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SENSITIVE FERROCENE REAGENTS FOR DERIVATIZATION OF AMINES FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

Six reagents possessing ferrocene as an electrophore were prepared and evaluated for pre-column derivatization of amino compounds for their determination by high-performance liquid chromatography with electrochemical detection. The utility of these reagents was investigated employing phenethylamine as a model compound. Among these six, N-succinimidyl 3-ferrocenylpropionate was the best with respect to reactivity, stability and electrochemical properties. The developed method was applied to the determination of putrescine formed from ornithine by ornithine decarboxylase.

INTRODUCTION

High-performance liquid chromatography (HPLC) with electrochemical detection (ED) is a useful method for the trace determination of various compounds in biological fluids [1]. In recent years, pre- and post-column labelling methods have been developed to extend its applicability [2–9]. In previous papers we proposed novel ferrocene reagents for pre-column labelling of hydroxy [4], carboxylic [5–8] and thiol [9] groups in HPLC–ED. As the ferrocene derivative undergoes facile oxidation and the product is in turn readily reduced, it can be detected selectively in the presence of other electroactive compounds such as phenols, catechols and aromatic amines.

In a preliminary report we proposed N-succinimidyl 3-ferrocenylpropionate as a pre-column labelling reagent of amino groups in HPLC–ED [10]. This paper

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deals with the preparation and properties of six derivatization reagents having ferrocene as an electrophore, including the above reagent, for the determination of amino compounds. In addition, the application of this method to the determination of putrescine formed from ornithine by ornithine decarboxylase is described.

EXPERIMENTAL

Materials

(Dimethylaminomethyl)ferrocene and ferrocenecarboxaldehyde were purchased from Aldrich (Milwaukee, WI, U.S.A.). Ferrocenecarboxylic acid and 1,1′-dimethylferrocene were supplied by Tokyo Kasei Kogyo (Tokyo, Japan), the latter being purified by chromatography on Toyo Pak ODS (TOSOH, Tokyo, Japan) to remove monomethylferrocene contaminant. Sep-Pak C_{18} cartridges were supplied by Waters Assoc. (Milford, MA, U.S.A.). Ornithine decarboxylase (from *Escherichia coli*, 0.63 U/mg of protein) was obtained from Sigma (St. Louis, MO, U.S.A.). All other reagents and chemicals were purified by recrystallization or distillation prior to use. Silica gel 60 and silica gel HF_{254} (E. Merck, Darmstadt, F.R.G.) were used for column chromatography and thin-layer chromatography (TLC), respectively.

Instruments

HPLC was carried out on a Waters Assoc. 202 chromatograph equipped with a Yanagimoto (Kyoto, Japan) VMD 501 electrochemical detector. The applied potential was set versus an Ag/AgCl reference electrode. A YMC-GEL C_8 (5 μm) column (15 cm$\times$0.4 cm I.D.) (Yamamura Chem. Lab., Kyoto, Japan) was used at a flow-rate of 1 ml/min at ambient temperature. Acetonitrile and 0.05 *M* sodium perchlorate were used as an organic modifier and electrolyte, respectively, unless stated otherwise.

Preparation of derivatization reagents

Melting points were measured on a micro hot-stage apparatus (Yanagimoto) and were uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a JEOL (Tokyo, Japan) FX-100 spectrometer at 100 MHz with deuteriochloroform solutions using tetramethylsilane as an internal standard (I.S.). Abbreviations used are: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad. Low- and high-resolution mass spectral (MS) measurements were run on Hitachi (Tokyo, Japan) M-52G and JEOL JMS-01SG-2 spectrometers, respectively. The structures of the reagents and their synthetic intermediates are shown in Fig. 1.

