# Diaminodiphenylmethane Derivatives as a New Chromogenic Hydrogen Donor in Peroxidase-Catalyzed Oxidation Reaction with Hydrogen Peroxide

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**New chromogenic hydrogen donors (1a—c) for peroxidase-catalyzed oxidation were synthesized and demonstrated to be potentially useful for spectrometric determination of hydrogen peroxide. Under oxidative conditions these compounds exhibit, without an additional component, intense blue colors at *ca.* 650 nm with higher sensitivities toward hydrogen peroxide than those of conventional reagents. The detection limit of hydrogen peroxide using these compounds was 0.1 μM under serum-free conditions.**

**Keywords** chromogenic hydrogen donor; peroxidase; hydrogen peroxide; diaminodiphenylmethane derivative; methane base

The spectrometric determination of hydrogen peroxide using a chromogenic hydrogen donor is among the most important analytical method which has widespread applications in clinical chemistry. Although various types of phenol or aniline derivatives were demonstrated to be useful as hydrogen donors in the peroxidase (POD)-catalyzed oxidative condensation reaction with 4-aminoantipyrine (4-AA) in the presence of hydrogen peroxide,[1] most of these compounds suffer from several disadvantages due to their poor water solubilities and relatively short absorption wavelengths of the resulting chromogens (500—520 nm), which are most likely interfered by serum bilirubin. New water-soluble aniline derivatives that produce chromogens with the absorption maxima ($\lambda_{max}$) of longer than 600 nm under oxidative conditions have found wide applications in the assay of clinically important analytes since they were proposed in 1982 by Tamaoku and co-workers.[2,3] In automated clinical assays, however, a chromogenic hydrogen donor that allows a simplified assay procedure with a higher sensitivity would be preferable. When designing such a compound, the following criteria should be satisfied: 1) the compound is water soluble and produces a stable chromogen having a large molar absorptivity ($\varepsilon$) at $\lambda_{max}$ longer than 600 nm, and 2) the compound yields the chromogen without an additional component ("coupler") such as 4-AA, which makes the assay less complex and thereby less costly. A highly sensitive detection of hydrogen peroxide was reported using water-souble 4,4′-tetramethyldiaminodiphenylmethane (methane base) derivative with 3-methylbenzothiazolinonehydrazone (MBTH) as the coupler in the presence of POD.[4] Although the above-mentioned criteria were half met, these derivatives were less stable in an aqueous solution, which motivated us to exploit an alternative compound. Since the methane base has been known to form a blue pigment, without any coupler, in the presence of a suitable oxidant although the sensitivity is too low to be applied in clinical practices,[5] we expected that introducing electron-donating functionalities such as a methyl group into the molecule would accelerate the POD-catalyzed oxidation with hydrogen peroxide through reducing the oxidation potential.

We have synthesized three types of bis[4-(*N*-alkyl-*N*-sulfopropyl)amino-2,6-dimethylphenyl]methane derivatives, disodium salts (**1a**—**c** in Chart 1) and wish to describe herein our preliminary results concerning their reactivities toward hydrogen peroxide and also demonstrate their merits as a chromogenic hydrogen donor in POD-catalyzed oxidations.

## Experimental

**Apparatus** Thin-layer chromatography (TLC) was performed with Kieselgel 60 $F_{254}$ (Merck, Darmstadt, Germany); *n*-butanol saturated with 0.2 M aqueous ammonia was used as the developing solvent. Absorbance spectra were taken on a Shimadzu UV-210A spectrophotometer. $^1$H Nuclear magnetic resonance ($^1$H-NMR) spectra were measured on a Bruker AC-200P, operating at 200 MHz, with 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) as an internal standard. The splitting patterns were designated as follows: s, singlet; t, triplet; m, multiplet. IR spectra were recorded in KBr disks on a Hitachi 270-30 spectrometer. Uncorrected melting points were obtained on a Yamato MP-21 melting-point apparatus.

