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APPLICATION OF HIGHLY ACTIVE TEMPO DERIVATIVES FOR ELECTROCHEMICAL ANALYSIS

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Abstract – The electrochemical properties of 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO) derivatives with improved oxidative capability upon introducing electron-withdrawing groups were evaluated by cyclic voltammetry. Synthesized TEMPO derivatives, including 4-acetamido-2,2,6,6-tetramethylpiperidine 1-oxyl (A-TEMPO), 4-trifluoroacetamido-2,2,6,6-tetramethylpiperidine 1-oxyl (F-TEMPO), and dinitroxide condensed with TEPMO exhibiting a 1,2-dicarbonyl structure, 4,4′-[(1,2-dioxo-1,2-ethanediyl)diimino]bis[2,2,6,6-tetramethyl-1-piperidinyloxy] (di-TEMPO), were used as electrocatalysts. Ethanol and vancomycin response currents were compared, demonstrating the potential of specific TEMPO derivatives for quantification. TEMPO derivatives showed enhanced response current increase, suggesting their molecular recognition ability.

INTRODUCTION

2,2,6,6-Tetramethylpiperidine 1-oxyl (TEMPO, **1**), a stable nitroxyl radical, exhibits unique properties and catalyzes the selective oxidation of primary alcohols to aldehydes when combined with a co-oxidant.[1] TEMPO (**1**) is also used in various fields, such as labeling agents that utilize the scavenging ability of radical compounds and fluorescent probes for the oxidation and reduction of substances that utilize the fluorescence quenching effect.[2–4] TEMPO (**1**) can effectively oxidize alcohols by applying an electrical potential.[5,6] This oxidation process measures the oxidation current, which is proportional to the alcohol concentration in the system. Consequently, this reaction makes the electrochemical measurements of alcohols and compounds containing hydroxy groups feasible.[7,8]

However, TEMPO (**1**) suffers from limited reactivity owing to the steric hindrance caused by the four adjacent methyl groups that stabilize the radicals in the molecule. For example, sterically bulky compounds

(e.g., secondary alcohols) are difficult to quantify electrochemically because they cannot access the TEMPO (**1**) radical sites. To overcome this limitation, radicals can be stabilized using bicyclo and tricyclo structures, and highly active nitroxyl radical compounds such as 2-azaadamantane *N*-oxyl (AZADO) and 9-azabicyclo[3.3.1]nonane *N*-oxyl (ABNO).[9–11] Although these compounds were initially developed as oxidation catalysts in organic synthesis, our previous work demonstrated their potential in electrochemical analysis.[12–14] In particular, 3-hydroxy-8-azabicyclo[3.2.1]octane *N*-oxyl (3-HO-ABOO) (previously referred to as nortropine *N*-oxyl (NNO)) is suitable for electrochemical analysis, enabling the oxidation of substrates in neutral aqueous solutions, which is impossible with TEMPO. Moreover, this method has been successful in the electrochemical detection of sugars and pharmaceuticals.[12]

However, the high reactivity of these nitroxyl radical compounds poses a challenge in electrocatalytic activity when compounds containing primary amines or thiol groups are present or coexist.[15,16] Therefore, compounds comprising only simple alcohols (e.g., glucose) are suitable analytes for electrochemical analytical probes. In contrast, TEMPO (**1**) is more stable compared to these highly reactive radical compounds and is a useful sensing probe in electrochemical applications, even when amino and thiol groups coexist.[15,16]

Various reports suggest that an electron-withdrawing group can be introduced into the molecule to enhance the oxidizing ability of nitroxyl radical compounds and reduce the bulkiness near the nitroxyl radical site, as described above.[17,18] 4-Acetamido-2,2,6,6-tetramethylpiperidine 1-oxyl (A-TEMPO, **2**) and 4-trifluoroacetamido-2,2,6,6-tetramethylpiperidine 1-oxyl (F-TEMPO, **3**) have been synthesized and reported to exhibit enhanced oxidation catalytic ability compared to that of TEMPO (**1**).[17] In addition, in electrochemical measurements, A-TEMPO (**2**) comprising an electron-withdrawing group, such as the acetamide group, showed enhanced reactivity compared to that of TEMPO (**1**) and yielded high response currents.[15,18]

