Lembehsterols A and B, Novel Sulfated Sterols Inhibiting Thymidine Phosphorylase, from the Marine Sponge *Petrosia strongylata*

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Lembehsterols A (1) and B (2), two novel sulfated sterols, were isolated from the marine sponge *Petrosia strongylata*. Both sterols showed inhibitory activity against thymidine phosphorylase, which is an enzyme related to angiogenesis in solid tumors. The structures of these sulfated sterols were established on the basis of chemical and physicochemical evidence.

Key words lembehsterol; thymidine phosphorylase; angiogenesis; sulfated sterol; sponge

In the growth and metastasis of solid tumors, angiogenesis has an important role. Thymidine phosphorylase (TP) is an enzyme catalyzing the reversible phosphorolysis of thymidine and is identical with the platelet-derived endothelial cell growth factor (PD-ECGF), which has been shown to be an angiogenic factor.[1,2] TP also catalyzes transport of deoxyribose from one deoxynucleoside to another nucleo-base to form a secondary deoxynucleoside.[3—5] It was shown that TP was expressed at higher levels in a wide variety of solid tumors than in the adjacent nonneoplastic tissues[6] and the increased expression of TP promotes angiogenesis, tumor growth, invasiveness and ability to metastasize.[7,8] This evidence suggests that a compound which inhibits TP activity may delay abnormal angiogenesis and progression of various tumors. In fact, several inhibitors of TP showed suppression of angiogenesis and tumor growth.[9]

In the course of our study of bioactive substances from marine organisms,[10] we focused on a search for selective inhibitors of TP and isolated novel sulfated sterols, lembehsterols A (**1**) and B (**2**), from the marine sponge *Petrosia strongylata*. This paper describes the elucidation of their absolute stereostructure.

Results and Discussion

A crude TP was obtained from the lysate of the cultured KB/TP cells,[9] which was produced by transfection of PD-ECGF cDNA from human KB epidermoid carcinoma cells (KB 3-1). Using this crude TP, we have constructed the bioassay system by HPLC to search inhibitors of TP. The MeOH extract of the marine sponge *Petrosia strongylata*, collected at Bitung, Indonesia, showed inhibitory activity against TP and was subjected to bioassay-guided separation. The extract was partitioned into an AcOEt–water mixture to obtain an AcOEt soluble portion. The aqueous phase was further partitioned with *n*-BuOH to obtain an *n*-BuOH soluble portion, and then the aqueous phase was evaporated *in vacuo* to give an H_2O soluble portion. This H_2O soluble portion was separated by Diaion HP-20 column, Sephadex LH-20 column, and reversed-phase HPLC to furnish lembehsterol A (**1**) (4.1% yield from the H_2O soluble portion) and ibisterol sulfate B (**3**)[11] (2.2% yield) as major active components. Then, the *n*-BuOH soluble portion was separated by SiO_2 column, ODS column, and reversed-phase HPLC to afford lembehsterol B (**2**) (4.6% yield from the *n*-BuOH soluble portion) as a major active component.

Lembehsterol A (**1**) was obtained as a colorless amorphous solid: $[\alpha]_D$ +50.1°. The molecular formula of **1** was determined as $C_{28}H_{45}O_{12}S_3Na_3$ by negative ion high resolution (HR)-FAB-MS in conjunction with NMR analysis. The presence of a sulfate group in **1** was suggested by the potassium rhodizonate reagent.[12] The IR spectrum of **1** was also consistent with the presence of the sulfate group from the absorbance at 1258 cm^{-1}. The ^1H- and ^{13}C-NMR data of **1** indicated the presence of three tertiary methyls, an olefinic proton, and three oxymethine protons. All the proton and carbon signals of **1** were assigned by two-dimensional (2D)-NMR (correlation spectroscopy (COSY), ^1H-detected heteronuclear multiple quantum coherence (HMQC)) analysis of **1** (Table 1) and the COSY spectrum of **1** revealed the presence of two partial structures (fragment A: C-1 to C-9, C-11 and C-12, fragment B: C-15 to C-27) as shown in Fig. 1. The connectivities between these two partial structures, three quaternary carbons, and three tertiary methyls (C-18, C-19 and C-28) have been figured out on the basis of the following heteronuclear multiple bond connectivity (HMBC) correlations. Thus, the connectivity of fragments A and B through C-12 and C-15 was clarified by the cross peaks between H-18 and C-12; H-11, H-12, H-17, H-18 and C-13; H-15, H-28 and C-14; H-28 and C-8, C-15. Based on the accumulated evidence, the planar structure of lembehsterol A has been elucidated as **1** (Chart 1).

