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Antioxidant Constituents in Distillation Residue of Awamori Spirits

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Constituents in a distillation residue of Awamori (millet spirits) and their antioxidant activity are investigated in this study. The supernatant of the distillation residue obtained by centrifugation was partitioned with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol against water to afford the corresponding solubles. Among them, *n*-hexane and chloroform solubles showed higher antioxidant potency than L-ascorbic acid by the bleomycin−Fe method. In chloroform solubles, seven cyclic dipeptides were identified along with ethyl 2-pyrrolidione-5-carboxylate, tyrosol, and ethyl *p*-hydoroxyphenyllactate. Antioxidant activity of ethyl *p*-hydoroxyphenyllactate was 4.2 times that of L-ascorbic acid, whereas cyclic dipeptides showed activity 0.89−1.29 times as strong as that of L-ascorbic acid. On the other hand, scavenging effect of cyclic dipeptides against $O_2^{-\bullet}$ and $OH^\bullet$ by using electron spin resonance was also investigated. In the results, cyclo(L-Ile-L-Pro) showed significantly strong inhibitory effect against $OH^\bullet$ (95.4% at 2.5×10^{-3} M) and cyclo(L-Phe-L-Pro), cyclo-(L-Pro-L-Val), and cyclo(L-Leu-L-Pro) inhibited $OH^\bullet$ 64.9, 54.1, and 51.0%, respectively, whereas α-tocopherol showed 37.7% inhibition, though only a few cyclic dipeptides weakly inhibited $O_2^{-\bullet}$.

KEYWORDS: Awamori; distillation residue; *Aspergillus awamori*; cyclic dipeptide; antioxidant; bleomycin; ESR; hydroxyl radical; superoxide

INTRODUCTION

There are many distilled liquors in the world, and they are produced from various ingredients such as rice, barley, corn, potato, grape, and so on. These materials are fermented with yeast and/or fungi, and then the product is distilled followed by maturation. It is well understood that the micro-organisms used for brewing those liquors produce a variety of secondary metabolites along with alcohol. Those substances should be contained in beer, wine, Japanese sake, and so on. In the production process of distilled liquors, we always fractionate only alcohol and volatiles by distillation and we use only the product for drinking, but most of these metabolites are usually discarded as a distillation residue. As antibiotics were found in culture broths of fungi and bacteria, some compounds in the residue would have some functions which will be utilized in the near future, and it must be worthwhile to explore them from the viewpoint of human health and environment problems.

In Japan, accompanied with the well-known sake, several kinds of traditional millet spirits, so-called "shochu," are also very popular. Most of them are produced from rice, sweet potato, barley, and buckwheat. Among them, in the Ryukuan Islands (Okinawa Prefecture) in the south of Japan, awamori spirits, a kind of shochu, have been produced for more than several hundred years. Boiled Thai rice (*Oryza sativa* L. ssp. *indica*) is fermented with black yeast (*Aspergillus awamori*), and then the resulted mash is distilled to produce the awamori spirits. In Okinawa, a small amount of the distillation residue is used as a feed to hogs, but most of them are discarded without any utilization. We can assume that the distillation residue would contain some bioactive compounds estimating from its traditional usage, though no exact clinical evidence has been reported. But as for the residue, analyses of only amino acids, organic acids and minerals were made, and few other compounds and no biological activity has been investigated yet.

As mentioned above, we are interested in such biological effects of the distillation residue of awamori spirits. In this study, to get some information on them, we examined antioxidant activities of solubles obtained from a supernatant of the residue by centrifugation. Free radical and active oxygen are thought to be triggers of various diseases such as carcinogenesis, mutagenesis, diabetes mellitus and arteriosclerosis (*1*). A variety of methods are known to evaluate antioxidant (radical scavenging) activity. The mechanisms of these methods are based on the lipid peroxidation, radical scavenging ($^\bullet OH$, $O_2^{-\bullet}$, $^\bullet O_2$, stable radicals like DPPH, etc.), oxidative enzyme inhibition, and so

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on. Even if a sample exhibits high activity with one of these methods, it does not always show similar good result with all other methods. Accordingly, we should evaluate samples accurately with several methods. As the first screening to find the difference of antioxidant potency of the fractions obtained by solvent patitions, we examined antioxidant activity according to a modified bleomycin−Fe (BLM−Fe) method (*2*). This method gives results with high reproducibility in a short time, though bleomycin is expensive. We also examined antioxidant activity of some compounds isolated from the chloroform solubles of the supernatant by the same method. Moreover, we used electron spin resonance (ESR) to evaluate $OH^\bullet$ and $O_2^{-\bullet}$ radicals scavenging activity (*3*, *4*). We describe herein the constituents in the chloroform solubles and the antioxidant activity of the compounds.

