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Studies on the Antioxidants. XV.[1] Combination Effects of Butylated Hydroxyanisole, Butylated Hydroxytoluene and Their Analogs on Hydrogen Donation to 2,2-Diphenyl-1-picrylhydrazyl

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Combination effects of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and their analogs on hydrogen donation to 2,2-diphenyl-1-picrylhydrazyl (DPPH) were investigated. A synergistic effect was observed when 2,6-di-*tert*-butylphenol derivatives were combined with 4-methoxyphenol derivatives such as BHA and 4-methoxyphenol. This effect might arise from the regeneration of 4-methoxyphenol derivatives from possible intermediates formed from DPPH and these phenols, with enhanced loss of the 2,6-di-*tert*-butylphenol derivatives. The formation of the cross coupling compound from a pair in combination might decrease the regeneration of the phenols and thus influence the synergistic effect.

Keywords——antioxidants; butylated hydroxyanisole; butylated hydroxytoluene; 4-methoxyphenol derivatives; hindered phenols; 2,2-diphenyl-1-picrylhydrazyl; synergistic effect

Hydrogen donation of antioxidants to the free radicals formed during propagation reactions is known to be one of the most important reactions inhibiting the autoxidation of oils and fats.[2,3] In the previous paper,[4] the combination effects of pairs of several antioxidants on hydrogen donation to 2,2-diphenyl-1-picrylhydrazyl (DPPH), a stable free radical, were examined, and a synergistic effect was observed with the combination of butylated hydroxyanisole (BHA)+butylated hydroxytoluene (BHT). In this reaction, an intermediate which may be formed by the interaction of BHA and DPPH seemed to play an important role in the synergism, but the mechanism has not been elucidated. This paper deals with the combination effects of BHA, BHT and their analogs on hydrogen donation to DPPH, and with the interactions between pairs of these phenols. The relationship between the synergistic effect and the structure of phenols in combination is discussed.

Experimental

Materials——DPPH (Tokyo Kasei Kogyo Company, Ltd.) was recrystallized repeatedly from benzene-ether mixture.[5] BHA and BHT, both supplied by Nikki-Universal Company, Ltd., were recrystallized from petroleum ether[6] and ethanol, respectively. 4-Methoxyphenol (MP) and 2-*tert*-butyl hydroquinone (TBHQ) were products of Tokyo Kasei Kogyo Company, Ltd. and Wako Pure Chemical Industries, Ltd., respectively. 2,6-Di-*tert*-butylphenol (DBP) and 2,4,6-tri-*tert*-butylphenol (TBP), both obtained from Tokyo Kasei Kogyo Company, Ltd., were recrystallized from petroleum ether and ethanol, respectively. 2,2′-Dihydroxy-5,5′-dimethoxy-3,3′-di-*tert*-butylbiphenyl (A-I) and 2′,3-di-*tert*-butyl-2-hydroxy-4′,5-dimethoxybiphenyl ether (A-II) were isolated by silica gel column chromatography from UV-irradiated BHA-benzene solution.[6] 1,2-Bis(3,5-di-*tert*-butyl-4-hydroxyphenyl) ethane (T-I) and 3,5,3′,5′-tetra-*tert*-butylstilbenequinone (T-II) were obtained by oxidation of BHT according to the method of Cook *et al.*[7] 4,4′-Dihydroxy-3,5,3′,5′-tetra-*tert*-butylbiphenyl (D-I) and 3,5,3′,5′-tetra-*tert*-butyl-4,4′-diphenoquinone (D-II) were obtained by oxidation of DBP according to the method of Kharash and Joshi.[8] 3,3′,5-Tri-*tert*-butyl-5′-methoxy-2,4′-dihydroxybiphenyl methane (AT-I) was isolated by silica gel column chromatography from a UV-irradiated equimolar mixture of BHA and BHT in benzene.[9] 2,6-Di-*tert*-butylquinone methide (QM) was obtained by debromination[10] of 2,6-di-*tert*-butyl-4-bromo-4-methylcyclohexa-3,5-dienone, which was itself prepared by bromination of BHT.[11]

