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Natural Antioxidants Isolated from *Eucalyptus* Leaf Waxes

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In the course of our search for novel types of natural antioxidants from leaf waxes of *Eucalyptus globulus*, 4-hydroxytritriacontane-16,18-dione was newly isolated and identified in addition to the known 16-hydroxy-18-tritriacontanone. 4-Hydroxytritriacontane-16,18-dione showed strong antioxidative activity in a water/alcohol system measured by thiocyanate and TBA methods, but had no activity in an oil system. The diketones with long alkyl side chains showed strong activity in comparison with other β-diketone analogues.

Recently, much attention has been focused on natural antioxidants which are preferred over synthetic antioxidants, such as BHA and BHT, from a food safety viewpoint. Although many natural antioxidants have been found in numerous plant materials (Dugan, 1979), tocopherols only are now widely used as the safe natural antioxidants. However, they have the limitation that they are not as effective as synthetic antioxidants when used alone (Emanuel and Lyaskovskaya, 1967) and also involve high manufacturing costs.

With these objectives, we started our new project to isolate a new type of antioxidant from natural resources, in particular, from *Eucalyptus* leaf waxes. This attempt was mainly with the assumption that *Eucalyptus* leaf waxes may provide chemical protection to endogenous essential oils from oxidative degradation as well as physical protection and may be a good source for natural antioxidants, and we have isolated and identified the novel β-diketone type antioxidant, *n*-tritriacontane-16,18-dione, in the leaf wax extracted from *Eucalyptus globulus* and also suggested the presence of other minor antioxidative substances (Osawa and Namiki, 1981).

This paper reports the isolation and structural elucidation of two new β-diketone analogues (S-2A and S-2B) and their antioxidative activities in model systems. The relationship between structure and antioxidative activity of β-diketone derivatives has also been investigated.

MATERIALS AND METHODS

Extraction of Leaf Wax. *Eucalyptus globulus* leaves (13.4 kg), collected from the Botanical Garden in Hekinan City, Aichi, Japan, in Aug, 1981, were extracted three times with 3 L of chloroform. The chloroform extract was filtered and concentrated in vacuo to give 36.7 g of leaf wax.

Purification of Antioxidative Substances. The leaf wax was charged on a silica gel column (Wako Gel C-100, Wako Chemicals Co. Ltd.) and eluted with hexane with stepwise increase in the volume of ethyl acetate. Two fractions eluted with *n*-hexane–ethyl acetate in the ratio of 9:1 (v/v) and 8:2 (v/v) showed antioxidative activity in a water/alcohol system measured by the thiocyanate method. The fraction eluted with *n*-hexane–ethyl acetate (9:1, v/v) was expected to contain some minor active components besides the known *n*-tritriacontane-16,18-dione and purified by preparative TLC (Merck 60 F_{254}, 0.5-mm thickness) with *n*-hexane–acetone (9:1, v/v) as the solvent system. Fraction at *Rf* 0.8 was scraped off and further purification of this fraction was carried out by preparative HPLC [Waters associates M-6000, column, Develosil 60-10/20 (i.d. 8 diameter × 500 mm); detector, UV_{254nm}; solvent system, *n*-hexane–ethyl acetate (20:1 v/v); flow rate, 4.0 mL/min. The main peak, whose retention time was 32.0 min, was collected and it gave S-2A as pure crystal (yield, 100 mg). The fraction eluted with *n*-hexane–ethyl acetate (8:2, v/v) exhibited the strongest activity and was purified by preparative TLC and HPLC. *n*-Hexane–acetone (5:1, v/v) was used as the solvent system for separation of S-2B by preparative TLC (*Rf* value of S-2B was 0.6), *n*-hexane–ethyl acetate–acetic acid (1000:40:1, v/v) was used for preparative HPLC, and other conditions were the same as those for separation of S-2A. Pure S-2B (80 mg) was obtained from the peak (retention time, 18.0 min) by preparative HPLC.

