Isolation, Structural Elucidation, and Biological Evaluation of a 5-Hydroxymethyl-2-furfural Derivative, Asfural, from Enzyme-Treated Asparagus Extract

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Ⓢ *Supporting Information*

ABSTRACT: A novel 5-hydroxymethyl-2-furfural (HMF; **1**) derivative, which is named asfural (compound **2**), was isolated from enzyme-treated asparagus extract (ETAS) along with HMF (**1**) as a heat shock protein 70 (HSP70) inducible compound. The structure of compound **2** was elucidated on the basis of its spectroscopic data from HREIMS and NMR, whereas the absolute configuration was determined using chiral HPLC analysis, compared to two synthesized compounds, (S)- and (R)-asfural. As a result, compound **2** derived from ETAS was assigned as (S)-(2-formylfuran-5-yl)methyl 5-oxopyrrolidine-2-carboxylate. When compound **2**, synthesized (S)- and (R)-asfural, and HMF (**1**) were evaluated in terms of HSP70 mRNA expression-enhancing activity in HL-60 cells, compound **2** and (S)-asfural significantly increased the expression level in a concentration-dependent manner. HMF (**1**) also showed significant activity at 0.25 mg/mL.

KEYWORDS: asfural, asparagus, Asparagus officinalis L., enzyme-treated asparagus, ETAS, heat shock protein, HSP70, 5-hydroxymethyl-2-furfural, HMF, antistress function

■ INTRODUCTION

Heat shock proteins (HSPs) are called "stress proteins" due to their increased expression induced by physical, chemical, and biological stresses such as fever, infections, inflammation, reactive oxygen species, ultraviolet radiation, starvation, and hypoxia.[1] HSPs are intracellular proteins having molecular weights ranging from 10 to 150 kDa and are divided into various families according to their molecular weights (e.g., HSP10, HSP27, HSP40, HSP60, HSP70, HSP90, HSP110, etc.). HSPs protect cells and simultaneously possess molecular chaperon properties, which regulate folded proteins and suppress aggregation of mutated proteins, including Alzheimer's disease amyloid-β protein and mad cow disease prion protein.[2−4]

HSP70, one of the most well-studied HSPs, is constitutively expressed in various organs such as skin and the gastrointestinal tract. HSP70 has apoptosis-suppressive and anti-inflammatory activities, leading to cytoprotective functions from various stresses.[5−8] To develop a functional food ingredient showing HSP70 induction activity, we studied various crops of Hokkaido, Japan, and identified particularly high induction activity from asparagus (*Asparagus officinalis* L.), which is a popular vegetable cultivated in Hokkaido. After harvesting, only the top approximately 25 cm of the asparagus shoots are sent to consumers, and although some residual lower parts of the stem are used as animal feed, most of them are discarded as scrap. To utilize the unused resources effectively and result in an asparagus product as a functional food ingredient, we established a manufacturing procedure of enzyme-treated asparagus extract (ETAS) from the residual lower parts. The current study demonstrated the isolation and structural elucidation of a novel 5-hydroxymethyl-2-furfural (HMF; **1**) derivative named asfural (**2**) from ETAS, which showed significant HSP70 mRNA expression-enhancing activity. HMF (**1**) is known to be produced by heating hexoses such as glucose and has several biological activities including antioxidant, antimyocardial ischemia, and hemorheology ameliorative effects.[9−11] Furthermore, various HMF derivatives are produced by heating HMF and organic acids under an acidic condition. Mumefural was isolated from a fruit juice concentrate of Japanese apricot (*Prunus mume* Sieb. et Zucc) and found to be a condensation reaction product formed from HMF and citric acid in the course of heating.[12] The functional investigation reported that mumefural is an effective compound improving blood fluidity. So far, malate, tartarate, and succinate as well as citrate have been known as constituent organic acids of HMF derivatives.[13] Here, we report the novel HMF derivative that shows significant HSP70 mRNA expression-enhancing activity.

