148.35 (C-18), 49.3 (C-14), 33.55 (C-20). The final assignments of ^{1}H and ^{13}C NMR spectra were accomplished with COSY, HSQC, HMBC and NOE experiments. Irradiation at H-27 showed NOE with H-7, H-12α and H-14, which implied that these protons are α. When H-23 was irradiated, NOE observed at H-2β, H-6β and H25, which indicated that these are β. The missing connections from C-1 to C-3 and C-15 to C-16 in the HMBC spectrum were determined by COSY. The results of HMBC and NOE experiments were summarized in Table 1. Thus the structure of **1** was determined as illustrated (Fig. 1) including relative stereochemistry. Lucidenic acid O (**1**) is related to terpene, which is a new compound having a rare exomethylene group on C-18 and an acid on C-22.

The HR-FABMS of lucidenic lactone (**2**) indicated the molecular formula $C_{27}H_{40}O_7$, while the ^{1}H NMR showed the presence of a primary alcohol (-CH_2OH) at δ 3.58 (2H, s), two secondary hydroxy groups (-C*H*–OH) at 3.15 (1H, dd) and 4.54 (1H, dd) and five methyl groups (-CH_3) at 0.83 (3H, s), 0.98 (3H, s), 1.01 (3H, s), 1.24 3H, (s) and 1.28 (3H, s). ^{13}C NMR suggested one α,β-unsaturated carbonyl (*C*=*C*–*C*O) at δ 160.93, 143.02 and 201.49, one primary alcohol (-*C*H_2OH) at 68.37, and five methyl groups (-*C*H_3) at 28.68, 20.62, 19.64, 19.54 and 16.35. The ^{1}H and ^{13}C NMR were very similar to those of lucidenic acid O (**1**), however, the olefinic moiety of **1** had disappeared in the case of **2**. From the analysis of HMBC spectrum of **2**, two of five methyls at 28.68 (C-24) and 16.35 (C-23) had relationships to a secondary hydroxylated proton at 3.15 (H-3), and the primary alcohol protons at 3.5 (H-19) had cross peaks with 47.55 (C-14), 27.30 (C-20) and 94.31 (C-18), which was further correlated with the proton at 2.64 (H-14). The analyses of HMBC and NOE spectra lead to the structure of lactone moiety on D-ring. When H-25 was irradiated, NOE were observed at H-2β and H-23. Irradiation of H-24 showed NOE with H-3, H-6α and H-23. H-27 showed NOE with H-7α, H-12α, H-14 and H-15. The missing connections from C-1 to C-3, C-5 to

Table 1. Assignments of HMBC and NOE spectra[a]

	Lucidenic acid O (**1**)		Lucidenic lactone (**2**)	
	HMBC	NOE	HMBC	NOE
Position	Carbon	Proton	Carbon	Proton
3	4, 23, 24		23, 24	
5				3, 7
6	4, 5, 7, 10		7, 8, 10	
7	6, 8, 9		6, 8, 9	
12	9, 11, 13		9, 11, 13, 26	
14	15, 18, 19		13, 15, 18, 19	
15			16	
16	27		27	
19	14, 18, 20	20	14, 18, 20	12b, 14
20	18, 19, 22			
21	22		22	
23	3, 4, 5, 24	2b, 6b, 24, 25	3, 4, 5, 24	24, 25
24	3, 4, 5, 23		3, 4, 5, 23	3, 6a, 23
25	1, 9, 10	2b, 23, 6b	1, 5, 9, 10	2b
26	12, 13, 14, 17	2b	12, 13, 14, 17	12b, 19
27	8, 13, 16, 17	7, 12a, 14	8, 13, 16, 17	12a, 14, 15a

[a] Numbering should be referred to Figure 1.

