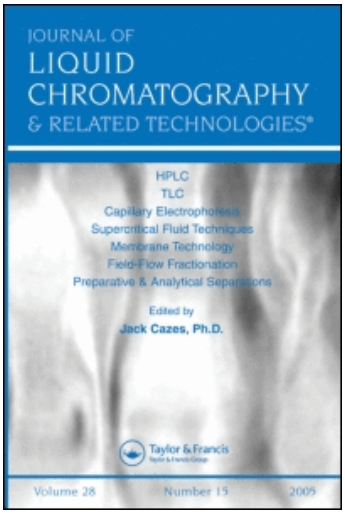

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A NEW ELECTROGENERATED CHEMILUMINESCENCE DERIVATIZATION REAGENT, 3-(DIETHYLAMINO) PROPIONIC ACID FOR ALCOHOL IN HPLC USING *TRIS*(2,2′-BIPYRIDINE)RUTHENIUM(II)

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INTRODUCTION

Recently, the electrogenerated chemiluminescent (ECL) system, using *tris*(2,2′-bipyridine)ruthenium(II) [$Ru(bpy)_3^{2+}$] has become a powerful tool for the determination of compounds containing tertiary amine or diketone groups because of its high sensitivity and selectivity. The reaction mechanism between $Ru(bpy)_3^{2+}$ and tertiary amine is postulated to be as follows:

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^-$$

$$Ru(bpy)_3^{3+} + \text{reductant} \rightarrow \text{product} + [Ru(bpy)_3^{2+}]^*$$

$$[Ru(bpy)_3^{2+}]^* \rightarrow Ru(bpy)_3^{2+} + h\nu$$

$Ru(bpy)_3^{3+}$, obtained from electrochemical oxidation, reacts with amines to give cation radicals. It is thought to produce neutral radicals by eliminating α-protons.

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These unstable radical compounds immediately react with $Ru(bpy)_3^{3+}$ to give the excited state of the ruthenium complex, $[Ru(bpy)_3^{2+}]$.* Its emission to the background state gives phosphorescence, with the maximum wavelength at 620 nm.

The tertiary amine group can be selectively detected by this system and a variety of drugs and endogenous compounds containing tertiary amine have been sensitively determined with simple, minimal pretreatment of biological samples. Some applications for high-performance liquid chromatography (HPLC) or flow injection analysis (FIA) have been developed in recent years. Compounds to which $Ru(bpy)_3^{3+}$ chemiluminescence (CL) has been applied include amino acids,[1–4] antihistamines,[5] clindamycin antibiotics,[6] glyphosate,[7] NADH,[8,9] dansyl derivatized amino acids,[10,11] oxalate,[10,12] erythromycin,[13,14] and erythromycin derivatives,[15] 4-hydroxyproline,[16] ascorbic acid,[17] antidepressants,[18] codeine,[19] and alkaloid-type drugs.[20] Nevertheless, compounds detectable with this system are still limited. Therefore, we attempted to extend the system to a wider range of compounds containing non-suitable functional groups by converting a variety of compounds into derivatives suitable for ECL detection by tagging appropriate molecules with an analyte.

Many derivatization reagents for HPLC have been developed;[21] however, excess reagent or disintegrated compounds resulting from it often interfere with the determination of the analyte. An ideal derivatization reagent should possess the ability to: (i) completely and quantitatively convert an analyte to a single derivative under mild reaction conditions in a short time; (ii) minimize side reactions; (iii) maintain reasonable stability at room temperature (this also applies to the derivatives); (iv) allow simple chromatographic separation of the reagent and derivative; (v) be easy to obtain or synthesize; and (vi) allow highly sensitive and widely applicable detection of compounds with the same sensitivity. It would be very difficult to synthesize an ultraviolet or fluorescence reagent that fulfills all these criteria. However, as the ECL system can sensitively detect small molecules containing tertiary amine groups as a tagging molecule, it should be relatively easy to establish an ideal derivatization reagent without interference by degradation products, thus enabling easy separation from the analyte. Two reagents, dansyl chloride[11] and divinylsulfone (DVS),[22] have been reported for the ECL system, with the detection limits of dansyl and DVS derivatives of 2 pmol (signal-to-noise ratio of 2) for amino acids and 1–30 pmol for primary amines, respectively. As the derivatization reaction with DVS proceeds to change primary amines to tertiary amines, the detection limits of DVS derivatives depend on the structure of the analyte. To develop reagents that are more sensitive and have a constant intensity for a wide range of compounds, small molecules were considered as potential candidates for compounds containing tagging groups that display strong intensity in this system.