Next, we elucidated the stereostructure of lembehsterol A (**1**). As shown in Fig. 2, the relative stereostructure of the ring part in **1** was elaborated on the basis of the nuclear Overhauser effect spectroscopy (NOESY) correlations and the $^3J_{HH}$ couplings. The small coupling constants (br s) for H-2 and H-3 suggested equatorial orientation of H-2 and H-3, respectively. Furthermore, the W-type coupling between H-1β and H-3 also indicated equatorial orientation of H-3. The β-axial orientation of H-6 was deduced from the large coupling constants between H-6 and H-5, H-7α, and the NOESY correlations between H-6 and H-8, H-19. The stereochemistry of C-17 and C-20 were determined by the NOESY correlations between H-17 and H-21, H-28; H-28

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and H-12α; H-12β and H-21; H-16β and H-20.[13] Hydrolysis of **1** in dioxane and 10% aq. H_2SO_4 mixture gave a tri-desulfated derivative **4**, and then modified Mosher's method[14] was applied to the 6-hydroxyl group of **4**. Thus, **4** was treated with *S*-(+)- or *R*-(−)-2-methoxy-2-phenyl-2-trifluoromethylacetic acid (MTPA), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), and *N*,*N*-dimethylaminopyridine (DMAP) to furnish the 6-*O*-*R*-(+)-MTPA ester **5a** and the 6-*O*-*S*-(−)-MTPA ester **5b**, respectively. The characteristic proton signals of **5a** and **5b** were assigned and the absolute configuration at C-6 of **4** was determined as *S* by the analysis of the $\Delta\delta$ values (Fig. 3). Consequently, the total structure of lembehsterol A was established to be 5α-cholest-9(11)-en-2β,3α,6α-triol-2,3,6-trisulfate (**1**) (Chart 1).

The molecular formula of lembehsterol B (**2**) was determined as $C_{28}H_{44}O_8S_2Na_2$ by negative ion HR-FAB-MS. The IR spectrum of **2** exhibited an absorption band due to a sulfate group (1211 cm^{-1}). The ^{1}H- and ^{13}C-NMR spectra of **2** were closely similar to those of **1**, except for the additional signals assignable to olefin [δ 5.32 (d-like, J=4.9 Hz), δ_C 120.6 (d), 136.2 (s)] and disappearance of the oxygenated methine at C-6 of **1** (Table 1). On the basis of 2D-NMR analysis of **2**, the planar structure of lembehsterol B was as

