# Water-soluble chromogenic reagent for colorimetric detection of hydrogen peroxide—an alternative to 4-aminoantipyrine working at a long wavelength

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**A newly synthesized water-soluble phenylenediamine derivative, *N*-methyl-*N*-phenyl-3-sulfophenylenediamine sodium salt, proved to be of value as a reagent for the colorimetric determination of hydrogen peroxide in clinical analyses. The compound reacted with Trinder's reagents to give chromogens with a higher sensitivity, a longer wavelength (> 750 nm) as well as a higher water solubility compared with a commonly used reagent.**

The enzymatic method that utilizes a specific enzyme for a target analyte is a central technique for quantifying biogenic compounds in biological fluids and has been used widely in clinical analyses because of its high specificity and rapidity. Oxidase enzymes are most popular in the method where a family of reagents, so-called hydrogen donors, are used which produce an intense color upon oxidation followed by condensation with a cofactor molecule (coupler) in the presence of hydrogen peroxide and an oxidase such as peroxidase (POD). Trinder's reagents[1] and 4-aminoantipyrine (4-AA)[2] are the typical hydrogen donor and coupler, respectively, which condense to give a chromogen having an absorption at 500–550 nm. Although numerous aniline derivatives have been developed as Trinder's reagents, little has been documented as an alternative to 4-AA, which has disadvantages associated with the relatively short absorption wavelength (when coupled with Trinder's reagents) and poor water solubility. 3-Methyl-2-benzothiazolinone hydrazone (MBTH)[3] is one of few such compounds but is reported to be unstable in solution and less reactive than 4-AA.

We reported in a previous paper[4] *N*-methyldiphenylamine derivatives as couplers whose chromogens with Trinder's reagents absorb in the near-infrared wavelength region. The poor water solubility of these derivatives, however, largely limits their clinical applications. We describe herein the synthesis and the applicability of a new derivative, *N*-methyl-*N*-phenyl-3-sulfophenylenediamine sodium salt (**1**, Scheme 1), as a water-soluble coupler for use in clinical analyses which allows assays to be performed at 750 nm where no biogenic compounds interfere.

## Experimental

### *Apparatus*

Thin-layer chromatography (TLC) was performed on Merck Kieselgel 60 $F_{254}$ (Darmstadt, Germany). Absorption spectra were taken on a Shimadzu UV-210A spectrophotometer (Kyoto, Japan). Proton nuclear magnetic resonance ($^1$H-NMR) spectra were measured on a Bruker AC-200P spectrometer (Rheinstetten, Germany) operating at 200 MHz, using tetramethylsilane or sodium 3-(trimethylsilyl)-1-propanesulfonate as an internal standard. The IR spectra were recorded in KBr disks on a Hitachi 270-30 spectrometer (Tokyo, Japan). Fast atom bombardment mass spectra (FAB-MS) were measured on a JEOL JMS-AX 505W instrument (Tokyo, Japan).

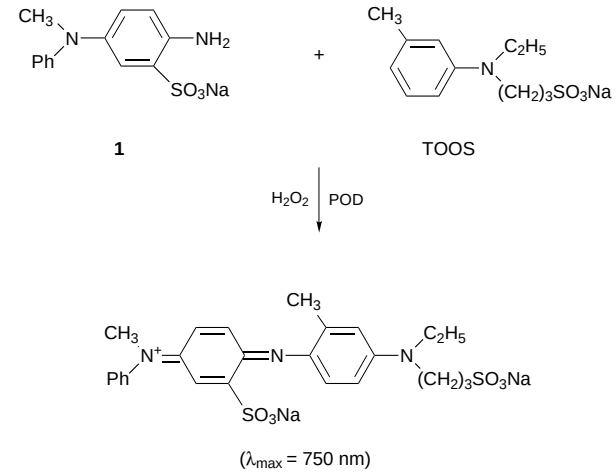
### *Materials*

*N*-Ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline sodium salt (TOOS) and *N*-ethyl-*N*-(3-sulfopropyl)aniline sodium salt (ALPS) were obtained from Dojindo Laboratories (Kumamoto, Japan). 4-Aminoantipyrine (4-AA), horseradish POD (192 I.U. $mg^{-1}$) and glucose oxidase (GOD, 162 I.U. $mg^{-1}$) were purchased from Wako Pure Chemicals (Osaka, Japan).