Various types of derivatization reagents for alcohol by fluorescence, ultraviolet absorbance, electrochemical and chemiluminescence detection

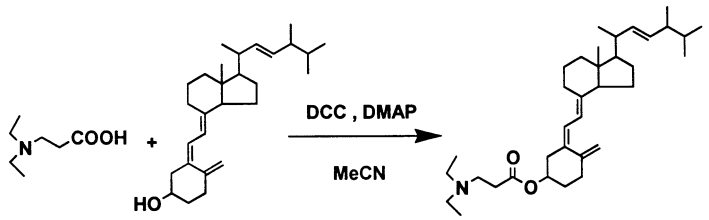

Figure 1. Reaction scheme of D_2 with DEAP.

have been developed, e.g., carbonyl chloride,[23] carbonyl nitrile,[24] carbonyl azide,[25,26] sulfonyl chloride[27,28] and isocyanate[29] types. However, most of these reagents require drastic reaction conditions because of their low reactivity. In the present study, we evaluated DEAP as the derivatization reagent for alcohol. To confirm that DEAP reacts under mild reaction conditions, without degradation of the analyte, we selected, as a model compound, vitamin D_2 (D_2), which is a relatively unstable and very lipophilic compound. The reaction of DEAP and D_2 is shown in Fig. 1.

EXPERIMENTAL

Reagents

Tris(2,2′-bipyridine)ruthenium(II) chloride pentahydrate ($Ru(bpy)_3Cl_2 \cdot 5H_2O$) and *N*,*N*′-dicyclohexylcarbodiimide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). D_2 was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). 4-Dimethylaminopyridine (DMAP) was obtained from Wako Pure Chemical Indutries, Ltd. (Osaka, Japan). DEAP hydrochloride was from Aldrich Chemical Co., Inc. (WI, USA). Acetonitrile and methanol were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan) for HPLC grade. All the other chemicals were of guaranteed grade and used without further purification. Twice-distilled deionized water was used throughout the study.

Instrumentation

The chemiluminescent intensity was observed by modifying a commercially available system. The HPLC system is shown in Fig. 2. All the solutions were purged with two types of degassers (DGU-10B for helium gas purge type and DGU-3A for membrane type, Shimadzu, Japan) and were delivered with

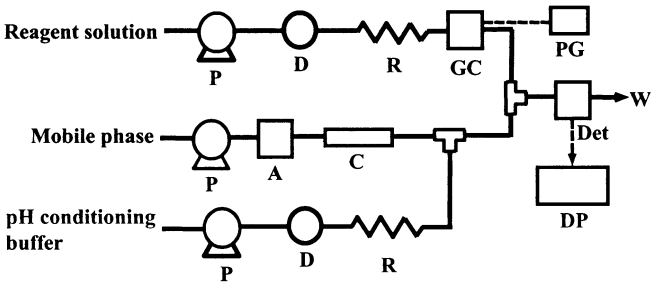

Figure 2. Flow diagram of ECL detection. Abbreviations: P = pump; D = damper; R = resistance coil; GC = guard cell; PG = potentio-galvanostat; A = autosampler; C = column; Det = chemiluminescence detector; DP = data processor; W = waste.