**Materials** Horseradish peroxidase (193 units/mg) was purchased from Wako Pure Chemicals (Osaka, Japan) and a solution of 1000 units/ml was freshly prepared prior to use. Stock solutions of compounds **1a**—**c** were prepared as 10 mM aqueous solutions. The following buffers were used for the respective pH range: 100 mM McIlvaine (pH 3.0—5.0), 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 5.5—7.0), 50 mM HEPES (pH 7.5—8.0) and 50 mM 2-(cyclohexylamino)ethanesulfonic acid (CHES, pH 8.5—9.0).

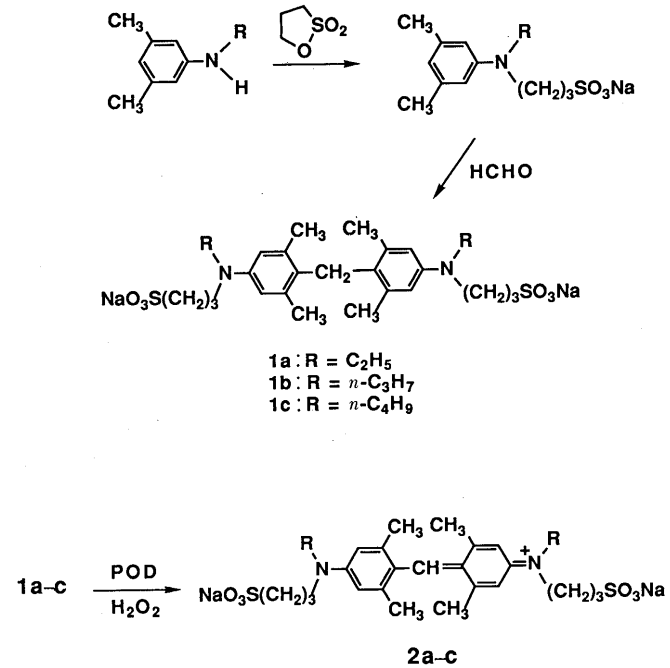


Chart 1

**Bis[4-(*N*-ethyl-*N*-sulfopropyl)amino-2,6-dimethylphenyl]methane Disodium Salt (1a)** To *N*-ethyl-*N*-(3-sulfopropyl)-3,5-dimethylaniline sodium salt[2] (2.0 g, 6.82 mmol) in water (12 ml) were added, under nitrogen, 37% formaldehyde (0.28 ml) and 90% formic acid (0.03 ml). After being warmed at 40 °C for 4 h, the reaction mixture was poured into acetone (200 ml). The resulting precipitates were collected and recrystallized from water to give 1.05 g (52%) of compound **1a** as white crystals. mp 232—233 °C (dec.). IR ($\nu_{max}$ cm$^{-1}$): 3560, 2960 (Ar), 1610 (Ar), 1493 ($CH_2$). TLC: $Rf$=0.35. $^1$H-NMR ($D_2O$) $\delta$: 0.98 (6H, t, $J$=7.0 Hz, $CH_3$), 1.82—1.94 (4H, m, $CH_2$), 1.99 (12H, s, $CH_3$), 2.84 (4H, t, $J$=7.6 Hz, $CH_2$), 3.10—3.23 (8H, m, $CH_2$), 3.79 (2H, s, $CH_2$), 6.49 (4H, s, ArH). *Anal.* Calcd for $C_{27}H_{40}N_2Na_2O_6S_2$: C, 54.2; H, 6.7; N, 4.7. Found: C, 54.3; H, 6.8; N, 4.6.

