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Synthesis of cobaltocenium salts for use as redox labels and their incorporation into Nafion films

Chantal Degrand,* Benoit Limoges,* Arnaud Gautier* and Ronald L. Blankespoor†‡

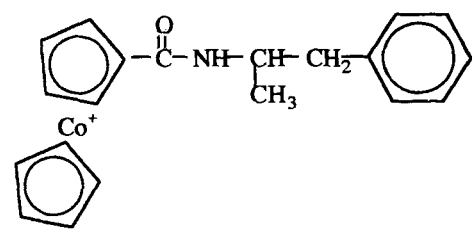
*Université Blaise Pascal de Clermont-Ferrand, Laboratoire Thermodynamique et Electrochimie en Solution (URA 434), Equipe Electrochimie Organique, 24, Avenue des Landais, 63177 Aubiere Cedex, France, and †Department of Chemistry, Calvin College, Grand Rapids, Michigan 49546, USA

A cobaltocenium label was covalently attached to two antidepressants, nortriptyline and desipramine, via an amide linkage, and also to the hydrazine derivative of the biologically important compound biotin (vitamin H), again via an amide linkage. Analytically pure samples of these new cobaltocenium salts could be obtained by chromatography on silica gel followed by elution with aqueous acetone solutions containing sodium chloride (NaCl). These positively charged cobaltocenium ions preconcentrate in a polyanionic Nafion film coated on a glassy carbon surface, albeit at different concentration levels. One factor which seems to influence the amount of cobaltocenium ion that enters the film is polarity since the cobaltocenium ion containing the rather polar biotin preconcentrates at the lowest level in the relatively hydrophobic Nafion. Square-wave voltammograms of Nafion films containing these cobaltocenium cations exhibit a one-electron, reversible, reduction wave at approximately −1.1 V (vs Ag/AgCl) with peak currents that are sufficiently large to permit detection of 10^{-8} M quantities of these substances in the bulk solution.

Keywords: Cobaltocenium, Nafion, nortriptyline, biotin, desipramine, square-wave voltammetry

INTRODUCTION

We have recently developed an analytical technique[1] that combines immunoassay with electrochemical detection at an electrode surface modified with a film of Nafion, a perfluorinated anionic polyelectrolyte corresponding to $(C_2F_4)_x(C_2F_3)_y(OC_3F_6)_zOC_2F_4SO_3Li$ that is stable and exhibits permselectivity towards different cations.[2–6] An important feature of this methodology is that the substance to be assayed is covalently attached to a positively charged redox label and, as a consequence, the labeled substance accumulates in the Nafion, which possesses negatively charged sulfonate groups. This preconcentration enhances the sensitivity of the immunoassay considerably and we were able to show, using the amphetaminecarbonylcobaltocenium cation **1** as analyte, that very dilute solutions of **1** (i.e. 10^{-7}–10^{-8} M) can be routinely analyzed. The cobaltocenium ion can serve as an excellent redox label for electrochemical detection. Since it is reversibly reduced at approx. −1.1 V (vs Ag/AgCl),[7] both aqueous and nonaqueous electrolytes can be employed. The cobaltocenium ion is stable not only to oxygen, but to a wide variety of reagents used in organic synthesis. This has led to the preparation of cobaltocenium ions with organic moieties that possess a broad spectrum of functional groups.[8–14]



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In this paper we show that this methodology can be used to assay both secondary amines and carboxylic acids. We describe the synthesis of cobaltocenium salts in which the redox label is attached to two antidepressants, desipramine (**2**)

‡ To whom correspondence should be addressed.

§ Nafion is a trademark registered by E. I. Dupont de Nemours, Inc.

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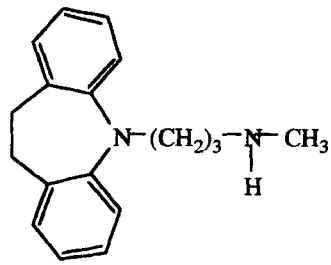

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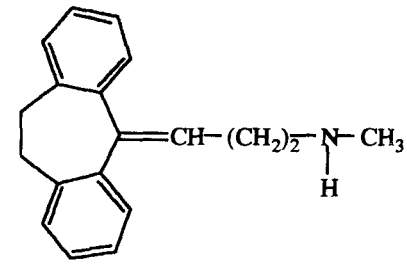

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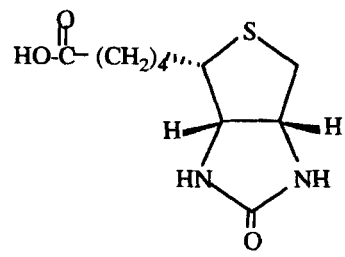

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and nortriptyline (**3**), and to the biologically important molecule biotin (**4**) and then go on to show that these cations can be preconcentrated in Nafion films.