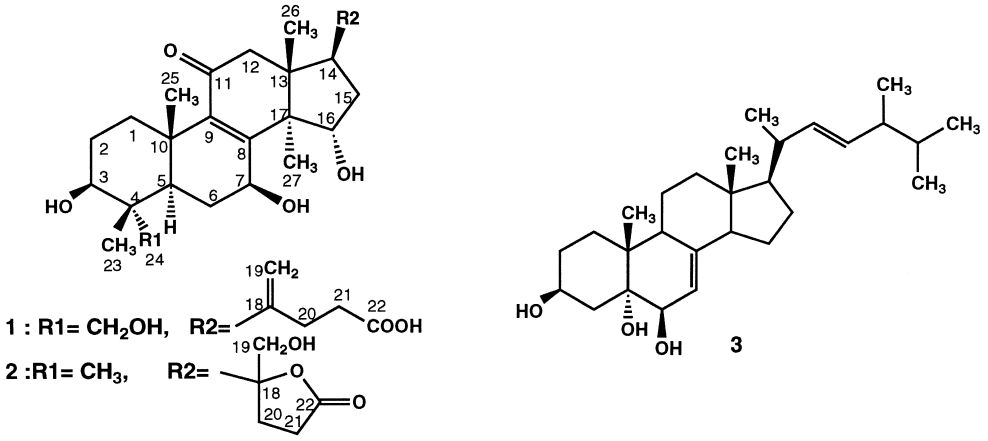

Figure 1. Structure of terpenoid compound **1**–**3** from the fruiting body of a basidiomycete, *Ganoderma lucidum*.

C-6 and C-20 to 21 in the HMBC spectrum were determined by COSY. These observations implied that the relative stereochemistry of **2** is similar to that of **1**. The results of HMBC and NOE experiments were summarized in the Table 1. Thus the structure of **2** was determined as a structurally new compound, lucidenic lactone (**2**).

The physicochemical property and spectral data of steroid (**3**) were identical to those of cerevisterol[28] isolated from fungi, *Fusarium moniliforme* and *Agaris blazei*, showing cytotoxic activity.[28]

Inhibition by terpenoids of the activities of DNA polymerases and other DNA metabolic enzymes

Both lucidenic acid O (**1**) and lactone (**2**) at 100 μM were found to significantly inhibit the activities of calf DNA polymerase α, rat DNA polymerase β and human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (Fig. 2). The steroid (**3**) only inhibited DNA polymerase α (Fig. 2), but these compounds did not influence the activities of the prokaryotic DNA polymerases, i.e. the Klenow fragment of DNA polymerase I, T4 DNA polymerase and Taq polymerase, the DNA

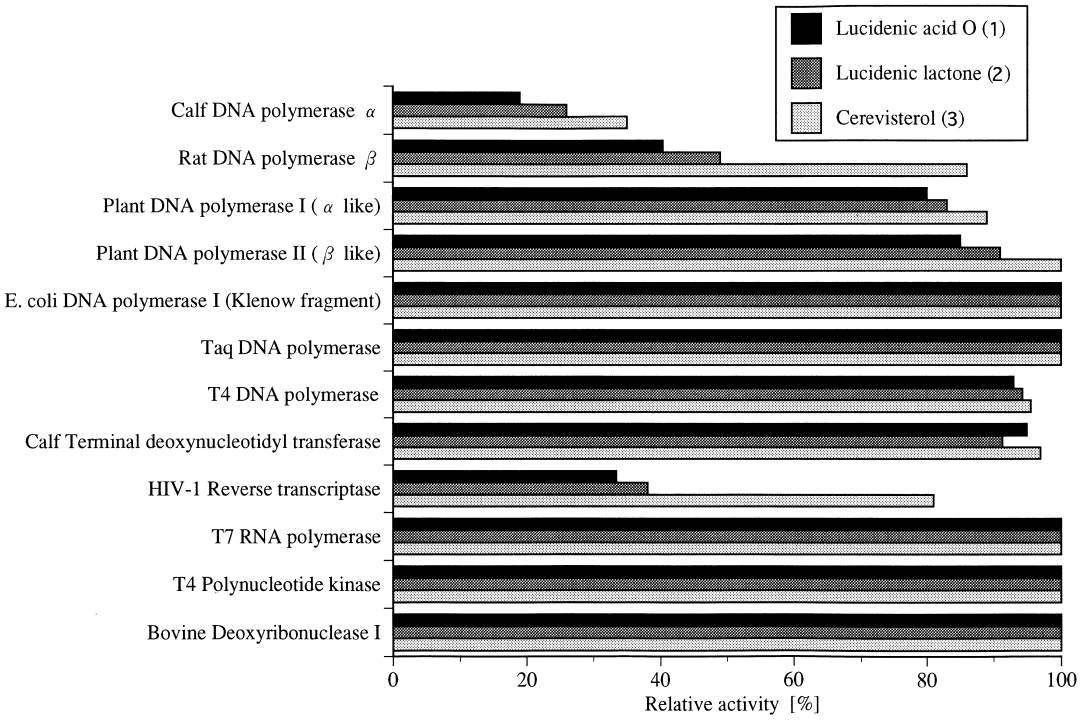

Figure 2. Effect of terpenoid compounds on the activities of various DNA polymerases and other enzymes. % of relative activity. Each terpenoid (100 μM) was incubated with each enzyme (0.05 units). The enzymatic activity was measured as described previously.[15,23,29] Enzyme activity in the absence of terpenoids was taken as 100%.

