New Dimeric Naphthopyrones from *Aspergillus niger*

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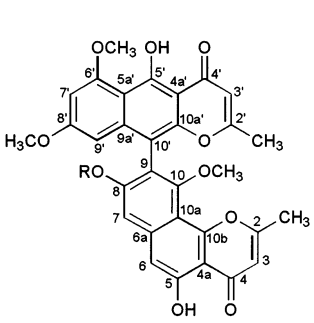
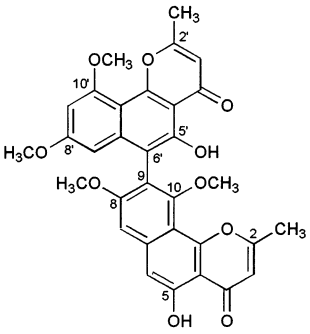
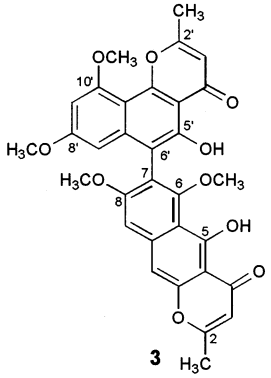
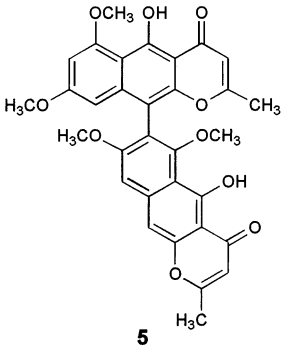
Three new dimeric naphthopyrones, asperpyrones A (**1**), B (**2**), and C (**3**), together with two known compounds, fonsecinone A (**4**) and aurasperone A (**5**), have been isolated from okara that was fermented with *Aspergillus niger* JV-33-48. Compounds **1**, **4**, and **5** showed inhibitory activity on *Taq* DNA polymerase.

In our continuing studies on bioactive fungal metabolites,[1,2] we isolated the fungus *Aspergillus niger* van Tieghem strain JV-33-48 from soil samples collected in Sakai. The methanol extract of okara (the insoluble residue of whole soybean) fermented with the title strain was found to exhibit inhibitory activity in the TRAP (telomeric repeat amplification protocol) assay, a polymerase chain reaction (PCR)-based assay originally developed for detecting telomerase activity in human cells and tissues.[3] The methanol extract was concentrated in vacuo, and the resulting aqueous concentrate was extracted with CH_2Cl_2. TRAP assay-guided purification of the CH_2Cl_2 extract by repeated column chromatography over silica gel and semipreparative ODS HPLC resulted in the isolation of three active compounds, together with two inactive ones. These active compounds were found to show inhibitory activity not on telomerase, but on *Taq* DNA polymerase in the TRAP assay. Spectroscopic analysis of the five compounds revealed that three of them (one active and two inactive) are new compounds, which were named asperpyrones A (**1**), B (**2**), and C (**3**). The other two active compounds were identified as the dimeric naphtho-γ-pyrones, fonsecinone A (**4**)[4] and aurasperone A (**5**),[5,6] by detailed analysis of spectral data and comparison with literature values.