EXPERIMENTAL

Infrared spectra were obtained with a Nicolet 205 FT spectrometer. NMR spectra were measured using a 300 MHz Bruker MSL spectrometer. Electrochemical measurements were made with a Princeton Applied Research 273 Potentiostat/Galvanostat. The working electrode was glassy carbon (GC) or GC modified with a Nafion film; the reference electrode was Ag/AgCl; and the counter-electrode was a platinum (Pt) wire. Elemental analyses were performed by the Centre National de la Recherche Scientifique (CNRS) at Vernaison. Mass-spectral analyses were performed by CNRS at Lyon and at the University of Rennes.

Electrode preparation

GC rods were sanded flat with 1200-grit silicon carbide paper and polished with 0.05 μm aqueous alumina suspension (Escil). Immediately after polishing, the electrodes were ultrasonically cleaned in ethanol, rinsed with doubly distilled water, and dried at 100 °C in an oven. In the preparation of Nafion-coated GC, 0.4 cm^3 of a Nafion solution (Aldrich, catalog ref. 27 470-4) was combined with 19.28 cm^3 DMF and 0.32 cm^3 of aqueous 0.05 M lithium hydroxide (LiOH) to give the Li^+ salt of Nafion. The Nafion coating was made by applying 5 μl of this diluted solution to the pretreated GC surface[15] and removing the bulk of the solvent at 140 °C for 5 min under an atmosphere saturated with DMF vapor. To assure complete removal of solvent, the electrode was placed in an oven for 10 min at 140 °C. For each measurement, a GC/Nafion rod was pressure-fitted into a narrow cylindrical hole of a Teflon tube in such a way that only the modified surface was exposed to the analyte containing solution. A film thickness of 0.4 μm was calculated by assuming a density of 1.58 g cm^{-3}.

Square-wave voltammetry

Electrochemical measurements were made at 22 °C in a one-compartment cell (2 cm^3 working volume) using a Princeton Applied Research 273 Potentiostat/Galvanostat interfaced to a IBM XT 286 computer system with PAR M270 software. The working electrode was GC or GC/Nafion mounted on a Tacussel rotating-disk electrode; the reference electrode was Ag/AgCl (0.05 M Cl^-); and the counter-electrode was a Pt wire. The potential step increment (dE) was 2 mV; the square-wave amplitude (ESW) was 50 mV; and the frequency (f) was 100 Hz.

Nortriptylinecarbonylcobaltocenium hexafluorophosphate (6)

Chlorocarbonylcobaltocenium salt was prepared *in situ* from carboxycobaltocenium hexafluorophosphate (300 mg, 0.80 mmol) and excess $SOCl_2$.[13] After the bulk of the excess $SOCl_2$ was removed by distillation, the resulting yellow–orange solid was washed with hexane and dried using nitrogen. To the crude chlorocarbonylcobaltocenium salts (Cl^- and PF_6^-) obtained in this manner was added, under nitrogen, a solution of nortriptyline (267 mg, 1.02 mmol) and triethylamine (300 mg) in 30 cm^3 of dry THF (freshly distilled from calcium hydride). After the reaction mixture had been stirred for 12 h, the THF was removed under reduced pressure (rotary evaporator), giving a yellow solid residue. To this residue was added 25 cm^3 dichloromethane (CH_2Cl_2) and the mixture was extracted with cold saturated $NaHCO_3$ (20 cm^3), cold 1.0 M hydrochloric acid (HCl) (20 cm^3), and saturated sodium chloride (NaCl) (20 cm^3). The CH_2Cl_2 solution was dried over calcium chloride and the CH_2Cl_2 was removed under reduced pressure giving a yellow oil that solidified on drying. Chromatography of this nortriptylinecarbonylcobaltocenium salt (presumably Cl^-) on silica gel followed by elution with water–acetone–NaCl (250 cm^3 : 200 cm^3 : 250 mg) gave a single yellow band that was collected. After the bulk of the acetone in this solution was removed under reduced pressure, a solution of 2.0 g of $NaPF_6$ in 5 cm^3 of water was added, resulting in the formation of a precipitate of **6** which was collected by vacuum filtration and dried (60 °C, 1 h, 0.1 Torr) giving a light yellow solid (276 mg, 55%) that did not have a well-defined melting point and consistently oiled out when attempts were made to recrystallize it: IR (Nujol) 1640 (s), 1120 (w), 1092 (w), 1070 (w), 1031 (w), 843 (w), 777 (w), 760 (w), 740 (w), 718 (w) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$, shows restricted rotation about the amide bond) δ 2.28–2.38 (m, 1H), 2.38–2.50 (m, 1H), 2.70–3.00 (m, 5H), 3.10–3.40 (m, 3H), 3.55 (broad s, 1H), 5.50–6.00 (m, 10H), 6.90–7.32 (m, 8H); ^{13}C NMR (300 MHz, $CDCl_3$) δ 27.3, 28.8, 32.0, 32.2, 33.6, 33.8, 34.2, 37.0, 48.3, 50.9, 84.0, 84.4, 85.0, 86.7, 101.6, 103.0, 126.0, 126.1, 126.3, 126.4, 127.4, 127.5, 127.7, 127.9, 128.3, 128.4, 128.6, 128.7, 130.3, 130.4, 137.1, 137.2, 139.3, 139.4, 139.6, 139.7, 140.4, 141.1, 145.2, 146.4, 162.3. Analysis: Calcd for $C_{30}H_{29}CoF_6NOP$: C, 57.80; H, 4.69; Co, 9.45; F, 18.29; N, 2.25; P, 4.96. Found: C, 57.76; H, 4.76; Co, 9.80; F, 17.99; N, 2.27; P, 5.16%.