In this study, we synthesized trifluoroacetic acid-modified F-TEMPO (**3**) and 4,4′-[(1,2-dioxo-1,2-ethanediyl)diimino]bis[2,2,6,6-tetramethyl-1-piperidinyloxy] (di-TEMPO, **4**) in addition to A-TEMPO (**2**). These compounds were obtained by condensing two TEMPOs using a 1,2-dicarbonyl structure. Furthermore, the synthesized compounds were evaluated for their electrolytic oxidation abilities in an aqueous solution to assess their potential as electrochemical analytical probes. However, F-TEMPO (**3)** and di-TEMPO (**4)** were not subjected to electrochemical evaluation (Figure 1).

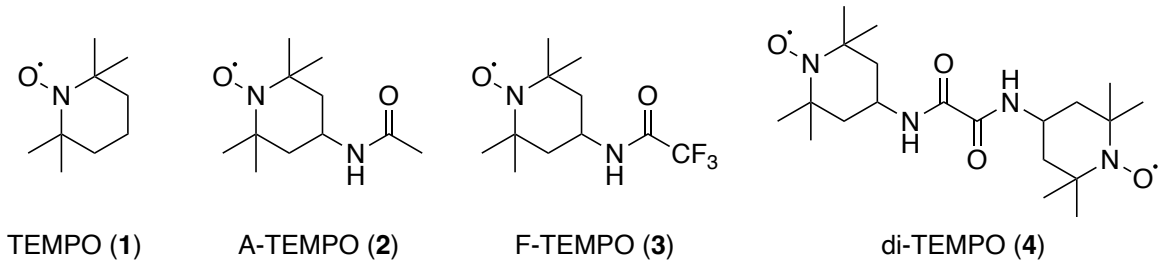

Figure 1. Chemical structures of nitroxyl radical compounds

RESULTS AND DISCUSSIONS

Cyclic voltammetry (CV) was performed using nitroxyl radical compounds A-TEMPO (**2**) (1 mM), F-TEMPO (**3**) (1 mM), and di-TEMPO (**4**) [0.5 mM, equivalent to TEMPO (**1**)] at a sweep rate of 10 mV/s (Figure 2). A-TEMPO (**2**) and di-TEMPO (**4**) exhibited reversible redox responses in a phosphate buffer (100 mM, pH 10), representing an essential condition where TEMPO (**1**) activity was high. The oxidation peak (E_{pa}) and reduction peak potentials (E_{pc}) of A-TEMPO (**2**) vs. Ag/AgCl were +630 and +560 mV, respectively. For di-TEMPO (**4**), E_{pa} and E_{pc} were +650 mV and +580 mV, respectively. The E_{pa} of TEMPO (**1**) was +500 mV, suggesting an enhanced oxidation potential. However, F-TEMPO (**3**) showed a much lower E_{pa} of +450 mV compared to the value of **1**. Consequently, no reduction peak was observed, indicating the absence of a reversible response attributed to the electron-withdrawing trifluoromethyl group in **3**, resulting in a higher δ+ character on the carbonyl carbon. Therefore F-TEMPO (**3**) is more susceptible to hydrolysis under basic conditions than A-TEMPO (**2**).[19] Therefore, this cyclic voltammogram was attributed to 4-amino-TEMPO. Conversely, under neutral pH conditions (pH 7.4), F-TEMPO (**3**) exhibited a reversible voltammogram with E_{pa} and (E_{pc}) of +650 and +580 mV, respectively. Under neutral pH