Asperpyrone A (**1**) has a molecular formula of $C_{31}H_{24}O_{10}$, as determined by HREIMS and NMR data (Table 1), being indicative of 20 degrees of unsaturation. The IR spectrum suggested the presence of hydroxyl (3435 cm^{-1}) and conjugated carbonyl (1668 cm^{-1}) groups. The UV spectrum showed absorption maxima at 230, 256, 280, and 399 nm, suggesting that **1** has the same chromophore as **4**.[4] The ^{13}C NMR and HSQC spectra showed 31 carbon signals including two methyls, three methoxy groups, six sp^2 methines, and 20 quaternary sp^2 carbons (two of which are carbonyls), indicating the compound to be hexacyclic. The ^1H NMR spectrum displayed signals for two singlet methyls (δ_H 2.13, 2.46), three methoxyls (δ_H 3.39, 3.58, 3.93), two *meta*-coupled aromatic protons (δ_H 6.14, 6.55, $J = 2.3$ Hz), four singlet aromatic or olefinic protons (δ_H 6.19, 6.50, 6.96, 7.07), and three phenolic hydroxyl protons (δ_H 10.10, 12.90, 15.10, two of which are intramolecularly hydrogen-bonded). These data indicated that **1** is a dimeric naphtho-γ-pyrone, consisting of an angular flavasperone and a linear rubrofusarin B, in which one out of the four methoxy groups was replaced by a phenolic hydroxyl group. This was also supported by the two hydroxyl protons at δ_H 12.90 and 15.10, which were characteristic for flavasperone- and rubrofusarin B-type naphtho-γ-pyrones, respectively.[4] The HMBC correlations of H-6 (δ_H 6.96) with C-4a (δ_C 108.1), C-5 (δ_C 155.5), C-7 (δ_C 105.2), and C-10a (δ_C 106.5) and of H-7 (δ_H 7.07) with C-6 (δ_C 104.4), C-8 (δ_C 158.4), C-9 (δ_C 116.2), and C-10a indicated an 1,2,3,6,7,8-hexasubstituted naphthalene structure unit in the dimeric molecule (Figure 1). The HMBC correlations of the hydroxyl proton at δ_H 12.90 with C-4a, C-5, and C-6 and of H-3 (δ_H 6.50) with C-2 (δ_C 167.9), C-4 (δ_C 182.3), and C-4a confirmed an angular naphtho-γ-pyrone structure having an intramolecularly hydrogen-bonded hydroxyl group at C-5. A methoxy group (δ_H 3.39, δ_C 60.9) was located at C-10 (δ_C 156.9) as determined by its HMBC correlations with C-10. The remaining two methoxy functionalities were attached to the other half monomer unit on the basis of the HMBC spectrum. This suggests that a phenolic hydroxyl group (δ_H 10.10) is attached to the oxygenated carbon at C-8 of the

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Table 1. ^{1}H and ^{13}C NMR Data for Asperpyrones A (**1**), B (**2**), and C (**3**)[a]

	1[b]		**2**[c]		**3**[c]	
position	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
2	167.9		166.8		167.5	
3	110.0	6.50 (1H, s)	110.6	6.29 (1H, s)	107.4	6.01 (1H, s)
4	182.3		182.9		184.5[e]	
4a	108.1		109.3		104.6	
5	155.5		156.4		161.9	
5a					111.5	
6	104.4	6.96 (1H, s)	106.1	7.02 (1H, s)	158.3	
6a	140.1[d]		140.7[d]			
7	105.2	7.07 (1H, s)	101.9	6.96 (1H, s)	118.3	
8	158.4		159.9		160.2	
9	116.2		117.8		101.7	6.96 (1H, s)
9a					140.6[d]	
10	156.9		156.5		101.2	7.10 (1H, s)
10a	106.5		108.1		153.2	
10b	154.7		155.0[e]			
CH_3-2	19.8	2.46 (3H, s)	20.6	2.46 (3H, s)	20.7	2.38 (3H, s)
OH-5		12.90 (1H, s)		12.79 (1H, s)		14.73 (1H, s)
OH-8		10.10 (1H, s)				
OCH_3-6					62.2	3.62 (3H, s)
OCH_3-8			56.1	3.78 (3H, s)	56.1	3.79 (3H, s)
OCH_3-10	60.9	3.39 (3H, s)	55.2	3.59 (3H, s)		
2′	168.4		166.5		166.5	
3′	106.7	6.19 (1H, s)	110.3	6.31 (1H, s)	110.2	6.30 (1H, s)
4′	183.9		183.0		183.0[e]	
4a′	103.3		108.4		108.3	
5′	162.0		154.3		154.2	
5a′	107.6					
6′	160.8		109.6		109.9	
6a′			140.8[d]		140.7[d]	
7′	96.4	6.55 (1H, d, 2.3)	96.3	6.18 (1H, d, 2.1)	96.6	6.24 (1H, d, 2.1)
8′	161.1		161.5		161.4	
9′	96.9	6.14 (1H, d, 2.3)	96.7	6.42 (1H, d, 2.1)	96.7	6.41 (1H, d, 2.1)
9a′	140.0[d]					
10′	105.1		159.5		159.4	
10a′	150.2		105.0		105.0	
10b′			155.8[e]		155.7	
CH_3-2′	20.8	2.13 (3H, s)	20.6	2.53 (3H, s)	20.5	2.52 (3H, s)
OH-5′		15.10 (1H, s)		13.18 (1H, s)		13.09 (1H, s)
OCH_3-6′	56.1	3.93 (3H, s)				
OCH_3-8′	55.0	3.58 (3H, s)	61.5	3.59 (3H, s)	55.2	3.59 (3H, s)
OCH_3-10′			56.0	3.99 (3H, s)	56.0	3.98 (3H, s)