Desipraminecarbonylcobaltocenium hexafluorophosphate (5)

To the crude chlorocarbonylcobaltocenium salts prepared from carboxycarbonylcobaltocenium hexafluorophosphate (300 mg, 0.80 mmol) as described above was added, under nitrogen, a solution of desipramine (267 mg, 1.00 mmol) and triethylamine (150 mg) in 25 cm^3 of dry THF. After stirring overnight, the THF was removed under reduced pressure to give a viscous oil which was combined with 30 cm^3 CH_2Cl_2 and extracted with cold saturated $NaHCO_3$ (20 cm^3), cold 1.0 M HCL (20 cm^3), and saturated NaCl (20 cm^3). The CH_2Cl_2 solution was dried over calcium chloride and the solvent was removed under reduced pressure. The residue was chromatographed (as described above for **6**) giving desipraminecarbonylcobaltocenium chloride as a hygroscopic, yellow oil: ^{13}C NMR (300 MHz, $CDCl_3$, restricted rotation about the amide bond) δ 24.8, 25.8, 31.8, 31.9, 33.6, 37.0, 46.0, 46.6, 47.2, 49.2, 84.0, 84.1, 84.8, 85.1, 86.6, 86.7, 100.9, 102.6, 119.3, 119.7, 122.5, 122.9, 126.3, 126.4, 129.8, 130.0, 133.7, 134.2, 147.3, 147.8, 162.2. To obtain an analytically pure sample the Cl^- ion was exchanged for PF_6^- by dissolving the above oil in 100 cm^3 of acetone–water (1 : 1, v/v) containing 2.5 g of $NaPF_6$. Removal of the acetone under reduced pressure followed by cooling in an ice bath gave **5** as a yellow–orange solid (275 mg, 54%). Further purification was accomplished by dissolving 70 mg of this solid in acetone (20 cm^3) and treating the solution with activated charcoal. After filtration, the solution was combined with a solution of 0.5 g of $NaPF_6$ in 20 cm^3 of water. The acetone was removed and the aqueous solution was extracted with CH_2Cl_2 (2 × 15 cm^3). The CH_2Cl_2 extracts were combined, the solvent was removed, and the residue was dried (50–60 °C, 30 min, 0.10 Torr) giving **5** as a yellow solid that did not give a well-defined melting point and consistently oiled out when attempts were made to recrystallize it: IR (Nujol) 1635 (s), 1295 (w), 1226 (w), 1167 (w), 1129 (w), 1103 (w), 1059 (w), 1029 (w), 1007 (w), 832 (w), 775, 754, 732, 716 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.72–1.84 (quintet, 1H), 1.85–1.97 (quintet, 1H), 2.80–2.98 (m, 5H), 3.20–3.32 (m, 3H), 3.45–3.58 (t, 1H), 3.59–3.68 (t, 1H), 3.82 (broad s, 1H), 5.60–6.00 (m, 9H), 6.90–7.25 (m, 8H); ^{13}C NMR (300 MHz, $CDCl_3$)

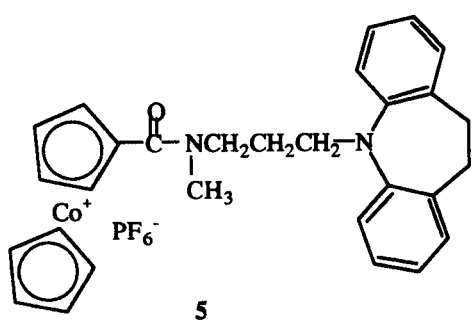
δ 25.3, 26.4, 32.5 (2), 34.3, 37.4, 46.5, 47.3, 47.7, 49.9, 84.1, 84.4, 84.7, 85.4, 87.0 (2), 101.7, 103.4, 120.0, 120.4, 123.6 (2), 127.2 (2), 130.8 (2), 134.4, 134.8, 148.0 (2), 162.8. MS (DEI, 70 eV), *m/z* (relative intensity) 481 (100), 234 (10), 216 (8), 208 (15), 194 (12), 193 (20), 188 (35). Analysis: Calcd for $C_{29}H_{30}CoF_6N_2OP$: C, 55.60; H, 4.83; Co, 9.41; F, 18.20; N, 4.47; P, 4.94. Found: C, 56.05; H, 4.76; Co, 9.35; F, 17.62; N, 4.69; P, 5.16%.