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Antioxidative Assays. Antioxidative assays were carried out by the methods described in the previous report (Osawa and Namiki, 1981).

(1) **Thiocyanate Method.** Each sample (200 μg dissolved in 100 μL of chloroform) was added to a solution mixture of linoleic acid/99.0% ethanol/0.2 M phosphate buffer. The mixed solution in a conical flask was incubated at 40 °C and the peroxide value was determined by reading the absorbance at 500 nm after a coloring reaction with $FeCl_2$ and thiocyanate at intervals during incubation (Mitsuda et al., 1966).

(2) **Thiobarbituric Acid (TBA) Method.** The mixed solution was prepared and incubated as described above. The formation of malonaldehyde was measured by reading the absorbance at 532 nm after the reaction with thiobarbituric acid (Ottolenghi, 1959).

(3) **Weighing Method.** Each sample (200 μg/100 μL chloroform) was placed in a petri dish and chloroform was completely removed by a stream of nitrogen. Linoleic acid (1 g) was added into the petric dish and the rate of oxidation of oil was determined by weighing the petri dish at definite intervals (Olcott and Einset, 1958).

Instruments for Structure Elucidation. UV: Hitachi 200-10 spectrometer. IR: Jasco-A-3. NMR: Jeol JNM-FX-100. Mass spectrum: Jeol JNM-D-100. High resolution mass spectrum: Jeol JMS-01SG.

Me_3Si Derivative of S-2A and S-2B. S-2A or S-2B (5 mg) was dissolved in anhydrous pyridine (0.3 mL) and mixed with 0.1 mL of hexamethyldisilazane. The mixed solution was kept at 80 °C for 30 min, dissolved in 10 mL of chloroform, washed with 5 mL of water twice, and concentrated in vacuo. The Me_3Si derivatives of S-2A and S-2B were purified by preparative HPLC using the same conditions for separation of S-2A and S-2B.

β-Diketone Analogues. Acetyl acetone and curcumin were purchased from Wako Chemical Co. Ltd. and *n*-duodecoylacetone from Dojin Chemical Co. Ltd. Syncarpic acid was synthesized from phloroglucinol in 30% yield by the method described previously (Crow et al., 1976). *n*-Duodecoylacetone was obtained in 29% yield by an established method (Morgan and Holmes, 1925).

RESULTS AND DISCUSSION

To determine the chemical structure of S-2A and S-2B, instrumental analyses were carried completed. S-2A: mp 89–90 °C. Elemental Anal. Calcd for $C_{33}H_{66}O_2$: C, 80.99; H, 13.44. Found: C, 80.15; H, 13.54. IR ν_{max} (chloroform) 3550 (OH), 2880 and 1460 (CH), and 1770 cm^{-1} (CO); UV ($CHCl_3$) spectrum showed only an end absorption; ^1H NMR δ ($CDCl_3$, Me_4Si) 0.9 (t, CH_3), 1.2 (m, $(CH_2)_n$), 2.3–2.6 (m, CH_2COCH_2), and 4.0 (m, CH(OH)); ^{13}C NMR δ ($CDCl_3$, Me_4Si) 14.0 (q, CH_3), 23.5 (q, CH_2CH_3), 29.0 (m, $(CH_2)_nCH_2CH_2CH_3$), 32.2 (t, $CH_2CH_2CH_3$), 43.0 (t, $COCH_2CH_2$), 48.5 (t, $COCH_2CH(OH)$), 68.2 (d, CH(OH)), and 217.0 (s, CO). S-2A gave a molecular ion peak at *m/z* 494.5091 (494.5120 calcd for $C_{33}H_{66}O_2$) by the high resolution mass spectrum and other fragment peaks were *m/z* 476 ($M^+ - H_2O$), 294, 283 ($M^+ - C_{15}H_{31}$), 281, 265 ($M^+ - H_2O - C_{15}H_{31}$), and 239 ($M^+ - C_{17}H_{35}O$). It was indicated that S-2A has no β-diketone moiety because it did not show any absorption due to β-diketone system in the UV spectrum (Osawa and Namiki, 1981) and has no protons of keto–enol tautomerism of β-diketone. These data suggested that one of the carbonyl groups of the β-diketone systems was reduced to a hydroxy group. In order to determine the position of the hydroxy group, the mass spectrum of the Me_3Si derivative of S-2A was measured. A strong peak was observed at *m/z* 355 ($M^+ - C_{15}H_{31}$) together with a molecular ion peak at *m/z* 566 and other fragment peaks at *m/z* 551, 522, and 239, and the hydroxy group was confirmed to be present at the C-16 position and that one of the carbonyl groups of *n*-tritriacontane-16,18-dione was reduced to a secondary hydroxy group.

