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Cleavage of α-Dicarbonyl Compounds by Terpene Hydroperoxide

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The highly reactive α-dicarbonyl compounds, glyoxal, methylglyoxal (MGO), and 3-deoxyglucosone, react with the amino groups of proteins to form advanced glycation end-products (AGEs) which have been implicated in diabetic complications, aging, and Alzheimer's disease. We found that a test sample of terpinen-4-ol (T4) containing hydroperoxides showed cleaving activity toward an α-dicarbonyl compound, but that the freshly isolated pure sample did not. Prepared terpinen-4-ol hydroperoxide (T4-H) also efficiently cleaved the C-C bond of the α-dicarbonyl compounds *via* Baeyer-Villiger-like rearrangement and subsequent hydrolysis of an acid anhydride moiety in the rearranged product to give carboxylic acids. Other terpene hydroperoxides, as well as T4-H, showed significant cleaving activities, and all these hydroperoxides protected RNase A from the lowering of enzyme activity induced by MGO. The cleaving mechanism *via* Baeyer-Villiger-like rearrangement was confirmed by time-interval NMR measurements of the reaction mixture of the symmetrical α-dicarbonyl compound, diacetyl with T4-H.

Key words: terpinen-4-ol; hydroperoxide; methylglyoxal; advanced glycation end-products (AGEs); RNase A

Advanced glycation end products (AGEs) are a group of compounds formed *via* a non-enzymatic reaction between reducing sugars and amine residues on proteins, and have been implicated as the cause of diabetic complication,[1] aging,[2,3] Alzheimer's disease,[4,5] and arteriosclerosis.[6] The major AGEs *in vivo* appear to be formed as highly reactive intermediate carbonyl groups, known as α-dicarbonyl compounds such as 3-deoxyglucosone, glyoxal, and methylglyoxal (MGO).[7,8] The first cleaving agent of an α-dicarbonyl compound appearing in the literature was *N*-phenacylthiazolium bromide (PTB), and the mechanism for cleaving the C-C bond has been proposed.[9] 3-Phenacyl-4,5-dimethylthiazolium chloride (ALT-711) was later introduced as a stable analogue of PTB.[10] These cleaving compounds might be useful as therapeutic agents for diabetes complications and ointment ingredients for skin care.[11,12] We have found that the volatile oil from *Citrus junos* Tanaka and certain terpenes could cleave 1-phenyl-1,2-propanedione (PPD), a model α-dicarbonyl compound like PTB, whereas we had difficulty replicating the phenomenon. To investigate this anomalous phenomenon, we focused our attention first on terpinen-4-ol (T4, **1**), one of the major components of tea tree oil used as an aromatherapy product. Although the pleiotropic biological activities of the essential oil have been reported, no logical explanation at the molecular level for the therapeutic effects has appeared in the scientific literature.[13] We were therefore interested in elucidating the mystery for the effects of the essential oil and found one possible answer. The proposed action mechanism involved the allylic hydroperoxide formation of T4 by autoxidation, the Baeyer-Villiger-like reaction of the α-dicarbonyl compound with the resulting hydroperoxide, and hydrolysis of the acid anhydride moiety of the rearranged product to give two carboxylic acid molecules.

Results and Discussion

Identification of the oxidative compounds from the reaction mixture of 1-phenyl-1,2-propanedione (PPD) and unpurified terpinen-4-ol

An unpurified test sample (unpurified T4 (**1**)), after exposing (−)-terpinen-4-ol (>95% by GC) to air for one month, showed moderate cleaving activity toward 1-phenyl-1,2-propanedione (PPD) to give benzoic acid as shown in Fig. 1A and C. To test the significance of the double bond, *trans*-4-methyl-1-(1-methylethyl)cyclohexanol, MMC (**2**) was prepared as indicated in Fig. 1B, and its cleaving activity was compared with that of unpurified T4. MMC lost the cleaving activity as shown in Fig. 1C. We next isolated the three oxidative derivatives of (−)-terpine-4-ol, 5-hydroxy-2-methyl-5-(1-methylethyl)cyclohex-2-enone (**3**), (1*R*,2*R*,4*R*)-1-methyl-4-(1-methylethyl)-1,2,4-cyclohexanetriol (**4**) and (2*Z*)-3,7-dimethyl-6-oxooct-2-enal (**5**), from the reaction mixture of unpurified T4 and PPD. The presence of these oxidized compounds suggested that allylic hydroperoxides of T4 in the active samples were the true cleaving agent of the α-dicarbonyl compound, and allylic hydroperoxide in the unpurified T4 sample triggered the formation of the oxidative products during the cleavage reaction of the α-dicarbonyl compound.