N-Succinimidyl 3-ferrocenylpropionate (IIb)

3-Ferrocenylpropionic acid (IIa) (50 mg), obtained from ferrocenecarboxaldehyde [11], was treated with N-hydroxysuccinimide (25 mg) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (50 mg) in dioxane (4 ml) at room temperature for 5 h. After extraction with ethyl acetate, the organic layer was washed with water and dried over anhydrous sodium sulphate. The organic

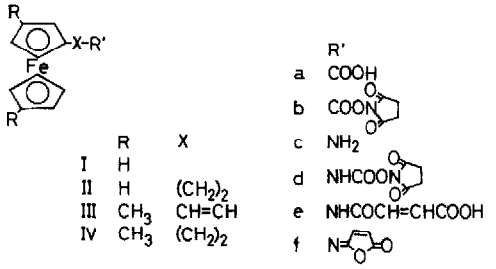

Fig. 1. Structures of derivatization reagents and their synthetic intermediates.

solvent was evaporated under vacuum and the residue was subjected to column chromatography (15 cm×1.2 cm I.D. column) with ethyl acetate–hexane (1:2) as eluent. The eluate was dried and recrystallized from ethyl acetate–hexane to give IIb (35 mg) as yellow needles (m.p. 141–142°C). Calculated for $C_{17}H_{17}FeNO_4$, C 57.49, H 4.82, N 3.94; found, C 57.38, H 4.76, N 3.94. Low-resolution MS: *m/z* 355 (M^+). ^1H NMR: δ 2.79 (4H, s, ferrocenyl-$(CH_2)_2$-), 2.85 (4H, s, -$CO(CH_2)_2CO$-), 4.15 (9H, br s, ferrocenyl-H).

N-Succinimidyl 3-(3,1′-dimethylferrocenyl)propionate (IVb)

3,1′-Dimethylferrocenylaldehyde (100 mg), obtained from 1,1′-dimethylferrocene [12], was dissolved in pyridine–piperidine (20:1) (2.1 ml) and treated with malonic acid (100 mg) at 80–90°C for 2 h. After extraction with ethyl acetate, the organic layer was washed with 5% (w/v) hydrochloric acid and water and then dried over anhydrous sodium sulphate. The organic solvent was evaporated and the residue obtained was subjected to column chromatography (12 cm×0.6 cm I.D. column) with benzene–ethyl acetate (4:1) as eluent. The eluate was dried and recrystallized from diethyl ether to give 3-(3,1′-dimethylferrocenyl)acrylic acid (IIIa) (48 mg) as a brown amorphous substance (m.p. 117–119°C). Calculated for $C_{15}H_{16}FeO_2$, C 63.41, H 5.68; found, C 63.70, H 5.72. High-resolution MS: *m/z* 284.0505 (M^+) (calculated for $C_{15}H_{16}FeO_2$, 284.0500). ^1H NMR: δ 1.87 (3H, s, CH_3), 2.01 (3H, s, CH_3), 3.96 (4H, br s, ferrocenyl-H), 4.09 (1H, br s, COOH), 4.20–4.34 (3H, m, ferrocenyl-H), 5.93 (1H, d, J = 15.7 Hz, ferrocenyl-CH=C*H*COOH), 7.48 (1H, d, J = 15.7 Hz, ferrocenyl-C*H*=CHCOOH).

A solution of IIIa (100 mg) in ethanol (5 ml) was shaken with 5% (w/w) palladium–charcoal (60 mg) under a stream of hydrogen for 4 h. After removal of the catalyst by filtration, the filtrate was evaporated to give 3-(3,1′-dimethylferrocenyl)propionic acid (IVa) as a yellow oily substance. High-resolution MS: *m/z* 286.0657 (M^+) (calculated for $C_{15}H_{18}FeO_2$, 286.0656). ^1H NMR: δ 1.77 (3H, s, CH_3), 1.87 (3H, s, CH_3), 2.45 (4H, br s, -$(CH_2)_2$-), 4.03 (7H, br s, ferrocenyl-H), 4.39 (1H, br s, COOH).

