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Nodulisporol and Nodulisporone, novel specific inhibitors of human DNA polymerase λ from a fungus, *Nodulisporium* sp.

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Abstract—Tetralols **1** and **2**, dihydroisocoumarins **3**–**6**, and chromone **7** are natural compounds isolated from cultures of fungi, and their structures were determined by spectroscopic analyses. Compounds **1** and **2** from *Nodulisporium* sp. are novel tetralols, 1,2,3,4-tetrahydro-5-methoxynaphthalene-1,4-diol (nodulisporol) and 3,4-dihydro-4-hydroxy-8-methoxynaphthalen-1(2*H*)-one (nodulisporone), respectively. All isolated compounds selectively inhibited the activity of human DNA polymerase λ (pol λ), and compound **5** (3,5-dimethyl-8-methoxy-3,4-dihydroisocoumarin) was the strongest inhibitor of pol λ in the tested compounds with an IC_{50} value of 49 μM. New tetralols (**1** and **2**) are the third and second strongest inhibitors of pol λ, but did not influence the activities of mammalian pols α to κ, and showed no effect even on the activities of plant pols α and β, prokaryotic pols, and other DNA metabolic enzymes such as calf terminal deoxynucleotidyl transferase, human immunodeficiency virus type-1 (HIV-1) reverse transcriptase, human telomerase, T7 RNA polymerase, and bovine deoxyribonuclease I. The structure–activity relationships of isolated compounds such as novel tetralols, dihydroisocoumarins, and chromone are discussed.

1. Introduction

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

The human genome encodes 14 pols to conduct cellular DNA synthesis.[3] Eukaryotic cells reportedly contain three replicative types: pols α, δ, and ε, mitochondrial pol γ, and at least 12 repair types: pols β, δ, ε, ζ, η, θ, ι, κ, λ, μ, σ, and REV1;[4] however, not all functions of eukaryotic pols have been fully elucidated. Selective inhibitors of pols are useful tools for distinguishing pols and clarifying the biological functions of pol each. We have searched for natural compounds that selectively inhibit each of these eukaryotic pols.[5–12] In this study, we report on newly found compounds that selectively inhibit only the activity of pol λ. The natural compounds were novel tetralols, nodulisporol (**1**) and nodulisporone (**2**), produced by a fungus (*Nodulisporium* sp.). To our knowledge, there have been no reports about such natural inhibitors specific to X-family pols such as β, λ, μ and terminal deoxynucleotidyl transferase (TdT), except for solanapyrone A as a pols β- and λ-inhibitor,[9] and prunasin as a pol β-inhibitor,[7] which we reported previously. The compound differed from solanapyrone A in that it inhibited only pol λ among the pols examined to date.[9] No such pol λ-specific inhibitors have been reported.

Two of the compounds, nodulisporol (**1**) and nodulisporone (**2**), are new tetralols and were purified by silica gel column chromatography. In addition, four dihydroisocoumarins (**3**–**6**) and chromone (**7**) were isolated and identified as 3-methyl-8-methoxy-3,4-dihydro-

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isocoumarin, 5-formyl-8-hydroxy-3-methyl-3,4-dihydroisocoumarin, 3,5-dimethyl-8-methoxy-3,4-dihydroisocoumarin, 3-methyl-8-hydroxy-3,4-dihydroisocoumarin (mellein), and 5-hydroxy-3-hydroxymethyl-2-methyl-7-methoxychromone, respectively, based on their physicochemical and spectroscopic data.

In this paper, we would like to report the isolation and structural determination of these tetralols, dihydroisocoumarins, and chromone.

2. Results

2.1. Extraction and purification of compounds from fungi

The culture (1 L) of the fungus (*Nodulisporium* sp.), which was collected in Chiba prefecture, Japan, was grown for 3 weeks without shaking, in the dark. Fungal mycelia were removed from the culture broth by filtering through cheesecloth. The filtrate was extracted with CH_2Cl_2. The organic layer was evaporated in vacuo to obtain 25.2 mg of crude residue. This crude extract was separated by silica gel column chromatography (Fuji Silysia Chemical FL60D, 1.0×20 cm) with hexane-EtOAc (9:1–1:9) to give two active fractions which inhibited pol activity, i.e., minimum inhibitory concentration was less than 1 mg/mL. The first active fraction was 4.2 mg of compound **1** and the latter active fraction was 5.5 mg of compound **2**.