**Bis[4-(*N*-propyl-*N*-sulfopropyl)amino-2,6-dimethylphenyl]methane Disodium Salt (1b)** Compound **1b** was synthesized in the same fashion for compound **1a** from *N*-propyl-*N*-(3-sulfopropyl)-3,5-dimethylaniline sodium salt[2] in 32% yield. mp 215 °C (dec.). IR ($\nu_{max}$ cm$^{-1}$): 3500, 2950 (Ar), 1625 (Ar), 1500 ($CH_2$). TLC: $Rf$=0.42. $^1$H-NMR ($D_2O$) $\delta$: 0.75 (6H, t, $J$=7.2 Hz, $CH_3$), 1.10—1.35 (4H, m, $CH_2$), 1.65—1.90 (4H, m, $CH_2$), 1.95 (12H, s, $CH_3$), 2.79—3.06 (8H, m, $CH_2$), 3.23 (4H, t, $J$=7.0 Hz, $CH_2$), 3.74 (2H, s, $CH_2$), 6.43 (4H, s, ArH). *Anal.* Calcd for $C_{29}H_{44}N_2Na_2O_6S_2 \cdot H_2O$: C, 54.0; H, 7.2; N, 4.3. Found: C, 54.0; H, 7.1; N, 4.4.

**Bis[4-(*N*-butyl-*N*-sulfopropyl)amino-2,6-dimethylphenyl]methane Disodium Salt (1c)** Compound **1c** was synthesized in the same fashion as compound **1a** from *N*-butyl-*N*-(3-sulfopropyl)-3,5-dimethylaniline sodium salt[2] in 53% yield. mp 239—241 °C (dec.). IR ($\nu_{max}$ cm$^{-1}$): 3500, 2950 (Ar), 1610 (Ar), 1495 ($CH_2$). TLC: $Rf$=0.42. $^1$H-NMR ($D_2O$) $\delta$: 0.76 (6H, t, $J$=7.1 Hz, $CH_3$), 1.06—1.20 (4H, m, $CH_2$), 1.31—1.49 (4H, m, $CH_2$), 1.85—1.97 (4H, m, $CH_2$), 1.98 (12H, s, $CH_3$), 2.82 (4H, t, $J$=7.6 Hz, $CH_2$), 2.90—3.20 (8H, m, $CH_2$), 3.73 (2H, s, $CH_2$), 6.37 (4H, s, ArH). *Anal.* Calcd for $C_{31}H_{48}N_2Na_2O_6S_2$: C, 56.9; H, 7.4; N, 4.3. Found: C, 54.0; H, 7.1; N, 4.4.

**Procedure** Thirty $\mu$l of hydrogen peroxide (0.01—1.0 mM) was added to a pre-incubated solution (37 °C) containing compound **1a** (30 $\mu$l) and POD (30 $\mu$l) in 50 mM MES buffer (3 ml, pH 5.5). After the mixture was incubated at 37 °C for 10 min, the absorbance was measured at 647 nm against the reagent blank that was prepared in the same manner without hydrogen peroxide.

## Results and Discussion

Compounds **1a—c** were synthesized in moderate overall yields based on a formic acid-mediated homocoupling reaction described in the literature,[4] employing the respective aniline derivatives as outlined in Chart 1. Commercially available *N*-alkyl-3,5-dimethylanilines were sulfopropylated with propanesultone in the first step to render the compounds water soluble. As expected, these compounds exhibit intense blue colors with $\lambda_{max}$ at *ca.* 650 nm under oxidative conditions with hydrogen peroxide and POD. The probable reaction scheme of this POD-catalyzed two-electron oxidation reactions, which is analogous to that of the methane base,[5] is illustrated in Chart 1, in which compounds **1a—c** are subjected to one-electron oxidation to form the cation radicals in the initial phase of the reactions, followed by further oxidation. The structures of the chromogens (**2a—c**) could be evidenced by NMR measurements of chemically oxidized products of compounds **1a—c** with lead dioxide in $D_2O$. The absorption spectra of these chemically oxidized products were identical to those obtained by the enzymatic oxidations with hydrogen peroxide and POD. The rates of these chromogen formations are much faster than that of the methane base nucleus presumably because of their lower oxidation potentials. The reagent blanks without hydrogen peroxide and POD, however, were found to be much more stable than the methane base.[4] When compounds **1a—c** (0.1 mM in 50 mM MES buffer at pH 5.5) were left at 15—20 °C without protecting them from lights for 60 d, the elevations of the absorbances at $\lambda_{max}$ of their respective chromogens were observed to be less than 0.02. Although this appears to be inconsistent with the above observations of the enhanced reactivities, the difference is attributable to the methyl groups which might play a key role in that it predominantly destabilizes the chromogenes, or that its steric bulkiness hinders an approach of the substrate to the active center of the enzyme (iron–porphyrin complex). The whole reaction mechanism is currently being investigated.