### *Synthesis*

*N*-Methyl-4-nitrosodiphenylamine: Concentrated HCl (50 ml, 0.60 mol) was added to a stirred suspension of *N*-methyldiphenylamine (7.5 g, 40.9 mmol) in water (25 ml), and the solution was cooled to 0 °C. To this was added sodium nitrite (2.8 g, 40.9 mmol) in water (20 ml) dropwise at 0–5 °C. After being stirred at ≈5 °C for 60 min, the reaction mixture was extracted with chloroform (150 ml). The chloroform layer was concentrated to ≈50 ml, and water (50 ml) was added. The aqueous layer was neutralized with 1 M NaOH at < 30 °C and extracted again with chloroform. The chloroform layer was concentrated to leave a dark brown oil which was taken up with hexane (500 ml). The hexane solution was washed with water and re-extracted with 1 M HCl. The aqueous layer was neutralized and then extracted with chloroform. Concentration of the chloroform layer after drying gave 3.9 g (38%) of the title compound as a dark green oil. TLC: $R_f$ = 0.49 ($CHCl_3$); FAB-MS: *m/z* = 213 [M + H].

*N*-Methyl-4-aminodiphenylamine: After a methanol solution (50 ml) containing *N*-methyl-4-nitrosodiphenylamine (3.9 g, 15.7 mmol) was hydrogenated with 5% Pd/C (0.1 g), the solution was filtered, concentrated and partitioned between ether and aqueous sodium carbonate. Addition of HCl-saturated dioxane caused precipitation of the hydrochloride salt of the title compound as a white powder. Yield 3.3 g (90%). TLC:




**Scheme 1** Chromogenic reaction with TOOS.

$R_f$ = 0.2 ($CHCl_3$); $^1$H-NMR ($CD_3OD$) δ: 3.29 (3 H, s, $CH_3$), 7.06–7.47 (9 H, m, ArH); IR ($cm^{-1}$): 3500, 2930, 1620, 1540; FAB-MS: *m/z* = 235 [M + H].

*N*-Methyl-*N*-phenyl-3-sulfophenylenediamine sodium salt (**1**): *N*-Methyl-4-aminodiphenylamine (3.3 g, 12.2 mmol) was slowly added to fuming sulfuric acid (20 ml) with stirring at < 40 °C, and the solution was warmed to 60 °C. After 2 h at this temperature, the solution was slowly poured onto ice water, the pH of which was adjusted to 8 with 2 M NaOH. The solution was concentrated under reduced pressure to leave a residue which was taken up with methanol. After filtration, the filtrate was concentrated to give a crude product. Chromatography on silica gel ($CHCl_3$ : MeOH = 7 : 3, v/v) afforded 2.1 g (50%) of **1** as a white powder. TLC: $R_f$ = 0.2 ($CHCl_3$ : MeOH = 7 : 3, v/v); $^1$H-NMR ($D_2O$) δ: 2.97 (3 H, s, $CH_3$), 6.53–7.36 (8 H, m, ArH); IR ($cm^{-1}$): 3450, 1607, 1525, 1190; FAB-MS: *m/z* = 301 [M + H].

### *Chromogenic reaction*

A typical procedure using TOOS as a Trinder's reagent is as follows. A mixture containing **1** (100 μM) TOOS (100 μM) and POD (10 I.U.) in 50 mM phosphate buffer (3 ml, pH 7.4) was pre-incubated at 37 °C. After 5 min, 2.0 mM hydrogen peroxide (30 μl) was added; the solution was incubated for an additional 5 min at 37 °C. The absorbance of the resulting chromogen was measured at 750 nm against a reagent blank prepared in a similar manner without hydrogen peroxide.