MATERIALS AND METHODS

Materials. Distillation residue of awamori spirits was collected in an awamori factory in Ishigaki Island, Okinawa, soon after distillation and cooling down. Then the residue was frozen and was transferred to our laboratory. Sodium arachidonate, 2-thiobarbituric acid (TBA), hypoxanthine, diethylenetriaminepentaacetic acid (DETAPAC), and xanthine oxidase from bovine milk were all purchased from Sigma-Aldrich Co. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Labotec (Tokyo). BLM hydrochloride and iron(II) sulfate were purchased form Wako Chemicals (Osaka). Silica gel (BW-820MH, Fuji Silysia, Nagoya) and a thin-layer chromatography (TLC) plate (Kieselgel 60 F_{254} 5715, Merck) were used for column chromatography and analytical TLC, respectively. A medium-pressure column chromatography (MPLC) was performed using silica gel (60K230, Katayama Chemical Co. Ltd., Nagoya) and ODS (Develosil ODS 30/50, Nomura Chemical Co. Ltd.). ODS, C8, and C30 columns for high-performance liquid chromatography (HPLC) were Develosil ODS-UG-5, Develosil C8-UG-5 and Develosil C30-UG-5 (all $\phi 20 \times 250$, Nomura Chemical Co. Ltd., Aichi, Japan), respectively. NMR spectra were recorded on JNM A-400, JNM ECP-500, and JNM A-600 spectrometers (JEOL). ESR spectra were recorded on JES FR-30 spectrometer (JEOL). The operation conditions for the ESR to estimate the concentration of superoxide anion radicals ($O_2^{-\bullet}$) were as follows: magnetic field, 335.7 ± 5 mT; power, 4 mW 9.414 GHz; modulation, 100 kHz·1 × 0.079 mT; response, 0.1 s; temperature, 25 °C; amplitude, 160; sweep time, 2 min. For the determination of hydroxyl radicals, the magnetic field, power, modulation, response, temperature, amplitude, and sweep time were 335.7 ± 5 mT, 4 mW 9.414 GHz, 100 kHz·1 × 0.079 mT, 0.1 s, 25 °C, 160 and 2 min, respectively. IR spectra, electron-impact mass spectroscopy (EIMS), and fast-atom bombardment mass spectroscopy (FABMS) were recorded on FT/IR-410 (JASCO), JMS MS-700 (JEOL) and JMS HX-110 spectrometers, respectively.

Antioxidant Assay (BLM−Fe Method). Antioxidant assay by the BLM−Fe(III) method was carried out in the same manner reported in our previous paper (*2*). The index of activity and the unit "VC" were also used to express the relative antioxidant activity as described in the reference *2*.

Antioxidant Assay (•OH). Antioxidant assay against •OH by ESR was carried out in the modified manner which we reported (*3*). An aqueous solution of 10 mM $FeSO_4$ (50 μL) was added to a mixture of 9.2×10^{-3} M DMPO (20 μL), 10 mM H_2O_2 (50 μL), sample solution (50 μL), and water (30 μL). The mixture was transferred to a quartz flat cell, and ESR spectra were recorded at 60 s after addition of $FeSO_4$.

Antioxidant Assay ($O_2^{-\bullet}$). Antioxidant assay against $O_2^{-\bullet}$ by ESR was carried out in the modified manner which we reported (*3*). A solution of 0.4 U/mL xanthine oxidase (50 μL) was added to a mixture of 0.92 M DMPO (20 μL), 2 mM hypoxanthine (50 μL), sample solution (50 μL), and 5.5 mM DETAPAC (30 μL). The mixture was transferred to a quartz flat cell, and ESR spectra were recorded at 45 s after addition of xanthine oxidase.

Fractionation of the Distillation Residue. The residue (10 L) was melted under running water followed by addition of 5 L of methanol. The suspension was submitted to centrifugation (5000 rpm, 15 min, 4 °C), and the resulting supernatant was then partitioned with *n*-hexane, chloroform, ethyl acetate, and *n*-butanol to afford the corresponding solubules. The yield of each solubles after concentration in vacuo was as follows: *n*-hexane solubles, 1.5 g; chloroform solubles, 5.6 g; ethyl acetate solubles, 22 g; *n*-butanol solubles, 216 g; water solubles, 535 g.