■ MATERIALS AND METHODS

General Experimental Procedures. Optical rotations were measured in MeOH with a JASCO P-2200 polarimeter. IR spectra were recorded on a JASCO FTIR-4100 spectrometer. NMR spectra

were recorded in DMSO-d_6 with a JEOL JNM-AL400 spectrometer at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR. EIMS and HREIMS data were obtained with a JEOL JMS-AX500 mass spectrometer. Diaion HP-20 (Mitsubishi Chemical Corp., Tokyo, Japan) and silica gel (63−200 μm particle size, Merck KGaA, Darmstadt, Germany) were used for column chromatography. A CAPCELL PAK C18 UG-120 column (5 μm, 4.6 × 250 mm, Shiseido Co., Ltd., Tokyo, Japan) and a CHIRALPAK IA column (5 μm, 4.6 × 150 mm, Daicel Corp., Osaka, Japan) were used for analytical HPLC, and a CAPCELL PAK C18 UG-120 column (5 μm, 20 × 250 mm, Shiseido Co., Ltd.) was used for preparative HPLC, respectively. Analyses were conducted with a Hitachi L-7100 system. UPLC was performed on a Waters Acquity UPLC system, which was equipped with a binary solvent delivery system. MS was performed on a Waters Micromass Quattro Premier tandem quadrupole mass spectrometer. The LC-MS system control was achieved by using MassLynx 4.0.

HPLC Conditions. The HPLC analysis was performed on a Hitachi L-7100 system using a CAPCELL PAK C18 UG-120 column (5 μm, 4.6 × 250 mm) at 40 °C with a flow rate of 1.0 mL/min at 280 nm. The analytes were eluted from the column with 20 mM phosphate buffer (pH 2.3, solvent A) and MeCN (solvent B) using a linear gradient mode (0−5 min, 5% B; 5−20 min, 5−10% B; 20−25 min, 10% B; 25−30 min, 10−50% B; 30−35 min, 50% B; 35−36 min, 50−5% B; and 36−40 min, 5% B).

Chiral HPLC Conditions. The HPLC analysis was performed on a Hitachi L-7100 system using a CHIRALPAK IA column (5 μm, 4.6 × 150 mm) at 40 °C with a flow rate of 1.0 mL/min at 280 nm. The analytes were eluted from the column with 20 mM phosphate buffer (pH 2.3, solvent A) and MeCN (solvent B) using a linear gradient mode (0−5 min, 5% B; 5−20 min, 5−10% B; 20−60 min, 10% B).
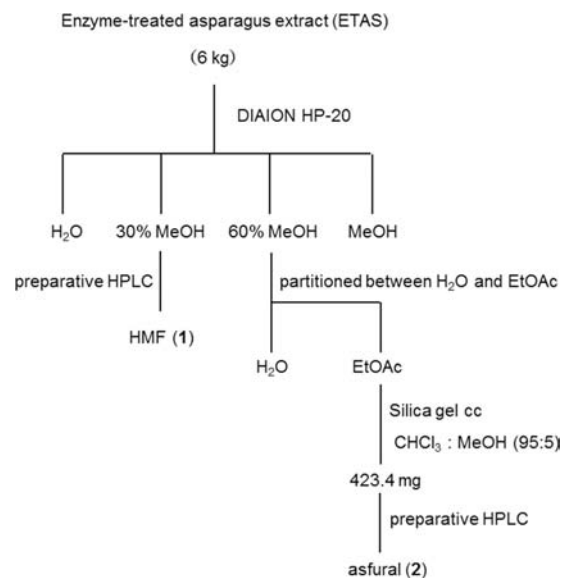
UPLC-MS/MS Conditions. All MS optimization experiments were performed in both the MS scan mode and the product scan mode.[14] All quantifications were performed in the multiple reaction monitoring (MRM) mode. The tune page parameters and conditions for each of the MRM transitions were optimized by infusing the neat standard solution into the mass spectrometer at 10 μg/mL. To ensure that the tune page parameters were compatible with the UPLC flow during the tuning, an UPLC flow of 0.3 mL/min at solvent A/B (1:1) was introduced into the mass spectrometer at the same time by utilizing a T unit (Upchurch Scientific, Oak Harbor, WA, USA). For MRM data collection during the LC experiments, the capillary voltage was 3.0 kV, the source temperature was 120 °C, the desolvation temperature was 350 °C, the desolvation gas flow was 800 L/h, and the cone gas flow was 50 L/h. During each LC injection, the mass spectrometer was set to collect data in MRM mode using electrospray ionization (ESI) in the positive ion mode. The MRM parameter for analyzing asfural (**2**) was as follows: pseudomolecular ion [M + H]$^+$, m/z 238.07, and transition ion, m/z 108.55; cone voltage, 23.0; collision energy, 14.50 eV.