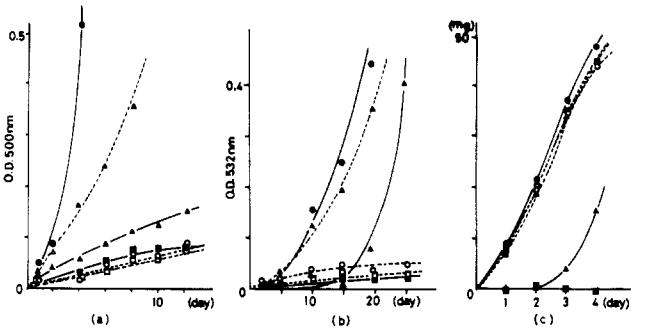

Figure 1. Antioxidative activity of the isolated antioxidants from *E. globulus*: (●) control; (▲) α-tocopherol; (■) BHA; (□) *n*-tritriacontane-16,18-dione; (△) S-2A; (○) S-2B. (a) Thiocyanate method. (b) Thiobarbituric acid test (TBA method). (c) Weighing method.

On the other hand, spectroscopic data of S-2B suggested the presence of a β-diketone system in its structure. S-2B: mp 77–78 °C. Elemental Anal. Calcd for $C_{33}H_{64}O_3$: C, 77.89; H, 12.67. Found: C, 77.80; H, 12.61. IR ν_{max} (chloroform) 3540 (OH), 2910, 2850 and 1460 (CH), and 1700 and 1600 cm^{-1} (β-diketone); UV λ_{max} (*n*-hexane) 275 nm (ϵ 8 × 10^3); ^1H NMR δ ($CDCl_3$, Me_4Si) 0.9 (t, CH_3), 1.2 (m, $(CH_2)_n$), 2.3 (t, $COCH_2CH_2$), 3.5 (s, $COCH_2CO$), 3.9 (m, CH(OH)), and 5.5 (s, COCH=C(OH)); ^{13}C NMR δ ($CDCl_3$, Me_4Si) 14.2 (q, CH_3), 23.0 (t, CH_2CH_3), 29.3 (m, $(CH_2)_n$), 32.2 (t, $CH_2CH_2CH_3$), 44.3 (t, $COCH_2CO$), 72.0 (d, CH(OH)), 99.5 (d, COCH=C(OH)), 115.0 (s, COCH=C(OH)), and 194.0 (s, CO). S-2B showed typical absorption of β-diketone in the IR and UV spectra and protons at δ 3.5 and 5.5 (1:6) in the ^1H NMR spectrum were ascribed to keto–enol tautomerism of β-diketone (Osawa and Namiki, 1981). In the ^{13}C NMR spectrum, methylene and carbonyl carbons of keto form were observed at δ 44.3 and 194.0, respectively, and olefinic carbons of the enol form were observed at δ 99.5 and 115.0. S-2B gave a molecular ion peak at *m/z* 508 (M^+) and other fragment peaks at *m/z* 490, 472, 466, 309, 298, 281, and 239 (base peak). By the high resolution mass spectrum, the molecular ion peak was not observed but the ($M^+ - H_2O$) peak was observed at *m/z* 490.4737 (490.4725 calcd for $C_{33}H_{62}O_2$) and other main fragment peaks at *m/z* 281 and 239 were ascribed to ($M^+ - C_{15}H_{31}O$) and ($M^+ - C_{17}H_{33}O_2$), respectively. A multiplet at δ 3.9 in the ^1H NMR spectrum and a doublet at δ 72.0 in the ^{13}C NMR spectrum suggested the presence of a secondary hydroxy group at one of the side chains of the β-diketone. The position of a hydroxy group was confirmed by the mass spectroscopic data of the Me_3Si derivative of S-2B. A strong fragment peak was observed at *m/z* 537 ($M^+ - C_3H_7$) and the position of the secondary hydroxy group was confirmed at C-4. From these spectroscopic data, the structure of S-2A was determined as 16-hydroxy-18-tritriacontane and that of S-2B was determined as 4-hydroxytritriacontane-16,18-dione.