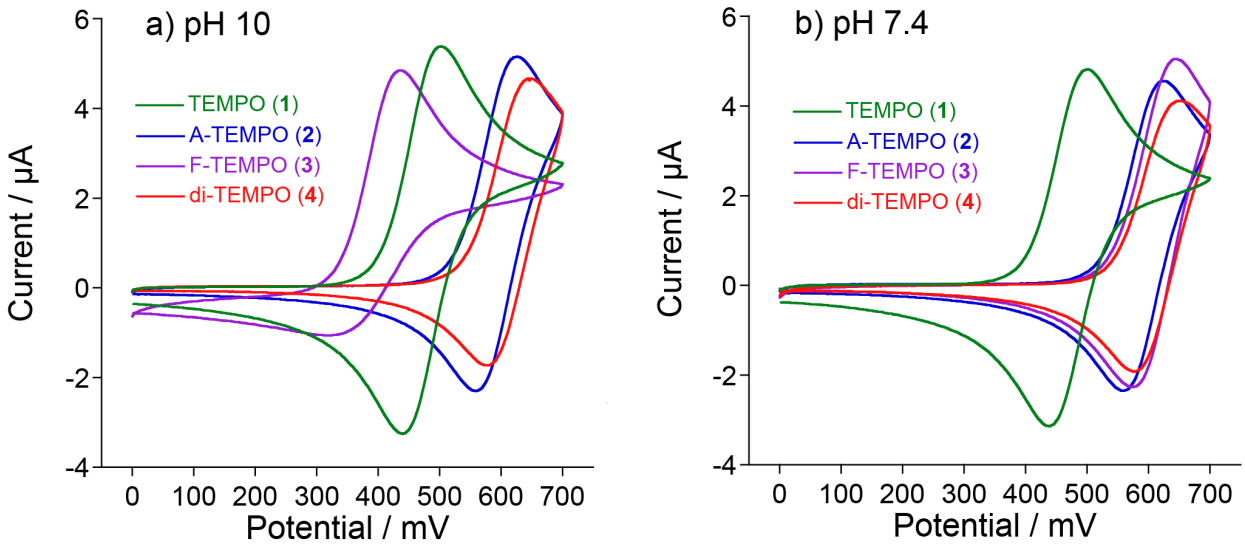

Figure 2. Cyclic voltammograms of A-TEMPO (**2**) (1 mM), F-TEMPO (**3**) (1 mM), di-TEMPO (**4**) (0.5 mM). a) pH 10, b) pH 7.4 phosphate buffer (100 mM), and sweep rate of 10 mV/sec.

conditions (pH 7.4), A-TEMPO (**2**) and F-TEMPO (**3**) exhibited similar behavior as observed under basic conditions. 4-Oxo-TEMPO also has an electron-withdrawing group, although it does not have the capacity for electrolytic oxidation of alcohols in aqueous solution.[18]

Figure 3 shows a cyclic voltammogram of di-TEMPO (**4**) in the presence of ethanol, demonstrating an increase in the oxidation current with increasing ethanol concentration, accompanied by the disappearance of the reduction peak. This mechanism is illustrated as follows. (1) The nitroxyl radical site of TEMPO (**1**) is oxidized at the electrode to obtain oxoammonium. (2) Oxoammonium is subsequently reduced by alcohol to obtain hydroxylamines. The alcohols are then oxidized to aldehydes. (3) Hydroxylamine is also oxidized at the electrode. Therefore, during the low to high potential sweep, TEMPO (**1**) is oxidized again, resulting in an increased peak current. Similarly, in the reverse sweep, the reduction peak disappears as the oxoammonium ions present on the electrode surface are reduced by the alcohol. Therefore, in the CV of the nitroxyl radical compound, the presence of an alcohol (hydroxy group) leads to an increased current value at the oxidation potential peak, proportional to the alcohol concentration, because of its electrocatalytic action. The increase in the current value (ΔI_p) at a given alcohol corresponds to the oxidation activity, which can be used as a measure to evaluate the oxidation activity of nitroxyl radicals.

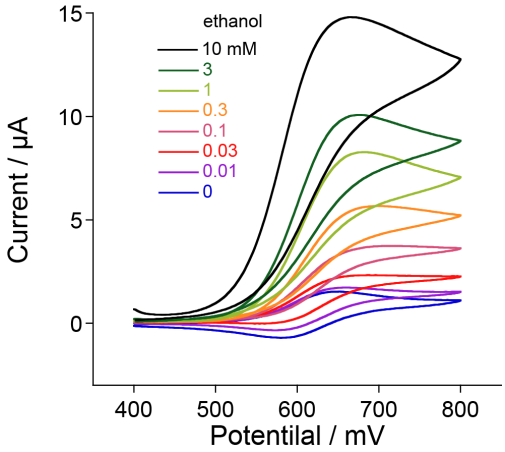

Figure 3. Cyclic voltammogram of di-TEMPO (**4**) (0.5 mM) in the presence of ethanol, pH 10 phosphate buffer (100 mM), and 10 mV/sec sweep rate.