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

	1			**2**	
No.	^{13}C δ_C	^{1}Hδ (mult., J (Hz))	No.	^{13}C δ_C	^{1}Hδ (mult., J (Hz))
1	35.7 (t)	1.48 (m): α, 2.06 (m): β	1	35.3 (t)	1.43 (m): α, 2.31 (m): β
2	72.5 (d)	4.54 (br s)	2	72.9 (d)	4.42 (d-like, 3.1)
3	72.2 (d)	4.34 (br s)	3	72.7 (d)	4.30 (d-like, 2.4)
4	24.1 (t)	1.98 (m): α, 1.38 (m): β	4	32.6 (t)	2.46 (m): α, 2.19 (m): β
5	41.9 (d)	1.34 (m)	5	136.2 (s)	—
6	74.2 (d)	4.05 (td, 11.0, 4.3)	6	120.6 (d)	5.32 (d-like, 4.9)
7	34.2 (t)	1.24 (m): α, 2.05 (m): β	7	36.9 (t)	1.83 (m): α, 2.05 (m): β
8	39.4 (d)	2.23 (d-like, 12.8)	8	37.7 (d)	2.30 (d-like, 12.2)
9	145.0 (s)	—	9	145.4 (s)	—
10	38.7 (s)	—	10	38.0 (s)	—
11	115.5 (d)	5.22 (d-like, 5.5)	11	116.4 (d)	5.37 (d-like, 6.1)
12	36.7 (t)	2.04 (m): α 1.90 (dd, 17.5, 5.5): β	12	36.9 (t)	2.10 (m): α 1.92 (dd, 17.1, 6.7): β
13	43.9 (s)	—	13	44.1 (s)	—
14	46.6 (s)	—	14	46.2 (s)	—
15	33.4 (t)	1.35 (m): α, 1.26 (m): β	15	33.3 (t)	1.35 (m): α, 1.26 (m): β
16	27.5 (t)	1.86 (m): α, 1.24 (m): β	16	27.5 (t)	1.83 (m): α, 1.25 (m): β
17	50.4 (d)	1.58 (ddd, 9.2, 9.2, 9.2)	17	50.5 (d)	1.61 (ddd, 9.8, 9.8, 9.8)
18	14.2 (q)	0.61 (s)	18	14.0 (q)	0.67 (s)
19	21.8 (q)	1.04 (s)	19	31.0 (q)	1.24 (s)
20	35.5 (d)	1.35 (m)	20	35.5 (d)	1.34 (m)
21	18.3 (q)	0.86 (d, 6.1)	21	18.2 (q)	0.87 (d, 6.7)
22	35.9 (t)	1.35 (m), 0.99 (m)	22	35.9 (t)	1.34 (m), 0.97 (m)
23	23.4 (t)	1.35 (m), 1.35 (m)	23	23.4 (t)	1.35 (m), 1.35 (m)
24	38.9 (t)	1.13 (m), 1.13 (m)	24	38.9 (t)	1.13 (m), 1.13 (m)
25	27.4 (d)	1.50 (m)	25	27.3 (d)	1.50 (m)
26	22.7 (q)	0.84 (d, 6.1)	26	22.6 (q)	0.84 (d, 6.7)
27	22.4 (q)	0.83 (d, 6.1)	27	22.3 (q)	0.84 (d, 6.7)
28	18.1 (q)	0.72 (s)	28	18.1 (q)	0.75 (s)

a) 600 MHz in DMSO-d_6.

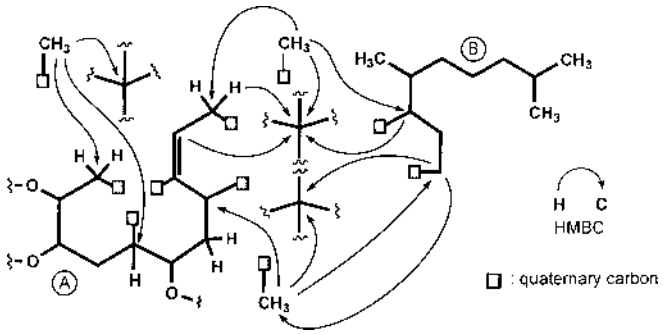


Fig. 1. HMBC Correlations between the Two Partial Structures of **1**

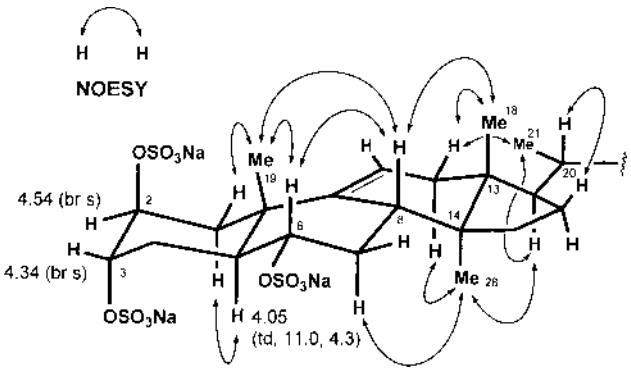


Fig. 2. Characteristic NOESY Correlations in **1**

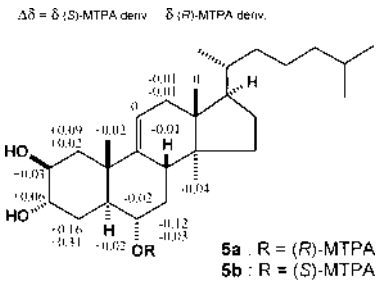
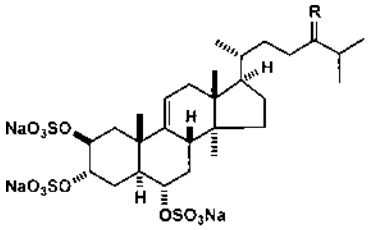
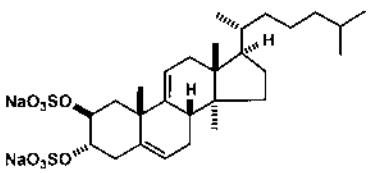