Fractionation of Chloroform Solubles. Chloroform solubles (5.5 g) were separated into four fractions by SiO_2 column chromatography using a mixed solvent of chloroform−methanol to give C1 (chloroform, 1.19 g), C2 (chloroform−methanol = 98:2, 985 mg), C3 (chloroform−methanol = 95:5, 787 mg), and C4 (chloroform−methanol = 70:30, 135 mg).

C3 was further fractionated using SiO_2 column and three fractions, C3−1 (hexane−ethyl acetate = 30:70, 123 mg), C3−2 (ethyl acetate, 359 mg), and C3−3 (ethyl acetate−methanol = 70:30, 195 mg), were obtained. From C3−1 ethyl *p*-hydroxyphenyllactate (**1**) (4.1 mg) was isolated by using SiO_2 column (hexane−diethyl ether) and HPLC with ODS column (methanol−H_2O). C3−2 (359 mg) was fractionated with SiO_2 column (hexane−ethyl acetate), HPLC equipped with ODS column (methanol−H_2O), and HPLC with C30 column (methanol−H_2O) to give ethyl pyroglutamate (**2**) (23 mg), cyclo(L-Pro-L-Val) (**3**) (36 mg), cyclo(L-Leu-L-Pro) (**4**) (26 mg), and cyclo(L-Ile-L-Pro) (**5**) (59 mg). Moreover, C3−3 (195 mg) was separated by column chromatography with SiO_2 gel (chloroform−methanol), MPLC with ODS column (methanol−H_2O), and HPLC with C8 column (methanol−H_2O) to afford cyclo(L-Phe-L-Pro) (**6**) (13 mg).

C4 (135 mg) was fractionated by MPLC using ODS column with a mixed solvent of methanol−H_2O, and three fractions, C4−1 (methanol−H_2O = 50:50, 712 mg), C4−2 (methanol−H_2O = 75:25, 292 mg), and C4−3 (methanol, 284 mg), were obtained. C4−1 was separated into two fractions (C4−1−1 and C4−1−2). From C4−1−1, tyrosol (**7**) (21 mg) was isolated by SiO_2 column chromatography purification. C−4−1−2 was submitted to repeated HPLC separation using ODS column, and then cyclo(L-Glu-L-Val) ethyl ester (**8**) (4 mg), and cyclo-(L-Glu-L-Leu) ethyl ester (**9**) (23 mg) were obtained. Moreover, C4−2 was separated by MPLC using ODS column followed by repeated HPLC using C8 and C30 columns to afford cyclo(L-Leu-L-Phe) (**10**) (0.4 mg)

Ethyl *p*-Hydroxyphenyllactate (1). Colorless oil; $[\alpha]_D$ −12.4° (*c* 0.04, MeOH); IR (film) ν_{max} 3382, 1727, 1515, 1455, 1223 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.07 (2H, d, *J* = 8.4 Hz; H-3′,5′), 6.73 (2H, d, *J* = 8.4 Hz; H-2′,6′), 4.37 (1H, ddd, *J* = 6.6, 5.9, 4.4 Hz; H-2), 4.20 (2H, q, *J* = 7.0 Hz; $-OCH_2CH_3$), 3.03 (1H, dd, *J* = 13.9, 4.4 Hz; H-3a), 2.89 (1H, dd, *J* = 13.9, 6.6 Hz; H-3b), 2.71 (1H, d, *J* = 5.9 Hz; −O*H*), 1.27 (3H, t, *J* = 7.0 Hz; $-OCH_2CH_3$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 174.2 (C-1), 154.5 (C-4′), 130.7 (C-2′,6′), 128.5 (C-1′), 115.2 (C-3′,5′), 71.3 (C-2), 61.7 ($-OCH_2CH_3$), 39.6 (C-3), 14.2 ($-OCH_2CH_3$); HR-EIMS *m/z* 210.0871 ($[M]^+$, 210.0892 calcd for $C_{11}H_{14}O_4$).