Similar measurements were performed using A-TEMPO (**2**) (1 mM) and F-TEMPO (**3**) (1 mM), and the changes in the response current (ΔI_p) to ethanol are shown in Figure 4. The measurements were performed at sweep rates of 10, 50, and 100 mV/s. At a sweep rate of 10 mV/s, A-TEMPO (**2**) (1 mM) and di-TEMPO (**4**) (0.5 mM) showed comparable ethanol responses; however, di-TEMPO (**4**) showed enhanced response with an increasing sweep rate. Although A-TEMPO (**2**) is a highly active TEMPO derivative, di-TEMPO (**4**) was more useful for ethanol quantification under these conditions. In contrast, F-TEMPO (**3**) did not exhibit ethanol response under basic conditions. However, under alkaline conditions, F-TEMPO (**3**)

hydrolyzes to amino-TEMPO. The amino group of amino-TEMPO was deactivated through a reaction with oxoammonium. Consequently, we did not anticipate F-TEMPO (**3**) to exhibit electrochemical reactivity due to this mechanism. Although F-TEMPO (**3**) has been reported to have excellent reactivity in organic synthesis,[7] it does not show an electrochemical response under basic aqueous solution conditions.

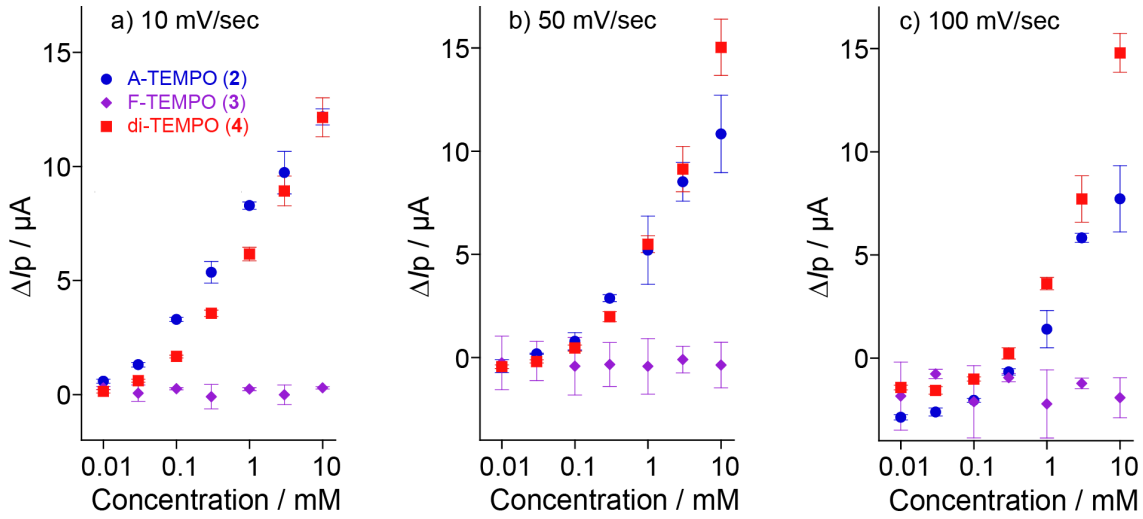

Figure 4. Ethanol calibration curve prepared using A-TEMPO (**2**) (1 mM), F-TEMPO (**3**) (1 mM), di-TEMPO (**4**) (0.5 mM), and pH 10 phosphate buffer (100 mM). A-TEMPO (**2**) (+630 mV), F-TEMPO (**3**) (+450 mV), and di-TEMPO (**4**) (+650 mV) current value changes were used. The average values of 3 electrodes with standard deviation are plotted.