2-(3′,5′-Di-*tert*-butyl-4′-hydroxyphenyl)-6-*tert*-butyl-4-methoxyphenol (AD-I)——A solution of potassium ferricyanide (3.3 g) and potassium hydroxide (0.5 g) in water (40 ml) was added to a solution of BHA (0.9 g)

and DBP (1.03 g) in benzene (20 ml). The mixture was stirred for 10 min at room temperature, then the benzene layer was separated and the solvent was removed *in vacuo*. The resulting oil was applied to a silica gel column (28 mm I.D. × 120 mm) and the column was eluted with hexane–toluene (2: 1) mixture. Eluates were subjected to TLC developed with hexane–benzene (1: 1) mixture and the eluate containing the compound of *Rf* 0.45 was evaporated to dryness *in vacuo*. A pale yellow plate-type residue (0.586 g, yield 31%) was obtained. Repeated recrystallization from ethanol gave white plates of AD-I, mp 167—168°C. Nuclear magnetic resonance spectrum (dimethylsulfoxide-d_6; internal standard, tetramethylsilane) δ, ppm: 1.35 (9H, s, 3-C$(CH_3)_3$), 1.40 (18H, s, 3′-C$(CH_3)_3 \times 2$), 3.64 (3H, s, OCH_3), 6.50 (1H, d, H_3 or H_5, J= 3 Hz), 6.63 (1H, d, H_3 or H_5, J=3 Hz), 7.16 (2H, s, $H_{3'}$ and $H_{5'}$). Mass spectrum (MS) m/e: 384 (M^+). *Anal.* Calcd for $C_{25}H_{36}O_3$: C, 78.08; H, 9.44. Found: C, 77.88; H, 9.45.

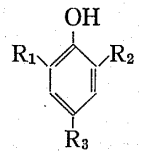
Analytical Methods——Gas chromatography (GLC) was carried out with a Yanaco G80 gas chromatograph equipped with a hydrogen flame ionization detector. The gas chromatograph with a glass column (3 mm I.D. × 2 m) of polyethylene glycol 20 M on 80—100 mesh Chromosorb W AW was operated isothermally at 190°C (column temperature) and at 210°C (injection temperature) with a carrier nitrogen flow of 25 ml/min for determination of BHA, and at 160°C (column temperature) and 170°C (injection temperature) with a carrier nitrogen flow of 25 ml/min for MP. For identification of QM, the gas chromatograph with a glass column (3 mm I.D. × 3 m) of silicone OV-17 on 60—80 mesh Chromosorb W AW was operated at 170°C (column temperature) and 190°C (injection temperature) with a nitrogen flow of 25 ml/min; the retention time was 5.2 min. For identification of AD-I, the gas chromatograph with a glass column (3 mm I.D. × 2 m) of silicone gum SE-30 on Chromosorb W AW was operated at 210°C (column temperature) and 230°C (injection temperature) with a nitrogen flow of 40 ml/min; the retention time was 8.5 min. High performance liquid chromatography (HPLC) was performed on a Shimadzu LC-2 liquid chromatograph with a Shimadzu SPD-1 spectrophotometric detector and a stainless steel column of Zorbax ODS (4.6 mm I.D. × 0.25 m). HPLC was carried out with ethanol–methanol–water (5: 3: 2) elution at 0.5 ml/min. QM was detected at 280 nm and its retention time was 26 min. Nuclear magnetic resonance spectra were taken with a JEOL PS-100 machine. Mass spectra were taken with a Hitachi RMU-7L mass spectrometer. A Hitachi 101 spectrophotometer or a Shimadzu UV-200S double-beam spectrophotometer was used for the measurement of absorbance. Thin layer chromatography (TLC) was performed with Wakogel B5 F (Wako Pure Chemical Industries, Ltd.). Silica gel (100 mesh, Kanto Chemical Campany, Ltd.) was used for column chromatography.