Cultures of five other species of fungi, which were also collected in Chiba prefecture, Japan, were extracted with CH_2Cl_2 and the crude extracts were separated by silica gel chromatography to give dihydroisocoumarins **3**–**6** and chromone **7**.

2.2. Structure determination of isolated compounds

The molecular formula of **1** was determined with HRESIMS (High Resolution Electrospray Ionization Mass Spectrometry) to be $C_{11}H_{14}O_3$. Resonances for six aromatic, one methoxy, two oxygenated methine, and two methylene carbons were present in the ^{13}C NMR spectrum. In the ^1H NMR spectrum, resonances for three aromatic protons (δ 6.85, δ 7.08, δ 7.30) indicated the presence of a trisubstituted benzene ring. The COSY spectrum of **1** suggested the partial structure of –CH(O)–CH_2–CH_2–CH(O)–. This partial structure and trisubstituted benzene ring were connected by HMBC correlations between oxygenated methine protons (H-1 and H-4) and aromatic carbons (C-8 and C-5). From these results, the structure of **1** was determined to be 1,2,3,4-tetrahydro-5-methoxynaphthalene-1,4-diol, and was named nodulisporol (Fig. 1). The molecular formula of **2** was determined with HRESIMS to be $C_{11}H_{12}O_3$. ^1H and ^{13}C NMR spectra of **2** were similar to those of **1** except for carbon resonances (δ 196.5) attributed to a ketone. The position of the ketone was determined from the HMBC spectrum; therefore, the structure of **2** was established to be 3,4-dihydro-4-hydroxy-8-methoxynaphthalen-1(2*H*)-one, and was named nodulisporone (Fig. 1). The assignments of ^1H and ^{13}C NMR signals of **1** and **2** are shown in Table 1.

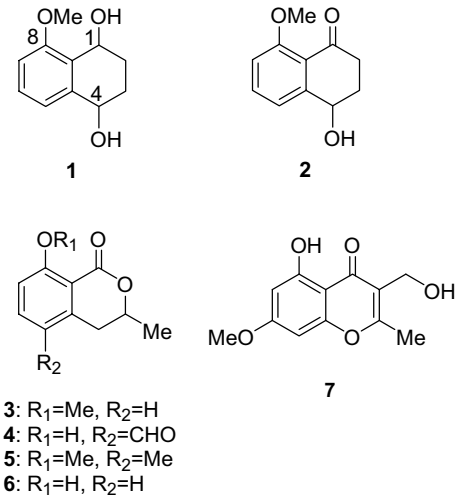

Figure 1. Structures of compounds **1**–**7** from the culture broth of fungi. (**1**) Nodulisporol, (**2**) nodulisporone, (**3**) 3-methyl-8-methoxy-3,4-dihydroisocoumarin, (**4**) 5-formyl-8-hydroxy-3-methyl-3,4-dihydroisocoumarin, (**5**) 3,5-dimethyl-8-methoxy-3,4-dihydroisocoumarin, (**6**) 3-methyl-8-hydroxy-3,4-dihydroisocoumarin (mellein), and (**7**) 5-hydroxy-3-hydroxymethyl-2-methyl-7-methoxychromone.