Compound IVa (50 mg) was condensed with N-hydroxysuccinimide (25 mg) in acetonitrile (2 ml) as described above. The crude product was subjected to column chromatography (18 cm×1 cm I.D. column) with benzene as eluent. The eluate was dried to give IVb (10 mg) as a yellow amorphous substance (m.p. 71–

75°C). High-resolution MS: *m/z* 383.0816 (M^+) (calculated for $C_{19}H_{21}FeNO_4$, 383.0818). ^1H NMR: δ 1.94 (3H, s, CH_3), 1.97 (3H, s, CH_3), 2.75 (4H, br s, ferrocenyl–$(CH_2)_2$–), 2.84 (4H, s, –$CO(CH_2)_2CO$–), 3.90 (7H, br s, ferrocenyl–H).

N-Succinimidyl ferrocenylcarbamate (Id)

N,N′-Disuccinimidyl carbonate (75 mg) in acetonitrile (2 ml) was added to a solution of ferrocenylamine (Ic) (47 mg), obtained from ferrocenecarboxylic acid [13], in acetonitrile (1 ml) at room temperature for 3 h. After evaporation of the solvent, the residue was subjected to preparative TLC using benzene–ethyl acetate (2:1, v/v) as developing solvent. Elution of the adsorbent corresponding to the spot of R_F 0.58 with ethyl acetate and evaporation of the eluate gave Id (5 mg) as a yellow amorphous substance. The physical properties of Id were not examined because of its instability.

N-Succinimidyl 2-ferrocenylethylcarbamate (IId)

2-Ferrocenylethylamine (IIc) (100 mg), obtained from (dimethylaminomethyl)ferrocene [7,14], was treated with N,N′-disuccinimidyl carbonate (140 mg) as described above. The crude product was subjected to preparative TLC using benzene–ethyl acetate (2:1, v/v) as developing solvent. The adsorbent corresponding to the spot of R_F 0.52 was eluted with ethyl acetate and the eluate was evaporated. The crude product was recrystallized from benzene to give IId (10 mg) as a yellow amorphous substance (m.p. 158–160°C). ^1H NMR: δ 2.57 (2H, br s, ferrocenyl–CH_2 or –CH_2N), 2.82 (4H, s, –$CO(CH_2)_2CO$–), 3.38 (2H, br s, ferrocenyl–CH_2 or –CH_2N), 4.20 (9H, br s, ferrocenyl–H), 5.29 (1H, br s, –NH–).

N-Ferrocenylisomaleimide (If)

N-(Ferrocenyl)maleamic acid (Ie) (100 mg), obtained from Ic [9], was treated with dicyclohexylcarbodiimide (100 mg) in dichloromethane (3 ml) at room temperature for 2 h. After evaporation of the solvent, the residue was subjected to preparative TLC using benzene–ethyl acetate (4:1, v/v) as developing solvent. Elution of the adsorbent corresponding to the spot of R_F 0.59 with ethyl acetate and evaporation of the eluate gave If as a blue amorphous substance (10 mg) (m.p. 98–108°C). High-resolution MS: *m/z* 281.0093 (M^+) (calculated for $C_{14}H_{11}FeNO_2$, 281.0138). ^1H NMR: δ 4.19 (5H, br s, ferrocenyl–H), 4.41 (2H, t, J = 2 Hz, ferrocenyl–H), 4.83 (2H, t, J = 2 Hz, ferrocenyl–H), 6.60 (1H, d, J = 5.7 Hz, vinyl–H), 7.21 (1H, d, J = 5.7 Hz, vinyl–H).