Efficient cleavage of the α-dicarbonyl compound by terpene hydroperoxides, and their protection of the enzyme function of RNase A from methylglyoxal

This hypothesis was verified by two experiments; pure T4 (**1**) freshly isolated from commercially available (−)-terpinen-4-ol (>95% by GC) did not cleave the α-dicarbonyl compound, while the hydroperoxide, T4-H (**6**) prepared from T4 with singlet oxygen,[14,15] demon-

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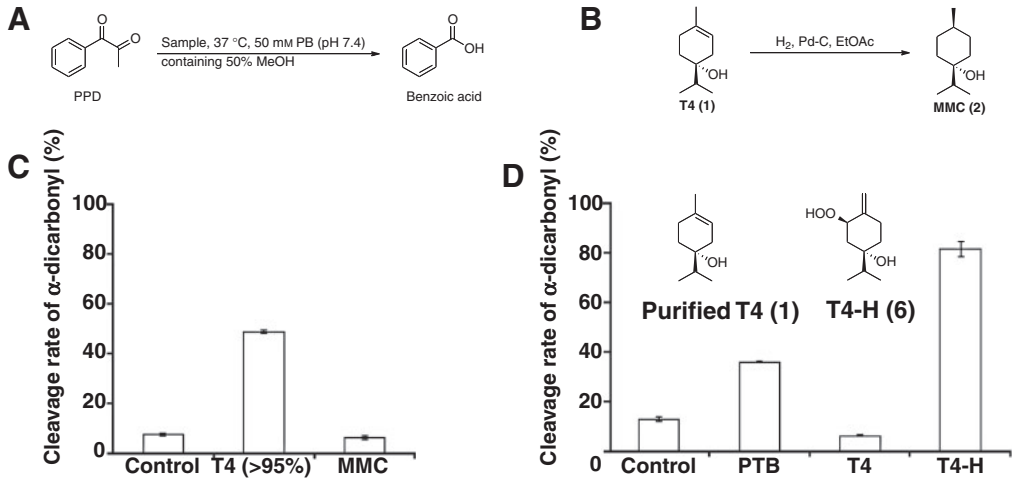

Fig. 1. Cleaving Effect of Unpurified T4 (>95%) with Conversion of 1-Phenyl-1,2-propanedione (PPD) to Benzoic Acid.

A: The cleaving activity of the α-dicarbonyl compound by unpurified T4 was evaluated by a modified procedure of Vasan's protocol.[9] B: *trans*-4-Methyl-1-(1-methylethyl)cyclohexanol (MMC) was prepared by hydrogenation of unpurified T4. C: MMC did not cleave PPD, suggesting the importance of the double bond for the cleavage of α-diketone. D: Cleavage rate of PPD with purified T4 or terpine-4-ol hydroperoxide (T4-H). Control, DMSO (vehicle); PTB, *N*-phenacylthiazolium bromide. Error bar: ±SD (n = 3).

strated significant cleaving activity of the α-dicarbonyl compound as shown in Fig. 1D. To prove the generality of the cleaving effect of the terpene hydroperoxide and its protective effect on glycation-induced oxidative damage of the enzyme, we next prepared additional hydroperoxides and evaluated the protective effects of these hydroperoxides on ribonuclease A (RNase A) in the presence of MGO,[16,17] a major precursor of AGEs. MGO is a highly reactive endogenous metabolite, and its levels are elevated in diabetic patients.[18,19] α-Terpineol hydroperoxide (T-ol-H,a 3:1 mixture of **8a** and **8b**) prepared from α-terpineol(T-ol, **7**), and α-terpinyl acetate hydroperoxide(T-ace-H, **10**) prepared from α-terpinyl acetate (T-ace, **9**) showed significant cleaving activity toward PPD, whereas highly purified T-ol and T-ace did not cleave the α-dicarbonyl compound as in the case of T4 (Fig. 2). PPD could be slightly cleaved by atmospheric oxygen in the sealed reaction tube,[9] while purified T4, T-ol, and T-ace rather inhibited the cleavage of PPD as shown in Figs. 1D and 2, probably because these pure terpenes rapidly consumed oxygen to give **4** as a major oxidative derivative, while **3** and **5** as minor oxidative derivatives, and the allylic hydroperoxide were not present in sufficient concentrations to cleave PPD. Terpene hydroperoxide in test sample was therefore the real active agent for cleaving the α-dicarbonyl compound. All these hydroperoxides, T4-H, T-ol-H and T-ace-H, protected ribonuclease A (RNase A) from highly reactive MGO, their protective effects being comparable with those of the positive controls, aminoguanidine (AG) and metformin (Met), in the RNase A-MGO assay as shown in Fig. 3.

Cleaving mechanism of the α-dicarbonyl compound verified by ^{1}H-NMR analysis

The cleaving mechanism of the α-dicarbonyl compound by terpene hydroperoxide could be explained by Baeyer-Villiger-like rearrangement and subsequent hydrolysis,[20] this hypothesis being reliably established by ^{1}H-NMR experiments as indicated in Fig. 4. To detect the reaction intermediates, 10 mM of diacetyl as a symmetrical α-dicarbonyl compound in an NMR tube