Estimation of Decrease in DPPH Concentration——A solution of BHA, BHT or an analog was added to a solution of DPPH (1 mM) in benzene (1.0 ml), and the volume of the mixture was adjusted to 20 ml with benzene. Each mixture was permitted to stand at 20°C in a thermostatically controlled water bath. The absorbance of the mixture at 520 nm was measured at selected times. The concentration of DPPH was calculated from the absorbance according to the equation reported by Boguth and Repges.[12]

Results and Discussion

Phenolic compounds having a methoxyl group on the *para*-position (MP) and having one *tert*-butyl group on the *ortho*-position (TBHQ) were chosen as analogs of BHA, and compounds having the two *tert*-butyl groups on the *ortho*-positions (DBP and TBP) were chosen as analogs of BHT. These compounds were treated with a 4-fold molar excess of DPPH at 20°C in benzene. The time courses of decrease in DPPH concentration are illustrated in Fig. 1. The DPPH concentration was decreased rapidly by TBHQ, reaching a minimum at 10 min after the addition of TBHQ, when 2 mol of DPPH was lost. MP gradually consumed about one mol of DPPH in 60 min. The losses of DPPH with DBP and TBP were very slow; less than 0.04 mol of DPPH was lost after 60 min.

Various pairs of BHA, BHT and their analogs were combined in a molar equivalent ratio, and these solution were treated with a 4-fold molar excess of DPPH. The ratio of the decrease in DPPH concentration caused by the combined phenols to the mean value of the decrease caused by each of the two phenols was calculated and the results for the various combinations are listed in Table I. Synergistic effect was observed in six combinations, BHA+BHT, BHA+DBP, BHA+TBP, MP+BHT, MP+DBP and MP+TBP. Like the combination of BHA+BHT,[4] the combination of MP+BHT showed a marked synergistic effect, the ratio being larger than 2.0. No synergistic effect was observed in the combinations of TBHQ with other phenols. The 2,6-di-*tert*-butylphenol derivatives such as BHT, DBP and TBP gave very slow losses of DPPH when they were treated alone, but the loss of DPPH was



	R_1	R_2	R_3
BHA;	H	*tert*-butyl	methoxy
MP;	H	H	methoxy
TBHQ;	H	*tert*-butyl	OH
BHT;	*tert*-butyl	*tert*-butyl	methyl
DBP;	*tert*-butyl	*tert*-butyl	H
TBP;	*tert*-butyl	*tert*-butyl	*tert*-butyl

Chart 1

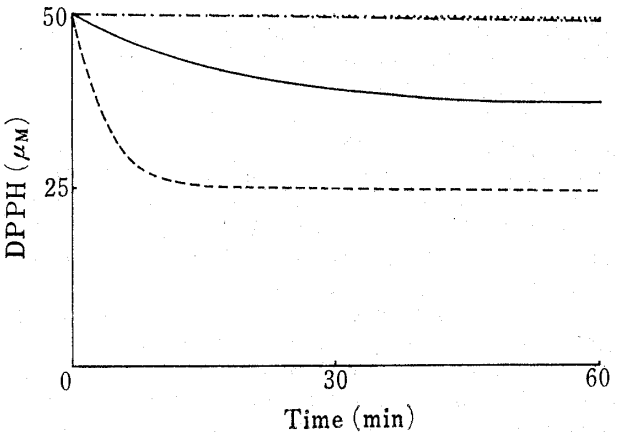


Fig. 1. Time Courses of Decrease in DPPH Concentration caused by Analogs of BHA and BHT

Mixtures of DPPH (50 μM) and each of the analogs (12.5 μM) were treated at 20°C in benzene.
MP; ——, TBHQ; – – –, DBP; —·—·, and TBP; ·····.