The electrolytic oxidation capacity was evaluated under neutral conditions [pH 7.4 (100 mM phosphate buffer)]. Figure 5 shows the changes in the response current (ΔI_p) to ethanol at pH 7.4 for A-TEMPO (**2**) (1 mM), F-TEMPO (**3**) (1 mM), and di-TEMPO (**4**) (0.5 mM), and at pH 10. When 10 mM ethanol and A-TEMPO (**2**) coexisted, ΔI_p at a 10 mV/sec sweep rate was 12.2 μA at pH 10 and 1.9 μA at pH 7.4. Di-TEMPO (**4**) showed a similar trend. TEMPO (**1**) is known to exhibit a reduced response current under neutral conditions.[13] However, because the hydrolysis of the trifluoromethyl group did not occur under neutral conditions, F-TEMPO (**3**) exhibited ethanol response and the highest activity among the three compounds. In the reversible redox reaction, the peak potential is theoretically proportional to v^(1/2) of the sweep rate but decreases inversely at 50 and 100 mV/s.

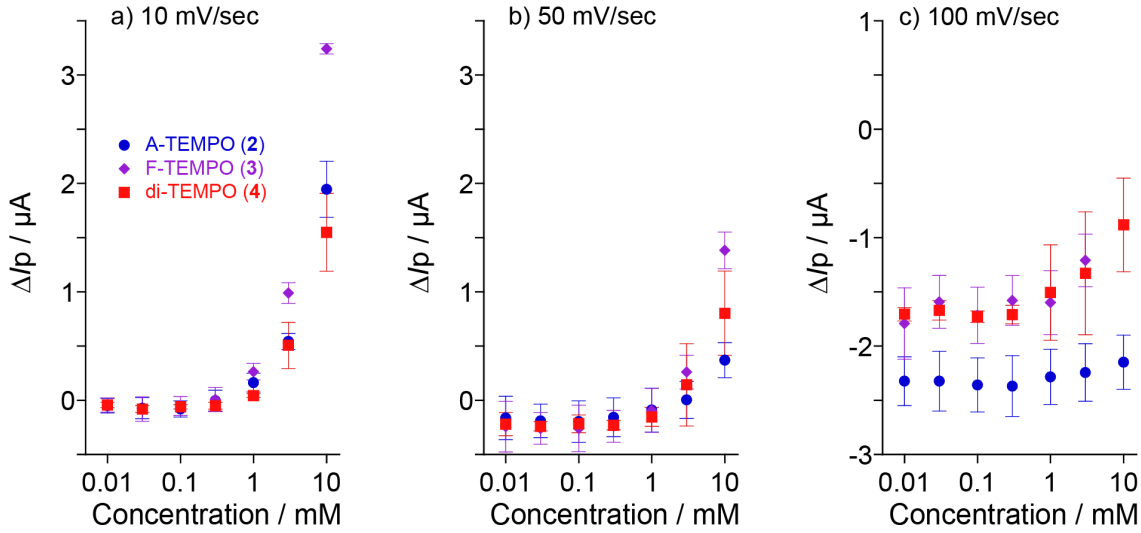

Figure 5. Ethanol calibration curve prepared using A-TEMPO (**2**) (1 mM), F-TEMPO (**3**) (1 mM), di-TEMPO (**4**) (0.5 mM), and pH 7.4 phosphate buffer (100 mM). A-TEMPO (**2**) (+630 mV), F-TEMPO (**3**) (+450 mV), and di-TEMPO (**4**) (+650 mV) current value changes were used. The average values of 3 electrodes with standard deviation are plotted.