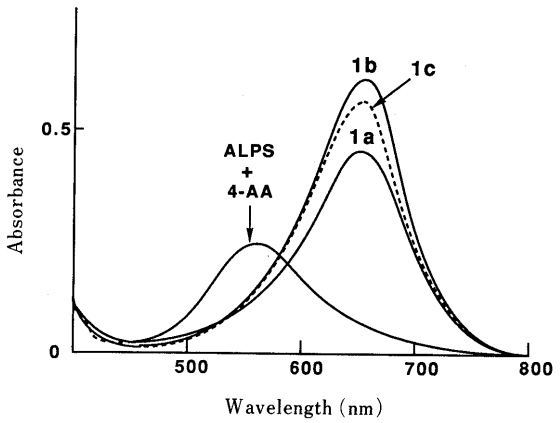


Fig. 1. Absorption Spectra of Compounds **1a—c** in Comparison with ALPS and 4-AA under Oxidative Conditions of Hydrogen Peroxide and POD

Compounds **1a—c** (0.1 mM) in 50 mM MES buffer (pH 6.5) were incubated with hydrogen peroxide (10 $\mu$M) and POD (10 units) at 37 °C for 10 min and the absorbances of the resulting chromogens were measured at 647 nm for **1a**, 649 nm for **1b** and 651 nm for **1c**, respectively. The reaction of ALPS (0.1 mM) with 4-AA (0.1 mM) was carried out in an identical manner and the absorbance was measured at 561 nm.

Figure 1 shows the absorption spectra of compounds **1a—c** when oxidized with hydrogen peroxide and POD. The figure also compares, under optimized conditions, with that obtained from 4-AA and *N*-ethyl-*N*-3-(sulfopropyl)aniline sodium salt (ALPS) which was reported to be one of the most sensitive chromogenic hydrogen donors.[2] Compounds **1a—c**, which absorb at *ca.* 250 nm, develop colors without any couplers under oxidative conditions, with higher sensitivities toward hydrogen peroxide (*e.g.*, 2.9-fold for **1b**) and longer wavelengths (647 nm for **1a**, 649 nm for **1b** and 651 nm for **1c**) than those of the chromogen produced with ALPS and 4-AA; $\varepsilon$ values based on the concentration of hydrogen peroxide (=absorbance/[$H_2O_2$]) range from 45000 to 65000, and the sensitivities are in the order of compound **1b**>**1c**>**1a**. The developed colors are stable without any decreases of the absorbances observed within at least 3 h.

The pH profiles of these POD-catalyzed oxidations of compounds **1a—c** were then examined (Fig. 2). The reactions of all these compounds have similar pH profiles with the optimum pH ranges between 5 and 7, where the absorbance intensities are fairly stable. There were no significant changes of absorbance observed when other buffer species were used (data not shown). The overall two-electron oxidation process can be expressed as:

$$DH + H_2O_2 + H^+ \rightarrow D^+ + 2H_2O$$

where DH is a hydrogen donor and $D^+$ is the resulting chromogen. The equation implies that the reaction favors