Ethyl Pyroglutamate (2). Colorless oil; $[\alpha]_D$ −6.2° (*c* 0.31, MeOH); IR (film) ν_{max} 3372, 3236, 1745, 1704, 1204 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 4.20 (1H, dd, *J* = 12.8, 8.8 Hz; Hα), 4.19 (2H, q, *J* = 7.3 Hz; $-OCH_2CH_3$), 2.45 (1H, m; Hβ1), 2.34 (2H, m; Hγ), 2.20 (1H, m; Hβ2), 1.27 (3H, t, *J* = 7.3; $-OCH_2CH_3$); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 177.9 (Cδ), 172.0 (CO), 61.7 ($-OCH_2CH_3$), 55.5 (Cα), 29.3 (Cγ), 24.9 (Cβ), 14.2 ($-OCH_2CH_3$); HR-EIMS *m/z* 157.0729 ($[M]^+$, 157.0739 calcd for $C_7H_{11}NO_3$).

Cyclo(L-Pro-L-Val) (3). Colorless amorphous solid; $[\alpha]_D$ −142.2° (*c* 0.16, MeOH); IR (film) ν_{max} 3209, 1666 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 4.05 (1H, br.t, *J* = 7.3 Hz; Pro-Hα), 3.91 (1H, br.s; Val-Hα), 3.62 (1H, dt, *J* = 12.1, 8.1 Hz; Pro-Hδ), 3.52 (1H, ddd, *J* = 12.1, 9.5, 2.9 Hz; Pro-Hδ), 2.61 (1H, d·sept, *J* = 2.6, 7.0 Hz; Val-Hβ), 2.35 (1H, m; Pro-Hβ1), 2.02 (2H, m; Pro-Hβ2 and Pro-Hγ1), 1.88 (1H, m; Pro-Hγ2), 1.04 (3H, d, *J* = 7.0 Hz; Val-Hγ1), 0.89 (3H, d, *J* = 7.0 Hz; Val-Hγ2); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 169.9, 165.0, 60.4, 58.8, 45.1, 28.5, 28.4, 22.4, 19.3, 16.1; HR-EIMS *m/z* 196.1212 ($[M]^+$, 196.1212 calcd for $C_{10}H_{16}N_2O_2$).

Cyclo(L-Leu-L-Pro) (4). Colorless amorphous solid; $[\alpha]_D$ −37.2° (*c* 0.22, MeOH); IR (film) ν_{max} 3265, 1670 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 4.09 (1H, t, *J* = 6.9 Hz; Pro-Hα), 3.99 (1H, dd, *J* = 9.5, 3.7 Hz; Leu-Hα), 3.57 (1H, dt, *J* = 12.1, 8.1 Hz; Pro-Hδ1), 3.51 (1H, ddd, *J* = 12.1, 8.8, 3.7 Hz; Pro-Hδ2), 2.32 (1H, ddt, *J* = 12.8, 2.9, 6.9

Hz; Pro-Hβ1), 2.10 (1H, m; Pro-Hβ2), 2.03 (1H, ddd, J = 14.7, 9.5, 3.7 Hz; Leu-Hβ1), 1.99 (1H, m; Pro-Hγ1), 1.87 (1H, m; Pro-Hγ2), 1.73 (1H, m; Leu-Hγ), 1.50 (1H, ddd, J = 14.7, 9.5, 4.8 Hz; Leu-Hβ2), 0.97 (3H, d, J = 6.6 Hz; Leu-Hδ1), 0.92 (3H, d, J = 6.6 Hz; Leu-Hδ2); ^{13}C NMR (CDCl$_3$, 150 MHz) δ 170.3, 166.3, 59.1, 53.5, 45.6, 38.7, 28.2, 24.8, 23.4, 22.8, 21.3; HR-EIMS m/z 210.1338 ([M]$^+$, 210.1368 calcd for $C_{11}H_{18}N_2O_2$).

Cyclo(L-Ile-L-Pro) (5). Colorless amorphous solid; $[\alpha]_D$ −27.6° (c 0.60, MeOH); IR (film) ν_{max} 3220, 1669 cm^{-1}; ^{1}H NMR (CDCl$_3$, 600 MHz) δ 4.04 (1H, br.t, J = 7.3 Hz; Pro-Hα), 3.94 (1H, br.s; Ile-Hα), 3.60 (1H, dt, J = 12.1, 8.1 Hz; Pro-Hδ1), 3.51 (1H, ddd, J = 12.1, 9.2, 2.9 Hz; Pro-Hδ2), 2.35 (1H, br.dt, J = 10.6, 6.6 Hz; Pro-Hβ1), 2.28 (1H, m; Ile-Hβ), 2.00 (2H, m; Pro-Hβ2 and Pro-Hγ1), 1.87 (1H, m; Pro-Hγ2), 1.40 (1H, m; Ile-Hγ11), 1.15 (1H, m; Ile-Hγ12), 1.03 (3H, d, J = 7.3 Hz; Ile-Hγ2), 0.90 (3H, t, J = 7.4 Hz; Ile-Hδ); ^{13}C NMR (CDCl$_3$, 125 MHz) δ 170.0, 165.1, 60.6, 58.9, 45.2, 35.4, 28.6, 24.1, 22.4, 16.0, 12.2; HR-EIMS m/z 210.1369 ([M]$^+$, 210.1368 calcd for $C_{11}H_{18}N_2O_2$).