UPLC Conditions for MRM. The analytes were eluted from the column (Acquity BEH column, 1.7 μm, C18, 2.1 × 100 mm i.d., Waters) with a mixed solvent of 20% MeOH with 0.05% AcOH (solvent A) and MeOH with 0.05% AcOH (solvent B) using a liner gradient mode (0−0.2 min, 10% B; 0.2−2.5 min, 10−90% B; 2.5−2.6 min, 90−100% B; 2.6−4.5 min, 100% B). The flow rate was 0.25 mL/min. The total UPLC cycle time was 5 min, including the column re-equilibration.

Plant Material. The fresh bottom parts (approximately 10 cm) of asparagus stems (*A. officinalis* L.) were collected from Kimobetsu, Hokkaido, Japan.

Production and Isolation. Asparagus (90 kg, fresh weight) was extracted with hot water (180 L) at 121 °C for 45 min (Scheme 1). The extract was cooled to 60 °C and treated with sucrase C as a cellulase (0.9 kg, Mitsubishi-Kagaku Foods Corp., Tokyo, Japan) for 1.5 h. Then the extract was cooled to 45 °C and treated with macerozyme A as a pectinase (0.9 kg, Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan) for 24 h. After inactivation of the enzymes (121 °C, 20 min), the extract was separated by centrifugation at 7200 rpm (room temperature) and mixed with dextrin (Pindex, 9.0 kg, Matsutani Chemical Industry Co., Ltd., Hyogo, Japan) as a filler. The supernatant containing the filler was then concentrated in vacuo at 105 °C and sterilized (121

Scheme 1. Scheme for Isolation of Active Compounds from ETAS

Enzyme-treated asparagus extract (ETAS)
(6 kg)
DIAION HP-20
H₂O | 30% MeOH | 60% MeOH | MeOH
preparative HPLC | partitioned between H₂O and EtOAc
HMF (1)
H₂O | EtOAc
Silica gel cc
CHCl₃ : MeOH (95:5)
423.4 mg
preparative HPLC
asfural (2)

°C, 45 min). Finally, the concentrate was spray-dried to produce ETAS (14.5 kg). A portion of ETAS (1.0 kg) was suspended in H$_2$O, applied on a Diaion HP-20 column (10.5 × 48 cm), and successively eluted with H$_2$O (12 L), 30% MeOH (12 L), and 60% MeOH (15 L) followed by MeOH (10 L). The 30% MeOH fraction was evaporated to dryness under reduced pressure (F1, 121.2 g). The 60% MeOH fraction was dried in vacuo (F2, 14.1 g). An aliquot of fraction F1 (100.0 mg) was purified by preparative HPLC [CAPCELL PAK C18 UG-120 column (5 μm, 20 × 250 mm); mobile phase (solvent A, H$_2$O; solvent B, MeOH; 0−10 min, 20% B; 10−30 min, 20−50% B; 30−40 min, 50% B; 40−41 min, 50−20% B; and 41−45 min, 20% B); flow rate, 8 mL/min; UV detection at 280 nm] to yield compound **1** (3.0 mg). The F2 fraction was suspended in H$_2$O and extracted with EtOAc (300 mL × 5). The EtOAc layer was evaporated to dryness under reduced pressure (2.5 g) and chromatographed on a silica gel column (4.0 × 42 cm) with CHCl$_3$/MeOH (95:5, 1.5 L). These purification steps from ETAS were repeated six times. The resulting residue (423.4 mg) was further purified by preparative HPLC [CAPCELL PAK C18 UG-120 column (5 μm, 20 × 250 mm); mobile phase (solvent A, H$_2$O; solvent B, MeOH; 0−10 min, 20% B; 10−30 min, 20−50% B; 30−40 min, 50% B; 40−41 min, 50−20% B; and 41−45 min, 20% B); flow rate, 8 mL/min; UV detection at 280 nm] to yield compound **2** (2.0 mg).