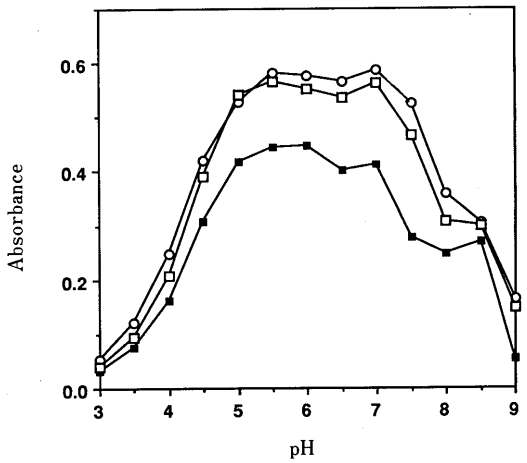


Fig. 2. pH Profiles of the POD-Catalyzed Oxidation Reactions of Compounds **1a**—**c** with Hydrogen Peroxide

—■—, **1a**; —○—, **1b**; —□—, **1c**. The procedures are described under Experimental except for the concentration of hydrogen peroxide which is 10 $\mu$M.

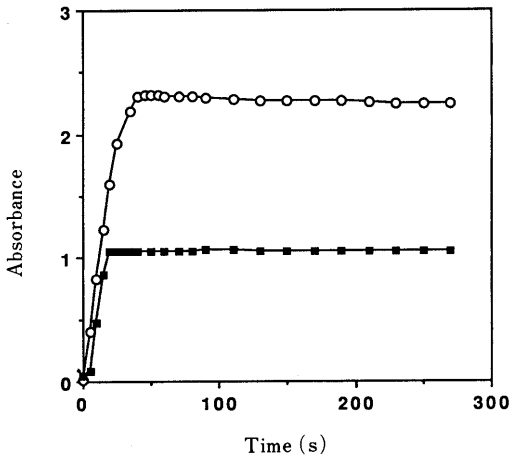


Fig. 3. Time Course of the Oxidation Reaction of Compound **1b** (—○—) with Hydrogen Peroxide in Comparison with that of ALPS with 4-AA (—■—)

Concentration of hydrogen peroxide is 50 $\mu$M; the other conditions are the same as described under Experimental. The reaction of ALPS (0.1 mM) with 4-AA (0.1 mM) was carried out in an identical manner and the absorbance was measured at 561 nm.

a weakly acidic pH where POD remains active, and also that the chromogen ($D^+$) is unstable at alkaline pHs because of possible formation of a colorless pseudo-base (*i.e.*, D–OH). These pH profiles, however, are very similar to that of POD whose optimum pH is in the range of 5 to 7, and perhaps are simply reflecting the POD activity *versus* pH rather than that of chromogens. pH was then fixed at 5.5 in the following experiments.

The time course of the oxidative reaction of compound **1b** is shown in Fig. 3 in which it is compared with that of ALPS and 4-AA under identical conditions. The reaction of **1b** is completed within 5 min at 37 °C with the initial velocity in terms of the increase of absorbance per minutes ($\Delta A/\Delta t$) being 4.9, which is slightly larger than that of ALPS with 4-AA (*ca.* 1.4-fold). This relatively fast color development, as well as its stability as described above, is advantageous, particularly from an economic point of view; it allows a rapid assay of a large number of samples.

The concentrations of compounds **1a**—**c** in these

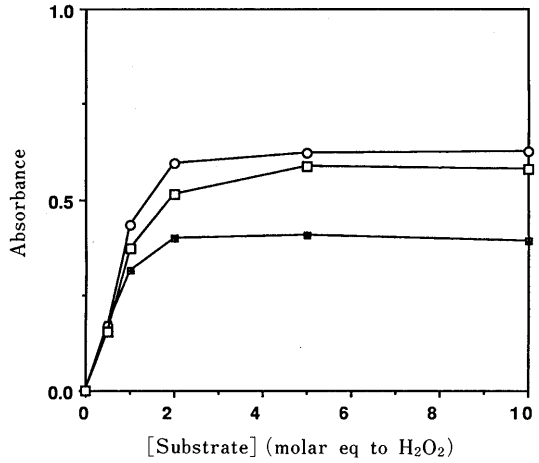


Fig. 4. Absorbance *versus* Substrate Concentrations in the Oxidative Reactions of Compounds **1a**—**c**

—■—, **1a**; —○—, **1b**; —□—, **1c**. Concentration of hydrogen peroxide is 10 $\mu$M; the other conditions are the same as described under Experimental.