Compounds **3**–**7** were identified by spectroscopic analysis to be known compounds, 3-methyl-8-methoxy-3,4-dihydroisocoumarin,[13] 5-formyl-8-hydroxy-3-methyl-3,4-dihydroisocoumarin,[14] 3,5-dimethyl-8-methoxy-3,4-dihydroisocoumarin,[14,15] 3-methyl-8-hydroxy-3,4-dihydroisocoumarin (mellein),[13] and 5-hydroxy-3-hydroxymethyl-2-methyl-7-methoxychromone,[16] respectively. The structures of these compounds are shown in Figure 1. ^1H and ^{13}C NMR resonance assignments for **3**–**7** were in good agreement with published values.[13–16]

Table 1. ^1H and ^{13}C NMR spectral data of compounds **1** and **2**

Position	1		2	
	δ_H (J Hz)	δ_C	δ_H (J Hz)	δ_C
1	5.06 (t, 4.8)	63.1		196.5
2	1.90 (m)	25.7	2.59 (ddd, 17.3, 8.7, 5.2)	36.3
	2.28 (m)		2.91 (ddd, 17.3, 7.7, 5.1)	
3	1.80 (m)	27.7	2.15 (m)	31.3
	2.21 (m)		2.33 (m)	
4	4.79 (t, 4.5)	67.1	4.91 (t, 3.5)	68.4
4a		139.9		147.8
5	7.08 (d, 7.9)	120.9	7.18 (d, 7.7)	118.7
6	7.30 (t, 7.9)	129.0	7.53 (dd, 8.5, 7.7)	134.7
7	6.85 (d, 7.9)	109.7	6.97 (d, 8.5)	111.8
8		157.5		160.0
8a		139.9		120.5
OMe-8	3.90 (s)	55.5	3.92 (s)	56.1

Recorded in $CDCl_3$ for TMS as an internal standard and chemical shifts are expressed as δ ppm. s, singlet; d, doublet; dd, doublet of doublets; t, triplet.

Table 2. IC_{50} values of compounds **1**–**7** for the activities of mammalian DNA polymerases

Compound	IC_{50} values of compounds (μM)						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Calf pol α	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Rat pol β	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Human pol γ	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Human pol δ	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Human pol ε	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Human pol λ	168	82	180	275	49	316	454

Enzymatic activity was measured as described in Section 4. Pol activity in the absence of the compounds was taken as 100%.

2.3. Inhibition by isolated compounds of the activities of DNA polymerases and other DNA metabolic enzymes

First, isolated compounds **1**–**7** were investigated as to whether they inhibit the activities of major mammalian pols such as replicative pols α, δ, and ε, repair-related pols β and λ, and mitochondrial pol γ. As shown in Table 2, all the compounds selectively inhibited human pol λ activity. Since these compounds (**1**–**7**) consist of bicyclic ring, this skeleton structure might be important for the selective inhibition of pol λ. The inhibitory effect of compound **5** was the strongest of all the compounds tested, with 50% inhibition observed at a concentration of 49 μM. The IC_{50} values of compounds **1**–**3**, and **5** were less than 200 μM, and these compounds have a methoxy group at position 8. On the other hand, the IC_{50} values of compounds **4**, **6**, and **7** were more than 250 μM, and these compounds have a hydroxyl group at the same position. These results suggested that the methoxy group at position 8 of these compounds must be essential for pol λ inhibition. Compound **5** has a methoxy group and a methyl group at positions 8 and 5, respectively, and compounds **4**–**6**, which were the weakest inhibitors of pol λ, have other groups at the same positions; therefore, these groups might be important for pol λ inhibitory activity. The IC_{50} values of novel tetralols (**1** and **2**) on pol λ were 168 and 82 μM, respectively, suggesting that the carbonyl group of **2** (Fig. 1) was slightly more effective for pol λ inhibition.

Both new tetralols, nodulisporol (**1**) and nodulisporone (**2**), at 200 μM were found to only inhibit the activities of pol λ in the nine mammalian pols (Fig. 2), and the same concentration of these compounds did not influence the activities of a higher plant, cauliflower, pols α and β, prokaryotic pols, i.e., the Klenow fragment of

