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Fatty Acids and Sterols of the Tunicate, *Salpa thompsoni*, from the Antarctic Ocean: Chemical Composition and Hemolytic Activity

TSUTOMU MIMURA,^a MASARU OKABE,^a MIKIO SATAKE,^a TSUTOMU NAKANISHI,^{*,b}
AKIRA INADA,^b YOSHINORI FUJIMOTO,^c FUMIKO HATA,^c
YASUKO MATSUMURA,^c and NOBUO IKEKAWA^c

Faculty of Pharmaceutical Sciences, Osaka University,^a 1-6 Yamada-oka, Suita, Osaka 565,
Japan, Faculty of Pharmaceutical Sciences, Setsunan University,^b 45-1
Nagaotoge-cho, Hirakata, Osaka 573-01, Japan, and Department
of Chemistry, Tokyo Institute of Technology,^c
O-okayama, Meguro-ku, Tokyo 152, Japan

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The fatty acid and sterol compositions of the marine tunicate *Salpa thompsoni* from the Antarctic Ocean are reported. Of the 21 fatty acids identified, the acids 16:0, 20:5 ω 3, and 22:6 ω 3 were present at high concentrations. The salpa also contained various C₂₆, C₂₇, C₂₈, and C₂₉ sterols with three Δ^5 -sterols, i.e., cholesterol (6), (22E)-(24 ξ)-24-methylcholesta-5,22-dien-3 β -ol (9), and 24-methylene-cholest-5-en-3 β -ol (11) as the main constituents. The presence of eight related pairs of Δ^5 -sterols and 5 α -stanols with identical side chains was characteristic. Desmosterol (8) and sitostanol (or C-24 epimer) (17) were found for the first time in salpas. Furthermore, the hemolytic active components of methanol extract of the salpa were examined. The total fatty acids fraction was strongly hemolytic, and detailed examination of each component acid showed the polyunsaturated acids 22:6 ω 3 and 20:5 ω 3 to be most potent.

Keywords—*Salpa thompsoni*; tunicate; Antarctic Ocean; fatty acid; marine sterol; composition; hemolytic activity

Salpa thompsoni (Salpidae) belongs to the subphylum tunicate. Several reports on the chemical components of marine tunicates, including salpas, have appeared, dealing with the sterol composition and chemistry.¹⁾ However, no work on the free fatty acid components of salpas and tunicates has been done to date. This paper is the first report to present a detailed analysis of the fatty acid and sterol composition of *S. thompsoni* from the Antarctic Ocean. In addition, the hemolytic active components in the methanol extract of the salpa were examined.

Fatty Acid Composition

The composition of the component fatty acids of *S. thompsoni* was determined by capillary gas chromatographic (GLC) analysis of the corresponding methyl esters (Table I). Fatty acids ranged from C₁₄ to C₂₂ and those of even chain length were predominant. The polyunsaturated acids 20:5 ω 3 and 22:6 ω 3 followed by the saturated and mono-unsaturated acids 14:0, 16:0, and 16:1 were present at high concentrations. The high contents of the acids 22:6 ω 3, 16:0, and 14:0 are reminiscent of the fatty acid composition of algae²⁾ and dinoflagellates.³⁾ However, the abundance of the acid 20:5 ω 3 and the absence of the acid 18:5 appear to be characteristic of the salpa composition. To our knowledge, this is the first report on the composition of the free fatty acids contained in salpas and also in tunicates.

Sterol Composition⁴⁾

The free sterol fraction from the salpa (see Experimental) was, in a usual manner,

TABLE I. Fatty Acid Composition of *Salpa thompsoni* from the Antarctic Ocean

Fatty acid	% composition	t_R	Fatty acid	% composition	t_R
12:0	0.3	9.23	20:0	0.1	19.10
14:0	8.8	9.95	20:1	0.8	19.94
14:1	0.7	10.28	20:2 ω 6	0.4	21.76
15:0	1.5	10.54	20:4 ω 6	0.5	23.64
16:0	14.6	11.38	20:5 ω 3	17.8	26.76
16:1	10.1	11.72	22:0	0.6	28.24
17:0	0.8	12.38	22:1	0.2	30.16
18:0	0.7	14.04	22:5 ω 3	0.7	43.20
18:1 ω 9	5.9	14.68	22:6 ω 3	15.6	44.96
18:2 ω 6	1.8	15.52	Others ^{a)}	10.4	
18:3 ω 3	1.6	16.92			
18:4 ω 3	6.1	17.72			

a) Sum (%) of minor and/or unidentified acids.