pumps (LC-10AD, Shimadzu). Shimadzu SIL-6A was used as the autosampler. Electrochemical oxidation was performed with a porous graphite working electrode (guard-cell, model 5020, ESA) and the current was controlled with a potentio-galvanostat (NPGS-2501, Nikkoh Keisoku, Japan). The chemiluminescence detector used was a Shimadzu CLD-10A equipped with a 80 μL spiral flow cell and R374HA photomultiplier tube (Hamamatsu Photonics, Japan). C-R4AX (Shimadzu) was used as the data processor. To avoid the permeation of atmospheric oxygen, a metal tube was used for the connection. A low-volume tee-tube was used for mixing of the mobile phase, the reagent solution and the pH-conditioning buffer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were obtained with a Unity-600 spectrometer (Varian) at 600 MHz using CD_3OD containing tetramethylsilane as an internal standard. Electrospray ionization mass spectra (MS) were recorded on a ThermoFinnigan TSQ Quantum spectrometer.

Synthesis of DEAP Derivatives of D_2

To a solution of D_2 (50 mg, 0.13 mmol) in *N*,*N*-dimethyformamide (DMF)/tetrahydrofuran (THF) (1 : 1) 0.8 mL were added DEAP hydrochloride (0.20 mmol), DCC (0.39 mmol) and DMAP (0.13 mmol) for 24 h at room temperature. The reaction mixture was filtered and evaporated to dryness in vacuo. The residue was dissolved with ethyl acetate and washed with brine. The organic layer was dried with anhydrous Na_2SO_4 and the filtrate was evaporated in vacuo. The residue was dissolved with a small portion of methanol and purified by thin-layer chromatography on silica-gel with ethyl acetate/*n*-hexane/triethylamine (2 : 6 : 1, v/v) as the developing solvent to afford 13.3 mg of the D_2-DEAP derivative. ^{1}H NMR (CD_3OD): δ 0.57 (3H, s), 0.85 (3H, d, $J = 6.4$ Hz),

0.86 (3H, d, $J = 6.4$ Hz), 0.93 (3H, d, $J = 7.3$ Hz), 1.03 (6H, d, $J = 7.2$ Hz), 1.04 (3H, d, $J = 7.2$ Hz), 1.33 (1H, m), 1.37 (1H, m), 1.44 (1H, m), 1.48 (1H, m), 1.56 (1H, m), 1.69 (1H, m), 1.76 (1H, m), 1.79 (1H, m), 1.84 (1H, m), 1.86 (1H, m), 1.94 (1H, m), 2.01 (1H, m), 2.02 (1H, m), 2.03 (1H, m), 2.04 (1H, m), 2.21 (1H, m), 2.38 (1H, m), 2.41 (1H, m), 2.46 (2H, dd, $J = 8.1$, 6.7 Hz), 2.54 (4H, q, $J = 7.3$ Hz), 2.56 (1H, m), 2.80 (2H, t, $J = 7.3$ Hz), 2.85 (1H, dd, $J = 11.8$, 3.9 Hz), 4.79 (1H, d, $J = 2.7$ Hz), 4.94 (1H, m), 5.08 (1H, m), 5.20 (1H, m), 5.24 (1H, m), 6.04 (1H, d, $J = 11.2$ Hz), 6.21 (1H, d, $J = 11.2$ Hz). MS: m/z 524.5 ($[M+H]^+$).