Table I. Combination Effects of BHA, and Their Analogs on Hydrogen Donation to DPPH

	MP	TBHQ	BHT	DBP	TBP
BHA	0.98	0.95	2.78	1.45	1.54
MP		0.93	2.07	1.47	1.71
TBHQ			1.06	1.02	1.02
BHT				—a)	—a)
DBP					—a)

Two phenols, separately or combined in an equivalent ratio (total concentration: 12.5 μM), were treated with DPPH (50 μM) at 20°C in benzene for 60 min. The ratio of the loss of DPPH caused by the combined antioxidants to the mean value of the losses obtained with each antioxidant alone are tabulated.
a) The losses of DPPH with the two phenols, separately and combined, were less than 4%.

significantly enhanced when they were combined with 4-metyoxyphenol derivatives such as BHA and MP.

Recovery of BHA in the reaction of BHA+BHT, DBP or TBP with DPPH was followed by GLC (Table II). When BHA (0.5 mM) was treated with DPPH (1 mM) ,BHA was rapidly lost and the concentration of BHA that remained after 10 min was 0.21 mM. When BHT, DBP and TBP (0.5 mM) were added 10 min after this reaction, the concentrations of BHA were increased to 0.44, 0.28 and 0.26 mM, respectively. Higher recoveries of BHA were obtained when 1 mM BHT, DBP and TBP were added. When BHT, DBP and TBP were added at the start of the reaction, the losses of BHA were markedly retarded as compared with the reaction of BHA alone. We suggested previously the formation of a possible intermediate from BHA and DPPH that can regenerate BHA upon addition of BHT.[4] Similar regeneration of BHA from the reaction mixture of BHA and DPPH was observed on addition of other 2,6-di-*tert*-butylphenol derivatives, but the amounts of BHA regenerated by BHT, DBP and TBP decreased in that order.

Losses of BHT and DBP and formation of their oxidation products were studied by TLC (Fig. 2). When these phenols (1 mM) were treated with DPPH (2 mM) for 10 min (a), two main spots corresponding to DPPH and each phenol added were observed, and oxidation products of the phenols were rarely found on the chromatograms. BHT and DBP were greatly diminished by the addition of BHA (1 mM) (b). Loss of TBP with DPPH was also

Table II. Recovery of BHA from the Reaction Mixture of DPPH and BHA upon Addition of BHT, DBP or TBP

Initial concentration		Phenol added (mM)		BHA recovered (%)	
DPPH (mM)	BHA (mM)			Reaction I	Reaction II
1.0	0.5	None		40	39
		BHT	(0.5)	97	88
		BHT	(1.0)	98	90
		DBP	(0.5)	65	56
		DBP	(1.0)	71	63
		TBP	(0.5)	54	52
		TBP	(1.0)	58	55

A phenol was added to the mixture of DPPH and BHA (20°C) at the start of the reaction (reaction I) or 10 min after DPPH and BHA had been mixed (reaction II). The concentration of BHA was determined by GLC 20 min after the phenol had been added.

enhanced by the addition of BHA. The enhancement of loss of DPPH observed in the combinations of BHA with the 2,6-di-*tert*-butylphenol derivatives may be explained by an increase in the rate of loss of the latter phenols, whose loss was very slow in reaction alone with DPPH. When BHA and BHT were treated with DPPH, the substance showing *Rf* 0.65 was identified as QM by comparing its *Rf* value on TLC and retention times in GLC and HPLC with those of an authentic sample.[7] When BHA and DBP were treated with DPPH, the dimers of DBP, D-I (*Rf*; 0.60) and D-II (*Rf*; 0.54), and a cross coupling compound of BHA and DBP, AD-I