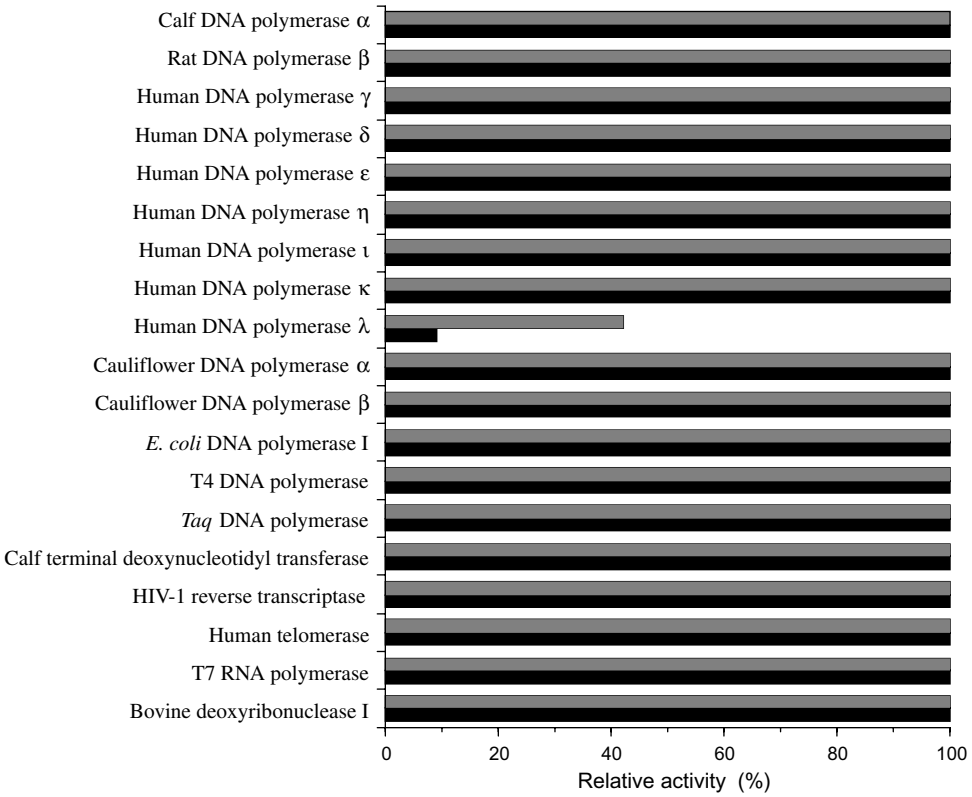

Figure 2. Effect of novel tetralols **1** (nodulisporol) and **2** (nodulisporone) on the activities of various DNA polymerases and other enzymes. Compound **1** (gray bars) and compound **2** (black bars) (200 μM each) were incubated with each enzyme (0.05 U). Percentage of relative activity. Enzymatic activity was measured as described previously.[5,6,9] Enzyme activity in the absence of the compounds was taken as 100%.

pol I, T4 pol, and Taq pol, or DNA metabolic enzymes such as calf terminal deoxynucleotidyl transferase, human immunodeficiency virus type-1 (HIV-1) reverse transcriptase, human telomerase, T7 RNA polymerase, and bovine deoxyribonuclease I.

3. Discussion

As described in this report, we found novel potent inhibitors specific to human pol λ from a fungus, *Nodulisporium* sp. The natural compounds were found to be tetralols, nodulisporol (**1**) and nodulisporone (**2**).

Although the biochemical function of pol λ is unclear as yet, pol λ appears to work in a similar manner to pol β.[17] Pol β, which is widely known to have roles in the short-patch base excision repair (BER) pathway,[17–22] plays an essential role in neural development.[23] Recently, pol λ was found to contain 5′-deoxyribose-5-phosphate (dRP) lyase activity, but no apurinic/apyrimidinic (AP) lyase activity,[21] and to be able to substitute pol β in in vitro base excision repair (BER), suggesting that pol λ also participates in BER. Northern blot analysis indicated that transcripts of pol β were abundantly expressed in the testis, thymus, and brain in rats,[24] but pol λ was efficiently transcribed mostly in the testis.[25] The reason why the testis and thymus require pol β activity has been suggested; both organs have DNA repair and recombination systems for meiotic crossing over and immunoglobulin production,[26,27] and the systems require the polymerase. The roles of pol β in the brain are unknown as yet; therefore, pol λ as well as pol β may also have a role in the testis. Since the DNA repair system at meiotic prophase requires pol β activity, the system must contain a process similar to BER. The system may also require pol λ activity, and pol λ may be an essential enzyme for nucleotide excision repair (NER). In this connection, the fact that the molecular target of the bio-antimutagen was a pol λ inhibitor is of great interest. The bio-antimutagen may lead to blockage of the mis-match error in BER, NER, and translesion synthesis of DNA-damaged cells. To determine why a bio-antimutagen is a pol λ-specific inhibitor, we are at present analyzing the structure and function of pol λ using an inhibitor.