N-(2-Ferrocenylethyl)isomaleimide (IIf)

N-(2-Ferrocenylethyl)maleamic acid (IIe) (200 mg), obtained from IIc [9], was treated with acetic anhydride (2 ml) and sodium acetate (20 mg) at 50–60°C for 15 min. After extraction with diethyl ether, the organic layer was washed with 5% (w/v) sodium hydrogencarbonate and water and then dried over anydrous sodium sulphate. After evaporation of the solvent, the crude product was recrystallized from diethyl ether to give IIf as a yellow amorphous substance (m.p. 59–

62°C). High-resolution MS: *m/z* 309.0471 (M^+) (calculated for $C_{16}H_{15}FeNO_2$, 309.0451). ^1H NMR: δ 2.69 (2H, t, J = 7.7 Hz, ferrocenyl–CH_2 or –CH_2N), 3.79 (2H, t, J = 7.7 Hz, ferrocenyl–CH_2 or –CH_2N), 4.10 (9H, br s, ferrocenyl–H), 6.58 (1H, d, J = 5.7 Hz, vinyl–H), 7.19 (1H, d, J = 5.7 Hz, vinyl–H).

Preparation of authentic putrescine and 1,6-diaminohexane derivatives

1,6-Diaminohexane (DAH) was used as a suitable I.S. Putrescine or DAH (0.5 mg) in water (0.2 ml) was treated with IIb (5 mg) in pyridine (0.2 ml) at room temperature for 12 h. After extraction with diethyl ether, the organic layer was washed successively with 5% (w/v) hydrochloric acid, 2.5% (w/v) sodium hydroxide and water and the organic solvent was evaporated under a nitrogen gas stream. Both derivatives were obtained as yellow needles. Low-resolution MS: *m/z* 568 (M^+) for the putrescine derivative and 596 (M^+) for the DAH derivative.

Derivatization of phenethylamine

Each derivatization reagent (ca. 20 equiv.) in pyridine (0.1 ml) was added to a solution of phenethylamine hydrochloride (1 μg) in water (0.1 ml) and the mixture was kept at room temperature. The reaction was terminated by the addition of excess of methylamine and an aliquot of the solution was subjected to HPLC–ED. The derivatization rate was determined by comparison of the peak area of the ferrocene equivalent to the phenethylamine used.

Procedure for the determination of putrescine formed from ornithine by ornithine decarboxylase

Incubation of the enzyme with ornithine was performed according to the procedure described by Haraguchi et al. [15]. The incubation mixture consisted of ornithine decarboxylase (50 μg) in citrate (20 m*M*)–phosphate (40 m*M*) buffer (pH 5.0, containing 0.1 m*M* dithiothreitol, 10 m*M* β-mercaptoethanol and 0.1 m*M* disodium ethylenediaminetetraacetate) (0.1 ml), 0.1 ml of 1 m*M* pyridoxal phosphate, 0.1 ml of 12.5 m*M* dithiothreitol, 0.2 ml of 5 m*M* ornithine and 0.1 ml of citrate (0.1 *M*)–phosphate (0.2 *M*) buffer (pH 5.0). The mixture was incubated at 37°C for 1 h. After addition of I.S. (DAH) (2.65 nmol), the incubation medium was diluted with sodium octanesulphonate (5 m*M*) in 0.1 *M* acetic acid (5 ml) and applied to a Sep-Pak C_{18} solid-phase cartridge which had previously been equilibrated with the above solvent. The cartridge was washed with a mixture of the above solvent with acetonitrile (17:3, v/v) (6 ml) and the putrescine was eluted with acetonitrile–water (2:1, v/v) (6 ml). After evaporation of the solvent, the residue was dissolved in borate buffer (0.1 *M*, pH 8.5) (0.2 ml) and treated with IIb (200 μg) in pyridine (0.2 ml). The solution was kept at room temperature for 1 h, then an aliquot was subjected to HPLC–ED. Acetonitrile–methanol–water (6:2:5, v/v) containing 0.05 *M* sodium perchlorate was used as the mobile phase. The applied potential of the electrochemical detector was set at +0.45 V.

Recovery test for putrescine in incubation medium

The spiked samples were prepared by addition of either 0.31 or 3.10 nmol of putrescine to a standard incubation medium containing bovine serum albumin

instead of the enzyme. Pretreatment and derivatization with IIb followed by HPLC–ED were carried out in the manner described above.