Biotinhydrazidocarbonylcobaltocenium chloride

To the crude chlorocarbonylcobaltocenium salts prepared from carboxycobaltocenium hexafluorophosphate (208 mg, 0.551 mmol) as described above was added, under nitrogen, a mixture of biotin hydrazide (270 mg, 1.05 mmol) in 20 cm^3 of dry DMF (treated with calcium hydride, then passed through a column of activated alumina). Complete dissolution of the biotin hydrazide occurred after several minutes. The reaction mixture was stirred overnight, after which time a white precipitate was obtained (presumably biotin hydrazide-HCl). The precipitate was removed by filtration and the DMF was removed from the filtrate under reduced pressure giving a viscous, yellow oil. This oil was chromatographed on silica gel and eluted with acetone–water (6:1 followed by 1:1, v/v). Unreacted biotin hydrazide and carboxycobaltocenium salts were eluted first. A band was then eluted which contained biotinhydrazidocarbonylcobaltocenium salts and changed color from yellow to orange–red as it passed through the column. The pH of this fraction was decreased from 7.5 to 5.0 with the addition of dilute HCl, resulting in a color change from orange–yellow to yellow (reversible). To the yellow solution was added 2.5 g of potassium chloride (KCl) and the solvent was removed under reduced pressure to give a solid residue from which biotinhydraxidocarbonylcobaltocenium chloride was extracted using methanol–acetone (1:1, v/v). Removal of solvent gave a mixture of the cobaltocenium and KCl (elemental analysis) as a yellow, hygroscopic solid which, even after heating at 150 °C for 5 h (0.1 Torr), retained some water: IR (Nujol) 3520 (s), 3450 (s), 1682, 1632 (s), 1614 (s), 1309 (w), 1268 (w), 1159 (w), 1015 (w), 944 (w), 874 (w), 721 (w) cm^{-1}; ^1H NMR (300 MHz, D_2O, acetone as internal standard) δ 1.23–1.65 (m, 6H), 2.23 (t, 2H), 2.54–2.67 (m, 1H), 2.84 (dd, $J = 4.8$ and 12.9 Hz, 1H), 3.16–3.24 (m, 1H), 4.27 (dd, $J = 4.8$ and 8.6 Hz, 1H), 4.44 (dd, $J = 5.2$ and 8.6 Hz, 1H), 5.70 (s, 5H), 5.77 (t, 2H), 6.12 (t, 2H); ^{13}C NMR δ 23.8, 26.7, 26.8, 32.4, 38.9, 54.3, 59.3, 61.1, 83.1, 85.4, 85.6, 89.4, 162.4, 164.0, 174.5.

Biotinhydrazidocarbonylcobaltocenium tetraphenylborate (7)

Biotinhydrazidocarbonylcobaltocenium salt (Cl^- and PF_6^- mixture) was prepared as described above starting with 102 mg (0.270 mmol) of carboxycobaltocenium hexafluorophosphate. Following chromatography of the reaction mixture on silica gel, the acetone in the fraction containing the cobaltocene was removed under reduced pressure and the remaining aqueous solution was combined with a solution of $NaBPh_4$ (150 mg, 0.440 mmol) in 3 cm^3 of water, resulting in the formation of a yellow precipitate. The precipitate was collected by vacuum filtration and dried (60 °C, 2 h, 0.10 Torr) to give 87 mg of the desired cobaltocenium **7**. Reducing the volume of the filtrate by one-half gave 25 mg of additional product for a combined yield of 112 mg (52%). Although attempts to recrystallize **7** were unsuccessful, an analytically pure sample was obtained by dissolving 77 mg of the cobaltocene in 50 cm^3 of acetone–water (1:1, v/v), adjusting the pH to 5 with the addition of HCl, reducing the volume of the solution by one-third, and collecting the resulting solid. After drying (60 °C, 2 h, 0.10 Torr), 68 mg of a light yellow solid was obtained: m.p. 130 °C (dec., yellow to red); IR (Nujol) 3340 (broad), 1682 (s, broad), 1579 (w), 1306 (w), 1267 (w), 1153 (w), 1023 (w), 863 (w), 733 (s), 702 (s), 608 cm^{-1}; ^1H NMR (300 MHz, acetone-d_6) δ 1.41–1.70 (m, 4H), 1.71–1.87 (m, 2H), 2.27–2.43 (m, 2H), 2.69 (d, $J = 12.2$ Hz, 1H), 2.91 (dd, $J = 5.0$ and 12.2 Hz, 1H), 3.03 (broad s, 4H), 3.16–3.24 (m, 1H), 4.27–4.35 (m, 1H), 4.45–4.52 (m, 1H), 5.62 (t, 2H), 5.26 (s, 5H), 6.27 (t, 2H), 6.81 (t, 4H), 6.96 (t, 8H), 7.84 (broad s, 8H); ^{13}C NMR (300 MHz, acetone-d_6) δ 25.5, 28.3, 28.5, 33.4, 40.4, 46.2, 55.9, 60.6, 60.8, 62.0, 62.1, 84.7 (2), 87.0, 91.1, 122.0, 125.8, 127.0, 136.6, 162.2, 163.5, 164.2, 164.8, 165.4, 173.5. MS (EI, 70 eV), *m/z* (relative intensity) 242 (10), 165 (14), 164 (26), 163 (16), 78 (100), 77 (69), 66 (49), 65 (29); MS (FAB), *m/z* (relative intensity) 475 (15), 474 (30), 473 (100). Analysis: Calcd for $C_{45}H_{46}BCoN_4O_3S$: C, 68.18; H, 5.85; B, 1.36; Co, 7.43; N, 7.07; S, 4.04.