The antioxidative activity of S-2A and S-2B was examined by several model systems by using the thiocyanate, TBA, and weighing methods. As shown in Figure 1 part a, S-2B showed a marked antioxidative activity, and it was as effective as *n*-tritriacontane-16,18-dione in the water/alcohol system whereas S-2A showed only a weak antioxidative activity measured by the thiocyanate method. It was also shown that S-2B inhibited the production of malonaldehyde as strong as *n*-tritriacontane-16,18-dione,

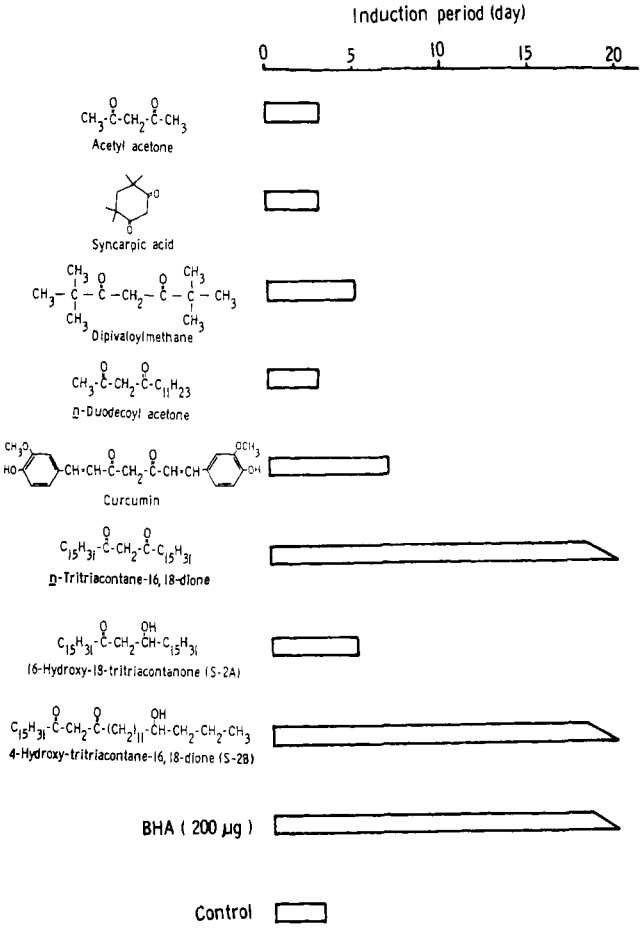

Figure 2. Antioxidative activity of β-diketone derivative determined by the thiocyanate method.