TABLE II. Sterols from the Marine Tunicate, *Salpa thompsoni*

Sterol No.	Compound ^{a)}	Mobility GC ^{b)}	M ⁺ (m/z) ^{c)}	% sterol fraction
1	Δ^5	0.64	442	5.1
2	5 α	0.66	444	1.3
3	Δ^5	0.87	456	5.0
4	Δ^5	0.90	456	6.6
5	5 α	0.92	458	1.2
6	Δ^5	1.00	458	16.2
7	5 α	1.02	460	1.5
8	Δ^5	1.08	456	1.3
9	Δ^5	1.11	470	18.1
10	5 α	1.13	472	10.5
11	Δ^5	1.26	470	13.3
12	5 α	1.29	472	2.8
13	Δ^5	1.38	484	1.9
14	5 α	1.42	486	0.9
15	Δ^5	1.60	484	7.7 ^{d)}
16	Δ^5	1.60	486	
17	5 α	1.65	488	7.7 ^{e)}
18	5 α	1.65	486	

a) The symbols Δ^5 and 5 α represent sterols having a Δ^5 and a 5 α nuclei, respectively. b) Retention time relative to cholesterol. c) M⁺ of the corresponding C₃-O-TMSi-ether. d) Total percentage of sterols 15 and 16. e) Total percentage of sterols 17 and 18.

transformed into the corresponding trimethylsilyl (TMSi)-ether fraction, which was analyzed by capillary gas chromatography-mass spectrometry (GC-MS). Eighteen component sterols were characterized (Table II).

The sterols **1**, **3**, **4**, **6–9**, **11**, **13**, and **15–17** were identified by comparison of their retention times and mass fragmentation patterns with those of authentic standards. The structures of the other sterols (**2**, **5**, **10**, **12**, **14**, and **18**) were established based on their retention times and on detailed and careful analyses of the mass spectral data.

Two sterols having 26 carbons, (22*E*)-24-norcholesta-5,22-dien-3 β -ol (**1**)⁴⁾ and (22*E*)-24-nor-5 α -cholest-22-en-3 β -ol (**2**) were characterized; these were previously found in the tunicate *Halocynthia roretzi*^{1a)} and are representatives of the widespread and unique marine C₂₆ sterols.⁵⁾

The six C₂₇-sterols identified were: (22*E*)-27-nor-(24 ξ)-24-methylcholesta-5,22-dien-3 β -ol (**3**)⁴⁾ (22*E*)-cholesta-5,22-dien-3 β -ol (= *trans*-22-dehydrocholesterol) (**4**)⁴⁾ (22*E*)-5 α -cholest-22-en-3 β -ol (= *trans*-22-dehydrocholestanol) (**5**), cholesterol (**6**)⁴⁾ 5 α -cholestan-3 β -ol (=cholestanol) (**7**)⁴⁾ and cholesta-5,24-dien-3 β -ol (=desmosterol) (**8**). In the present assigned structure for sterol **3** the configuration at C₂₄ remains undetermined, but of the two related possible diastereoisomers, the 24*S* isomer, i.e., ocellasterol⁶⁾ seems more likely, because only the 24*S* isomer has so far been found in a number of marine organisms including a tunicate.⁷⁾

The four C₂₈-sterols identified were: (22*E*)-(24 ξ)-24-methylcholesta-5,22-dien-3 β -ol (**9**)⁴⁾ (cf. 24*R*=brassicasterol; 24*S*=epibrassicasterol), (22*E*)-(24 ξ)-24-methyl-5 α -cholest-22-en-3 β -ol (**10**), 24-methylene-cholest-5-en-3 β -ol (= 24-methylenecholesterol) (**11**)⁴⁾ 24-methylene-5 α -cholestan-3 β -ol (= 24-methylenecholestanol) (**12**).

The following six C₂₉ sterols were detected. (22*E*)-(24 ξ)-24-ethylcholesta-5,22-dien-3 β -ol (**13**)⁴⁾ (cf. 24*S*=stigmasterol; 24*R*=poriferasterol), (22*E*)-(24 ξ)-24-ethyl-5 α -cholest-22-en-3 β -ol (**14**), (24*E*)-24-ethylcholesta-5,24(28)-dien-3 β -ol (= fucosterol) (**15**), (24 ξ)-24-ethylcholest-5-en-3 β -ol (**16**)⁴⁾ (cf. 24*R*=sitosterol; 24*S*=clionasterol), (24 ξ)-24-ethyl-5 α -cholest-3 β -ol (**17**) (cf. 24*R*=sitostanol), and (24*E*)-24-ethyl-5 α -cholest-24(28)-en-3 β -ol (= fucostanol) (**18**).