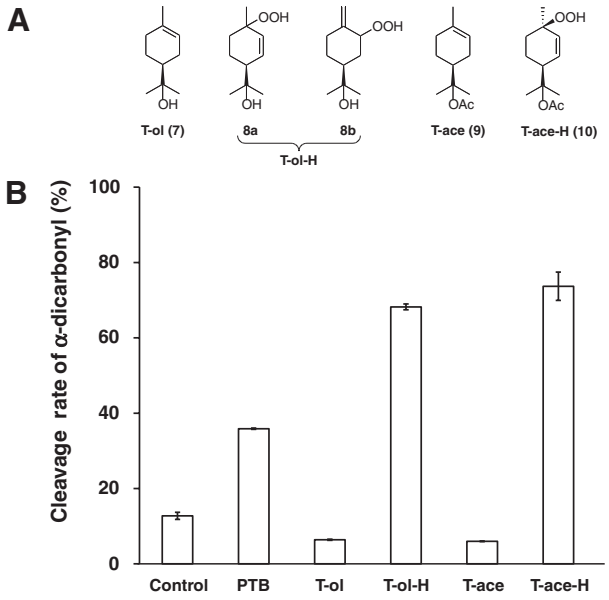

Fig. 2. Structures of Terpene Hydroperoxides and Their Effects on the Cleavage of PPD.

A: Structures of α-terpineol (T-ol), α-terpineol hydroperoxide (T-ol-H), α-terpinyl acetate (T-ace), and α-terpinyl acetate hydroperoxide (T-ace-H). B: Cleavage rate of PPD treated with purified monoterpene or its hydroperoxide. Control, DMSO (vehicle); PTB, *N*-phenacylthiazolium bromide. Error bar: ±SD (n = 3).

was treated with 20 mM of T4-H (**6**) for 0–48 h at 30 °C in $CDCl_3$. Changes in the NMR signals of the starting materials (T4-H and diacetyl), reaction intermediate (acetic anhydride), and cleavage product (acetic acid) were monitored by ^{1}H-NMR measurements. A decrease in the R-OO<u>H</u> signal of T4-H at 8.16 ppm was observed during the 8–48 h period as shown in Fig. 4A. Figure 4B shows that the CH_3 peak at 2.34 ppm of the diacetyl gradually decreased *via* the signal being split into three peaks at 8 h, suggesting that one ketone of the diacetyl had been attacked by T4-H to form a detectable intermediate until 8 h. Three peaks at around 2.34 ppm appearing at 8 h were tentatively assigned to a diastereomeric pair of methyl groups of a methyl ketone of the intermediate in the Baeyer-Villiger reaction, and the

methyl group of the diacetyl. The CH_3 peak at 2.23 ppm of acetic anhydride (authentic acetic anhydride was 2.23 ppm) as the Baeyer-Villiger rearranged product appeared after 24 h and gradually increased over 48 h. The acetic anhydride was hydrolyzed to acetic acid by residual H_2O (1.59 ppm), and the CH_3 peak at 2.10 ppm of acetic acid (authentic acetic acid was 2.11 ppm) appeared after 24 h and gradually increased over 48 h as shown in Fig. 4C. These observations validated the cleaving mechanism of the α-dicarbonyl compound treated with terpene hydroperoxide.

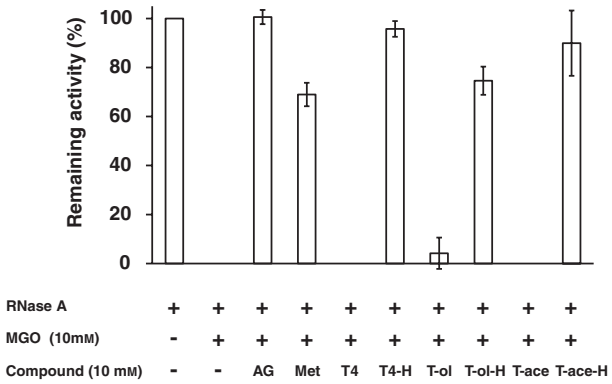

Fig. 3. Protective Effects of Terpene Hydroperoxides, T4-H, T-ol-H, and T-ace-H in the RNase A-MGO Assay.

MGO, methylglyoxal; AG, aminoguanidine (positive control 1); Met, metformin (positive control 2). The remaining activity of RNase A was determined as described in the Experimental section. Error bar: ±SD (n = 3).

Discussion on the biological activities of monoterpenes and their hydroperoxides

We clarified the cleaving mechanism of the α-dicarbonyl compound by terpene hydoroperoxide, although terpene hydroperoxides have only previously been recognized as skin sensitizers. *d*-Limonene itself gives no significant skin sensitization, and some of the oxidation products formed when *d*-limonene is exposed to air have been shown to be potent contact allergens, this being analogous to the oxidation products of Δ-carene as another monoterpene. The hydroperoxides of these compounds have been reported to be responsible for the main allergenic effect of turpentine.[21,22] We confirmed that fresh T4 easily reacted with air at room temperature for 30 d to give at least three hydroperoxy derivatives (R_f 0.3, 0.5, and 0.69) which were detected as yellow spots by a saturated aqueous KI solution, an indicator of hydroperoxides, on a silica gel TLC plate (EtOAc:hexane, 1:2). These spots, which can also be indicated as characteristic white spots on the TLC image