Asfural (**2**): amber oil; [α]$^{25}_D$ −12.2 (c 0.69, MeOH); IR ν_{max} 3340, 3136, 1773, 1682, 1205 cm^{-1}; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.58 (1H, s, H-6), 8.05 (NH, s), 7.52 (1H, d, J = 3.4 Hz, H-3), 6.82 (1H, d, J = 3.4 Hz, H-4), 5.26 (1H, d, J = 13.6 Hz, H-7a), 5.21 (1H, d, J = 13.6 Hz, H-7b), 4.24 (1H, dd, J = 9.1, 3.9 Hz, H-2′), 2.34 (1H, m, H-3′a), 2.14 (1H, m, H-4′a), 2.11 (1H, m, H-4′b), 1.98 (1H, m, H-3′b); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 178.7 (CH, C-6), 177.3 (C, C-5′), 172.6 (C, C-6′), 155.1 (C, C-5), 152.5 (C, C-2), 124.0 (CH, C-3), 113.3 (CH, C-4), 58.3 (CH$_2$, C-7), 54.7 (CH, C-2′), 28.9 (CH$_2$, C-4′), 24.6 (CH$_2$, C-3′); EIMS m/z 237 [M]$^+$ (5), 110 (12), 109 (27), 84 (100); HREIMS m/z 237.0612 (calcd for C$_{11}$H$_{11}$NO$_5$, 237.0637).

Preparation of (S)- and (R)-Asfural. (S)-Pyroglutamic acid (3.0 g) and D-fructose (1.5 g) were mixed and heated at 121 °C for 20 min. The mixture was suspended in H$_2$O, applied on a Diaion HP-20 column (2.5 × 25 cm), and eluted with H$_2$O (600 mL) and 30% MeOH (500 mL) followed by MeOH (500 mL). The MeOH fraction was dried in vacuo (465.6 mg) and purified by preparative HPLC [CAPCELL PAK C18 UG-120 column (5 μm, 20 × 250 mm); mobile phase (solvent A, H$_2$O; solvent B, MeOH; 0−10 min, 20% B; 10−30 min, 20−50% B; 30−40 min, 50% B; 40−41 min, 50−20% B; and 41−45 min, 20% B); flow rate, 8 mL/min; UV detection at 280 nm] to yield (S)-asfural (24.4 mg). (R)-Pyroglutamic acid (2.0 g) and D-fructose (1.0 g) were mixed and heated

at 121 °C for 20 min. The mixture was suspended in H_2O, applied on a Diaion HP-20 column (2.5 × 15 cm), and eluted with H_2O (300 mL) and 30% MeOH (300 mL) followed by 60% MeOH (400 mL). The 60% MeOH fraction was evaporated to 50 mL under reduced pressure and extracted with EtOAc (50 mL × 5). The EtOAc extract was evaporated to dryness under reduced pressure (80.8 mg), chromatographed on a Diaion HP-20 (1.5 × 4 cm), and eluted with H_2O (50 mL) and 30% MeOH (50 mL) followed by 60% MeOH (50 mL). The 60% MeOH fraction was evaporated to dryness under reduced pressure to yield (R)-asfural (24.6 mg).

Cell Culture. A human promyelocytic leukemia cell line, HL-60 (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), was cultured in RPMI-1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2.[15] The 24 h cell viability of HL-60 cells after addition of each compound was measured by a trypan blue exclusion test.