The sterols **15** and **16** have the same retention time and give a single peak under the present GLC conditions. The retention time and the mass spectrum originating from this peak were consistent with those due to a mixture of authentic fucosterol and sitosterol, supporting the proposed structures of **15** and **16**. Similarly, careful analysis of the mass spectrum originating from the peak of mobility 1.65 revealed that the major sterol **17** (consistent with sitostanol) was contaminated with a small amount of **18** (assigned as fucostanol). For the 5,6-dihydrosterols **2**, **5**, **10**, **12**, **14**, and **18**, the 5 α -structure can reasonably be assigned based on co-occurrence with two 5 α -stanols, cholestanol (**7**) and sitostanol (**17**) and on the biogenetic hypothesis that 5 α -stanols are more common than the corresponding 5 β -stanols in marine organisms.⁷⁾

An interesting feature of the *S. thompsoni* sterols is that each of the eight Δ^5 sterols (**1**, **4**, **6**, **9**, **11**, **13**, **15**, and **16**) is accompanied by the corresponding 5 α -stanol (**2**, **5**, **7**, **10**, **12**, **14**, **18**, and **17**). Thus, all the identified sterols except for ocellasterol (**3**) and desmosterol (**8**) were present as related pairs of Δ^5 -sterols and 5 α -stanols with identical side chains.

The major components in the total sterol fraction were brassicasterol (and/or its C-24 epimer) (**9**) (18.1%), its 5,6-dihydrostanol (**10**) (10.5%), cholesterol (**6**) (16.2%), and 24-methylenecholesterol (**11**) (13.3%). The sterol composition of *S. thompsoni* was roughly consistent with those of three other species of salpas (*Pegea confoederata*, *Salpa maxima*, and *S. fusiformis*) from the Western Mediterranean,^{1b)} but the present analysis provides more detailed information on the structures of salpa sterols. To our knowledge, this is the first time that desmosterol (**8**) and sitostanol (and/or its C-24 epimer) (**17**) have been detected in salpas. The C₂₇ sterols **4**, **5**, **7**, and **8** may be either precursors or metabolites of cholesterol (**6**) which may arise by “*de novo*” biosynthesis,⁴⁾ whereas the C₂₈ and C₂₉ sterols appear to be of dietary origin⁴⁾ and the 24-nor C₂₆ sterols are also reported to be of phytoplanktonic origin.⁸⁾ The C₂₆ sterols **1** and **2** and ocellasterol (**3**) may be biogenetically produced from the precursor C₂₈ sterols **9** and/or **10** by demethylation.⁴⁾

Hemolytic Activity

No study on biological active principles of *S. thompsoni* has so far been done. Therefore, as a first examination, the hemolytic activity of the salpa components was tested. The methanol extract from *S. thompsoni* was fractionated as shown in Chart 1, and the relative hemolytic activity of each separated fraction (fr. No. 1—6) was examined (Table III). Hemolytic activity was mainly distributed in fractions (fr. Nos. 1, 2, 3, and 5) which contained fatty acid components. The activity is directly proportional to the concentration of fatty acids, and accordingly, the total fatty acid fraction (fr. No. 5) together with the lipid mixture of fatty acids and sterols (fr. No. 3) showed the most potent activity.

Composition analysis of the total fatty acid fraction revealed that the acids 14:0, 16:0, 16:1, 18:1 ω 9, 18:4 ω 3, 20:5 ω 3, and 22:6 ω 3 were abundant components of the salpa. Therefore, the hemolytic activities of these predominant component acids and related model acids [myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (*trans*) (16:1 ω 7), oleic acid (*cis*) (18:1 ω 9), vaccenic acid (*trans*) (18:1 ω 7), petroselinic acid (*cis*) (18:1 ω 12), 4,8,12,15-

Chart 1. Extraction and Fractionation Procedures

TABLE III. Relative Hemolytic Activity of Each Fraction Separated from *Salpa thompsoni*

Fraction	fr. No.	Relative activity ^{a)}
MeOH ext.	1	42.4
AcOEt ext.	2	100.0
Sterols and fatty acids	3	134.0
Sterols	4	10.0 >
Fatty acids	5	131.0

^{a)} Relative activity represents the 50% hemolytic activity of each sample compared with that of a saponin standard (defined as 100).