### *Colorimetric determination of glucose*

A mixture containing **1** (100 μM), TOOS (100 μM), glucose oxidase (10 I.U.) and POD (10 I.U.) in 50 mM phosphate buffer (3 ml, pH 7.4) was pre-incubated at 37 °C. After 5 min, 30 μl of glucose solution of various concentrations (20–100 mg $dl^{-1}$ water) was added and the solution was incubated for an additional 5 min at 37 °C. The absorbance was measured at 750 nm with a glucose blank.

## Results and discussion

Compound **1** was designed so that the resulting chromogen with Trinder's reagents becomes extremely water-soluble and absorbs at a wavelength longer than 700 nm where endogenous substances have no absorptions. Water solubility is important for clinical applications for practical reasons, particularly for use in 'dry chemistry' where a high reagent concentration is required. Water solubility has a further advantage in that it tends to reduce toxicity. To achieve this, a sulfonyl group was introduced to the position *ortho* to the amino group of *N*-methyl-4-aminodiphenylamine, which also made the compound more stable and resistant to self-oxidation. Compound **1** was readily synthesized from commercially available *N*-methyldiphenylamine in three steps in 17% overall yield. The water solubility of **1** was found to be ≈1.0 M in 50 mM phosphate buffer (pH 7.0) at 20 °C, a value large enough for clinical applications.

Fig. 1 compares under the same conditions **1** with 4-AA in the absorption spectra of the chromogenic reactions with TOOS and ALPS as Trinder's reagents. With both TOOS and ALPS, **1** gave more than twice the absorbance as 4-AA (2.2-fold for TOOS and 2.8-fold for ALPS), with the $\lambda_{max}$ at 750 nm, which is 200 nm longer than with 4-AA. The $\lambda_{max}$ allows assays to be performed at a high signal-to-noise (S/N) ratio because there is no background absorption at this wavelength due to endogenous visible light-absorbing substances such as bilirubin and hemoglobin. The sensitivity of **1** with TOOS for hydrogen peroxide was determined to be 4.78 based on the apparent molar coefficient (log ε) per the concentration of hydrogen peroxide whereas that of 4-AA was 4.30.

The effect of the reaction time on the chromogenic reaction of **1** with TOOS measured at 750 nm is shown in Fig. 2 in comparison with 4-AA. Compound **1** reacts much faster with TOOS than does 4-AA under identical reaction conditions, completing within 2 s at 20 °C, whereas 4-AA takes more than 20 s to complete; it is as fast as the analogous compound that lacks the sulfonate group (data not given).[4] When the concentration of **1** was varied in this reaction system (0–5.0 equiv for TOOS), the sensitivity for hydrogen peroxide increased with an increasing concentration of **1** and reached a plateau at the concentration of 1.0 equiv, suggesting that the reaction is most likely 1 : 1 as shown in Scheme 1. The reaction, on the other hand, was not sensitive to pH, as the sensitivity remained almost constant when the pH was varied (pH 4–8). The fact that POD favors acidic pHs was not reflected in this sensitivity–pH profile, presumably because the absorbance was measured under conditions where reactions were completed (*i.e.*, POD activity controls the rate of the reaction rather than the final sensitivity).