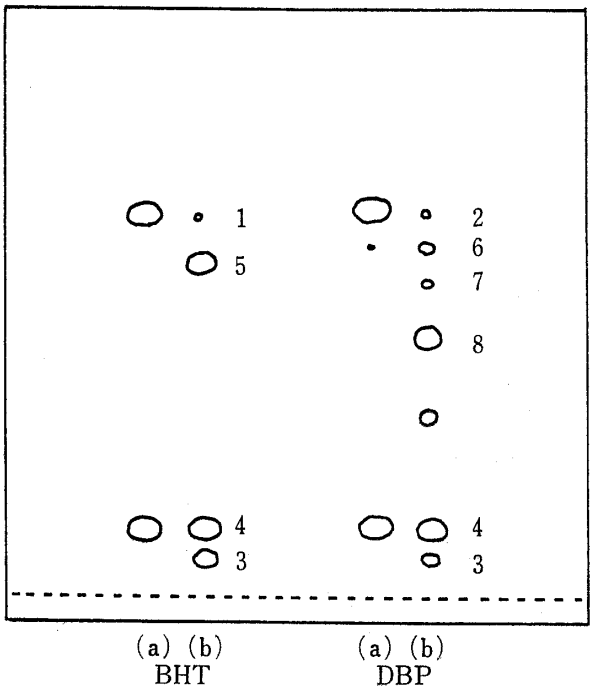


Fig. 2. Thin Layer Chromatograms of BHT and DBP treated with DPPH with or without BHA

DPPH (2.0 mM) and BHT or DBP (1.0 mM) were treated at 20°C for 10 min with (b) or without (a) BHA (1.0 mM). A 30 μl portion of the reaction mixture was applied to a silica gel thin layer chromatoplate and developed with benzene: hexane (1: 1) mixture. The phenols and their oxidation products were visualized by spraying 2,6-dichloroquinonechloroimide solution in ethanol. 1; BHT, 2; DBP, 3; BHA, 4; DPPH and $DPPH_2$, 5; QM, 6; D-I, 7; D-II and 8; AD-I.

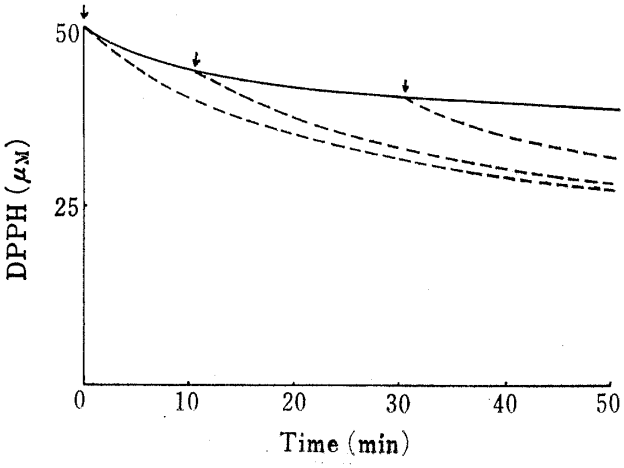


Fig. 3. Time Courses of Decrease in DPPH Concentration caused by the Combination of MP and BHT

A solution of MP and DPPH (20 ml) was treated at 20°C in benzene (——). A solution containing MP, BHT and DPPH (20 ml) was treated at 20°C in benzene (– – –); 1 ml of BHT solution was added to 19 ml of MP and DPPH solution at the times indicated by arrows. Final concentrations of MP, BHT and DPPH were 10, 10 and 50 μM, respectively.

(*Rf*; 0.45), were formed. Formation of AD-I was comfirmed by GLC, and the concentration in the reaction mixture was determined to be 0.11 mM. Formation of this coupling compound might decrease the regeneration of BHA and thus influence the synergistic effect on hydrogen donation to DPPH.