Fig. 3. Application of Modified Mosher's Method to the Tri-Desulfated Derivative **4**



lembehsterol A (**1**) : R = H, H
ibisterol sulfate B (**3**) : R = CH_2



lembehsterol B (**2**)

Chart 1

shown in Chart 1. The relative stereochemistry of the ring part and C-20 in **2** were also elaborated on the basis of the NOESY correlations and the $^3J_{HH}$ couplings. Consequently, lembehsterol B was elucidated to be cholest-5,9(11)-dien-2β,3α-diol-2,3-disulfate (**2**).

Lembehsterols A (**1**) and B (**2**) showed inhibitory activity against TP at 41 and 45 μM concentration (IC_{50} value) in this assay system, respectively. Tri-desulfated derivative **4** of **1** did not show inhibition at 230 μM concentration, suggesting the importance of sulfate group for the inhibitory activity against TP. The IC_{50} value of TPI,[9] which was a nucleoside analogue known as one of the most potent inhibitors, was 12 nM. Several sulfated sterols have been isolated from marine sponges, which show a variety of biological activities (*e.g.*, HIV inhibitor).[12,15] However, there is no report of sulfated sterols having inhibitory activity against TP. Most of the known TP inhibitors are nucleoside analogues, and lembehsterols A and B are the first examples having a non-nucleoside skeleton. The action mechanism of these sulfated sterols is under investigation.

Experimental

Isolation The titled dried marine sponge (300 g), which was collected in July, 1999 at Lembeh Island, Bitung, Indonesia, was initially steeped in MeOH. The residue obtained by evaporation of the solvent under reduced pressure was partitioned into an AcOEt–water mixture (1 : 1) to obtain an AcOEt soluble portion (1.8 g). The aqueous phase was further partitioned with *n*-BuOH to obtain an *n*-BuOH soluble portion (3.7 g), and then the aqueous phase was evaporated under reduced pressure to give an H_2O soluble portion (10 g). The H_2O soluble portion (1 g) was separated by Diaion HP-20 column to give an active MeOH eluate, which was further separated by Sephadex LH-20 column (MeOH : H_2O=2 : 1) to afford an active fraction (120 mg) [30% inhibition at 25 μg/ml]. Then, the active fraction was purified by reversed-phase HPLC (YMC-Pack A-323 RP-18, MeOH : H_2O=8 : 2, 0.1 M $NaClO_4$) to provide lembehsterol A (**1**, 41 mg, 4.1% yield from the H_2O soluble portion) and ibisterol sulfate B[12] (**3**, 22 mg, 2.2% yield). The *n*-BuOH soluble portion (1.42 g) was separated by SiO_2 column ($CHCl_3$–MeOH–H_2O) to afford an active fraction (200 mg) [33% inhibition at 25 μg/ml] and the active fraction was further purified by ODS column (MeOH–H_2O) and reversed-phase HPLC (YMC-Pack A-323 RP-18, MeOH : H_2O=9 : 1, 0.1 M $NaClO_4$) to afford lembehsterol B (**2**, 65 mg, 4.6% yield from the *n*-BuOH soluble portion).

Lembehsterol A (**1**): $[\alpha]_D$ +50.1° (*c*=0.41, MeOH). FAB-MS: *m/z* 715 $(M-Na)^-$, *m/z* 613 $(M-Na-NaSO_3+H)^-$, *m/z* 595 $(M-NaHSO_4-Na)^-$, *m/z* 493 $(M-NaHSO_4-NaSO_3-Na+H)^-$. HR-FAB-MS *m/z*: Calcd for $C_{28}H_{45}O_{12}S_3Na_2$: 715.1868; Found: 715.1849. IR (KBr): 3517, 1373, 1258 cm^{-1}. ^{1}H- and ^{13}C-NMR: as shown in Table 1.