Cyclo(L-Phe-L-Pro) (6). Colorless amorphous solid; IR (film) ν_{max} 3234, 1674, 1544, 1437, 1214 cm^{-1}; ^{1}H NMR (CDCl$_3$, 500 MHz) δ 7.33 (2H, t, J = 7.3 Hz; Phe-Hϵ), 7.27 (1H, t, J = 7.3 Hz; Phe-Hζ), 7.21 (2H, d, J = 7.3 Hz; Phe-Hδ), 4.25 (1H, dd, J = 10.6, 3.7 Hz; Phe-Hα), 4.06 (1H, t, J = 7.8 Hz; Pro-Hα), 3.63 (1H, m; Pro-Hδ1), 3.61 (1H, dd, J = 14.3, 3.7 Hz; Phe-Hβ1), 3.55 (1H, ddd, J = 15.1, 8.7, 2.8; Pro-Hδ2), 2.76 (1H, dd, J = 14.3, 10.6 Hz; Phe-Hβ2), 2.32 (1H, m; Pro-Hβ1), 2.00 (1H, m; Pro-Hγ1), 1.99 (1H, m; Pro-Hβ2), 1.89 (1H, m; Pro-Hγ2); ^{13}C NMR (CDCl$_3$, 150 MHz) δ 169.3, 165.0, 135.9, 129.3 (2C), 129.1 (2C), 127.6, 59.1, 56.1, 45.5, 36.8, 28.3, 22.5; HR-EIMS m/z 244.1215 ([M]$^+$, 244.1212 calcd for $C_{14}H_{16}N_2O_2$).

Tyrosol (7). Colorless oil; IR (film) ν_{max} 3386 cm^{-1}; ^{1}H NMR (CDCl$_3$, 600 MHz) δ 7.08 (2H, d, J = 8.4 Hz), 6.76 (2H, d, J = 7.8 Hz), 3.81 (2H, t, J = 6.6 Hz), 2.76 (2H, t, J = 6.6 Hz); ^{13}C NMR (CDCl$_3$, 150 MHz) δ 154.2, 130.5, 130.2 (2C), 115.4 (2C), 63.8, 38.2; HR-EIMS m/z 138.0674 ([M]$^+$, 138.0681 calcd for $C_8H_2O_2$).

Cyclo(L-Glu-L-Val) Ethyl Ester (8). Colorless amorphous solid; $[\alpha]_D$ −23.3° (c 0.064, MeOH); ^{1}H NMR (CD$_3$OD, 600 MHz) δ 4.294 (1H, d, J = 5.5 Hz; Val-Hα), 4.286 (1H, dd, J = 8.4, 4.4 Hz; Glu-Hα), 4.20 (1H, dq, 11.0, 7.0 Hz; −CH_2CH$_3$), 4.16 (1H, dq, 11.0, 7.0 Hz; −CH_2CH$_3$), 2.45 (1H, dddd, J = 12.5, 9.9, 8.4, 7.3; Glu-Hβ1), 2.38 (1H, ddd, J = 16.9, 9.9, 7.3 Hz; Glu-Hγ1), 2.28 (1H, ddd, J = 16.9, 9.9, 5.1; Glu-Hγ2), 2.17 (1H, sept, J = 6.2 Hz; Val-Hβ), 2.09 (1H, dddd, J = 12.5, 9.9, 5.1, 4.4; Glu-Hβ2), 1.26 (3H, t, J = 7.0 Hz; −CH$_2$CH_3), 0.97 (6H, d, J = 6.2 Hz; Val-Hγ); ^{13}C NMR (CD$_3$OD, 150 MHz) δ 181.6 (Glu-Cδ), 175.1 (Glu-CO), 172.9 (Val-CO), 62.2 (−CH$_2$CH$_3$), 59.5 (Val-Cα), 57.7 (Glu-Cα), 31.6 (Val-Cβ), 30.4 (Glu-Cγ), 26.8 (Glu-Cβ), 19.4 (Val-Cγ1), 18.5 (Val-Cγ2), 14.5 (−CH$_2$$CH_3$); HR-FABMS m/z 256.1432 ([M]$^+$, 256.1423 calcd for $C_{12}H_{20}N_2O_4$).