metabolic enzyme bovine deoxyribonuclease I, or the DNA polymerase I (α like) and II (β like) from a higher plant, cauliflower (Fig. 2). Lucidenic acid O (**1**) and lactone (**2**) appear to be selective inhibitors of mammalian DNA polymerases in vitro. The inhibition of lucidenic acid O (**1**) and lactone (**2**) were dose-dependent, and the 50% inhibition (IC_{50}) for DNA polymerase α was achieved at approximately 35 and 42 μM, respectively, for DNA polymerase β was 72 and 99 μM, respectively, and for human immunodeficiency virus type 1 (HIV-1) reverse transcriptase was 67 and 69 μM, respectively (Fig. 3(A) and (B)). Also, the inhibition of the known steroid (**3**) was dose-dependent for DNA polymerase α, with an IC_{50} of 84 μM (Fig. 3(C)). The R2 group of the chemical structure (Fig. 1) was slightly more effective on DNA polymerase β and HIV-1 reverse transcriptase than on DNA polymerase α.

Experimental

Materials

Nucleotides and chemically synthesized template-primers such as poly(dA) and oligo$(dT)_{12-18}$ were purchased from Pharmacia (Uppsala, Sweden). [^{3}H]-deoxythymidine 5′-triphosphate (dTTP) (43 Ci/mmol) was purchased from New England Nuclear Corp (Boston, MA). All other reagents were of analytical grade and were purchased from Wako Chemical Industries (Osaka, Japan). The fruiting caps of the mushroom *G. lucidum*, were purchased from Japan Microbe Chemical Co. (Tokyo, Japan). The mushrooms were collected in Gunma prefecture, Japan, and were identified by Forestry & Forest Products Res. Inst. (FFPRI), Japan, as *G. lucidum*.

Enzymes and DNA polymerase assays

The DNA polymerases and DNA metabolic enzymes used and the enzyme assay methods are the same as those described in previous reports.[15,23,29] EPO was dissolved in dimethyl sulfoxide (DMSO), and 4 μL of the dissolved sample was mixed with 16 μL of each enzyme (final 0.05 units) in 50 mM Tris–HCl (pH 7.5) containing 0.1 mM EDTA, 1 mM dithiothreitol and 50% glycerol, then kept at 0°C for 10 min. Eight μL of each of the preincubated solutions was added to 16 μl of each of the enzyme standard reaction mixtures, then each of the enzyme activities was measured.

Instrumental analyses

NMR spectra were collected on JEOL JNM Lambda 600 and Bruker DPX 300 spectrometers. All NMR spectra were recorded in $CDCl_3$ solutions, and the spectra are referenced to the residual CD_3OD peak at 3.30 ppm for ^{1}H or 49.8 ppm for ^{13}C. MS data were measured with a JEOL HX-110 mass spectrometer. Specific rotations were recorded on a Digital Polar meter, JASCO DIP-370. TLC plates were purchased from Merck (Darmstadt, Germany) and were silica gel $60F_{254}$, with a layer thickness of 0.5 mm, and the R_f values were determined using plates 20 cm in length. After being developed, the compounds were visualized with UV at 254 nm.

Structure determination

Compound 1 (Lucidenic acid O (1)). Amorphous solid; $[\alpha]_D^{23}$ +71° (c = 0.2, MeOH); R_f 0.49 (IPA:MeOH:H_2O 5:1:0.5); HR-FABMS (negative ion mode; glycerol matrix): m/z 475.2712 (M-H, $C_{27}H_{39}O_7$ requires 475.2696). UV λ_{max} (MeOH) nm (ε): 253 (7490).