Optimization of Detection pH

The detection pH was optimized by an HPLC method. The mobile phase of 50 mM Britton–Robinson (BR) buffer (pH 2.0) containing 60% acetonitrile and the reagent solution of 0.8 mM $Ru(bpy)_3Cl_2$ in 10 mM H_2SO_4 were pumped at the flow rates of 1.0 mL/min and 0.5 mL/min, respectively. The detection pH was controlled by 0.5 M BR buffer (pH 4.0–7.5) pumped at the flow rate of 0.3 mL/min. The 0.5 M BR buffer was prepared as an acid solution with 61.3 g of boric acid, 56.5 mL of acetic acid and 68 mL of phosphoric acid in 2 L of water. The pH was adjusted with 2.5 M NaOH to prepare pH 4–9 buffers. The column was a Cosmosil 5Ph (4.6 × 50 mm, Nacalai Tesque Inc.). An aliquot of 5 μL of methanol solution containing 100 pmol synthetic sample was injected. The electrochemical oxidation was done by controlled-current mode with the current maintained at 200 μA. The oxidation potential was approximately +1.3 V vs. an α-hydrogen/palladium reference electrode. The photomultiplier applied was biased at 0.5 kV.

Derivatization Procedure

To a solution of D_2 in 50 μL of acetonitrile was added 50 μL each of the 20 mM DEAP, 25 mM DCC and 10 mM DMAP solution in acetonitrile. After mixing with a vortex mixer for a few seconds, the reaction mixture was allowed to stand for 2 h at room temperature and 200 μL of methanol was added to stop the reaction. Of the resulting mixture, 20 μL was injected into the chromatograph.

HPLC Conditions

The mobile phase of 50 mM BR buffer (pH 2.0) containing 55% acetonitrile and reagent solution of 0.8 mM $Ru(bpy)_3Cl_2$ in 10 mM H_2SO_4

were delivered at the flow rates of 1.0 mL/min and 0.5 mL/min, respectively. To optimize the detection pH, 0.5 M BR buffer (pH 6.0) was used and pumped at the flow rate of 0.3 mL/min. The column was a Cosmosil 5Ph (4.6 × 50 mm). The column temperature was ambient (23 ± 2°C).

RESULTS AND DISCUSSION

Optimization of Detection pH

We selected DEAP as a derivatization reagent that included a reaction group for alcohol and showed strong intensity in small molecules. Figure 3 shows the pH-dependent ECL intensity of DEAP and its derivative of D_2. The ECL intensity of the DEAP derivative of D_2 decreased by 4-fold compared to that of the unbound DEAP. The maximum intensity was obtained at pH 6.0 for the DEAP derivative of D_2 and at pH 7.5 for the unbound DEAP. In this system, hydroxide ion raises the background level, therefore, a lower detection pH leads to a more stable analytical condition. Although the ECL intensity of the DEAP derivative of D_2 decreased compared to unbound DEAP, the detection limit of the DEAP derivative of D_2 was almost the same as that of unbound DEAP because of its high signal-to-noise ratio. This result shows that DEAP does not decrease the sensitivity by esterification with very lipophilic compounds such as D_2.

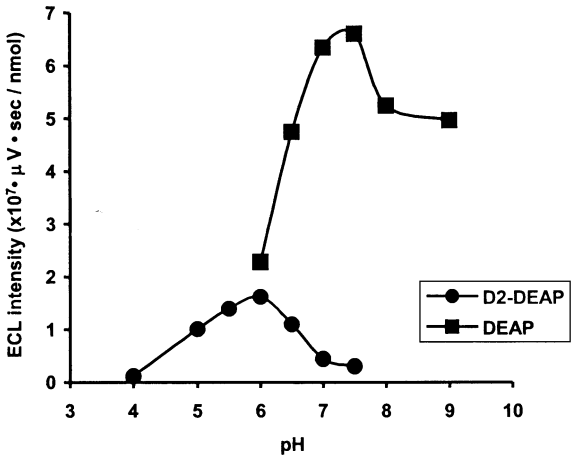

Figure 3. pH profile of DEAP and its derivative of D_2. Sample: 100 pmol in methanol solution was injected.

Optimization of the Derivatization Reaction

For the derivatization of alcohol, DCC and DMAP were selected as the condensation reagent and the catalyst, respectively. These reagents allowed the reaction to proceed with alcohol to produce the corresponding ester at room temperature.