Real-Time PCR Assay for HSP70 mRNA Expression. The cell density of HL-60 cells was adjusted to $5 × 10^5$ cells/mL with preheated (37 °C) RPMI-1640 culture medium, and 0.9 mL of the cell culture was transferred into a 1.5 mL microtube. Then 0.1 mL of test sample dissolved in the culture medium was added at final concentrations of 0.5, 0.25, and 0.125 mg/mL for compound 2 and (S)-asfural and at 0.25 mg/mL for HMF (1) and (R)-asfural. As a control (nontreated) sample, 0.1 mL of distilled water was used, and 0.1 mL of 100 μM geranylgeranylacetone (GGA) purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) was used as a positive control. Following a 4 h incubation, the cells were collected by centrifugation at 1000*g*, and total RNA was extracted using a TRIzol reagent (Ambion, Austin, TX, USA) according to the manufacturer's instruction. The RNA concentration and quality were determined spectrophotometrically by Nanodrop (Thermo Scientific, Wilmington, DE, USA), adjusted to 50 ng/μL with DEPC water. Complementary DNA (cDNA) was synthesized by ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) according to the manufacturer's instruction, and obtained cDNAs were diluted 10-fold with DEPC water. Quantitative PCR of HSP70 gene and β-2 microglobulin (B2M) gene (housekeeping control) was performed in 4 μL of cDNA, 5 μL of SsoAdvanced SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), and 0.5 μL of 10 μM of the following primers: HSP70 forward primer (5′-GCATTTCCTAGTATTTCTGTTTGT-3′); HSP70 reverse primer (5′-AATAGTCGTAAGATGGCAGTATA-3′); B2M forward primer (5′-TAGCTGTGCTCGCGCTACT-3′); B2M reverse primer (5′-AGTGGGGGTGAATTCAGTGT-3′). The reaction was run for an initial denaturation at 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 1 s, and annealing at 59 °C for 10 s on a CFX Connect Real-Time System (Bio-Rad). The normalized expression of the HSP70 gene was evaluated by using the ΔΔCq method in CFX Manager software (version 2.1; Bio-Rad) with automatic threshold settings.

RESULTS AND DISCUSSION

Unused bottom parts of asparagus stems (90 kg, fresh weight) were extracted with hot water, processed, and mixed with a filler to yield ETAS (14.5 kg). The active compounds 1 (3.0 mg) and 2 (2.0 mg) were isolated from the portion of ETAS (6.0 kg) as shown in Scheme 1. Compound 1 was identified as 5-hydroxymethyl-2-furfural (HMF) because the data for compound 1 agreed with those of ^1H and ^{13}C NMR and retention time (7.5 min) by HPLC of the authentic standard (Wako Pure Chemical Industries, Ltd.).[16] Compound 2 (Figure 1) was
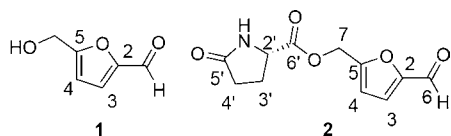
Figure 1. Chemical structures of HMF (1) and compound 2.

obtained as an amber oil. Its molecular formula was assigned as $C_{11}H_{11}NO_5$ on the basis of the $[M]^+$ ion peak at m/z 237.0612 (calcd 237.0637) in HREIMS. Its IR spectrum had absorption bands at 3340, 3136, 1773, and 1682 cm^{-1}, suggesting the presence of NH, lactam, and α,β-unsaturated aldehyde functionalities. The ^1H and ^{13}C NMR spectral data of compound 2 were similar to those of compound 1. In the ^1H and ^{13}C NMR spectra of compound 2, the characteristic signals of HMF (1) with two methines [$δ_H$ 7.52 d (*J* = 3.4 Hz, H-3); $δ_C$ 124.0 (C-3) and $δ_H$ 6.82 d (*J* = 3.4 Hz, H-4); $δ_C$ 113.3 (C-4)], an aldehyde [$δ_H$ 9.58 s (H-6); $δ_C$ 178.7 (C-6)], and quaternary carbons [$δ_C$ 155.1 (C-5), 152.5 (C-2)] were observed. The HMBC spectrum exhibited a cross-peak from an amide proton ($δ_H$ 8.05) to C-5′ ($δ_C$ 177.3). Furthermore, the HMBC correlations between H-4′ and C-5′, between H-3′ and C-5′, and between H-2′ and C-3′ and C-6′ indicated that compound 2 had a pyroglutamate moiety. The planar structure of compound 2 was confirmed by additional HMBC correlation of H-7 and C-6′. Therefore, compound 2 was assigned as (2-formylfuran-5-yl)methyl 5-oxopyrrolidine-2-carboxylate (Figure 2) and named asfural.
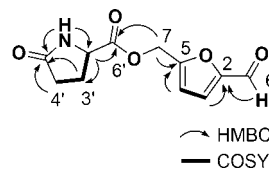
Figure 2. COSY and selected HMBC correlations for compound 2.