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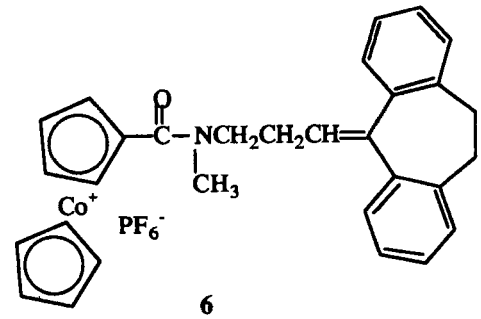

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Found: C, 67.55; H, 6.05; B, 1.45; Co, 6.96; N, 6.81; S, 3.68%.

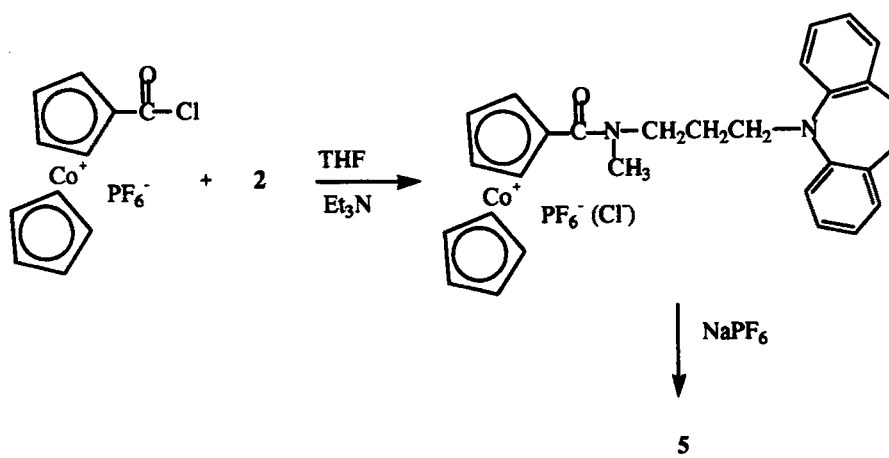
RESULTS AND DISCUSSION

Synthesis of cobaltoceniums 5 and 6

Cobaltocenium salts **5** and **6** were prepared from chlorocarbonylcobaltocenium hexafluorophosphate, as shown in Scheme 1 for **5**, in yields of 54 and 55%, respectively. Although attempts to recrystallize these compounds were unsuccessful, analytically pure samples were obtained using a chromatographic procedure that employed solutions of NaCl in aqueous acetone to elute the polar cobaltoceniums from columns of silica gel (see the Experimental section). Interestingly, NMR spectroscopy shows restricted rotation about the amide bond in both **5** and **6**. In Fig. 1 are shown the proton-decoupled ^{13}C spectra of desipramine and cobaltocenium **5**. As expected, 11 peaks are observed for desipramine. With the exception of the carbonyl carbon at 163 ppm, the spectrum of **5** shows that there are two peaks for each carbon position. This can be accounted for by the presence of two isomers that interconvert by rotation about the carbon–nitrogen bond at a rate that is slow relative to the NMR time scale.

Synthesis of cobaltocenium 7

Biotin was covalently attached to the cobaltocenium ion via its commercially available hydrazide **8** using the sequence of reactions outlined in Scheme 2. Although the hygroscopic chloride salt of the biotinhydrazidocarbonylcobaltocenium ion was prepared free of starting materials (see the Experimental section), it was necessary to replace the chloride anion with the more hydrophobic tetraphenylborate to obtain an analytically pure sample. This was easily accomplished by taking advantage of the different solubilites of the two salts. In contrast to the chloride salt, which is very soluble in aqueous media, the tetraphenylborate