but S-2A was slightly active when measured in the water/alcohol system by the TBA method (Figure 1 part b). A confirmatory experiment on the antioxidative activity in the oil system was carried out and the rate of oxidation of linoleic acid was measured. As shown in Figure 1 part c, all of the leaf wax components had no antioxidative activity measured by the weighing method. These data suggest that the presence of a hydroxy group at the side chain has no influence on antioxidative activity in the water/alcohol systems. However, the β-diketone moiety is essential for antioxidative activity in the aqueous system (Osawa and Namiki, 1981). To establish a relationship between the antioxidative activity and structure, antioxidative activity of different kinds of β-diketone analogues was measured by the thiocyanate method. As shown in Figure 2, strong antioxidative activity was observed in *n*-tritriacontane-16,18-dione and S-2B, however, acetyl acetone, the simplest β-diketone, and syncarpic acid, a cyclic β-diketone, had no antioxidative activity. Long hydrocarbon side chains on both sides of β-diketones seem to be essential for antioxidative activity because *n*-duodecoylacetone which has one long hydrocarbon side chain did not show any antioxidative activity. Although the minimum hydrocarbon length for revealing the antioxidative activity is not known, it is speculated that S-2B is active as the enol form in the presence of water and that hydrophobic side chains of S-2B are supposed to interact with the unsaturated moiety of linoleic acid and exhibited the antioxidative activity because acetylacetone, whose ratio of keto–enol tautomerism is also 1:6, had no antioxidative activity (Osawa and Namiki, 1981). It is also assumed that S-2B may act as the chelating agent (metal inactivator) to form a complex with transition metals contained in the system, but this assumption is denied because most β-diketones which have no hydrophobic side chains can also act as the strong chelating agents but they have no antioxidative activity. The reason why long-chain β-diketones showed no antioxidative activity in the oil system determined by the weighing method has not been clearly interpreted yet. The hydroxy group at the C-4 position of the hydrocarbon side chain in the structure of S-2B did not affect the antioxidative activity though it is expected that introduction of hydroxy groups to long alkane side chains by chemical synthesis would increase the solubility of long-chain β-diketones when used in oily foods. Although the possibility of utilization of long chain β-diketones for food systems is not clear, we hope that these β-diketone antioxidants can be used for oil/water food systems such as mayonaise, salad dressing, frozen meat, and fish products etc. In fact, we observed preliminarily that *Eucalyptus* β-diketones exhibited a strong antioxidative activity in the mayonaise system, and detailed experiments using other food systems are being undertaken. *Eucalyptus* essential oils are now widely used as additives for foods and cosmetics and for medicinal purposes. Many plant growth regulators have been isolated from *Eucalyptus* oils (Crow et al., 1973, 1977), which are also evaluated as the alternate source of renewable fuel (Nishimura et al., 1979, 1981).

β-Diketones and hydroxy β-diketones are common leaf wax components in many plants such as *Acacia* (Horn et al., 1964), *Rhododendron* (Evans et al., 1975), *Buxus* (Dierickx, 1973), *Triticum* (Tulloch and Hoffman, 1973a), *Barley* (Jackson, 1971), and *Oat* (Tulloch and Hoffman, 1973b). We have screened different kinds of leaf waxes for evaluation of antioxidative activity and observed that leaf waxes of various species of *Acacia* and *Rhododendron* also have strong antioxidative activity. By TLC and HPLC analyses, long chain β-diketones seem to be responsible for antioxidative activity and a detailed examination is being undertaken.

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Registry No. S-2A, 97191-42-9; S-2B, 97191-41-8; acetyl acetone, 123-54-6; syncarpic acid, 7181-79-5; dipivaloylmethane, 1118-71-4; *n*-duodecoylacetone, 53759-23-2; curcumin, 458-37-7; *n*-tritriacontane-16,18-dione, 24514-86-1.