The time courses of decrease in DPPH concentration by MP and BHT were followed (Fig. 3). When MP (10 μM) was treated with DPPH (50 μM), DPPH was gradually consumed and the concentration of DPPH was 39 μM after 60 min. When BHT (10 μM) was added to the reaction mixture of MP and DPPH at the start of the reaction, loss of DPPH was greatly enhanced and 27.5 μM DPPH remained at 60 min. Similar enhancement of loss of DPPH was observed upon addition of BHT at both 10 min and 30 min after the start of the reaction. Fig. 4 shows the time courses of change of MP. When MP (1 mM) was treated with DPPH (2 mM) for 10 min, the concentration of MP was 0.18 mM. Addition of BHT (5 mM) to this reaction mixture increased the concentration of MP to 0.48 mM. Much higher recovery (95%) of MP was observed when BHT was added at the start of the reaction. In the combination of MP and BHT, the synergistic effect and the regeneration of MP were similar to those observed in the combination of BHA and BHT.[4] There might be similar interactions between the pairs of phenols in the combinations of BHA+BHT and MP+BHT upon treatment with DPPH.

Dimers of BHA (A-I and A-II), dimers of BHT (T-I and T-II) and a cross coupling compound of BHA and BHT (AT-I) were also examined in this study, because many of the substi-

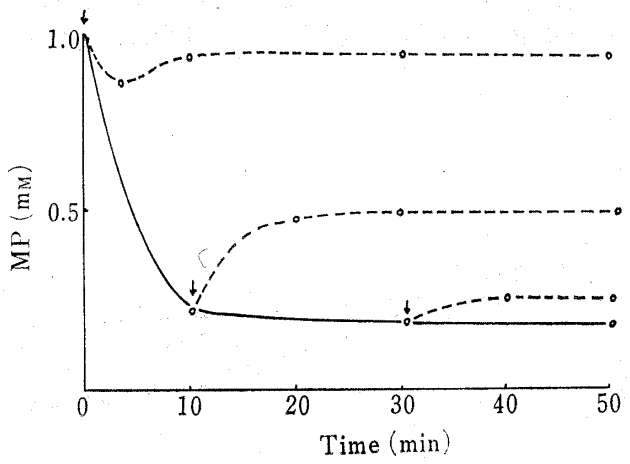


Fig. 4. Effect of BHT on the Recovery of MP in the Reaction with DPPH

A solution (10 ml) of MP (1.0 mM) and DPPH (2.0 mM) (——) was supplemented with 11 mg of BHT (----) at the times indicated by arrows. The concentration of MP was determined by GLC.

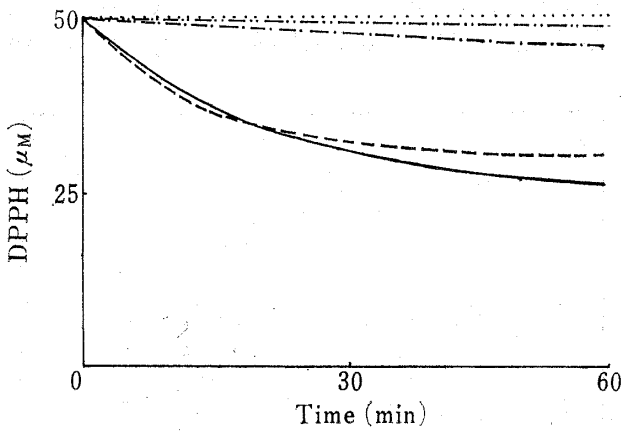


Fig. 5. Time Courses of Decrease in DPPH Concentration caused by the Dimers of BHA and BHT

Mixtures of DPPH (50 μM) and one of the dimers (12.5 μM) were treated at 20°C in benzene.
A-I; ——, A-II; —·—·, T-I; —··—··, T-II; ····· and AT-I; — — —.