[a] The spectra were taken at 500 MHz for ^{1}H and at 125 MHz for ^{13}C. [b] In DMSO-d_6. [c] In $CDCl_3$. [d,e] Interchangeable in the same column.

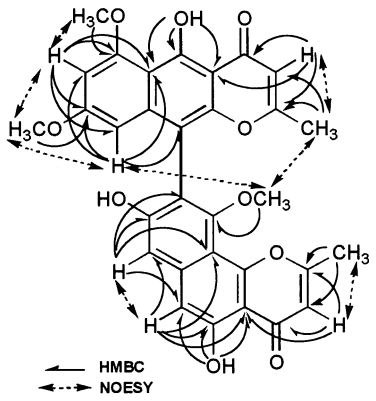

Figure 1. HMBC and NOESY correlations of **1**.

angular naphtho-γ-pyrone. A methyl group (δ_H 2.46, δ_C 19.8) was placed at C-2 as revealed by the HMBC correlations with C-2 and C-3. NOESY correlations between H-6 and H-7, and between CH_3-2 and H-3, supported these assignments. Therefore, half of the compound was determined to be an 8-*O*-desmethylated flavasperone linked to the other monomeric unit at C-9. Turning to the other half of the dimer, the HMBC correlations of two *meta*-coupled aromatic protons at H-9′ (δ_H 6.14) with C-5a (δ_C 107.6), C-7′ (δ_C 96.4), C-8′ (δ_C 161.1), and C-10′ (δ_C 105.1) and at H-7′ (δ_H 6.55) with C-5a′, C-6′ (δ_C 160.8) and C-8′, C-9′ (δ_C 96.9), and of the intramolecularly hydrogen-bonded proton at δ_H 15.10 with C-4a′ (δ_C 103.3), C-5′ (δ_C 162.0), and C-5a′, indicated a linear naphtho-γ-pyrone structure. The positions of the methyl group (δ_H 2.13, δ_C 20.8) and two methoxy groups (δ_H 3.58, δ_C 55.0, and δ_H 3.93, δ_C 56.1) were respectively located at C-2′, C-8′, and C-6′, as indicated by the HMBC spectrum. NOESY correlations observed between CH_3-2′ and H-3′, OCH_3-6′ and H-7′, H-7′, and OCH_3-8′, and OCH_3-8′ and H-9′ supported these assignments. Therefore, this monomeric unit was determined to be 10′-linked rubrofusarin B. Thus, the structure of **1** was determined to be a dimeric naphtho-γ-pyrone consisting of 8-*O*-desmethylated flavasperone and the linear rubrofusarin B linked at C-9 and C-10′, respectively. This was further supported by the NOESY correlations between OCH_3-10 and H-9′, and OCH_3-10 and CH_3 -2′, as shown in Figure 1.

Asperpyrone B (**2**) was shown to have a molecular formula of $C_{32}H_{26}O_{10}$ from the HREIMS and NMR data (Table 1), being indicative of 20 degrees of unsaturation. The IR spectrum indicated the presence of hydroxyl (3470 cm^{-1}) and conjugated carbonyl (1664 cm^{-1}) groups. The UV spectrum showed absorption maxima at 240, 284, and 374 nm, suggesting an angular flavasperone chromophore. The ^{13}C NMR spectrum showed 32 carbon signals including two methyls, four methoxyls, six sp^2 methines, and 20 quater-