Cyclo(L-Glu-L-Leu) Ethyl Ester (9). Colorless amorphous solid; $[\alpha]_D$ −43.9° (c 0.12, MeOH); ^{1}H NMR (CD$_3$OD, 600 MHz) δ 4.42 (1H, dd, J = 8.6, 6.8 Hz; Leu-Hα), 4.22 (1H, dd, J = 8.8, 4.4 Hz; Glu-Hα), 4.16 (2H, m; −CH_2CH$_3$), 2.46 (1H, m; Glu-Hβ1), 2.38 (1H, ddd, J = 17.0, 9.5, 7.3 Hz; Glu-Hγ1), 2.28 (1H, ddd, J = 17.0, 9.9, 5.5 Hz; Glu-γ2), 2.10 (1H, m; Glu-β2), 1.69 (1H, m; Leu-γ), 1.63 (2H, ddd, J = 8.1, 6.4, 1.9 Hz; Leu-β), 1.25 (3H, t, J = 7.2 Hz; −CH$_2$CH_3), 0.96 (3H, d, J = 6.6 Hz; Leu-δ1), 0.92 (3H, t, J = 6.5 Hz; Leu-δ2); ^{13}C NMR (CD$_3$OD, 150 MHz) δ 181.6 (Glu-Cδ), 175.1 (Leu-CO), 174.0 (Glu-CO), 62.4 (−CH$_2$CH$_3$), 57.8 (Glu-Cα), 52.4 (Leu-Cα), 41.2 (Leu-Cβ), 30.4 (Glu-Cγ), 26.7 (Glu-Cβ), 26.0 (leu-Cγ), 23.3 (Leu-Cδ1), 21.7 (Leu-Cδ2), 14.5 (−CH$_2$$CH_3$); HR-FABMS m/z 271.1656 ([M+H]$^+$, 271.1658 calcd for $C_{15}H_{20}N_2O_2$).

Cyclo(L-Leu-L-Phe) (10). Colorless amorphous solid; ^{1}H NMR (CD$_3$OD, 600 MHz) δ 7.31−7.18 (5H, m), 4.29 (1H, ddd, J = 5.0, 4.1, 1.1 Hz; Phe-Hα), 3.65 (1H, ddd, J = 8.9, 4.4, 1.0 Hz; Leu-Hα), 3.28 (1H, dd, J = 13.3, 3.9 Hz; Phe-Hβ1), 2.93 (1H, dd, J = 13.3, 4.6 Hz; Phe-Hβ2), 1.42 (1H, m; Leu-Hγ), 0.86 (1H, m; Leu-Hβ1), 0.72 (3H, d, J = 6.6 Hz; Leu-Hδ1), 0.68 (3H, d, J = 6.6 Hz; Leu-Hδ2), 0.07 (1H, m; Leu-Hβ2); HR-EIMS m/z 260.1518 ([M]$^+$, 260.1525 calcd for $C_{15}H_{20}N_2O_2$).

Table 1. Antioxidant Activity of Solubles and Fractions from the Distillation Residue of Awamori Spirits

fractions	antioxidant activity[a] (VC = 1)
n-hexane solubles	2.63
chloroform solubles	1.63
C1	1.21
C2	1.38
C3	1.71
C3–1	1.85
C3–2	1.42
C3–3	1.47
C4	2.07
C4–1	0.83
C4–2	2.44
ethyl acetate solubles	0.59
1-butanol solubles	0.35
water solubles	0.56

[a] Activity was examined at a concentration of 3 mg/mL.

RESULTS

Antioxidant Activity of Fractions of Partition with Solvents. Prior to the study of the antioxidant compounds contained in the distillation residue, antioxidant activities of the fractions obtained by solvent partition were investigated using the BLM−Fe(III) method (*2*) at a concentration of 3 mg/mL. **Table 1** shows the activities of the fractions. Among them, *n*-hexane solubles exhibited the highest activity (2.63 times as L-ascorbic acid). According to our previous study, methyl linolenate and linolenic acid were the antioxidant constituents from the hexane fraction of *Raphanus sativus* L. (radish sprout) (*2*). By the TLC analysis of the hexane solubles of the distillation residue, it was also estimated to contain similar fatty acids. Chloroform solubles also showed high antioxidant activity (1.63 VC). Moreover, high polar fractions, C3 and C4, obtained from chloroform solubles were more active (1.71 and 2.07 VC, respectively) than the original fraction, and specific spots with low R_f values on TLC were observed compared to those of ordinary compounds in chloroform solubles. Then, in this study, we examined the constituents in these fractions so as to identify the antioxidant constituent.