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Organic and Inorganic Bromide Residues in Spices Fumigated with Methyl Bromide

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Two warehouses containing imported spices were fumigated with methyl bromide to eradicate a khapra beetle infestation. Methyl bromide (MB) and inorganic bromide (INBR) residues were determined in spices before and after fumigation by gas-liquid chromatography/electron capture detection (GC/ECD). A private laboratory analyzed spices for INBR with identical lot numbers as those analyzed with GC/ECD by using an ashing–titration method. The highest MB residue found in a before fumigated spice sample was 14.85 ppm in parsley. After the fumigation, the highest MB residue was found in Yugoslavian sage (65.78 ppm). INBR residues in spices before fumigation which were analyzed by GC/ECD showed none with residues in excess of the 200 ppm INBR tolerance for spices. Analysis of fumigated spices revealed two samples with INBR residues greater than 200 ppm. Ashing–titration analysis by the private laboratory showed seven spice samples with INBR residues exceeding 200 ppm before the fumigation and only four fumigated spice samples with residues in excess of 200 ppm. A comparison of residue levels obtained by the two methods is shown. Residues for INBR in selected samples were confirmed qualitatively and quantitatively by gas-liquid chromatography/mass spectrometry (GC/MS).

INTRODUCTION

In the fall of 1980, two warehouses in the northeastern United States which contained various spices from 27 foreign countries were found to be infested with khapra beetle (*Trogoderma granarium*). Both warehouses were covered with tarpaulins and fumigated with methyl bromide (MB) to eradicate the pest. Before the fumigation, representative spice samples were collected for MB and INBR residues. Seventy-two hours after the fumigation, spice samples were again collected from identical lot numbers as those collected before the fumigation for MB and INBR analysis. The spices were subsampled and one group was shipped in dry ice to the National Monitoring and Residue Analysis Laboratory (NMRAL) for MB and INBR residue determination by GC/ECD. The other group was sent to a private laboratory for INBR residue determination by an ashing–titration method.

Warehouse Size and Methyl Bromide Fumigation. The area of warehouse no. 1 was 3 400 000 cubic feet. Methyl bromide was applied to its contents at a rate of 7.5 lbs/1000 cubic ft for 12 h. Warehouse no. 2 contained 312 000 cubic feet and methyl bromide was applied at a rate of 6 lbs/1000 cubic ft for 12 h.

Spice samples were collected according to a biometric design developed by the Technology Analysis and Development Staff (TADS) of Plant Protection and Quarantine of the United States Department of Agriculture.

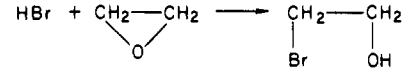
EXPERIMENTAL SECTION

(1) Methyl Bromide. Free methyl bromide (MB) was extracted and analyzed by using a modification of a method described by Scudamore and Heuser (1970). Ten grams of spice was extracted with 30–120 mL (extraction solvent volume was adjusted so that the entire sample was immersed) of a 5:1 V/V, distilled in glass, acetone–distilled water mixture in an amber bottle with a screw cap. All samples were allowed to stand at approximately 8 °C for 24 h before analysis to extract the MB.

Analyses were performed by using two dissimilar columns for qualitative and quantitative confirmation. The analytical instrument used was a Microtek MT-222 equipped with a Ni^{63} electron-capture detector. The columns employed were glass 1.8 m long × 4 mm i.d. One column was packed with 15% UCON LB-550X on 60/80 mesh on Chromosorb W-HP; the other was packed with 10% Carbowax 20M on 80/100 mesh, Gas Chrom Q. Instrument parameters were identical for both columns: injection port temperature, 160 °C; oven temperature, 55 °C; detector temperature, 325 °C; carrier gas, 95%/5% argon–methane; flow rate 20 mL/min.

The GC injection port contained a glass insert with a small piece of glass wool inside. The insert was cleaned and glass wool replaced daily since the samples were not subjected to a cleanup procedure before instrumental analysis.

(2) Inorganic Bromide (GC Procedure) Standard Preparation, Derivatization, and Determination in Samples. (A) Standard Preparation. 2-Bromomethanol was synthesized by the addition of hydrobromic acid to ethylene oxide as described in the following reaction.



The crude product was vacuum distilled and examined by GC/MS. Bromoethene and bis(2-bromoethyl) ether were formed in addition to 2-bromoethanol. Careful

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