^{1}H NMR (600 MHz in CD_3OD): δ 4.96 and 4.95 each 1H, br. s, H-19), 4.81 (1H, dd, J = 9.8, 7.3 Hz, H-16), 4.62 (1H, dd, J = 9.8, 7.3 Hz, H-7), 3.60 (1H, dd, J = 11.7, 4.9 Hz, H-3α), 3.52 and 3.31 (each 1H, d, J = 11.2 Hz, H-24), 2.91 (1H, d, J = 15.1 Hz, H-12α), 2.88 (1H, dd, J = 9.3, 9.3 Hz, H-14), 2.69 (1H, ddd, J = 13.7, 2.9, 2.9 Hz, H-1β), 2.44 (2H, m, H-21), 2.34 (1H, m, H-15β), 2.26 (1H, d, J = 15.1 Hz, H-12β), 2.21 and 2.31 (each 1H, m, H-20), 2.03 (1H, br. dd, J = 12.7, 7.3 Hz, H-6α), 1.70 (1H, m, H-15α), 1.69 (1H, m, H-2β), 1.62 (1H, m, H-2α), 1.57 (1H, ddd, J = 12.7, 12.7, 9.8 Hz, H-6β), 1.39 (1H, br. d, J = 12.7 Hz, H-5), 1.31 (3H, s, H-27), 1.27 (3H, s, H-25), 0.94 (1H, m, H-1α), 0.79 (3H, s, H-26), 0.72 (3H, s, H-23). ^{13}C NMR (125 MHz in CD_3OD): δ 202.05 (C-11), 177.40 (C-22), 161.32 (C-8), 148.35 (C-18), 142.89 (C-9), 111.34 (C-19),

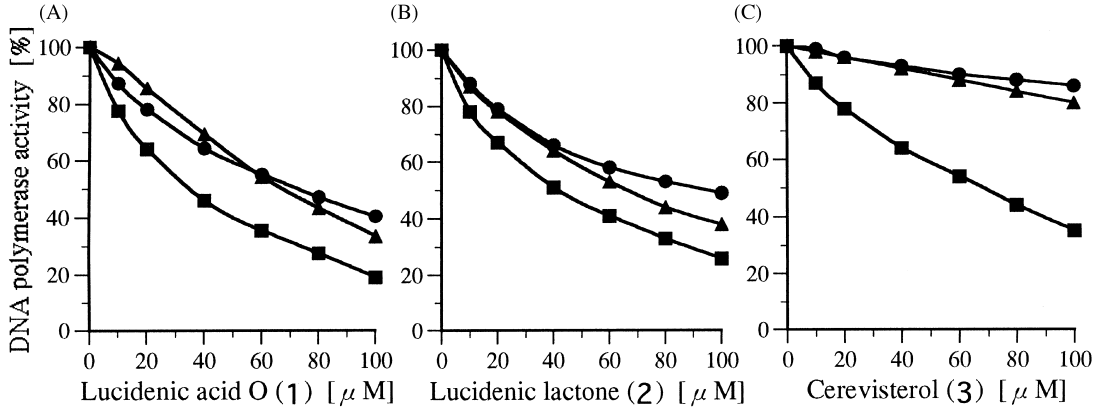

Figure 3. Dose curve of lucidenic acid O (**1**) (A), lucidenic lactone (**2**) (B) and cerevisterol (**3**) (C). The enzymes (0.05 units each) are calf DNA polymerase α (■), rat DNA polymerase β (●) and HIV-1 reverse transcriptase (▲).

73.63 (C-16), 72.41 (C-3), 69.75 (C-7), 66.16 (C-24), 54.95 (C-17), 52.16 (C-12), 49.3 (C-14), 48.5 (C13), 43.40 (C-4), 42.67 (C-5), 39.58 (C-10), 35.52 (C-1), 33.69 (C-15), 34.32 (C-21), 33.55 (C-20), 27.97 (C-2), 12.91 (C-23), 28.52 (C-6), 20.18 (C-25), 20.13 (C-27), 19.37 (C-26). HMBC (from H to C): δ 4.96 and 4.95 (148.35, 49.3, 33.55), 4.81 (20.13), 4.62 (161.32, 142.89, 28.52), 3.60 (66.16, 43.30, 12.91), 3.52 and 3.31 (72.41, 43.30, 42.67, 12.91), 2.91 and 2.26 (202.05, 142.89, 48.5), 2.88 (111.34, 148.35, 33.69), 2.44 (177.40), 2.34 and 2.21 (177.40, 148.35, 111.34), 2.03 and 1.57 (69.75, 43.30, 42.67, 39.58), 1.31 (161.32, 73.63, 54.95, 48.5), 1.27 (142.89, 42.67, 39.58, 35.52), 0.79 (54.95, 52.16, 49.3, 48.5), 0.72 (72.41, 66.16, 43.30, 42.67). NOE (from HA to HX): δ 4.94 (2.34), 1.31 (4.62, 2.91, 2.88), 1.27 (1.69, 1.57, 0.72), 0.79 (2.26), 0.72 (3.52, 3.31, 1.69, 1.57).