RESULTS AND DISCUSSION

The design of a useful derivatization reagent for amino compounds in HPLC–ED requires two structural features, viz., a functional group reactive toward the amino group and an electrophore responsive to an electrochemical detector with high sensitivity. In this study, each of two active esters, carbamates and isomaleimides possessing ferrocene as an electrophore, were prepared. The active esters N-succinimidyl 3-ferrocenylpropionate (IIb) and N-succinimidyl 3-(3,1′-dimethylferrocenyl)propionate (IVb), were prepared from ferrocenecarboxaldehyde and 3,1′-dimethylferrocenylaldehyde, respectively. N-Succinimidyl 2-ferrocenylethylcarbamate (IId) was obtained from 2-ferrocenylethylamine (IIc) as a stable compound. In contrast, N-succinimidyl ferrocenylcarbamate (Id), prepared from ferrocenylamine (Ic), was degraded rapidly to the starting material (Ic) when dissolved in the solvent. Based on the experimental data, Id was regarded as unsuitable as a derivatizing reagent for the amino compounds. N-Ferrocenylisomaleimide (If) and N-(2-ferrocenylethyl)isomaleimide (IIf) were prepared from Ic and IIc, respectively.

The reactivities of If, IIb, IId, IIf and IVb towards the amino group were examined employing phenethylamine as a model compound. Derivatization of phenethylamine was performed in pyridine–water (1:1, v/v) at room temperature. The reaction with IIb proceeded quantitatively to reach a plateau in 1 h, whereas those with IId and IVb reached 70 and 87%, respectively. The reactivities of isomaleimides (If, IIf) were not satisfactory (Fig. 2). The reactions of IIb with tryptamine and piperidine were also examined under the same conditions and gave the same results as phenethylamine. It is evident that IIb is the most favourable reagent with respect to reactivity and stability.

The electrochemical properties of the derivatives formed with IIb and IVb were investigated with a dual-electrode amperometric detector having twin electrodes in series. The hydrodynamic voltammograms of these derivatives are illustrated

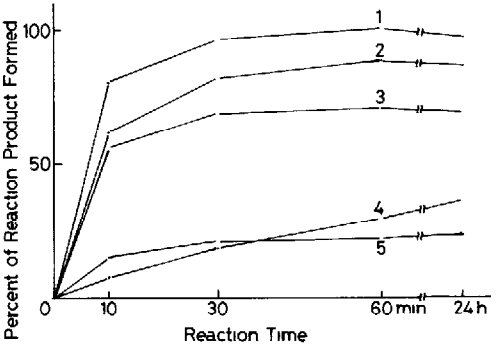

Fig. 2. Time courses for derivatization of phenethylamine. 1 = IIb; 2 = IVb; 3 = IId; 4 = IIf; 5 = If. The peak area of the ferrocene equivalent to phenethylamine was taken as 1.0.

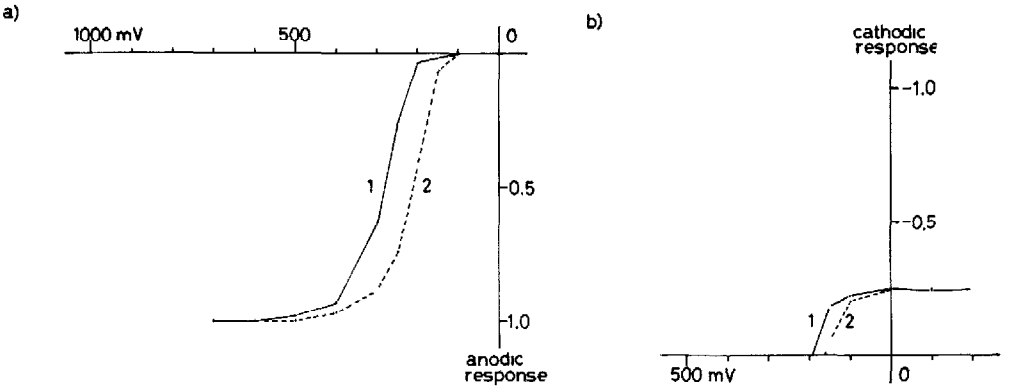

Fig. 3. Hydrodynamic voltammograms of phenethylamine derivatives. 1 = IIb; 2 = IVb. (a) Anodic response, (b) cathodic response. The maximum response of each compound was taken as 1.0.