Asfural (2) is thought to be a condensation reaction product of HMF and pyroglutamic acid derived from glutamine contained in asparagus, as with mumefural. The previous study reported that mumefural is produced during food processing, because it has not been detected in the fresh fruit by HPLC.[12] Similarly, asfural (2) was not detected in fresh asparagus by LC-MS/MS (Figure 3), demonstrating that asfural (2) was produced during the manufacturing process for ETAS. The fresh asparagus also contained a small amount of HMF (1) (<0.0005 w/w% in the components), which increased in the hot water extract (0.004 w/w% in the components). Furthermore, the enzyme treatments contributed to a remarkable increase of HMF (1) (0.14 w/w% in ETAS). These results suggest that the increased HMF (1) might be associated with generating and increasing asfural (2).

To determine the absolute configuration of compound 2, (S)-asfural and (R)-asfural were synthesized according to the method described by Ono[13] and Takashita.[17] A mixture of (S)-pyroglutamic acid and D-fructose or (R)-pyroglutamic acid and D-fructose was heated at 121 °C for 20 min. The reaction products were purified by column chromatography to yield (S)-asfural and (R)-asfural, and their structures were confirmed by NMR. The optical rotation of compound 2 ($[α]^{25}_D$, −12.2) was almost the same as that of synthesized (S)-asfural ($[α]^{25}_D$, −11.8), but not synthesized (R)-asfural ($[α]^{25}_D$, +11.3). Moreover, in the HPLC analysis using a chiral column (Figure 4), the retention time of compound 2 (18.9 min) was nearly coincidental with that of synthesized (S)-asfural (19.0 min). Consequently, compound 2 derived from ETAS was assigned as (S)-(2-formylfuran-5-yl)methyl 5-oxopyrrolidine-2-carboxylate.

Finally, the biological activities of HMF (1), compound 2, synthesized (S)-asfural, and synthesized (R)-asfural were evaluated for HSP70 mRNA induction activity in HL-60 cells
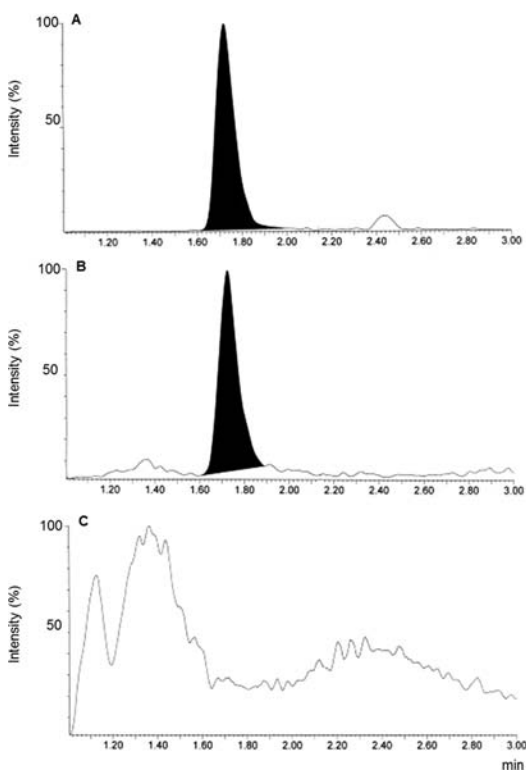
Figure 3. UPLC-MS/MS MRM chromatograms: (A) synthesized (*S*)-asfural; (B) ETAS; (C) fresh asparagus.
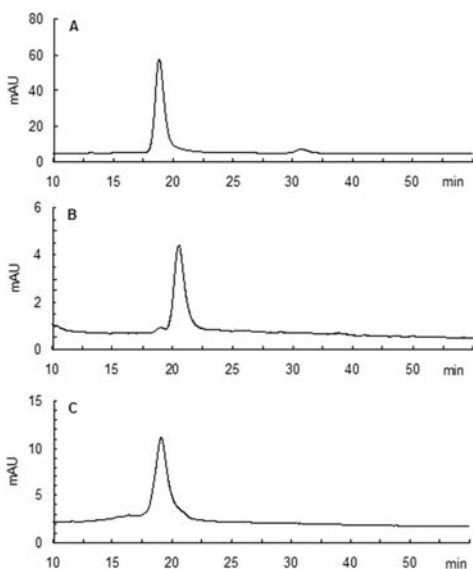
Figure 4. HPLC chromatograms for synthesized (*S*)- and (*R*)-asfural and compound **2**: (A) synthesized (*S*)-asfural; (B) synthesized (*R*)-asfural; (C) compound **2**.