Compound 2 (Lucidenic lactone (2)). Amorphous solid; $[\alpha]_D^{24}$ +13° (c = 0.1, MeOH); R_f 0.40 ($CHCl_3$:MeOH:H_2O 5:1:0.5); HR-FABMS (positive ion mode; glycerol matrix): m/z 477.2810 (M + H, $C_{27}H_{41}O_7$ requires 477.2830); UV λ_{max} (MeOH) nm (ε): 255 (6050).

^{1}H NMR (600 MHz in $CDCl_3$): δ 4.87 (1H, dd, J = 9.3, 7.3 Hz, H-16), 4.54 (1H, dd, 9.8, 7.3 Hz, H-7), 3.58 (2H, s, H-19), 3.15 (1H, dd, J = 11.7, 4.4 Hz, H-3), 2.94 (1H, d, J = 15.1 Hz, H-12α), 2.74 (1H, ddd, J = 18.1, 11.2, 8.8 Hz, H-21), 2.73 (1H, d, J = 15.1 Hz, H-1β), 2.64 (1H, dd, J = 11.0, 7.8 Hz, H-14), 2.55 (1H, ddd, J = 18.1, 10.7, 3.4 Hz, H-21), 2.39 (1H, d, J = 15.1 Hz, H-12β), 2.34 (1H, ddd, J = 13.2, 10.7, 8.8 Hz, H-20), 2.20 (1H, ddd, J = 13.2, 11.2, 3.4 Hz, H-20), 2.20 (1H, ddd, J = 14.2, 9.3, 7.8 Hz, H-15β), 2.12 (1H, ddd, J = 12.7, 7.3, 1.0 Hz, H-6α), 1.77 (1H, ddd, J = 14.2, 11.0, 7.3 Hz, H-15), 1.65 (1H, m, H-2α), 1.60 (1H, dd, 12.7, 11.7, 9.8 Hz, H-6β), 1.58 (1H, m, H-2β), 1.28 (3H, s, H-27), 1.24 (3H, s, H-25), 1.01 (3H, s, H-24), 0.98 (3H, s, H-26), 0.96 (1H, m, H-1α), 0.94 (1H, dd, 11.7, 1.0 Hz, H-5), 0.83 (3H, s, H-23). ^{13}C NMR (125 MHz in CD_3OD): δ 201.49 (C-11), 160.93 (C-8), 143.02 (C-9), 93.41 (C-18), 79.55 (C-22), 78.91 (C-3), 72.77 (C-16), 70.01 (C-7), 68.37 (C-19), 53.09 (C-12), 50.43 (C-5), 55.05 (C-17), 47.95 (C-13), 47.55 (C-14), 39.76 (C-10), 39.68 (C-4), 35.90 (C-1), 30.82 (C-21), 32.07 (C-15), 29.02 (C-6), 28.35 (C-2), 16.35 (C-23), 28.68 (C-24), 27.30 (C-20), 20.62 (C-27), 19.64 (C-25). HMBC (from H to C): δ 4.54 (160.93, 143.02, 29.02), 3.58 (93.41, 27.30), 3.15 (28.68, 16.35), 2.94 and 2.34 (201.49, 143.02, 47.95, 19.54), 2.74 and 2.50 (179.55), 2.64 (93.41, 47.95, 32.07, 19.54), 2.20 and 1.70 (72.77, 47.95), 4.87 (20.62), 2.12 and 1.60 (160.93, 70.01, 39.76), 1.28 (160.93, 72.77, 55.05, 47.95), 1.01 (78.91, 50.43, 39.68, 16.35), 0.98 (143.02, 50.43, 39.76, 35.90), 0.98 (55.05, 53.09, 47.95, 47.55), 0.83 (78.91, 50.43, 39.68, 28.68). NOE (from H_A to H_X): δ 3.58 (2.64, 2.39), 1.28 (4.54, 2.94, 2.64, 1.77), 1.24 (2.73, 0.83), 1.01 (3.15, 2.12, 0.83), 0.98 (3.58, 2.38), 0.94 (4.54, 3.15), 0.83 (1.24, 1.01).

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