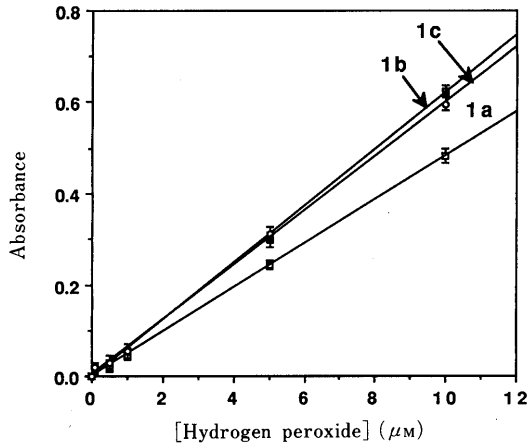


Fig. 5. Standard Curves of Hydrogen Peroxide Using Compounds **1a**—**c**

See Experimental for details. Each point and bar show the mean and standard deviation ($n=5$).

reactions were next varied to find a minimum concentration effective for maximum sensitivity (Fig. 4). As shown in the figure, the maximum sensitivities are obtained when more than 2 molar eq of the substrates to hydrogen peroxide are used, whereas more than a 15-fold excess of the substrate concentrations is normally required in the case of ALPS. Considering that only 1 eq of the hydrogen donor is sufficient for the oxidation to be completed in the above equation, this observation is best rationalized by assuming that a competitive reaction of an intermediate species (presumably cation radical $DH^{+\bullet}$ or radical $D^{\bullet}$) is operative in these reactions.

Under these optimized conditions, standard curves of the absorbance intensities obtained from these reactions of compounds **1a**—**c** *versus* various concentrations of hydrogen peroxide were measured (Fig. 5). Linear responses of the absorbance intensities of the formed chromogens were observed over a range of 0.1 to 10.0 $\mu$M of hydrogen peroxide; the detection limit of 0.1 $\mu$M is much higher than that of ALPS with 4-AA (7 $\mu$M), and even higher than the value of 3 $\mu$M reported by Shiga *et al.*[4] employing an identical compound to **1a** except that the four methyl groups

on the phenyl rings are lacking. Linear regression analysis afforded the correlation coefficients ($r$) of 1.000, 0.999 and 0.999 for **1a**, **1b** and **1c**, respectively. The presence of serum, however, was found to substantially suppress the color development. The addition of horse serum (1% (w/v)) to **1a** (0.1 mM) in 50 mM Tris–HCl buffer (pH 7.0) in the presence of POD (10 units) and hydrogen peroxide of various concentrations, for example, causes color fading with the sensitivity decreased to 65%; it drops to 15% when the amount of serum is increased to 5% (w/v). This lowered sensitivity might be due to bilirubin, transferin and/or glutathione, which are well known for producing negative interferences in the assays of serum samples presumably due to the consumption of hydrogen peroxide by a chemical reaction.

In summary, compounds **1a—c** have proved to permit a highly sensitive assay of hydrogen peroxide with the sensitivity being 2.9-fold of that of ALPS with 4-AA. In addition, the resulting chromogens, **2a—c**, absorb at *ca.* 650 nm where biological samples have no background absorbance. Furthermore, the present approach using these compounds requires no coupler reagents, eliminating an extra step of the procedure. Thus, the potential utility of these compounds as chromogenic hydrogen donors was demonstrated under serum-free conditions. Our current efforts are being focused on an elaboration to make these molecules less susceptible to color fading and will be reported in due course.

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