The effect of the reaction temperature was examined and no significant difference was observed at 25–80°C (data not shown). At high temperature, degradation products of D_2 were observed, and therefore, the reaction temperature was set at room temperature. DMF, THF, dioxane, acetonitrile and ethyl acetate were examined to determine the reaction solvent, and the highest reactivity was displayed by acetonitrile, leading to its selection. DMAP, pyridine and triethylamine were tested as basic catalysts. As triethylamine reacts with $Ru(bpy)_3^{3+}$, it raised the background level, but pyridine did not show a significant effect. DMAP was selected as the catalyst. Figures 4–6 show the effect of the concentration of DEAP, DCC or DMAP on the reactivity to D_2, respectively.

The effect of the concentration of DEAP on the reactivity to D_2 was examined at 0–20 mM and then set at 20 mM. The effect of the concentration of DCC and DMAP on the reactivity to D_2 was examined at 0–100 mM. A maximum peak area was obtained at 25 mM for DCC and 10 mM for DMAP. Figure 7 shows the time course of the reaction under these conditions. The maximum peak area was observed at 2 h. Many derivatization reagents for alcohol need heating or drastic reaction conditions because of their low reactivity. DEAP reacts with alcohol at room temperature, and thus compounds unstable to heating, such as vitamin D, can be determined without degradation.

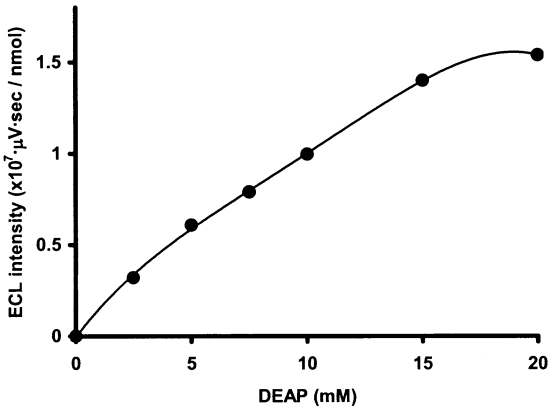

Figure 4. Effect of DEAP concentration on ECL intensity. Conditions: 10 μM D_2, 25 mM DCC, 5 mM DMAP, room temperature for 1 h.

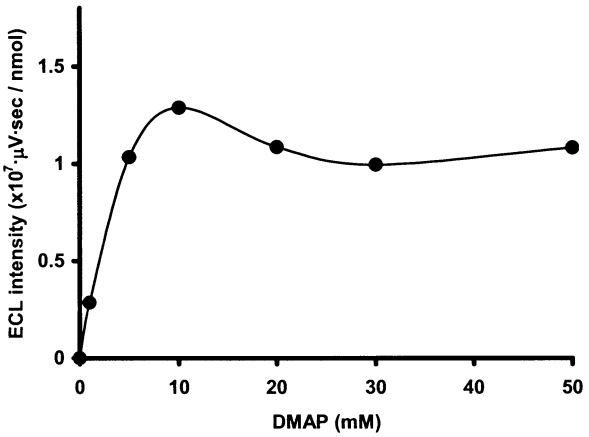

Figure 5. Effect of DCC concentration on ECL intensity. Conditions: 10 μM D_2, 20 mM DEAP, 5 mM DMAP, room temperature for 1 h.