TABLE IV. Relative Hemolytic Activity of Major Component Fatty Acids of *Salpa thompsoni* and of Related Fatty Acids

Fatty acid		Relative activity ^{a)}
14:0	Myristic acid	93.7
16:0	Palmitic acid	10.0>
16:1	Palmitoleic acid (ω 7; <i>trans</i>)	32.5
18:0	Stearic acid	10.0>
18:1 ω 9	Oleic acid (<i>cis</i>)	115.0
18:1	Vaccenic acid (ω 7; <i>trans</i>)	55.0
	Petroselinic acid (ω 12; <i>cis</i>)	129.0
18:4 ω 3	4,8,12,15-Octadecatetraenoic acid	76.0
20:5 ω 3	Eicosapentaenoic acid (EPA)	226.0
22:6 ω 3	Docosahexaenoic acid (DHA)	382.0

a) Relative activity represents the 50% hemolytic activity of each acid compared with that of a saponin standard (defined as 100).

Fig. 1. Hemolytic Activity of Various Component Fatty Acids from the *Salpa* and of Related Fatty Acids

—▲—, 22:6; —△—, 20:5; —●—, 18:1 (petroselinic acid); —●—, 18:1 (oleic acid); —○—, 18:1 (vaccenic acid); —■—, 16:0, 16:1, 18:0; —■—, 18:4; —□—, 14:0; —x—, saponin (standard).

octadecatetraenoic acid (18:4 ω 3), eicosapentaenoic acid (EPA) (20:5 ω 3), and docosahexaenoic acid (DHA) (22:6 ω 3)] were examined as shown in Table IV and Fig. 1.

Of the acids used in this study, EPA and DHA were freshly isolated from the total fatty acid fraction of the salpa and the others were commercial products.

Among the acids, DHA and EPA showed the most potent and the second most potent activity, respectively. Slight changes in the structure of the fatty acid, *i.e.*, in the position and the geometry of the double bond, seem to influence the potency of hemolytic activity of the fatty acid, as indicated by comparison of the three different 18:1 acids. However, it is not yet clear what structure is required for potent hemolytic activity.

Experimental

Material—*Salpa thompsoni* used in this study was collected off South Orkney Island (Lat. 59°S.; Long. 18°W.) in the Antarctic Ocean in January, 1985 and identified by Prof. T. Nemoto, University of Tokyo. The animals collected were frozen on site and stored frozen until analysis.

Extraction and Isolation—The whole body (600 g) of the frozen salpa was extracted with MeOH (2 l) at room temperature for one week and the same extraction procedure was repeated twice more. The combined MeOH solution was evaporated under reduced pressure. The extract (41 g) was suspended into H₂O and extracted twice with AcOEt (each 200 ml) to give the AcOEt (5.9 g) and H₂O (32.3 g) residues, respectively. A portion (3.2 g) of the organic residue was chromatographed on SiO₂ (230–400 mesh; 500 g), and eluted successively with *n*-hexane–AcOEt (3:1), (2:1), (1:1), and AcOEt. The initial fraction obtained with *n*-hexane–AcOEt (3:1) gave a mixture (590 mg) of sterols and fatty acids. The subsequent fraction with the same eluent afforded a mixture (560 mg) of various fatty acids, which was used for the biological study and was, after usual methylation, subjected to composition analysis. The

mixture of sterols and fatty acids was treated with CH_2N_2 in Et_2O . In this methylation reaction, the fatty acid components were methylated to the corresponding methyl esters and the sterols did not react. Separation on the reaction products by SiO_2 (50 g) column chromatography gave the fatty acid methyl esters (290 mg) and the sterols (220 mg), respectively, on elution with *n*-hexane–AcOEt (5:1 and 3:1). The sterol mixture thus obtained was subjected to composition analysis.

Fatty Acid Analysis—Fatty acid methyl esters were analyzed on a Shimadzu 7A gas chromatograph with flame ionization detector using a Silar 5CP (50 m $\times$ 0.27 mm i.d.) capillary column. Nitrogen was used as the carrier gas at a flow rate of 51 ml/min, and the column temperature was 200 °C. Fatty acid methyl esters were identified by co-chromatography with authentic standards and by comparison with standard acids previously characterized from fish oils.