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Scheme 1

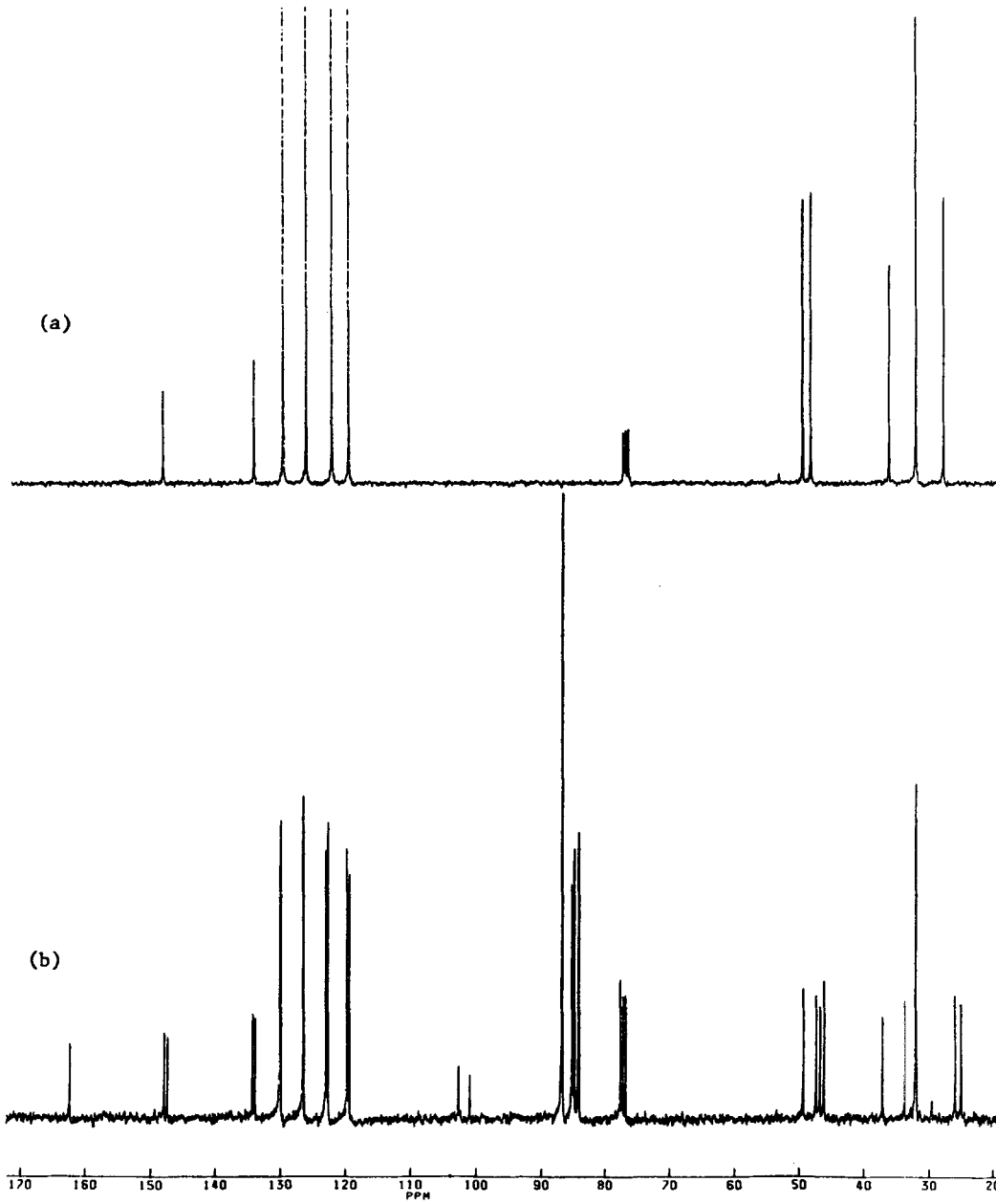
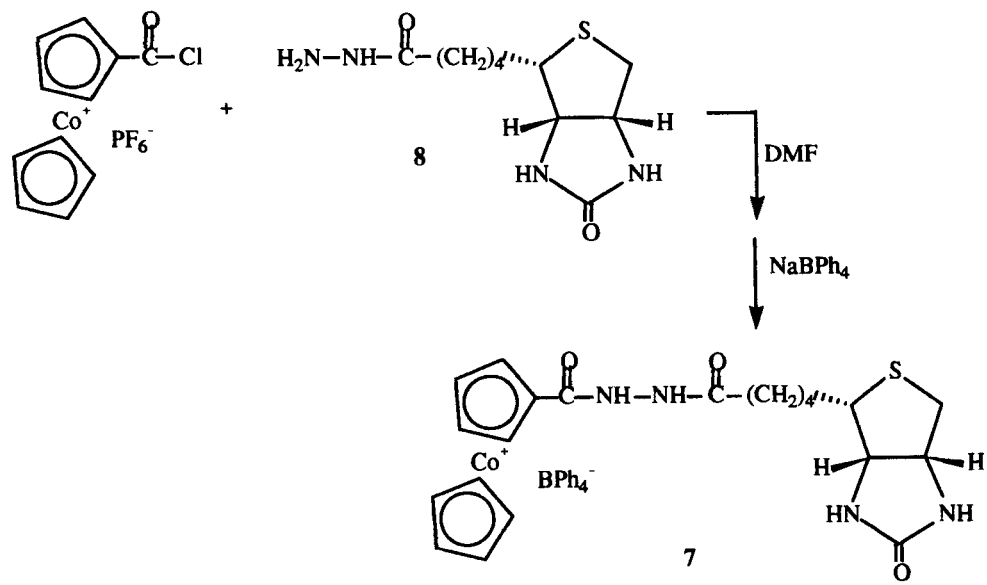


Figure 1 Proton-decoupled ^{13}C spectra ($CDCl_3$) of (a) desipramine and (b) desipraminecarbonylcobaltocenium hexafluorophosphate (**5**).

salt can be precipitated from aqueous solution by the addition of sodium tetraphenylborate.