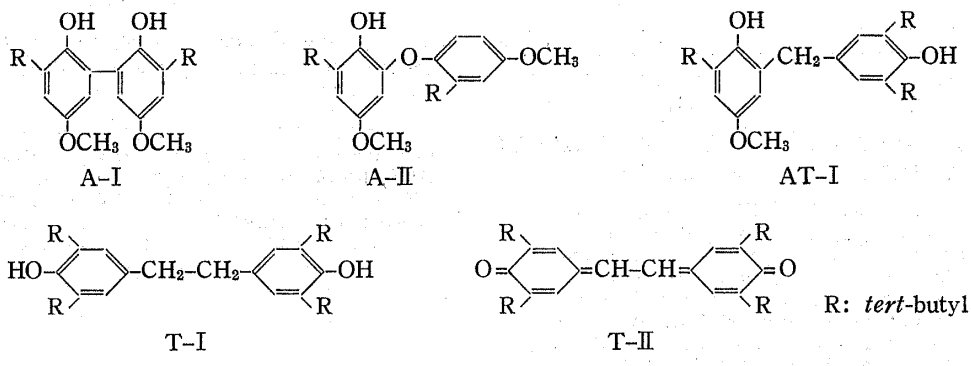


Chart 2

tuents of the parent antioxidants remain in these compounds. They were each treated with a 4-fold molar excess of DPPH. The time courses of decrease in DPPH concentration are illustrated in Fig. 5. A-I and AT-I gradually consumed 2 mol and 1.8 mol of DPPH during 60 min, respectively. The losses of DPPH with A-II and T-I were very slow and only 0.2 mol and 0.05 mol of the reagent were lost after 60 min. T-II did not consume a significant amount of DPPH.

Each of these dimers was combined with BHA or BHT in a molar ratio of 1: 2 and the mixture was treated with DPPH for 60 min at 20°C. The ratio of the decrease in DPPH concentration caused by a pair to the mean value of the decrease caused by each of them separately was calculated and the results are listed in Table III. Among 10 combinations, BHA+A-I, BHA+T-I, BHA+AT-I, BHT+A-I and BHT+AT-I gave ratios higher than 1, and marked synergistic effects were observed in the combinations of BHA+T-I (2.28) and BHT+AT-I (4.06).

Table III. Combination Effects of BHA and BHT with Their Dimers on Hydrogen Donation to DPPH

	A-I	A-II	T-I	T-II	AT-I
BHA	1.10	0.98	2.28	1.00	1.22
BHT	1.73	1.03	—a)	—a)	4.06

Mixtures of BHA or BHT (12.5 μM) and each of the dimers (6.25 μM) were treated with DPPH (50 μM) at 20°C in benzene for 60 min. The ratio of the loss of DPPH was calculated as described in Table I.
a) The losses of DPPH caused by the pair, separately and combined, were less than 4%.

Table IV. Recovery of BHA from the Reaction Mixture of DPPH and BHA upon Addition of A-I, T-I or AT-I

Initial concentration		Dimer added (mM)		BHA recovered (%)	
DPPH (mM)	BHA (mM)			Reaction I	Reaction II
2.00	1.00	None		39	37
		A-I	5.0	96	85
		T-I	5.0	97	83
		AT-I	5.0	94	80

A dimer was added to the mixture of DPPH and BHA (20 C) at the start of the reaction (reaction I) or 10 min after DPPH and BHA had been mixed (reaction II). The concentration of BHA was determined by GLC 20 min after the dimer had been added.

Recoveries of BHA in the reactions of BHA+A-I, T-I and AT-I with DPPH were measured and the reasults are listed in Table IV. When A-I, T-I or AT-I was added to the reaction mixture of BHA and DPPH at the start of the reaction, 94—97% of BHA was recovered after 20 min, while BHA was rapidly lost (about 40% remaining after 10 min) without addition of these dimers. High recoveries of BHA were also obtained when these dimers were added to the reaction mixtures of BHA and DPPH 10 min after the reaction.