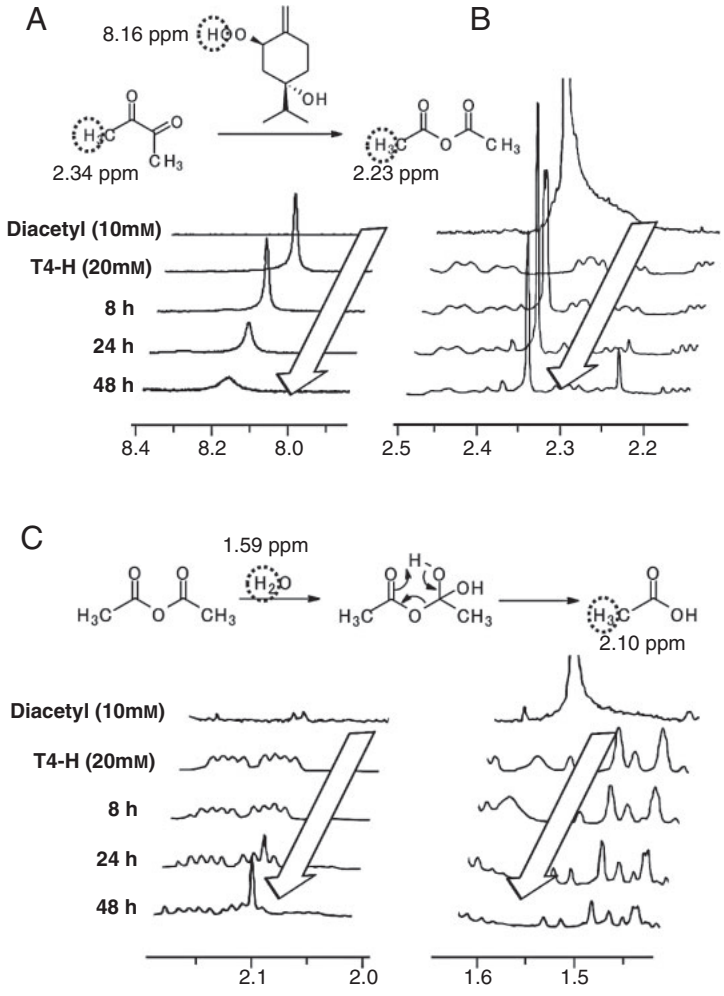

Fig. 4. Time-Interval ^{1}H-NMR Spectra of the Reaction Mixture of Diacetyl and T4-H.

Changes in signal intensity of (A) the hydroperoxide proton in T4-H (8.16 ppm), (B) the methyl protons in diacetyl (2.34 ppm) and acetic anhydride (2.23 ppm) as the Baeyer-Villiger oxidation products, and (C) acetic acid (2.10 ppm) as the cleavage product and the proton of H_2O (1.59 ppm).

before turning to blue spots on a green-yellow background by a phosphomolybdic acid solution, were detected in an active T4 sample as the α-dicarbonyl cleaving agent. Autoxidation of 1,2-dimethylcyclohexene with triplet oxygen gives the hydroperoxide with an unshifted double bond as the major product, while singlet oxygen-mediated oxidation of the same cyclohexene produces the hydroperoxide derivative having exomethylene as the major product.[15] It has recently been reported that α-terpinene was rapidly degraded to form allylic epoxides and p-cymene as the major oxidation products.[23] Australian tea tree oil containing T4 as a major component has exhibited such activities as antibacterial,[24] antifungal,[25] and antiviral,[26] and has been used topically to treat such conditions as acne, cold sores, dandruff, onychomycosis, oral candidiasis, and tinea pedis.[27,28] Although studies on human skin penetration of T4 have appeared,[29,30] no molecular mechanism for such biological activities has been reported. The biological activities of monoterpenes including T4 might therefore need to be reevaluated in consideration of the effects caused by a trace amount of hydroperoxy derivatives contained in the test samples. All terpene hydroperoxides synthesized in this study showed significant cleaving activities of α-dicarbonyl compounds and converted potentially toxic MGO into non-toxic or less toxic carboxylic acids to protect the enzyme function. The present findings are important to provide insight into the quality control of essential oils for traditional remedies and aromatherapy, and might contribute to the development of a novel class of inhibitors of AGE formation and/or skin care products whose mechanism is different from that of such thiazolium-type inhibitors as PTB and ALT-711.

Experimental

Materials. (−)-Terpinen-4-ol and diacetyl were purchased from Tokyo Kasei Co. (Tokyo, Japan). 1-Phenyl-1,2-propanedione (PPD), thionine-acetate, molecular sieves catalyst support Na–Y zeolite, methylglyoxal (MGO, 40% in H_2O), RNase A from bovine pancreas and transfer RNA (tRNA) from baker's yeast (*S. cerevisiae*) were purchased from Sigma Aldrich (St. Louis, MO, USA). Benzoic acid, trifluoroacetic acid (TFA), (*S*)-α-terpineol, aminoguanidine, metformin, perchloric acid and lanthanum nitrate hexahydrate were purchased from Wako Co. (Osaka, Japan). PTB was purchased from Prime Organics (Woburn, MA, USA). Thin-layer and silica gel column chromatography were respectively performed with Silica Gel 60 F_{254} (Merck, Darmstadt, Germany) and Silica Gel 60N (spherical, neutral) (Kanto Chemical, Tokyo, Japan). ^{1}H, ^{13}C, HH-COSY, HMBC, HMQC, DIF-NOE and NOESY NMR spectra were measured with an AMX-500 instrument (Bruker, Billeria, MA, USA) or JNM-EX270 instrument (Jeol, Tokyo, Japan). Chemical shifts are reported in δ ppm, using tetramethylsilane as an internal standard, and coupling constants (*J*) are given in Hertz. Mass spectra were acquired with a JMS-T100GCV instrument (Jeol, Tokyo, Japan). Some of the NMR and MS data were measured at the GC-MS and NMR Laboratory of Faculty of Agriculture at Hokkaido University. Optical rotation data were determined with a P-2000 polarimeter (Jasco, Tokyo, Japan). Singlet oxygen photo-oxygenation was performed for 1 h at 0 °C with a UM-102 high-pressure mercury lamp (Ushio, Tokyo, Japan).