Because electrochemical sensing was possible for ethanol, the electrochemical detection of a more complex compound, vancomycin (**5**), was performed. Vancomycin can be detected electrochemically using a nitroxyl radical because of the presence of a hydroxy group in its molecule (Figure 6). However, because of the presence of primary amines in the molecule, the highly active bicyclic nitroxyl radical inhibits the catalytic action, rendering electrochemical measurements impossible.[15] Figure 7 shows the changes in the response current (ΔI_p) to vancomycin (**5**) for A-TEMPO (**2**) (1 mM), F-TEMPO (**3**) (1 mM), and di-TEMPO (**4**) (0.5 mM) at pH 10. The measurements were performed at 10, 50, and 100 mV/sec sweep rates. Similar to ethanol, A-TEMPO (**2**) and di-TEMPO (**4**) exhibited vancomycin-concentration-dependent response currents. However, unlike ethanol, A-TEMPO (**2**) showed a higher response current than di-TEMPO (**4**) (Figure 4). Vancomycin (**5**) has a complex structure, and the steric hindrance makes it challenging for di-TEMPO (**4**), a combination of two TEMPOs, to access the hydroxy group of vancomycin (**5**). Consequently, A-TEMPO (**2**), with its compact molecular size, exhibited a significant response current. Therefore, the molecular design of TEMPO might impart molecular recognition capability for its oxidative action.

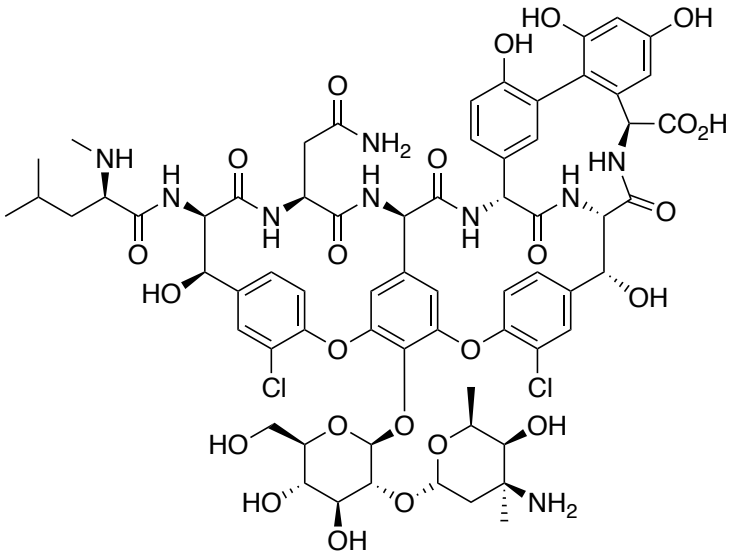

Figure 6. Chemical structure of vancomycin (**5**)

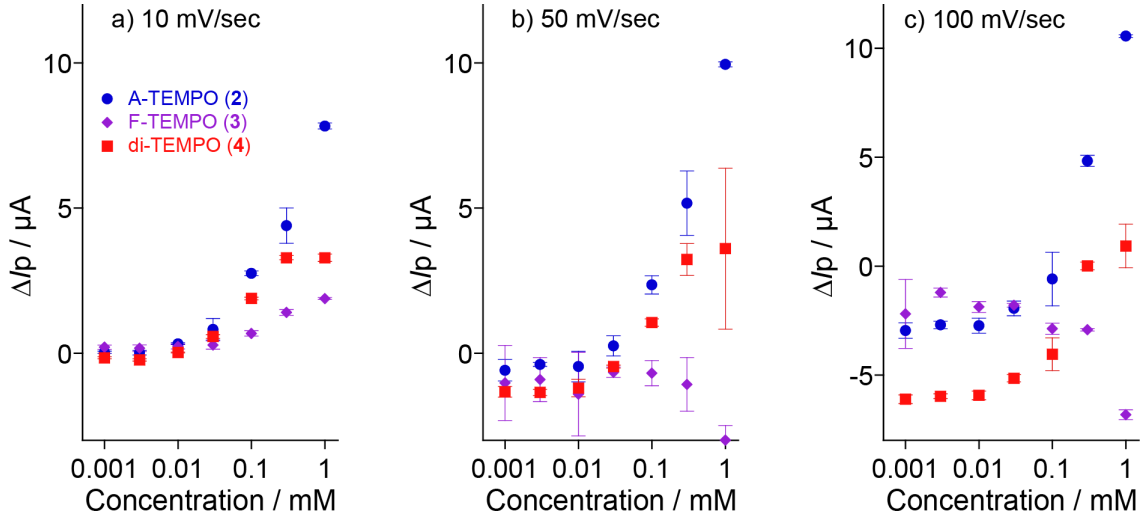

Figure 7. Vancomycin calibration curve prepared using A-TEMPO (**2**) (1 mM), F-TEMPO (**3**) (1 mM), di-TEMPO (**5**) (0.5 mM), and pH 10 phosphate buffer (100 mM). A-TEMPO (**2**) (+630 mV), F-TEMPO (**3**) (+450 mV), and di-TEMPO (**4**) (+650 mV) current value changes were used. The average values of 3 electrodes with standard deviation are plotted.