Thin layer chromatograms of BHA+T-I and BHT+AT-I treated with DPPH are shown in Fig. 6. When BHA (1 mM) and T-I (0.5 mM) were treated with DPPH (2 mM) for 10 min (b), the spot corresponding to T-I was rarely found and an orange-colored spot corresponding to T-II was formed. It is evident that the loss of T-I was significantly enhanced by BHA when compared with that of T-I alone. When BHA was combined with T-I, (which resembles BHT in having 2,6-di-*tert*-butyl and 4-alkyl groups), the syntergisic effect and the regeneration of BHA with enhanced loss of T-I were observed as in the combination of BHA and BHT. There might be similar interactions between the pairs of phenols in the combinations of BHA+

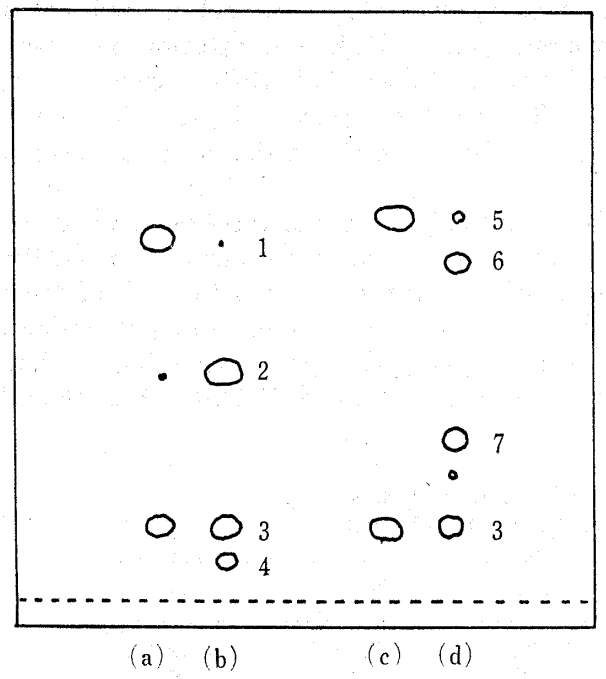


Fig. 6. Thin Layer Chromatograms of BHA+T-I and BHT+AT-I treated with DPPH

(a); T-I (0.5 mM), (b); T-I (0.5 mM)+BHA (1.0 mM), (c); BHT (1.0 mM) and (d); BHT (1.0 mM)+AT-I (0.5 mM) were treated with DPPH (2.0 mM). Other conditions were the same as in the legend to Fig. 1. 1; T-I, 2; T-II, 3; DPPH and $DPPH_2$, 4; BHA, 5; BHT, 6; QM and 7; AT-I.

T-I and BHA+BHT when they were treated with DPPH. A synergistic effect was also observed in the combination of BHA with each of the phenols having two substituents, such as *tert*-butyl and phenyl groups (A-I) and *tert*-butyl and benzyl groups (AT-I), on *ortho* positions, but not in the combination of BHA with A-II having an ether linkage on the *ortho* position. When BHT (1 mM) and AT-I (0.5 mM) were treated with DPPH (2 mM), the spot corresponding to BHT was rarely found and a spot corresponding to QM was observed (d). These results indicated that the loss of BHT was significantly enhanced by AT-I on treatment with DPPH. A similarly enhanced loss of BHT was observed when BHT+A-I were treated with DPPH.

A synergistic effect was observed when hindered phenols, such as BHT, DBP and TBP and T-I, were combined with 4-methoxyphenol derivatives such as BHA and MP. This effect might arise from the regeneration of 4-methoxyphenol derivatives from the possible intermediates formed from DPPH and these phenols, with enhanced loss of the hindered phenols. With the combination of BHA and BHT, an excellent synergistic antioxidative effect has been demonstrated in lard.[13] MP also retarded the oxidation of hydrocarbon and showed a synergistic effect in combination with 2,6-di-*tert*-butylphenols such as BHT, DBP and TBP.[14] All of these combinations showed a synergistic effect on hydrogen donation to DPPH. Mahoney and DaRooge[14] have suggested on the basis of kinetic experiments that the synergistic effect of MP+BHT arose as a result of regeneration of MP by means of its radical reacting with the hindered phenols. Our observations suggest that stable intermediates might be formed from DPPH and BHA or MP. Futher work is in progress on the reaction of the intermediates.

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