Cleavage rate of the α-dicarbonyl compound. The cleavage rate of PPD was determined by a previously described method[9] with slight modifications. Briefly, 1-mL solution of 10 mM of PPD with various concentrations of the test compound in 50 mM PB (pH 7.4) containing 50% methanol was incubated for 24 h at 37 °C while shaking at 300 rpm. To the reaction mixture, 200 μL of 2 M HCl was added, and the resulting solution was diluted 5-fold with a combined solution of equal parts of 100 mM sodium phosphate buffer (pH 7.4) and methanol. The components were analyzed by HPLC (Kanto Chemical ODS Mightysil RP-18 column, 4.6 × 250 mm (5 μm) and mobile phase of a 35% methanol/0.1% TFA solution), and the yield of benzoic acid was calculated by area integration (A_{254}) and interpolation of a calibration curve obtained by injecting standard amounts of pure benzoic acid. The percentage of cleaving activity was calculated as 100 × [concentration (mM) of generated benzoic acid/10 mM].

Synthesis of trans-*4-methyl-1-(1-methylethyl)cyclohexanol (MMC, **2**).* To 153 mg of Pd–C was added 2.31 g (15.0 mmol) of T4 (**1**) dissolved in 20 mL of EtOAc. The mixture was stirred in a hydrogen atmosphere at room temperature for 24 h. After filtering through a Celite pad, the filtrate was evaporated *in vacuo* and subjected to silica gel column chromatography (EtOAc:hexane, 1:9) to afford MMC (**2**, 1.19 g, 51%) as a syrup together with *cis*-4-methyl-1-(1-methylethyl)-cyclohexanol (0.56 g, 24%) as crystals.

*Compound **2** (MMC).* ^{1}H-NMR (500 MHz, $CDCl_3$): 0.91 (9H, d, $J = 6.9$ Hz, isopropyl-CH_3 × 2, and 4-CH_3), 1.03 (1H, s, OH), 1.23–1.39 (5H, m), 1.51–1.59 (5H, m). ^{13}C-NMR (125 MHz, $CDCl_3$): 16.9 (isopropyl-CH_3 × 2), 22.4 (4-CH_3), 30.4 (3- and 5-CH_2), 32.4 (4-CH), 33.7 (2- and 6-CH_2), 38.6 (isopropyl-CH), 72.6 (COH). HR-FI-MS: *m/z* $[M]^+$; calcd. for $C_{10}H_{20}O$, 156.1514; found, 156.1506.

*Isolation and structural determination of (5S)-5-hydroxy-2-methyl-5-(1-methylethyl)cyclohex-2-enone (**3**), (1R,2R,4R)-1-methyl-4-(1-methylethyl)-1,2,4-cyclohexanetriol (**4**), and (2Z)-3,7-dimethyl-6-oxooct-2-enal (**5**).* A 40-mL solution of 10 mM of PPD with 150 mM T4 (**1**) in 50 mM PB (pH 7.4) containing 50% methanol was stirred for 72 h at 37 °C. To the reaction mixture, 8 mL of 2 M HCl was added, and the resulting solution was extracted three times with EtOAc. The combined organic layer was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to give a crude product. Silica gel column chromatography (EtOAc: hexane, 1:2) of the crude product afforded compound **4** (13.4 mg) and crude compounds **3** and **5**. Compounds **3** (1.5 mg) and **5** (1.4 mg) were purified by further preparative silica gel TLC (MeOH:$CHCl_3$, 1:99, developed twice).

*Compound **3**.* ^{1}H-NMR (500 MHz, $CDCl_3$): 0.97 (6H, d, $J = 6.9$ Hz, isopropyl-CH_3), 1.75 (1H, sep, $J = 6.8$ Hz, isopropyl-CH), 1.82 (3H, d, $J = 1.4$ Hz, 2-CH_3), 2.41 (1H, dd, $J = 18.6$, 5.4 Hz, 4-CH_2), 2.52–2.60 (3H, m, 4- and 6-CH_2), 6.62 (1H, m, 3-CH). ^{13}C-NMR (125 MHz, $CDCl_3$): 15.5 (2-CH_3), 16.6 (isopropyl-CH_3), 16.8 (isopropyl-CH_3), 35.7 (4-CH_2), 37.4 (isopropyl-CH), 48.0 (6-CH_2), 76.5 (5-C), 135.3 (2-C), 141.2 (3-CH). HR-FD-MS: *m/z* $[M]^+$; calcd. for $C_{10}H_{16}O_2$, 168.1151; found, 168.1160.