Fractionation of Chloroform Solubles. Antioxidant activities of the four fractions (C1−C4) separated from the chloroform solubles are also shown in **Table 1**. The activity increased according to the polarity. Fraction C3 was further separated to give ethyl *p*-hydroxyphenyllactate (**1**), ethyl pyroglutamate (**2**), cyclo(L-Pro-L-Val) (**3**), cyclo(L-Leu-L-Pro) (**4**), cyclo(L-Ile-L-Pro) (**5**), and cyclo(L-Phe-L-Pro) (**6**). From fraction C4, tyrosol (**7**), cyclo(L-Glu-L-Val) ethyl ester (**8**), cyclo(L-Glu-L-Leu) ethyl ester (**9**), and cyclo(L-Leu-L-Phe) (**10**) were isolated (**Figure 1**).

Antioxidant Activity of the Compounds from Chloroform Solubules. Antioxidant activity of the compounds with sufficient amounts was evaluated by BLM−Fe method. The results at 1 mM are listed on **Table 2**. Ethyl *p*-hydroxyphenyllactate (**1**) showed the highest activity (4.01 VC), and cyclic dipeptides and other compounds (**2** and **7**) were as strong as L-ascorbic acid used as a positive control. But no significant difference of the activity was found among the cyclic dipeptides.

Radical Scavenging Activity of the Cyclic Dipeptides Using ESR. We investigated scavenging activities of the cyclic dipeptide against $O_2^{-\bullet}$ and $OH^\bullet$ using ESR, since most of the cyclic dipeptides we tested showed high potency by the BLM−Fe method. As the result, cyclo(Pro-Val) (**3**), cyclo(Leu-Pro) (**4**), cyclo(Ile-Pro) (**5**), and cyclo(Phe-Pro) (**6**) exhibited high radical scavenging activity against $OH^\bullet$ at a concentration of

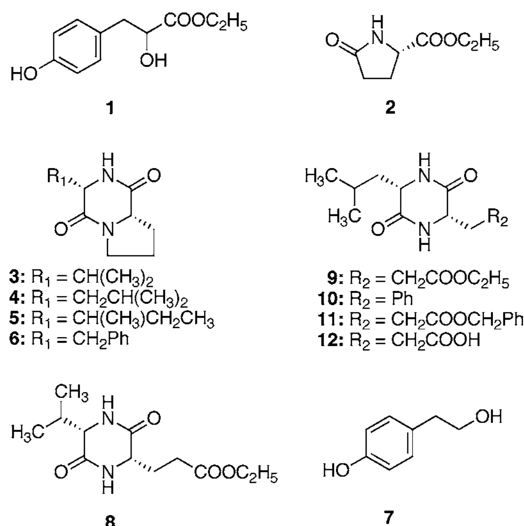

Figure 1. Constituents of the distillation residue of awamori spirits.

Table 2. Antioxidant Activity of the Constituents Isolated from the Chloroform Solubles of the Distillation Residue of Awamori Spirits

	antioxidant activity[a]			
	BRM-Fe[b]	$O_2^{-\bullet}$	OH•	
compounds	(VC = 1)	2.5×10^{-3} M	2.5×10^{-3} M	IC_{50} (M)
ethyl 4-hydroxyphenyllactate (**1**)	4.01			
ethyl pyroglutamate (**2**)	0.60			
tyrosol (**7**)	1.32			
cyclo(Pro-Val) (**3**)	1.01	0.0	88.4 ± 0.1	1.1×10^{-3}
cyclo(Leu-Pro) (**4**)	0.83	0.4	86.2 ± 0.0	1.1×10^{-3}
cyclo(Ile-Pro) (**5**)	0.86	4.9	97.0 ± 0.1	6.6×10^{-4}
cyclo(Phe-Pro) (**6**)		0.0	85.2 ± 0.1	9.0×10^{-4}
cyclo(Glu-Val) ethyl ester (**8**)	0.92			
cyclo(Glu-Val) ethyl ester (**9**)	1.29			
cyclo(Leu-Phe) (**10**)		8.3	23.5 ± 0.1	$>2.5 \times 10^{-3}$
cyclo(Glu-Leu) benzyl ester (**11**)		0.0	96.0 ± 0.3	7.8×10^{-3}
cyclo(Glu-Leu) (**12**)		0.0	97.8 ± 0.2	8.0 × 10-5
L-ascorbic acid	1.00			
α-tocopherol		68.1	37.7 ± 0.6	

[a] BRM–Fe, $O_2^{-\bullet}$, and OH• denote OH•-scavenging activity by the BLM–Fe method, $O_2^{-\bullet}$ scavenging activity by ESR, and OH• scavenging activity by ESR, respectively. [b] The relative activity was expressed as that of L-ascorbic acid was 1.0 at the concentration of 1 mM.