Since pol species-specificity was extremely high, these tetralols, dihydroisocoumarins, and chromone could be useful molecular tools as pol λ-specific inhibitors in studies to determine the precise roles of pol λ in vitro, and also might be useful to develop a drug design strategy for cancer chemotherapy agents for clinical radiation therapy or cancer chemotherapy.

4. Experimental

4.1. Materials

Nucleotides and chemically synthesized template-primers such as poly(dA), oligo(dT)$_{12–18}$, and [^{3}H]-deoxythymidine 5′-triphosphate (dTTP) (43 Ci/mmol) were purchased from GE Healthcare Bio-Sciences (Little Chalfont, UK). All other reagents were of analytical grade and were purchased from Wako Chemical Industries (Osaka, Japan). The fungi were collected in Chiba prefecture, Japan, and were identified by TechnoSuruga Co. Ltd., Japan.

4.2. DNA polymerase and other DNA metabolic enzyme assays

Pols from mammal and plant were purified, and prokaryotic pols and other DNA metabolic enzymes were purchased as described in our previous report.[5,6,9] The activities of all pols and other DNA metabolic enzymes were measured as described in previous reports.[5,6,9] The substrates of the pols were poly(dA)/oligo(dT)$_{12–18}$ and dTTP as the DNA template-primer and dNTP (2′-deoxyribonucleoside 5′-triphosphate) substrate, respectively. Compounds **1**–**7** were dissolved in dimethylsulfoxide (DMSO) at various concentrations and sonicated for 30 s. The sonicated samples (4 μl) were mixed with 16 μl of each enzyme (final amount, 0.05 U) in 50 mM Tris–HCl (pH 7.5) containing 1 mM dithiothreitol, 50% glycerol, and 0.1 mM EDTA, and kept at 0 °C for 10 min. These inhibitor–enzyme mixtures (8 μl) were added to 16 μl of each of the standard enzyme reaction mixtures, and incubation was carried out at 37 °C for 60 min, except for *Taq* pol, which was incubated at 74 °C for 60 min. Activity without the inhibitor was considered to be 100%, and the remaining activity at each concentration of the inhibitor was determined relative to this value. One unit of pol activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of dNTP (i.e. dTTP) into the synthetic DNA template-primer (i.e., poly(dA)/oligo(dT)$_{12–18}$, A/T = 2/1) in 60 min at 37 °C under normal reaction conditions for each enzyme.[5,6]

4.3. Instrumental analyses

^{1}H and ^{13}C NMR spectra were recorded at 400 MHz with Bruker DRX-400 spectrometer, using tetramethylsilane as the internal standard. IR spectra were recorded with a JASCO FT/IR-410 spectrophotometer. High-resolution mass spectra were obtained on an Applied Biosystems QSTAR Mass Spectrometer using the electron spray ionization (ESI) method. Merck precoated silica gel 60 F_{254} 0.25 mm thickness was used for analytical thin-layer chromatography.

4.4. Structure determination

4.4.1. Nodulisporol (1,2,3,4-tetrahydro-5-methoxynaphthalene-1,4-diol) (1). Oil; IR (film) ν_{max} 3389, 3004, 2941, 1727, 1587, 1473, 1259, 1019, 993, 754 cm^{-1}; HR-ESIMS *m*/*z* found 217.0853 [M+Na]$^+$, calcd. for $C_{11}H_{14}O_3Na$: 217.0835; ^{1}H and ^{13}C NMR data, see Table 1.