*Compound **4**.* $[\alpha]_D^{27} = -14.3$ (*c* 1.00, EtOH). ^{1}H-NMR (270 MHz, DMSO-d_6): 0.82 (6H, d, $J = 6.9$ Hz, isopropyl-CH_3 × 2), 1.10 (3H, s, CH_3), 1.20–1.52 (4H, m, CH_2), 1.59–1.81 (3H, m, isopropyl-CH, CH_2), 3.30 (1H, m, 2-CH), 4.14 (1H, s, 4-OH), 4.55 (1H, s, 1-OH), 5.12 (1H, d, $J = 6.9$ Hz, 2-OH). ^{13}C-NMR (67.5 MHz, CD_3OD): 17.1 (isopropyl-CH_3), 17.2 (isopropyl-CH_3), 27.1 (1-CH_3), 30.3 (5-CH_2), 30.4 (6-CH_2), 34.8 (2-CH_2), 39.0 (isopropyl-CH), 72.0 (1-C), 75.7 (4-C and 2-CH). HR-FD-MS: *m/z* $[M]^+$; calcd. for $C_{10}H_{20}O_3$, 188.1412; found, 188.1423.

*Compound **5**.* ^{1}H-NMR (270 MHz, $CDCl_3$): 1.11 (6H, d, $J = 6.9$ Hz, isopropyl-CH_3 × 2), 2.18 (3H, s, CH_3), 2.45 (1H, sep, $J = 6.9$ Hz, 7-CH), 2.66 (2H, t, $J = 8.2$ Hz, 4-CH_2), 2.84 (2H, t, $J = 8.2$ Hz, 5-CH_2), 5.90 (1H, d, $J = 7.6$ Hz, 2-CH), 9.99 (1H, d, $J = 7.6$ Hz, 1-CHO). ^{13}C-NMR (67.5 Hz, $CDCl_3$): 21.4 (isopropyl-CH_3 × 2), 24.5 (3-CH_3), 29.9 (4-CH_2), 35.8 (5-CH_2), 43.6 (7-CH), 125.2 (2-CH), 172.1 (3-C), 191.3 (1-CHO), 206.4 (6-C=O). HR-FI-MS: *m/z* $[M]^+$; calcd. for $C_{10}H_{20}O_3$, 168.1151; found, 168.1157.

*Preparation of (1R,3S)-3-hydroperoxy-1-(1-methylethyl)-4-methylene cyclohexanol (T4-H, **6**), 2-(4-hydroperoxy-4-methylcyclohex-2-ene-1-yl) propan-2-ol (**8a**), 2-(3-hydroperoxy-4-methylenecyclohexyl)-propan-2-ol (**8b**, (T-ol-H, a 3:1 mixture of **8a** and **8b**), and 2-((1S4R)-4-hydroperoxy-4-methylcyclohex-2-en-1-yl)propan-2-yl acetate (T-ace-H, **10**).* T4-H (**6**), T-ol-H (**8a** and **8b**), and T-ace-H (**10**) were prepared

by the previously described method[14,15] with slight modifications. The preparation of **10** as an example of the general procedure started by drying thionine-supported Na–Y zeolite (3.0 g) in a two-neck flask at 120 °C for 1.5 h *in vacuo*. To the flask, 30 mL of distilled hexane and 30 µL of pyridine were added, and the mixture was stirred for 5 h in an argon atmosphere. To the resulting mixture, 30.7 mg of (*S*)-α-terpinyl acetate (**9**) prepared from (*S*)-α-terpineol (**7**) was added, and the mixture was photooxygenated at 0 °C for 1 h in a slow stream of oxygen. After stirring for 12 h with 50 mL of CH_3CN, the mixture was passed through a Celite pad, and the filtrate was evaporated *in vacuo* and subjected to silica gel column chromatography ($CHCl_3$: Et_2O, 16:1) to give 17.1 mg of **10** as a white solid in a 48% yield.

*T4-H (**6**).* ^{1}H-NMR (270 MHz, $CDCl_3$): 0.93 (1H, d, $J = 7.6$ Hz, isopropyl-CH_3), 1.25 (1H, s, OH), 1.39–1.53 (2H, m, 2-CH_2-a, 6-CH_2-a), 1.61–1.71 (2H, m, isopropyl-CH, 2-CH_2-b), 2.14 (1H, ddd, $J = 12.8$, 4.9, 2.6 Hz, 6-CH_2-b), 2.27 (1H, ddd, $J = 13.5$, 4.9, 2.9 Hz, 5-CH_2-a), 2.44 (1H, dt, $J = 13.5$, 4.2 Hz, 5-CH_2-b), 4.76 (1H, dd, $J = 11.5$, 4.9 Hz, 3-CH), 4.86 (1H, d, $J = 1.3$ Hz, olefinic-CH_2-a), 4.96 (1H, d, $J = 1.3$ Hz, olefinic-CH_2-b), 8.37 (1H, s, OOH). HR-FD-MS: m/z $[M]^+$; calcd. for $C_{10}H_{18}O_3$, 186.1256; found, 186.1226.