Sterol Analysis—The sterol mixture (*ca.* 0.1 mg) was treated with *N*-trimethylsilylimidazole (10 μl) at *ca.* 50 °C for a few min to afford the corresponding sterol-trimethylsilyl (TMSi) ethers, which were diluted with AcOEt (*ca.* 0.5 ml) and subjected to analysis. GC-MS was performed on a Shimadzu 9020 DF gas chromatograph-mass spectrometer equipped with a data-processing system using a Shimadzu HiCap CBP1-S25-050 (25 m $\times$ 0.33 mm i.d.) capillary column under the following conditions: injection temperature, 260 °C; column oven temperature, 245 °C; separator temperature, 285 °C; ionization source temperature, 290 °C; ionization energy, 70 eV; trap current, 60 A; accelerating voltage, 3.5 kV. Samples (0.2–0.5 μl) were injected using a moving needle-type solvent cut sample injection apparatus. The flow rates of split and make-up gas were 50 and 25 ml/min, respectively.

Main and significant MS ions of the 3-O-TMSi derivatives of the component sterols (**1**–**18**) were as follows:

1: m/z 442 (M^+), 352 [$\text{M}^+ - (\text{Me})_3\text{SiOH}$ (= TMSiOH)], 337 ($\text{M}^+ - \text{TMSiOH} - \text{Me}$), 313 ($\text{M}^+ - 129$), 255, 129, 97 [C_7H_{13} (side chain), 100%].

2: m/z 444 (M^+), 429 ($\text{M}^+ - \text{Me}$), 374 [$\text{M}^+ - \text{C}_5\text{H}_9$ (fission between C_{20} and C_{22}) – H], 345 [$\text{M}^+ - \text{C}_7\text{H}_{13}$ (side chain) – 2H], 257, 75 (100%).

3: m/z 456 (M^+), 441 ($\text{M}^+ - \text{Me}$), 366 ($\text{M}^+ - \text{TMSiOH}$), 351 ($\text{M}^+ - \text{TMSiOH} - \text{Me}$), 327 ($\text{M}^+ - 129$), 255, 129, 111 [C_8H_{15} (side chain), 100%], 69.

4: m/z 456 (M^+), 366 ($\text{M}^+ - \text{TMSiOH}$), 351 ($\text{M}^+ - \text{TMSiOH} - \text{Me}$), 327 ($\text{M}^+ - 129$), 255, 129, 111, 69 (100%).

5: m/z 458 (M^+), 443 ($\text{M}^+ - \text{Me}$), 368 ($\text{M}^+ - \text{TMSiOH}$), 353 ($\text{M}^+ - \text{TMSiOH} - \text{Me}$), 329 ($\text{M}^+ - 129$), 255, 69 (100%).

6: m/z 458 (M^+), 443 ($\text{M}^+ - \text{Me}$), 368 ($\text{M}^+ - \text{TMSiOH}$), 353 ($\text{M}^+ - \text{TMSiOH} - \text{Me}$), 329 ($\text{M}^+ - 129$), 255, 129 (100%).

7: m/z 460 (M^+), 445 ($\text{M}^+ - \text{Me}$), 355 ($\text{M}^+ - \text{Me} - \text{TMSiOH}$), 215, 75 (100%).

8: m/z 456 (M^+), 366 ($\text{M}^+ - \text{TMSiOH}$), 351 ($\text{M}^+ - \text{TMSiOH} - \text{Me}$), 343 [$\text{M}^+ - \text{C}_8\text{H}_{15}$ (side chain) – 2H], 327 ($\text{M}^+ - 129$), 253, 129, 69 (C_5H_9 , 100%).

9: m/z 470 (M^+), 455 ($\text{M}^+ - \text{Me}$), 380 ($\text{M}^+ - \text{TMSiOH}$), 365 ($\text{M}^+ - \text{TMSiOH} - \text{Me}$), 255, 129, 125 [C_9H_{17} (side chain)], 69 (C_5H_9 , 100%).

10: m/z 472 (M^+), 457 ($\text{M}^+ - \text{Me}$), 374 [$\text{M}^+ - \text{C}_7\text{H}_{13}$ (fission between C_{20} and C_{22}) – H], 345 [$\text{M}^+ - \text{C}_9\text{H}_{17}$ (side chain) – 2H], 257, 75 (100%), 69 (C_5H_9).