HPLC Conditions and Calibration Curve

Figure 8 shows a typical chromatogram of D_2 prepared by the derivatization procedure. The DEAP derivative gave a single peak on the reversed-phase column. For the mobile phase, 50 mM BR buffer (pH 2.0)/acetonitrile was used. The retention time of the DEAP derivative was 12.5 min, and the detection limit

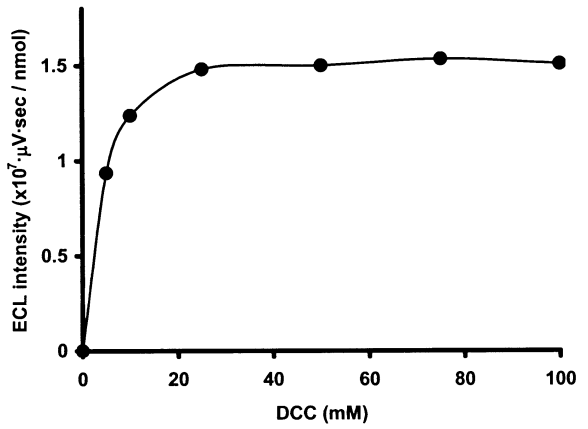

Figure 6. Effect of DMAP concentration on ECL intensity. Conditions: 10 μM D_2, 25 mM DCC, 20 mM DEAP, room temperature for 1 h.

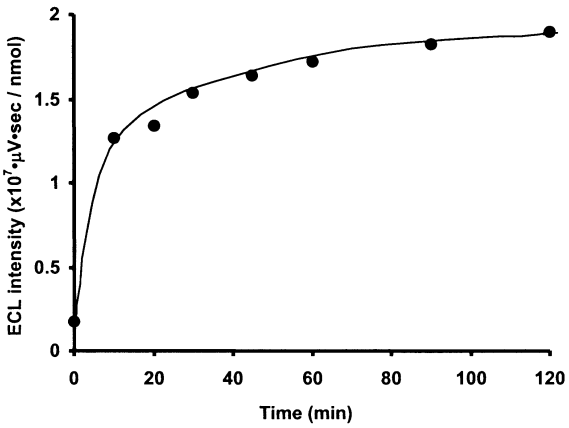

Figure 7. Time course of derivatization reaction of DEAP with D_2 in the presence of DCC and DMAP. Conditions: 10 μM D_2, 20 mM DEAP, 25 mM DCC, 10 mM DMAP, room temperature.

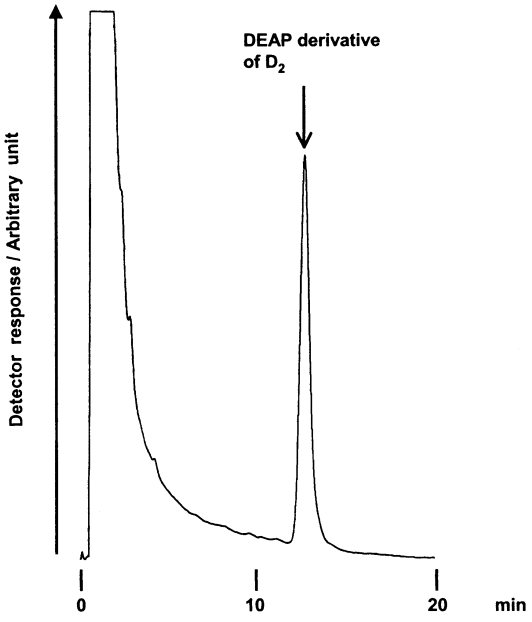

Figure 8. Chromatogram of DEAP derivative of D_2. HPLC conditions are given in the experimental section. Sample: 25 pmol on column.

of the DEAP derivative of D_2 was found to be 40 fmol at a signal-to-noise ratio of 3. The calibration curves ranging from 3.8–750 nM ($n = 9$) were linear with a correlation coefficient of 0.9999.

CONCLUSIONS

A new derivatization reagent specifically for the ECL system, was developed for alcohol. No decrease of sensitivity and no degradation were observed in the derivatization reaction, which could be completed under mild conditions. Separation of the analyte with excess reagent was readily achieved. DEAP is commercially available, inexpensive and highly sensitive. In case of applying to hydrophilic compounds, excess reagent should be removed, however, hydrophobic compounds could be used without the removal of the excess reagent. This reagent should be used for alcoholic compounds that are unstable under drastic derivatization reaction conditions.

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