*T-ol-H (**8a**).* ^{1}H-NMR (270 MHz, $CDCl_3$): 1.18 (3H, s, 1- or 3-CH_3), 1.24 (3H, s, 1- or 3-CH_3), 1.33 (3H, s, 3′-CH_3), 1.52–1.92 (2H, m, 5′-CH_2-a and 6′-CH_2-a), 2.05–2.36 (3H, m, 5′-CH_2-b, 6′-CH_2-b, and 1′-CH), 5.69 (1H, ddd, $J = 10.2$, 2.3, 1.7 Hz, 3′-olefinic-CH), 6.08 (1H, ddd, $J = 10.2$, 2.3, 1.0 Hz, 2′-olefinic CH), 7.97 (1H, s, OOH). ^{13}C-NMR (67.5 Hz, $CDCl_3$), 20.4 (6′-CH_3), 24.8 (4′-CH_3), 26.1 (1- or 3-CH_3), 28.1 (1- or 3-CH_3), 47.2 (1-C), 72.7 (2-C), 78.5 (4′-C), 130.3 (3′-olefinic-CH), 134.1 (2′-olefinic-CH). HR-FI-MS: m/z $[M+H]^+$; calcd. for $C_{10}H_{18}O_3$, 186.1256; found, 186.1252.

*T-ol-H (**8b**).* ^{1}H-NMR (270 MHz, $CDCl_3$): 1.14 (3H, s, 1- or 3-CH_3), 1.19 (3H, s, 1- or 3-CH_3), 1.38–1.92 (7H, m, 1′-CH, 2′-, 5′-, and 6′-CH_2), 4.53 (1H, t, $J = 2.9$ Hz, 3′-CH), 4.98 (2H, br d, $J = 0.7$ Hz), 8.74 (1H, s, OOH). ^{13}C-NMR (67.5 MHz, $CDCl_3$): 26.1 (2′-CH_2), 27.5 (1- or 3-CH_3), 28.4 (1- or 3-CH_3), 30.4 (6′-CH_2), 31.2 (5′-CH_2), 42.0 (1′-CH), 72.7 (2-C), 85.2 (37-CH), 113.8 (4′-olefinic-CH_2), 145.2 (4′-olefinic-CH). FI-MS: m/z 187 $[M + H]^+$.

*T-ace-H (**10**).* ^{1}H-NMR (270 MHz, $CDCl_3$): 1.34 (3H, s, 4′-CH_3), 1.39 and 1.44 (each 3H, each s, 1- and 3-CH_3), 1.54–1.62 (2H, m, 5′-CH_2-a, 6′-CH_2-a), 1.98 (1H, m, 5′-CH_2-b), 2.00 (3H, s, –OCOCH_3), 2.17–2.23 (1H, m, 6′-CH_2-b), 2.77–2.84 (1H, m, 1′-CH), 5.65 (1H, dt, $J = 10.2$, 2.1, 1.8 Hz, 3′-olefinic-CH), 5.92 (1H, dd, $J = 10.2$, 1.9 Hz, 2′-olefinic-CH), 7.48 (1H, s, 4′-OOH). ^{13}C-NMR (67.5 MHz, $CDCl_3$): 22.1 (6-CH_2), 22.5 (OCO$\underline{C}H_3$), 23.0 (4′-CH_3), 23.7 (1- or 3-CH_3), 24.8 (1- or 3-CH_3), 31.6 (5-CH_2), 44.3 (1′-CH), 78.6 (2-C), 84.8 (4′-C), 129.9 (3′-olefinic-CH), 133.7 (2′-olefinic-CH). HR-FD-MS: m/z $[M + H]^+$; calcd. for $C_{12}H_{20}O_4$, 228.1362, found 228.1346.

Assay for protective effects of terpene hydroperoxides on RNase A in the presence of methylglyoxal (MGO). The enzyme activity of RNase A was determined by the previously described method[16,17] with slight modifications. Briefly, 200 µL of the reaction mixture in a PCR tube containing 1 mg mL^{-1} RNase A and 10 mM MGO with or without the test compound dissolved in 2 µL of EtOH in a 20 mM phosphate buffer (pH 7.2) was incubated for 24 h at 37 °C. After 200-fold dilution with a 5% glycerol solution, the mixture was transferred into a 1.5-mL Eppendorf tube to be incubated with 0.1 mg mL^{-1} of tRNA in a 60 mM phosphate buffer (pH 8.0) with a total volume of 250 µL. The enzyme reaction was carried out by incubating this reaction mixture at 37 °C for 20 min while shaking (300 rpm), and then quenched with 250 µL of a stop solution (0.86 g of $La(NO_3)_3$ and 7.1 mL of $HClO_4$ in 100 mL of H_2O). The resulting solution was centrifuged at 13,000 rpm for 10 min at 4 °C, and the absorbance at 260 nm was measured by a GeneQuant pro RNA/DNA calculator spectrometer (Cambridge, England). The remaining activity (%) = $[(A_s - A_b) - (A_{c+} - A_b)]/[(A_{c-} - A_b) - (A_{c+} - A_b)] \times 100$, where A_{c-} is the absorbance of the control (RNase A and tRNA solution), A_{c+} is the absorbance of the AGEs control (RNase A, MGO and tRNA solution), A_b is the absorbance of a blank (MGO and tRNA solution), and A_s is the absorbance of the sample (RNase A, MGO, sample, and tRNA solution).