2.5×10^{-3} M, especially cyclo(Ile-Pro) (**5**) trapped 97.0% OH•. On the other hand, only cyclo(Ile-Pro) (**5**) and cyclo(Leu-Phe) (**10**) showed very weak activity against $O_2^{-\bullet}$ (4.9% and 8.3%, respectively) at the same concentration. But we could not examine the activity of the ethyl esters (**8** and **9**) because of the insufficient amount of samples. Then we synthesized cyclo-(Glu-Leu) benzyl ester (**11**) and cyclo(Glu-Leu) (**12**) and investigated their radical scavenging activity at the same concentration mentioned above. **Table 2** shows the results that the radical scavenging potencies of these compounds were negligible against $O_2^{-\bullet}$ like other cyclic dipeptides, but these compounds possessed significantly strong scavenging activity against OH•.

DISCUSSION

As mentioned above, we mainly focused on the cyclic dipeptides contained in the distillation residue of awamori spirits and examined their radical scavenging activity (antioxidant activity) with three kinds of methods. Cyclic dipeptides are known to be in various kinds of foods and beverages such as cocoa (*5*), coffee (*6*), chicken essence (*7*), beer (*8*), and Japanese sake (*9*). So we do not think it strange that these cyclic dipeptides were contained in the distillation residue of awamori spirits, and we assumed that they were generated during fermentation, accompanied by heat of distillation. These compounds were reported to contribute to the metallic and bitter tastes of the foods, though the threshold concentration seemed to depend on the amino acid residues, which compose the cyclic dipeptides. Furthermore, biological activities of several cyclic dipeptides were reported, (*10*) and many bioactive compounds possessing the 2,5-diketopiperazine moiety, which is the common skeleton to cyclic dipeptides, were isolated from microorganisms and marine sponges. (*11*, *12*) But it is easily assumed that the bioactivity of cyclic dipeptides is a relatively unexplored field, compared to many other types of peptides which act as hormones or exhibit antimicrobial activity. In this study, we found the radical scavenging activity of the constituents of awamori spirits. Among them, cyclic dipeptides, especially cyclo(Ile-Pro) (**5**), cyclo(Glu-Leu) benzyl ester (**11**), and cyclo-(Glu-Leu) (**12**), exhibited high inhibiting potency against hydroxyl radical formation detected by using ESR. There are several reports on the radical oxidation of cyclic dipeptides (*13*−*15*). According to the papers, a radical would generate at the α position of amino acids, and the radical reacts with another reagent to form a product such as hydroperoxide. The antioxidant activity of the cyclic dipeptides may be partially performed by the same mechanism as that of the oxidation. But the participation of the side chains of the cyclic dipeptides should be more critical for the antioxidation, since the activities varied according to the amino acids composing the compound. For instance, carboxylic group of **11** and ester group of **12** may be involved in the radical scavenging activity, though the action of isoleucine of **5**, leucine of **4**, and valine of **3** is not clear. Now we are investigating the mechanism of antioxidant activity of cyclic dipeptides, and structure−activity relationships using synthetic cyclic dipeptides. Moreover, we are submitting one of the cyclic dipeptides to a pharmacological evaluation of disease involving active oxygen. We will report these results elsewhere.

From the viewpoint of the utilization of food wastes, the strong antioxidant activity of the distillation residue of awamori would be favorable. The sake cake, lees of sake mash, has been used as an ingredient traditionally in Japan for a long time. It was reported that it mainly consisted of fiber and crude protein (*16*). Recently, several pharmacological activities of the sake cake were reported, and some of them involved peptide to exhibit activity (*17*−*19*). As for the distillation residue of awamori, we can assume that it contains similar constituents, though the production process of awamori spirits is different from that of sake, and that it would perform some bioactivities. Identification of cyclo(L-Leu-L-Pro) (**4**) from aged sake is a good example of it (*20*).

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