in Fig. 3. The half-wave potential ($E_{1/2}$) of the IVb derivative (+0.28 V) was lower than that of the IIb derivative (+0.21 V). This may be ascribable to the negative inductive effect due to alkyl groups in IVb as reported previously [9]. However, the IVb derivative could be detected only by reduction (applied potential 0.0 V). Part of the IVb derivative would be spontaneously oxidized and hence its response in the oxidation mode (+0.5 V) was depressed to 70% of that of the IIb derivative. The detection limit of the derivative formed with IIb was 0.5 pmol (signal-to-noise ratio = 5, 4 nA full-scale) at the applied potential of +0.45 V vs. Ag/AgCl. These results prompted us to use IIb as a derivatization reagent for amino compounds.

The utility of IIb was tested for the determination of putrescine formed from ornithine by ornithine decarboxylase. The substrate was incubated with an enzyme preparation derived from *E. coli* and the incubation medium was applied to a Sep-Pak C_{18} cartridge, previously equilibrated with sodium octanesulphonate in acetic acid. Putrescine was retained on the stationary phase by forming an ion pair with sodium octanesulphonate whereas the substrate was not [16]. The putrescine was then eluted with acetonitrile–water, derivatized with IIb and then subjected to HPLC–ED.

A typical chromatogram is illustrated in Fig. 4b. Each derivative of putrescine and the I.S. exhibited a single peak of Gaussian shape without any interfering peaks. When putrescine added to the control incubation medium at two concentrations (0.31 and 3.1 nmol) the recovery was > 82.7% (Table I). When the ratio of the amount of putrescine to that of the I.S. (2.65 nmol) was plotted against the peak-height ratio, a linear relationship was observed in the range 0.31–31 nmol, the regression equation being $y = 1.64x$. The detection limit of putrescine and DAH was 3 pmol (signal-to-noise ratio = 5, 4 nA full-scale) at the applied potential of +0.45 V vs. Ag/AgCl. The peak broadening resulting from adsorption of these lipophilic substances on the column may give a higher value than that of phenethylamine. The amount of putrescine enzymatically formed at 37 °C increased linearly with increasing incubation time up to 1.5 h. The enzymic activity of ornithine decarboxylase was estimated to be 12.6 nmol/mg of protein

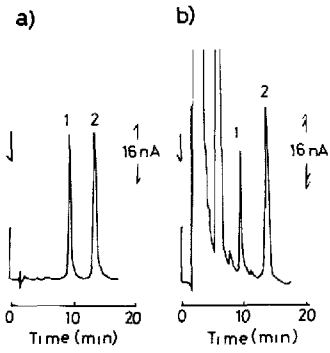

Fig. 4. High-performance liquid chromatograms of putrescine derivative. 1 = Putrescine; 2 = DAH (I.S). (a) Authentic sample: (1) 1.2 ng; (2) 2 ng; (b) enzymic reaction mixture: one tenth of derivatization mixture was injected.

TABLE I

RECOVERY OF PUTRESCINE ADDED TO THE INCUBATION MEDIUM

Recovery of DAH (1.06 nmol per tube) under the same conditions was 94.3 ± 3.3% (n = 6)

Putrescine (nmol per tube)		Recovery (mean ± S.D., n = 6) (%)
Added	Found	
0.31	0.26	82.7 ± 3 8
3.10	2.80	90.2 ± 3.8

per min. Previous methods for the pretreatment of polyamines in biological fluids [17] are tedious and time-consuming. The proposed procedure proved to be satisfactory with respect to simplicity, accuracy and reproducibility, as shown by the recovery test. Further applications of this method to the determination of polyamines in biological fluids are being conducted and the details will be reported elsewhere.

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