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References

1) Thomas MC, Baynes JW, Thorpe SR, and Cooper ME, *Curr. Drug Targets*, **6**, 453–474 (2005).
2) Vlassara H, Fuh H, Makita Z, Krungkrai S, Cerami A, and Bucala R, *Proc. Natl. Acad. Sci. USA*, **289**, 12043–12047 (1992).
3) Semba RD, Nicklett EJ, and Ferrucci L, *J. Gerontol. A Biol. Sci. Med. Sci.*, **65**, 963–975 (2010).
4) Sasaki N, Fukatsu R, Tsuzuki K, Hayashi Y, Yoshida T, Fujii N, Koike T, Wakayama I, Yanagihara R, Garruto R, Amano N, and Makita Z, *Am. J. Pathol.*, **153**, 1149–1155 (1998).
5) Reddy VP, Zhu X, Perry G, and Smith MA, *J. Alzheimers Dis.*, **16**, 763–774 (2009).
6) Forbes JM, Yee LTL, Thallas V, and Lassila M, *Diabetes*, **53**, 1813–1823 (2004).
7) Brownlee M, *Nature*, **414**, 813–820 (2001).
8) Thornalley JP, *Endocrinol. Metab.*, **3**, 149–166 (1996).
9) Vasan S, Zhang X, Kapurnitotu A, Bernhagen J, Teichberg S, Basgen J, Wagle D, Shih D, Terlecky I, Bucala R, Cerami A, Egan J, and Ulrich P, *Nature*, **382**, 275–278 (1996).
10) Wolffenbuttel BHR, Boulanger CM, Crijns FRL, Huijberts MSP, Poitevin P, Swennen GNM, Vasan S, Egan JJ, Ulrich P, Cerami A, and Levy BI, *Proc. Natl. Acad. Sci. USA*, **95**, 4630–4634 (1998).
11) Goh SY and Cooper ME, *J. Clin. Endocrinol. Metab.*, **93**, 1143–1152 (2008).
12) Vasan S, Foiles P, and Founds H, *Arch. Biochem. Biophys.*, **419**, 89–96 (2003).
13) Adorjan B and Buchbauer G, *Flavour Fragr. J.*, **25**, 407–426 (2010).
14) Stratakis M, Sofikiti N, Baskakis C, and Raptis C, *Tetrahedron Lett.*, **45**, 5433–5436 (2004).
15) Shailaja J, Sivagaru J, Robbins RJ, Ramamurty V, Snoj RB, and Chandrasekhar J, *Tetrahedron*, **56**, 6927–6943 (2000).
16) Bergmeyer HU, "Methods of Enzymatic Analysis" third ed., Academic Press, New York, pp. 136–139 (1984).
17) Li D, Shinya M, and Ubukata M, *Pharmaceut. Biol.*, Early Online: 1–5 (2012).
18) Lu J, Randell E, Han Y, Adeli K, Krahn J, and Meng QH, *Clin. Biochem.*, **44**, 307–311 (2011).
19) Beisswenger PJ, Howell SK, Touchette AD, Lal S, and Szwergold BS, *Diabetes*, **48**, 198–202 (1999).
20) Yan J, Travis BR, and Borhan B, *J. Org. Chem.*, **69**, 9299–9302 (2004).
21) Nilsson U, Bergh M, Shao LP, and Karlberg AT, *Chromatographia*, **42**, 199–205 (1996).
22) Matura M, Goosens A, Bordalo O, Garcia-Bravo B, Magnusson K, Wrangsjo K, and Karlberg AT, *Contact Dermatitis*, **49**, 15–21 (2003).
23) Rudbäck J, Bergström MA, Börje A, Nilsson U, and Karlberg AT, *Chem. Res. Toxicol.*, **25**, 713–721 (2012).
24) Carson CF and Riley TV, *J. Appl. Bacteriol.*, **78**, 264–269 (1995).
25) Hammer KA, Carson CF, and Riley TV, *J. Antimicrob. Chemother.*, **42**, 591–595 (1998).
26) Carson CF, Ashton L, Dry L, Smith DW, and Riley TV, *J. Antimicrob. Chemother.*, **48**, 450–451 (2001).
27) Seyed TA, Qureshi ZA, Ali SM, Ahned S, and Ahmed SA, *Trop. Med. Int. Health*, **4**, 284–287 (1999).
28) Bassett IB, Pannowitz DL, and Barnetson RS, *Med. J. Aust.*, **153**, 455–458 (1990).
29) Cross SE, Russell M, Southwell I, and Roberts MS, *Eur. J. Pharm. Biopharm.*, **69**, 214–222 (2006).
30) Reichling J, Landvatter U, Wagner H, Kostka KH, and Schaefer UF, *Eur. J. Pharm. Biopharm.*, **64**, 222–228 (2008).