11: m/z 470 (M^+), 455 ($\text{M}^+ - \text{Me}$), 386 [$\text{M}^+ - \text{C}_6\text{H}_{12}$ (fission between C_{22} and C_{23} ; McLafferty rearrangement)], 380 ($\text{M}^+ - \text{TMSiOH}$), 365 ($\text{M}^+ - \text{TMSiOH} - \text{Me}$), 341 ($\text{M}^+ - 129$), 296 (386 – TMSiOH), 257, 255, 253, 129 (100%).

12: m/z 472 (M^+), 457 ($\text{M}^+ - \text{Me}$), 388 [$\text{M}^+ - \text{C}_6\text{H}_{12}$ (see the data for **11**)], 382 ($\text{M}^+ - \text{TMSiOH}$), 367 ($\text{M}^+ - \text{TMSiOH} - \text{Me}$), 255, 215, 75 (100%).

13: m/z 484 (M^+), 469 ($\text{M}^+ - \text{Me}$), 394 ($\text{M}^+ - \text{TMSiOH}$), 351, 255, 129, 83 (100%), 69.

14: m/z 486 (M^+), 471 ($\text{M}^+ - \text{Me}$), 386, 381 ($\text{M}^+ - \text{Me} - \text{TMSiOH}$), 374, 353, 257, 75, 55 (100%).

15 and 16: Fragment ions from **15**; m/z 484 (M^+), 469 ($\text{M}^+ - \text{Me}$), 394 ($\text{M}^+ - \text{TMSiOH}$), 386 [$\text{M}^+ - \text{C}_7\text{H}_{14}$ (fission between C_{22} and C_{23} ; McLafferty rearrangement)], 355, 353, 296 (386 – TMSiOH), 257, 255, 129 (100%), 73, and those from **16**; m/z 486 (M^+), 471 ($\text{M}^+ - \text{Me}$), 396 ($\text{M}^+ - \text{TMSiOH}$), 357 ($\text{M}^+ - 129$), 129 (100%).

17 and 18: Fragment ions from **17**; m/z 488 (M^+), 473 ($\text{M}^+ - \text{Me}$), 398 ($\text{M}^+ - \text{TMSiOH}$), 383 ($\text{M}^+ - \text{Me} - \text{TMSiOH}$), 255, 129, 75 (100%), and those from **18**; m/z 486 (M^+), 471 ($\text{M}^+ - \text{Me}$), 388 [$\text{M}^+ - \text{C}_7\text{H}_{14}$ (see the data for **15**)], 75 (100%).

Preparation of the Biological Test Samples and Measurement of Hemolytic Activity—All of the free fatty acids used in this study were obtained from Sigma Co., Ltd., except for the acids 20:5 ω 3 and 22:6 ω 3 which were freshly isolated from the total fatty acid fraction of the salpa by high-performance liquid chromatography on a Yamamura Chemical YMC gel ODS S 10/50 column (1 m $\times$ 5 cm i.d.) using a Soken chemical instrument with MeOH as the mobile phase (flow rate, 30 ml/min).

To a solution (or a suspension) of an accurately weighed sample (*ca.* 1.2 mg) in $(\text{Me})_2\text{SO}$ (0.1 ml), 0.15 M phosphate-buffered saline of pH 7.4 (PBS) (2.9 ml) was added. If the resulting mixture was heterogeneous, the mixture was homogenized with a Biomixer (Nissei Co., Ltd.). Each of the resulting homogeneous solutions was diluted with appropriate amounts of PBS to make 6 sample solutions (10, 20, 30, 40, 50, and 60 $\mu\text{g}/\text{ml}$). Each of the sample solutions was preincubated at 37 °C and subjected to biological analysis. Sheep erythrocytes (SRBC) in Alsever solution were obtained from the Research Institute for Microbial Diseases, Osaka University. On the day of

use, the SRBC (1×10^{10} cells) were washed three times with PBS, and re-suspended in 40 ml of PBS. Hemolytic activity was measured by mixing the SRBC suspension (1 ml) with the sample solution (1 ml) at 37°C. After incubation for 30 min at 37°C, the optical density of the centrifuged supernatant was read spectrophotometrically at 550 nm to measure the release of hemoglobin. The results are given as the relative activity with respect to a saponin standard used as a positive control.

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