Square-wave voltammetry (SWV)

This is one of the most sensitive electroanalytical techniques that can be employed to detect reversible redox systems.[16] In Fig. 2 are SW voltammograms of 73.0 μM **7** (phosphate buffer at pH 7.4) at naked and Nafion-coated glassy carbon (GC). Peak potentials (E_p) of −0.973 and −1.075 V (vs Ag/AgCl) are obtained for the one-electron reduction of **7** at the naked and modified electrode surfaces, respectively, corresponding to a difference of almost 100 mV in E_p. The more negative potential at the Nafion-coated electrode



Scheme 2

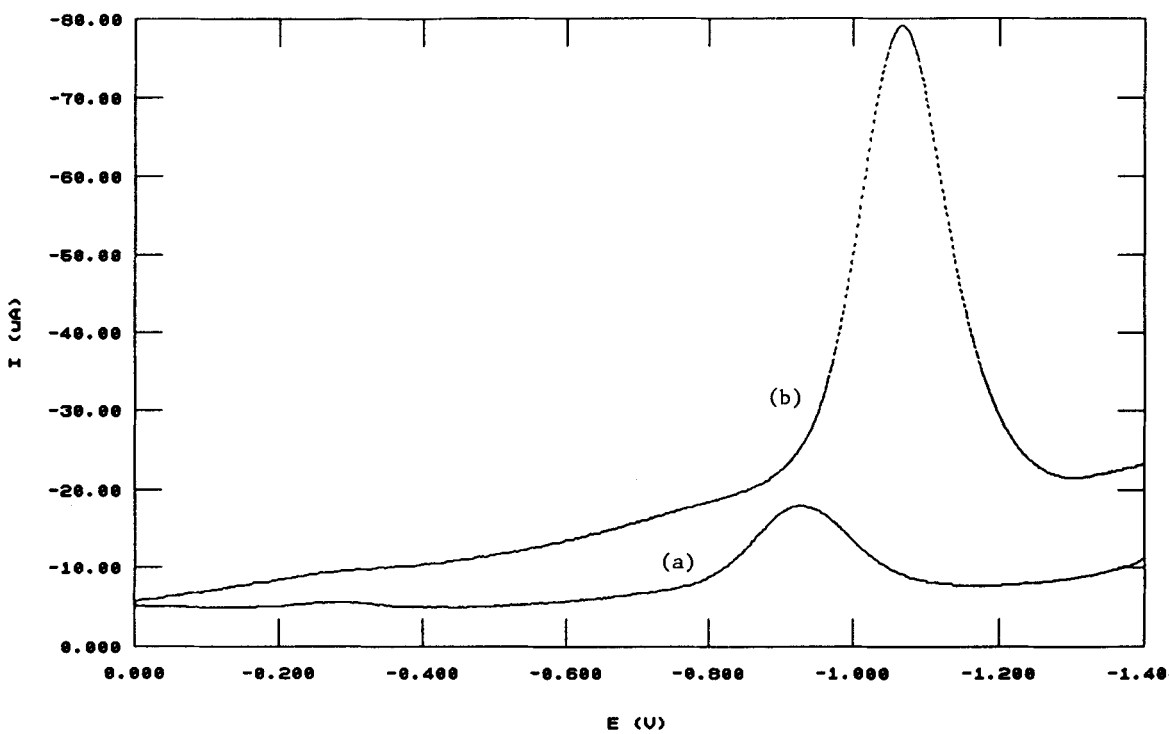


Figure 2 Square-wave voltammograms of 73.0 μM 7 (phosphate buffer at pH 7.4) at (a) a naked GC electrode and (b) a Nafion-coated electrode. Reference electrode is Ag/AgCl.