4.4.2. Nodulisporone (3,4-dihydro-4-hydroxy-8-methoxynaphthalen-1(2*H*)-one) (2). Oil; IR (film) ν_{max} 3425, 3011, 2942, 2840, 1669, 1593, 1469, 1273, 960, 754 cm^{-1}; HR-ESIMS *m*/*z* found 215.0678 [M+Na]$^+$,

calcd. for $C_{11}H_{12}O_3Na$: 215.0678; ^1H and ^{13}C NMR data, see Table 1.

4.4.3. 3-Methyl-8-methoxy-3,4-dihydroisocoumarin (3). ^1H NMR (400 MHz, $CDCl_3$): δ 7.46 (1H, dd, J = 7.4, 8.6 Hz), 6.92 (1H, d, J = 8.6 Hz), 6.79 (1H, d, J = 7.4 Hz), 4.56 (1H, m), 3.95 (3H, s), 2.88 (2H, m), 1.48 (3H, d, J = 6.3 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.7, 161.2, 142.0, 134.4, 119.1, 113.7, 110.8, 74.1, 56.1, 36.1, 20.7; ESIMS m/z 193 $[M+H]^+$.

4.4.4. 5-Formyl-8-hydroxy-3-methyl-3,4-dihydroisocoumarin (4). ^1H NMR (400 MHz, $CDCl_3$): δ 11.94 (1H, s), 10.02 (1H, s), 7.93 (1H, d, J = 8.7 Hz), 7.06 (1H, d, J = 8.7 Hz), 4.73 (1H, m), 3.95 (1H, dd, J = 3.3, 17.8 Hz), 3.06 (1H, dd, J = 11.8, 17.8 Hz), 1.59 (3H, d, J = 6.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 190.5, 169.7, 166.4, 143.2, 140.9, 124.3, 116.8, 109.0, 75.7, 30.9, 20.8; ESIMS m/z 205 $[M-H]^-$.

4.4.5. 3,5-Dimethyl-8-methoxy-3,4-dihydroisocoumarin (5). ^1H NMR (400 MHz, $CDCl_3$): δ 7.31 (1H, d, J = 8.5 Hz), 6.83 (1H, d, J = 8.5 Hz), 4.51 (1H, m), 3.92 (3H, s), 2.87 (1H, dd, J = 2.9, 16.5 Hz), 2.69 (1H, dd, J = 11.5, 16.5 Hz), 2.23 (3H, s) 1.50 (3H, d, J = 6.3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.2, 159.5, 140.1, 135.7, 126.0, 113.8, 110.4, 73.4, 56.1, 33.1, 20.8, 18.5; ESIMS m/z 205 $[M-H]^-$.

4.4.6. 3-Methyl-8-hydroxy-3,4-dihydroisocoumarin (mellein) (6). ^1H NMR (400 MHz, $CDCl_3$): δ 11.03 (1H, s), 7.41 (1H, dd, J = 7.7, 8.3 Hz), 6.88 (1H, d, J = 8.3 Hz), 6.69 (1H, d, J = 7.7 Hz), 4.73 (1H, m), 2.93 (2H, d, J = 7.2 Hz), 1.53 (3H, d, J = 6.5 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.9, 162.1, 139.3, 136.1, 117.8, 116.2, 108.2, 76.0, 34.5, 20.7; ESIMS m/z 201 $[M+Na]^+$.

4.4.7. 5-Hydroxy-3-hydroxymethyl-2-methyl-7-methoxychromone (7). ^1H NMR (400 MHz, $CDCl_3$): δ 12.52 (1H, s), 6.35 (1H, d, J = 2.2 Hz), 6.34 (1H, d, J = 2.2 Hz), 4.61 (2H, s), 3.86 (3H, s), 2.46 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 182.3, 165.6, 164.5, 162.0, 157.7, 118.3, 104.9, 98.0, 92.3, 56.8, 55.8, 18.0; ESIMS m/z 259 $[M+Na]^+$.

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