Lembehsterol B (**2**): $[\alpha]_D$ +39.0° (*c*=0.32, MeOH). FAB-MS: *m/z* 595 $(M-Na)^-$, *m/z* 493 $(M-Na-NaSO_3+H)^-$, *m/z* 475 $(M-NaHSO_4-Na)^-$. HR-FAB-MS *m/z*: Calcd for $C_{28}H_{44}O_8S_2Na$: 595.2375; Found: 595.2391. IR (KBr): 3436, 1373, 1211 cm^{-1}. ^{1}H- and ^{13}C-NMR: as shown in Table 1.

Hydrolysis of Lembehsterol A (1) A solution of **1** (1.0 mg) in dioxane (1.0 ml) and 10% aq. H_2SO_4 (1.0 ml) was refluxed at 140 °C for 3 h. The reaction mixture was poured into saturated aq. $NaHCO_3$, then the whole was evaporated *in vacuo*. The residue was purified by Diaion HP-20 column (H_2O–MeOH) to give a crude product. After evaporation of the solvent, the residue was purified by SiO_2 column ($CHCl_3$: MeOH=3 : 1) to furnish a tri-desulfate derivative **4** (0.5 mg).

4: FAB-MS: *m/z* 455 $(M+Na)^+$, *m/z* 439 $(M+Li)^+$. HR-FAB-MS *m/z*: Calcd for $C_{28}H_{48}O_3Na$: 455.3502; Found: 455.3488. IR (KBr): 3333 cm^{-1}. ^{1}H-NMR (DMSO-d_6, δ): 5.20 (1H, d-like, *J*=5.5 Hz, H-11), 4.41 (1H, d, *J*=3.1 Hz, 2-OH), 4.32 (1H, d, *J*=3.1 Hz, 3-OH), 4.19 (1H, d, *J*=5.5 Hz, 6-OH), 3.67 (1H, m, H-2), 3.61 (1H, m, H-3), 3.30 (1H, m, H-6), 2.25 (1H, d-like, *J*=12.2 Hz, H-8), 2.02 (1H, m, H-12a), 2.01 (1H, m, H-7a), 1.86 (1H, m, H-12b), 1.82 (1H, m, H-4a), 1.66 (1H, m, H-7b), 1.45 (1H, m, H-4b), 1.35 (1H, m, H-5), 1.03 (3H, s, H-19), 0.85 (3H, d, *J*=6.7 Hz, H-21), 0.84 (3H, d, *J*=6.7 Hz, H-26), 0.84 (3H, d, *J*=6.7 Hz, H-27), 0.71 (3H, s, H-28), 0.60 (3H, s, H-18). ^{13}C-NMR (DMSO-d_6, δ_C): 146.1 (s), 111.7 (d), 69.8 (d), 68.6 (d), 67.2 (d), 50.4 (d), 46.4 (s), 43.8 (s), 43.4 (d), 39.9 (d), 38.9 (t), 38.5 (s), 38.1 (t), 36.9 (t), 36.7 (t), 35.9 (t), 35.4 (d), 33.3 (t), 27.4 (t), 27.3 (d), 25.3 (t), 23.3 (t), 22.6 (q), 22.4 (q), 22.3 (q), 18.1 (q), 18.1 (q), 14.2 (q).

6-*R*-MTPA Ester 5a and 6-*S*-MTPA Ester 5b of Lembehsterol A (4) A solution of **1** (1.0 mg) in CH_2Cl_2 (0.5 ml) was treated with (*R*)-(+)-MTPA (4.6 mg), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDCI, 11.4 mg), and *N*,*N*-dimethylaminopyridine (DMAP, 1.2 mg) at room temperature for 20 h under an Ar atmosphere. The reaction was quenched by saturated aq. NH_4Cl and the whole was extracted with AcOEt. The AcOEt phase was washed by 5% HCl, saturated aq. $NaHCO_3$, and saturated aq. NaCl. After evaporation of the solvent, the residue was purified by SiO_2 column (*n*-hexane : AcOEt=3 : 1) to furnish 6-*R*-MTPA ester **5a** (0.8 mg). A solution of **1** (1.0 mg) in CH_2Cl_2 (0.5 ml) was similarly treated with (*S*)-(−)-MTPA (4.6 mg), EDCI (11.4 mg), and DMAP (1.2 mg) for 12 h to afford 6-*S*-MTPA ester **5b** (0.5 mg).