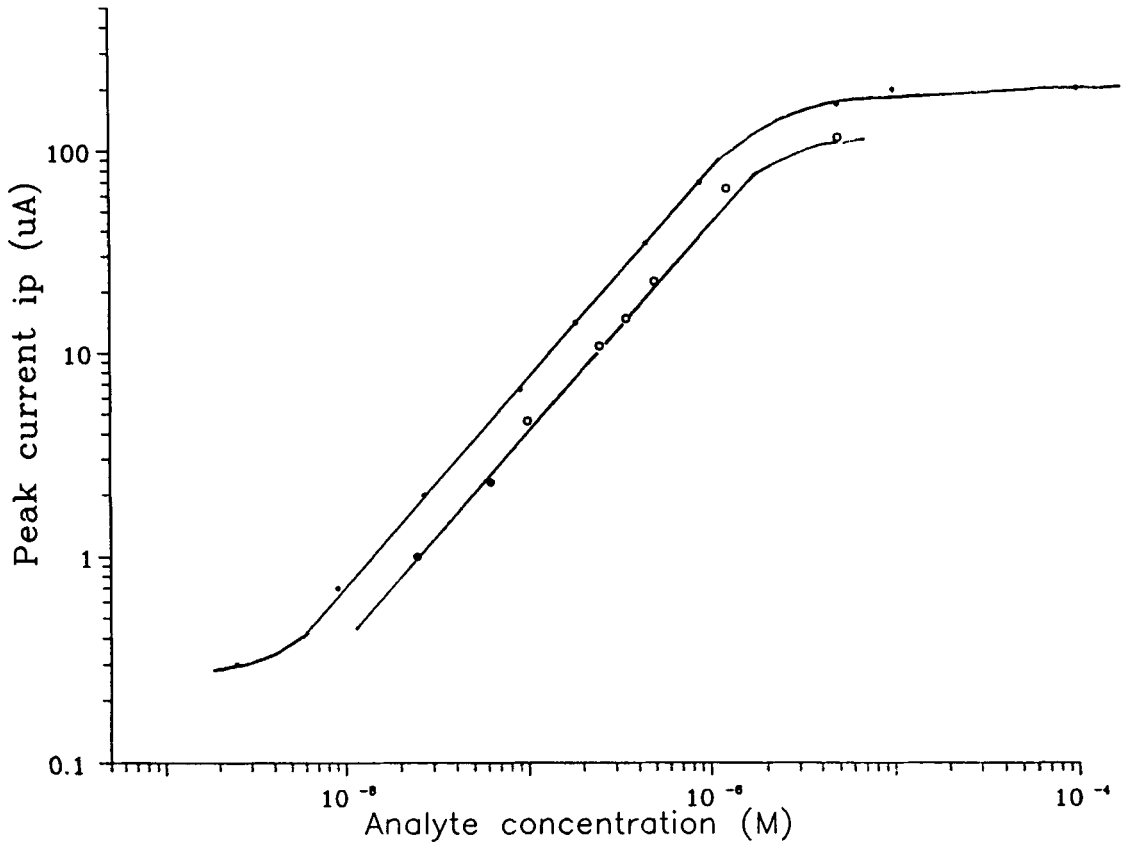


Figure 3 Peak currents (SWV) of cobaltoceniums **1** (•), **5** (●), and **6** (○) as a function of their concentration in the bulk solvent. The square voltammograms were recorded after each of the Nafion-coated GC electrodes was exposed to the bulk solution for 5 min.

has also been observed for the ferrocene/ferricinium couple.[17] The E_p values for cobaltoceniums **1**, **5** and **6** at Nafion-coated GC are −1.080, −1.130, and −1.130 V, respectively.

Preconcentration in Nafion

Figure 2 shows that the Nafion coating not only makes E_p more negative for the reduction of the cobaltocenium ion in **7**, but also enhances the peak current, I_p, from 11.4 at the naked electrode to 60.9 μA, i.e. by a factor of 5.3. It seems quite apparent then that the polyanionic Nafion film concentrates the positively charged cobaltocenium ion by cationic exchange. The preconcentration of cobaltoceniums **1**, **5** and **6** is quantified in Fig. 3 where plots of I_p vs concentration are shown. Note that these plots are logarithmically linear over a wide range of analyte concentrations (C) from 10^{-8} to 10^{-6} M. At analyte concentrations above 10^{-6} M the peak current rapidly levels off, presumably because of saturation of the anionic sites in the Nafion. One way to measure the ability of Nafion to preconcentrate analytes is to look at their ρ values ($\rho = I_p/C$). The ρ values for **1**, **5**, **6** and **7** are 78, 51, 51, and 0.83 μA/μM, respectively. It is clear from these data that the cobaltocenium ion containing biotin (i.e. **7**) does not preconcentrate nearly as much as the other three. This is not surprising since biotin is much more polar than nortriptyline, desipramine or amphetamine. As a consequence, the partition coefficient of **7** between the polar aqueous buffer and the more hydrophobic Nafion film is larger.

SUMMARY AND CONCLUSIONS

In this work we have shown that the cobaltocenium redox label can be covalently attached to two antidepressants and biotin (vitamin H) via their secondary amine and carboxylic acid groups. Although attempts to recrystallize these new cobaltoceniums were unsuccessful, analytically

pure samples could be obtained using a chromatographic method which employs an aqueous solution containing NaCl to elute the ionic cobaltoceniums from a silica gel column. These positively charged ions preconcentrate in a polyanionic Nafion film coated on a GC electrode surface, allowing these substances to be detected at concentrations as low as 10^{-8} M when reduced using square-wave voltammetry. One factor that determines the level to which these cobaltocenium ions preconcentrate in a Nafion film appears to be polarity. The cobaltocenium ion containing the relatively polar biotin preconcentrates at a much lower level in the hydrophobic film than those that contain hydrocarbon analytes. Work is in progress aimed at using this redox label in a new technique that we have recently developed that combines immunoassay with Nafion-modified electrodes.

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