(Figure 5). When the proliferation of HL-60 cells was measured following an addition of each sample, only (*R*)-asfural, which is not detected in ETAS, strongly inhibited the cell growth at 0.5 mg/mL (Table 1). Compound **2** and (*S*)-asfural showed no suppressive effect on the cell viability and significantly enhanced the expression level of HSP70 mRNA in a concentration-dependent manner. The intensities (% of control) of compound **2** were 131 and 334% at 0.25 and 0.5 mg/mL, respectively. Similarly, the intensities of (*S*)-asfural were 207% at 0.25 mg/mL and 286% at 0.5 mg/mL. HMF (**1**) also showed significant
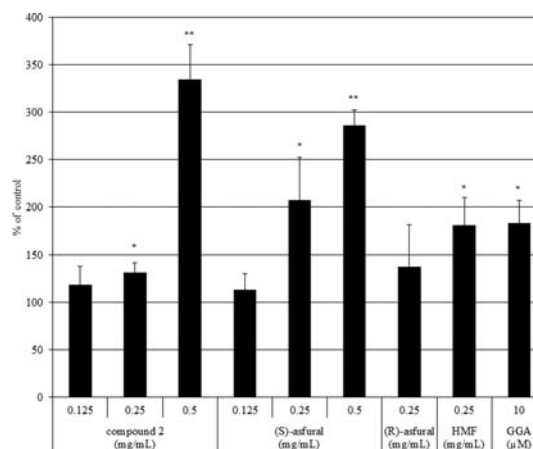
Figure 5. Induction of HSP70 mRNA. The expression levels of HSP70 mRNA were measured by a real-time PCR method after addition of each compound to HL-60 cells. Values represent the mean $\pm$ SE and percentage (%) of HSP70 level in compound-treated cells to that of nontreated cells (control). $*$, $p < 0.05$ versus control; $**$, $p < 0.01$ versus control.

Table 1. Trypan Blue Cell Viability Assay

	viable cell ratio (%)		
	0.5 mg/mL	0.25 mg/mL	0.125 mg/mL
compound **2**	75.9	95.0	127.5
(*S*)-asfural	106.6	114.6	130.8
(*R*)-asfural	37.2	92.5	111.7
HMF	94.9	85.8	120.8

activity at 0.25 mg/mL. Interestingly, (*R*)-asfural did not enhance the expression level at the concentration showing no cytotoxicity (0.25 mg/mL). It was suggested that the steric hindrance of (*R*)-asfural might affect the induction activity of HSP70 mRNA, although further studies are needed to elucidate the underlying mechanism as well as to clear the effect on HSP70 protein using Western blot and ELISA methods. The current HSP70 mRNA assay system also responded to GGA, which is widely used for HSP70-related studies.

Heat shock factor 1 (HSF1) is the HSF family member responsible for stress-induced expression of HSPs.[18−21] Heat stress and other physiological stimuli promote HSF1 trans-activation and heat shock element (HSE) acquisition via nuclear translocation,[22−24] homotrimerization,[25,26] and enhanced phosphorylation status at serine (Ser) residues Ser230 and Ser326.[27,28] We investigated the effect of compound **2** and (*S*)-asfural for HSF1 mRNA levels by using real-time PCR. In the result, neither substance influenced the HSF1 mRNA levels (data not shown), leading us to speculate that asfural might act during the steps of trimerization and/or phosphorylation.

Supplementation of ETAS containing HMF and asfural appears to exert a cytoprotective effect attributable to HSP70 elevation, which could result in increased resistance to various stresses and diseases. From another point of view, consumption of ETAS can contribute to the effective utilization of waste. The effective utilization of postharvest asparagus waste, combined with a unique processing technique, is consistent with the high-tech "green philosophy" of Hokkaido, Japan. The resulting ETAS displayed significant HSP70 induction in vitro and featured two compounds. ETAS may be a valuable material to help reduce the effects of fatigue and stress in Japan and

9158

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elsewhere. Further animal and clinical studies are warranted for proof of these functional concepts and safety.

■ ASSOCIATED CONTENT

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Notes
The authors declare no competing financial interest.

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