Under the optimized conditions as above, hydrogen peroxide was determined with a good response linearity ($r$ = 0.997 in the linear regression equation: $y = 0.0436x + 0.018$, where $x$ and $y$ mean, respectively, the concentration of hydrogen peroxide in μM and the absorbance at 750 nm) over the concentration range 5 to 25 μM. The detection limit was 1.0 μM at S/N = 3. Unlike for hydrogen peroxide and as described above, the sensitivity for POD (based on the maximum absorbance) was not proportional to its amount. There were no significant differences in the absorbance obtained with different POD concentrations (0.0033–33.3 I.U. $ml^{-1}$) under conditions where reactions were completed (see experimental section). The reaction rate, however, determined from the time to reach the

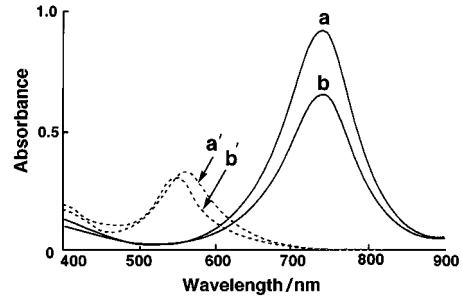



**Fig. 1** Absorption spectra of **1** and 4-AA when reacted with typical Trinder's reagents. The reactions were carried out as described in the experimental section using ALPS (spectra a, a′ for **1** and 4-AA, respectively) or TOOS (spectra b, b′ for **1** and 4-AA, respectively) as Trinder's reagent.

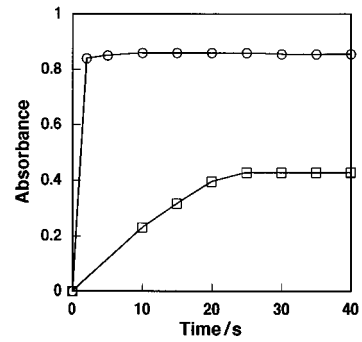



**Fig. 2** Time course of the chromogenic reaction of **1** with TOOS. The reaction was carried out with **1** (○) or 4-AA (□) at 20 °C, otherwise under conditions as described in the experimental section, which were monitored at 750 and 555 nm, respectively.

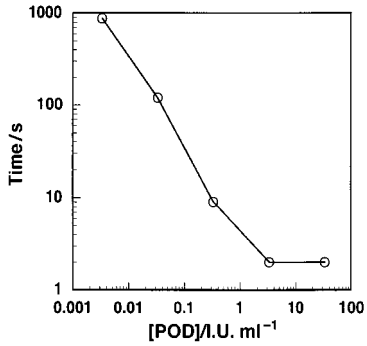



**Fig. 3** Plots of the time(s) to reach the maximum absorbance *versus* the concentration of POD. The reactions were carried out as described in the experimental section with various POD concentrations (0.0033–33.3 I.U. ml$^{-1}$).

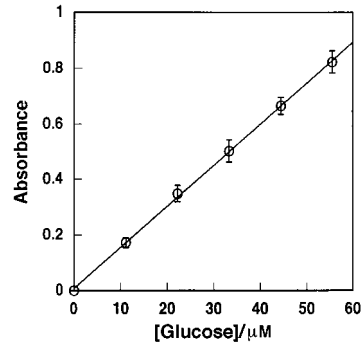



**Fig. 4** Standard curve of colorimetric determination of glucose using **1** and TOOS. The conditions are detailed in the experimental section. The between-assay constant of variation was less than 5% with $n = 5$.

maximum absorbance, was dependent on the POD concentration (Fig. 3). The rate was maximum at concentrations higher than 3.33 I.U. ml$^{-1}$.

We finally tested the applicability of **1** to the glucose assay based on the colorimetric determination of hydrogen peroxide produced from the GOD-catalyzed oxidation of glucose (Fig. 4). A linear response of the absorbance of the resulting chromogen to the amount of glucose was observed ($r = 0.9997$) over the concentration range 11.1–55.5 μM (equivalent to 0.2–1.0 mg dl$^{-1}$). Although a high detection sensitivity is not needed to measure glucose, which is relatively abundant in biological samples, compound **1** proved to be useful as a coupler in the enzymatic method, with higher sensitivity, longer wavelength and higher water solubility than 4-AA.

## References

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