When performing TDM of drugs, performing measurements under neutral conditions is desirable to simplify pretreatment. Therefore, vancomycin levels were measured at pH 7.4. Figure 8 shows the changes in ΔI_p vs. vancomycin using A-TEMPO (**2**) (1 mM), F-TEMPO (**3**) (1 mM), and di-TEMPO (**4**) (0.5 mM). A-TEMPO (**2**) and F-TEMPO (**3**) exhibited vancomycin concentration-dependent responses at all sweep rates. For A-TEMPO (**2**), a faster sweep rate results in higher ΔI_p. Comparing the values at 1 mM vancomycin, ΔI_p was 6, 11, and 12 μA for sweep rates of 10, 50, and 100 mV/sec, respectively. In the presence of 1 mM vancomycin (**5**), F-TEMPO (**3**) exhibited response currents of 5, 9, and 8 μA for sweep rates of 10, 50, and 100 mV/sec, respectively, with large error bars. Unlike ethanol measurements, a large

value of ΔI_p was observed at pH 7.4 for A-TEMPO (**2**) and F-TEMPO (**3**). In di-TEMPO (**4**), a vancomycin (**5**) concentration-dependent response was observed only at 10 mV/s; however, the response current remained below 2 μA even at 1 mM. Moreover, steric hindrance prevents the easy access of di-TEMPO (**4**) to the hydroxyl group of vancomycin, as observed at pH 10.

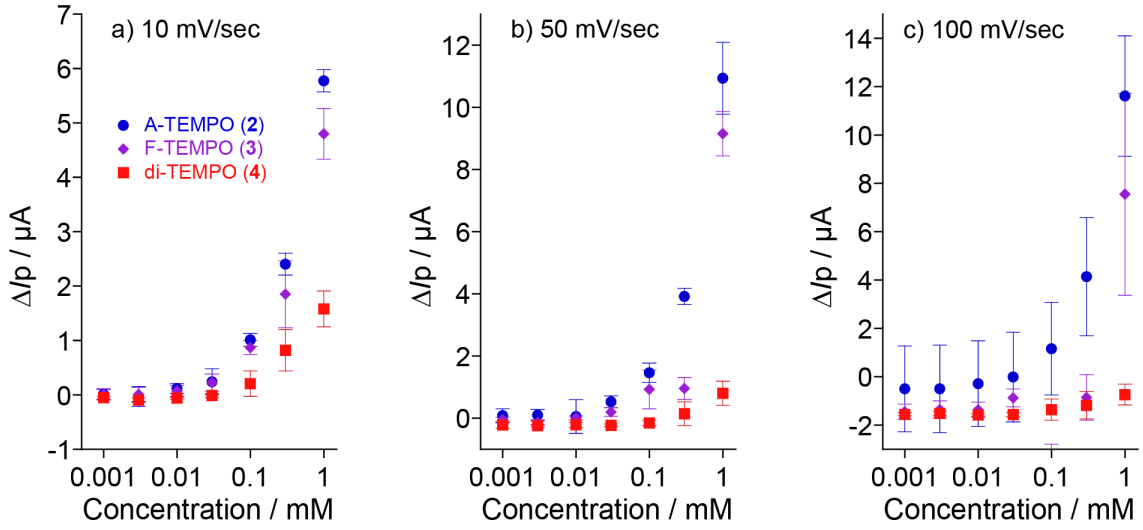

Figure 8. Vancomycin calibration curve prepared using A-TEMPO (**2**) (1 mM), F-TEMPO (**3**) (1 mM), di-TEMPO (**4**) (0.5 mM), and pH 7.4 phosphate buffer (100 mM). A-TEMPO (**2**) (+630 mV), F-TEMPO (**3**) (+450 mV), and di-TEMPO (**4**) (+650 mV) current value changes were used. The average values of 3 electrodes with standard deviation are plotted.