5a: HR-FAB-MS *m/z*: Calcd for $C_{38}H_{55}O_5F_3Na$: 671.3899; Found: 671.3900 $(M+Na)^+$. ^{1}H-NMR ($CDCl_3$, δ): 5.28 (1H, d, *J*=6.1 Hz, H-11), 3.89 (1H, br s, H-2), 3.74 (1H, d-like, *J*=2.4 Hz, H-3), 2.38 (1H, d-like, *J*=11.6 Hz, H-8), 2.02 (1H, m, H-12a), 1.94 (1H, m, H-7a), 1.89 (1H, m, H-12b), 1.77 (1H, m, H-1b), 1.76 (1H, m, H-5), 1.61 (1H, m, H-4a), 1.46 (1H, m, H-7b), 1.25 (1H, m, H-1a), 1.25 (1H, m, H-4b), 1.14 (3H, s, H-19), 0.81 (3H, d, *J*=6.7 Hz, H-21), 0.80 (3H, d, *J*=6.1 Hz, H-26), 0.80 (3H, d, *J*=6.1 Hz, H-27), 0.70 (3H, s, H-28), 0.59 (3H, s, H-18).

5b: HR-FAB-MS *m/z*: Calcd for $C_{38}H_{55}O_5F_3Na$: 671.3899; Found: 671.3917 $(M+Na)^+$. ^{1}H-NMR ($CDCl_3$, δ): 5.28 (1H, d, *J*=5.5 Hz, H-11), 3.92 (1H, br s, H-2), 3.80 (1H, d-like, *J*=2.4 Hz, H-3), 2.37 (1H, d-like, *J*=14.0 Hz, H-8), 2.01 (1H, m, H-12a), 1.91 (1H, m, H-7a), 1.88 (1H, m, H-12b), 1.79 (1H, m, H-1b), 1.78 (1H, m, H-5), 1.77 (1H, m, H-4a), 1.34 (1H, m, H-7b), 1.34 (1H, m, H-1a), 1.56 (1H, m, H-4b), 1.16 (3H, s, H-19), 0.81 (3H, d, *J*=6.7 Hz, H-21), 0.80 (3H, d, *J*=6.1 Hz, H-26), 0.80 (3H, d, *J*=6.1 Hz, H-27), 0.66 (3H, s, H-28), 0.59 (3H, s, H-18).

Preparation of Crude TP Human KB epidermoid carcinoma cells (KB 3-1) were maintained in minimal essential medium containing 10% fetal bovine serum. Transfection of PD-ECGF cDNA into KB 3-1 cells was performed as described in the previous report.[9] KB/TP cells, in which PD-ECGF cDNA was transfected, were also cultured under the above conditions. After 7 d, KB/TP cells were harvested and homogenized in the lysis buffer, which consisted of Tris–HCl (pH 7.5), 2 mM (*p*-amidinophenyl)-methanesulfonyl fluoride, and 0.02% 2-mercaptethanol (buffer A). The lysate was centrifuged at 15000×***g*** for 20 min at 4 °C, and the supernatant was used as a crude TP for enzyme assay. The protein concentrations were determined by Bradford's method.[16]

Enzyme Assay The enzyme activity of the crude TP was measured by HPLC. Thus, 0.05 mM buffer A solution (100 μl) of thymidine containing 5 μg of the crude TP was incubated with 5 μl of the DMSO solution of the testing sample at 37 °C. After 1 h, the enzyme reaction was stopped by heat-treatment at 95 °C for 5 min. Then, the remaining thymidine and the thymine produced were quantified on reversed-phase HPLC (column: mightysil RP-18(H), elution: CH_3CN : H_2O=5 : 95 containing 0.1 M aq. sodium dihydrogenphosphate, detection: UV absorption at 260 nm). The activity of the crude TP was calculated from the production amount of thymine and the remaining thymidine. The inhibitory activity of the testing sample was evaluated by the inhibitory-ratio of the enzyme reaction in compared with that of the control (without testing sample).

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