nary sp^2 carbons (two of which are carbonyls), indicating the compound to be hexacyclic. The ^1H NMR spectra displayed signals for two singlet methyls (δ_H 2.46, 2.53), four methoxyls (δ_H 3.59, 3.59, 3.78, 3.99), two *meta*-coupled aromatic protons (δ_H 6.18, 6.42, $J = 2.1$ Hz), four singlet aromatic or olefinic protons (δ_H 6.29, 6.31, 6.96, 7.02), and two hydrogen-bonded hydroxyl protons (δ_H 12.78, 13.18). These data suggested the compound to be an asymmetric dimer of flavasperone. The two hydroxyl protons (δ_H 12.79 and 13.18) were diagnostic and correlated, respectively, with C-4a (δ_C 109.3), C-5 (δ_C 156.4), and C-6 (δ_C 106.1) and with C-4a′ (δ_C 108.4), C-5′ (δ_C 154.3), and C-6′ (δ_C 109.6) in the HMBC spectrum, indicating an angular structure in the two moieties. The four methoxy groups (δ_H 3.78, δ_C 56.1; δ_H 3.59, δ_C 55.2; δ_H 3.59, δ_C 61.5; δ_H 3.99, δ_C 56.0) were determined to be located at C-8 (δ_C 159.9), C-10 (δ_C 156.5), C-8′ (δ_C 161.5), and C-10′ (δ_C 159.5), respectively, by a HMBC experiment. The two methyl groups (δ_H 2.46, δ_C 20.6; δ_H 2.53, δ_C 20.6) were placed, respectively, at C-2 (δ_C 166.8) and C-2′ (δ_C 166.5), as determined by the HMBC correlations of the former protons with C-2 and C-3 (δ_C 110.6) and of the latter protons with C-2′ and C-3′ (δ_C 110.3). This structure was confirmed by the NOESY correlations between CH_3-2 and H-3 (δ_H 6.29), H-6 (δ_H 7.02) and H-7 (δ_H 6.96), H-7 and OCH_3-8, CH_3-2′ and H-3′ (δ_H 6.31), H-7′ (δ_H 6.18) and OCH_3-8′, OCH_3-8′ and H-9′ (δ_H 6.42), and H-9′ and OCH_3-10′. The structure of **2** was determined to be an asymmetric dimer of flavasperone linked at C-9 and C-6′.

The molecular formula of asperpyrone C (**3**) was assigned as $C_{32}H_{26}O_{10}$ from the HREIMS and NMR data (Table 1). The IR spectrum also suggested the presence of hydroxyl (3434 cm^{-1}) and conjugated carbonyl (1657 cm^{-1}) groups. The UV spectrum showed absorption maxima at 224, 248, 280, and 386 nm, suggesting linear rubrofusarin B and angular flavasperone chromophores. The ^1H and ^{13}C NMR spectra showed that the C-9-linked flavasperone moiety in **2** was isomerized to a linear rubrofusarin B structure in **3**, as indicated by the downfield shift of H-10 (δ_H 7.10) and OH-5 (δ_H 14.73) and the upfield shift of CH_3-2 (δ_H 2.38) and H-3 (δ_H 6.01).[4] A hydroxyl proton at δ_H 14.73 had long-range correlations with C-4a (δ_C 104.6), C-5 (δ_C 161.9), and C-5a (δ_C 111.5) in the HMBC spectrum, confirming the linear structure in the 7-linked moiety. Further analysis of the HMBC and NOESY experiments showed the structure of **3** to be a dimer of the linear rubrofusarin B and the angular flavasperone linked at C-7 and C-6′, respectively.

The optical rotation values of asperpyrones A (**1**) and C (**3**), fonsecinone A (**4**), and aurasperone A (**5**) isolated in this study were $[\alpha]_D$ +89°, +18°, −1.8°, and +97° in chloroform, respectively. This atropisomerism is ascribed to restricted rotation of the C−C linkage between two monomers as reported in the literature.[7] The optical purity of these compounds was not examined further in this study. Aurasperone A (**5**), previously isolated from the same species but a different strain, *A. niger* BL-5-1, showed an optical rotation value of $[\alpha]_D$ −136°, in the same solvent.[5] This indicates that the ratio of a pair of two atropisomers of aurasperone A (**5**) produced by the fungus *A. niger* JV-33-48 differs considerably from that produced by the strain BL-5-1.