CONCLUSION

This study reports the synthesis of highly activated TEMPO (**1**) derivatives by introducing electron-withdrawing groups and investigated their applications in electrochemical analysis. Although the expected results were not obtained with F-TEMPO (**3**) due to hydrolysis at pH 10, ethanol and vancomycin were quantifiable at pH 7.4. However, when A-TEMPO (**2**) and di-TEMPO (**4**) were used, ethanol and vancomycin (**5**) were quantifiable at pH 7.4 and 10. In contrast, di-TEMPO (**4**) showed decreased reactivity compared to the other derivatives when vancomycin was measured due to steric hindrance. The findings suggest that TEMPO's molecular design may impart molecular recognition capability for its oxidative action.

EXPERIMENTAL

Materials

TEMPO (**1**) and A-TEMPO (**2**) were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). F-TEMPO (**3**) and di-TEMPO (**4**) were synthesized as described below. All other reagents were used as received without further purification.

*Synthesis of F-TEMPO (**3**):*

To 4-amino-TEMPO (343 mg, 2.0 mmol) was dissolved in dichloromethane (DCM) (15 mL), followed by the addition of pyridine (0.48 mL, 6.0 mmol) and 4-dimethylaminopyridine (DMAP) (cat.) at 0 °C. The resulting solution was stirred for 5 min, followed by the addition of trifluoroacetic anhydride (0.56 mL, 4.0 mmol) at 0 °C. Subsequently, the reaction mixture was stirred for 12 h at room temperature, and the reaction was quenched using 1M HCl. The resulting solution was extracted using DCM (10 mL × 3). The combined organic layers were washed using a sat. aq. $NaHCO_3$ (10 mL) and brine (10 mL). After drying over anhydrous $MgSO_4$, the solution was filtered, and the solvent was evaporated under vacuum. Purification via column chromatography (SiO_2) elution (using a 1:1 EtOAc: hexane mixture) yielded 4-trifluoroacetamido-2,2,6,6-tetramethylpiperidine 1-oxyl (F-TEMPO) (**3**) (394 mg, 74%).[19]

*Synthesis of di-TEMPO (**4**):*

4-Amino-TEMPO (2.00 g, 11.6 mmol) was dissolved in DCM (60 mL), followed by the addition of triethylamine (4.01 mL, 29.0 mmol), and DMAP (cat.) at 0 °C. The resulting solution was stirred for 5 min, followed by the addition of oxalyl chloride (0.24 mL, 2.90 mmol) at 0 °C. After stirring for 19 h at room temperature, the reaction was quenched using sat. aq. NH_4Cl. The mixture was extracted using DCM (3 × 30 mL). The combined organic layers were washed using a sat. aq. $NaHCO_3$ (50 mL) and brine (50 mL). After drying over anhydrous $MgSO_4$, the solution was filtered, and the solvent was evaporated under vacuum. Purification via column chromatography (SiO_2) elution (using 1;1 AcOEt: hexane mixture) yielded 4,4′-[(1,2-dioxo-1,2-ethanediyl)diimino]bis[2,2,6,6-tetramethyl-1-piperidinyloxy] (di-TEMPO) (**4**) (878.3 mg, 76%).[20]

Electrochemical measurement

Electrochemical measurements were performed using an electrochemical analyzer (ECstat-400, ec-frontier, Kyoto, Japan). The measurements were performed using a three-electrode system comprising a GC electrode (3 mm in diameter), a platinum wire, and Ag/AgCl (3 M KCl) as the working, counter, and reference electrodes, respectively. Various nitroxyl radical compounds (1 mM TEMPO) and ethanol or vancomycin dissolved in phosphate buffer (100 mM, pH 10.0, and 7.4) were used as electrolytes. Measurements were performed at sweep rates of 10, 50, and 100 mV/s, and the increase (ΔI_p) in the peak current upon the addition of the substrate in the cyclic voltammogram of the first cycle was calculated as the response current. All experiments were performed at room temperature (approximately 20 °C).

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