Asperpyrones A (**1**), B (**2**), and C (**3**), together with fonsecinone A (**4**) and aurasperone A (**5**), were tested for inhibitory activity in the TRAP assay with PCR. Naphthopyrones **1**, **4**, and **5** showed weak inhibitory activity in the TRAP assay, whereas **2** and **3** did not show significant activity at 100 μg/mL. The percentages of inhibition of compounds **1**, **4**, and **5** were 41, 40, and 52, respectively. It was found that these active compounds inhibited PCR (*Taq* DNA polymerase), as shown by adding these compounds after the telomerase reaction. Compounds **1**, **4**, and **5** inhibited PCR by 40, 40, and 49%, respectively, at 100 μg/mL. The data suggest that the C-10′-linked linear rubrofusarin B moiety is required for the inhibitory activity on *Taq* DNA polymerase. None of the compounds inhibited telomerase at 100 μg/mL (data not shown).

Monomeric and dimeric naphtho-γ-pyrones have been isolated from a wide variety of fungi belonging to the genera *Aspergillus* and *Fusarium*.[4−7] These compounds were first found as yellow pigments in the mycelia of these fungi[5] and were shown to exhibit acute toxicity to mice and rats, acting mainly as central nervous system depressants.[8] Monomeric rubrofusarin B and dimeric dianhydroaurasperone C isolated from *Aspergillus* strain M-39 were found to reverse multidrug resistance of human epidermal KB carcinoma cells, possibly by interacting with P-glycoprotein and inhibiting its role as an active efflux pump.[9] In this study, we have shown that dimeric naphtho-γ-pyrones asperpyrone A (**1**), fonsecinone A (**4**), and aurasperone A (**5**) inhibit *Taq* DNA polymerase. The C-10′-linked rubrofusarin B moiety seems to be required for their inhibitory activity on the polymerase. Further study is needed to resolve the inhibition mechanism.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Horiba SEPA-300 polarimeter. IR spectra were recorded with a Perkin-Elmer 1760X FT-IR spectrophotometer, and UV spectra were measured with a Hitachi U-3210 instrument. ^1H and ^{13}C NMR spectra were obtained with a JEOL JNM-A500 NMR spectrometer. Chemical shifts were referenced to the solvent peaks (DMSO-d_6 δ_H 2.49, δ_C 39.5; $CDCl_3$ δ_H 7.24, δ_C 77.0) as an internal standard. Mass spectra were recorded with a JEOL JMS-600 instrument. Column chromatography was performed with Wakogel C-200 (Wako Pure Industries, Japan), Kieselgel 60 (Merck, Germany), and Inertsil ODS (ϕ 10 × 250 mm, 5 μm, GL Sciences, Japan). The okara used as a medium in this study was kindly supplied by Kitagawa Tofu (bean-curd) shop (Sakai, Osaka, Japan).

Fermentation, Extraction, and Isolation. A strain of *Aspergillus niger* JV-33-48 was isolated from a soil sample collected in Sakai (Osaka, Japan) in the usual manner. Identification of this strain was carried out at Centraalbureau voor Schimmelcultures (The Netherlands). Fermentation of okara was carried out in the same manner as previously described.[1,2] The okara (20 kg) that had been fermented with strain JV-33-48 was soaked in MeOH. The methanol extract was concentrated in vacuo, and the resulting aqueous concentrate was extracted with CH_2Cl_2. Evaporation of the solvent gave an CH_2Cl_2 extract (25 g). This extract was first chromatographed on a Wakogel C-200 column by eluting stepwise from *n*-hexane to EtOAc. The 100% EtOAc eluate (5 g) was subsequently subjected to a Wakogel C-200 column chromatography employing stepwise elution from $CHCl_3$ to acetone. The fraction eluted with 20% acetone (1.2 g) was rechromatographed on Kieselgel 60 by eluting stepwise with *n*-hexane to acetone. The fractions eluted with 50−60% acetone (400 mg) were further chromatographed on Kieselgel 60 by eluting with $CHCl_3$ to give two active fractions, I (52 mg) and II (34 mg). Fraction II was subjected to ODS HPLC, employing linear gradient elution within 30 min from 70% MeOH−H_2O to 90% MeOH−H_2O at a flow rate of 3 mL/min. The compounds eluted from the column were monitored at 254 nm. Asperpyrone A (**1**, 5.2 mg) eluted as a single peak was collected. Fraction I was subjected to ODS HPLC, employing isocratic elution with 70% MeOH−H_2O at a flow rate of 3 mL/min and monitoring

at 254 nm to give asperpyrone B (**2**, 5.0 mg), asperpyrone C (**3**, 4.8 mg), fonsecinone A (**4**, 13.1 mg), and aurasperone A (**5**, 19.3 mg).

Asperpyrone A (1): yellow powder; $[\alpha]^{25}_{D}$ +89° (*c* 0.13, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 230 (4.39), 256 (4.35), 280 (3.53), 399 (3.63) nm; IR (KBr) ν_{max} 3435, 1668, 1615, 1589, 1419, 1272, 1213, 1164 cm^{-1}; ^1H and ^{13}C NMR (DMSO-d_6), Table 1; HMBC and NOESY correlations, Figure 1; EIMS *m*/*z* 556 $[M]^+$ (100), 499 (12); HREIMS *m*/*z* 556.1351 (calcd for $C_{31}H_{24}O_{10}$, 556.1369).

Asperpyrone B (2): yellow powder; $[\alpha]^{25}_{D}$ 0° (*c* 0.012, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 240 (4.87), 284 (4.37), 374 (3.90) nm; IR (KBr) ν_{max} 3470, 1664, 1616, 1428, 1320, 1201 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$, Table 1); EIMS *m*/*z* 570 $[M]^+$ (32), 539 (100), 524 (9), 299 (9), 270 (17); HREIMS *m*/*z* 570.1542 (calcd for $C_{32}H_{26}O_{10}$, 570.1525).

Asperpyrone C (3): yellow powder; $[\alpha]^{25}_{D}$ +18° (*c* 0.011, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 224 (4.36), 248 (4.45), 280 (4.35), 386 (3.72) nm; IR (KBr) ν_{max} 3434, 1657, 1622, 1423, 1169 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$, Table 2); EIMS *m*/*z* 570 $[M]^+$ (28), 539 (100), 524 (14), 299 (13), 270 (20); HREIMS *m*/*z* 570.1508 (calcd for $C_{32}H_{26}O_{10}$, 570.1525).

TRAP Assay. The TRAP assay was performed as previously described[3] with slight modifications. TRAP assay tubes were prepared in advance by pipetting 2 μL of 50 μg/mL of a CX primer (5′-CCCTTACCCTTACCCTTACCCTAA-3′) to the bottom of 0.5 mL tubes containing wax gems. The tubes were heated at 65 °C for 3 min to melt the wax and then cooled to 20 °C to seal the CX primer under the wax barrier. One microliter of each test sample or 1 μL of DMSO (control) was assayed in the tube in 50 μL reaction mixtures containing 0.1−0.6 μg of HT1080 cell extract, reaction buffer [20 mM Tris-HCl (pH 8.3), 68 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.05% Tween 20], 0.1 μg of TS primer (5′-AATCCGTCGAGCAGAGTT-3′), 1 μg of T4 gene 32 protein, 50 μM of each of deoxynucleoside triphosphate, 2 units of *Taq* DNA polymerase, and 2 μCi of 10 μCi/μL [α-^{32}P] dCTP (3000 Ci/mmol). After 30 min incubation at 23 °C for the telomerase-mediated extension of the TS primer, the reaction mixture was heated at 90 °C for 90 s to inactivate telomerase and to liberate the CX primer sequestered under the wax. The sample was then subjected to 31 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s. The PCR reaction products were adsorbed onto a Whatman DE-51 paper. The radioactivity was measured by a liquid scintillation counter after the paper was washed with 0.5 M Na_2HPO_4 three times. The percent inhibition was calculated as follows: inhibition (%) = [1 − test sample (cpm) − control reaction using RNase-treated HT1080 cell extracts (cpm)/control (cpm) − control reaction using RNase-treated HT1080 cell extracts (cpm)] × 100. Test samples were added after the telomerase reaction when their inhibitory activities on *Taq* DNA polymerase were evaluated.

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