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Hydroquinone-Based Derivatization Reagents for the Quantitation of Amines Using Electrochemical Detection

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Two new reagents, NDTE (2,5-dihydroxyphenylacetic acid, 2,5-bis-tetrahydropyranyl ether *p*-nitrophenyl ester) and HLTE (homogentisic *γ*-lactone tetrahydropyranyl ether), are described for the chemical derivatization of primary and/or secondary amines to form an electrochemically active product. These reagents undergo reaction with the aforementioned analytes to form a product possessing the hydroquinone moiety, thus allowing for reversible electrochemical detection at mild oxidation potentials. The reactivity of each reagent was demonstrated by using *N*-ethylbenzylamine (EBzA) and the dipeptide isoleucine leucine methyl ester as model analytes. The investigation included the isolation and identification of the intermediates and final products from derivatization of EBzA. These isolated standards were subsequently characterized with respect to electrochemical properties by means of cyclic voltammetry. In LC-EC experiments, the concentration limit of detection (CLOD) of the purified EBzA product was determined to be 5 nM (100 fmol) at a detection potential of +200 mV vs Ag/AgCl ([Cl$^-$] = 3 M). The CLOD values obtained by LC-EC after derivatization of aqueous solutions of EBzA and Ile-Leu-OMe with NDTE were 25 nM (250 fmol) and 250 nM (2.5 pmol), respectively.

For the past two decades instrumentation has been available for the routine application of liquid chromatography (LC) with electrochemical (EC) detection. With the advent, growth, and now widespread practice of LC-EC, this technology provides the bioanalytical chemist with a valuable tool for the sensitive and selective determination of numerous substances present in biofluids and tissues. Among the first described applications of LC-EC were methods for the determination of various endogenously occurring catecholeamines and indoleamines,[1−6] where mass limits of detection in the low femtomole region were frequently achieved. These analytes represented an excellent technique−problem match due to their inherent electrochemical activity and, notwithstanding the numerous advances in analytical methodologies and instrumentation, at present LC-EC usually remains the method of choice for the aforementioned substances.

While some substances exhibit suitable electrochemical reactivity and thus are amenable to determination at trace concentrations by LC-EC, there are unfortunately a large number of important analytes (e.g., various drug substances, their associated metabolites, amino acids, peptides, etc.) that are not amenable to high sensitivity detection techniques such as fluorescence and electrochemistry in their native state. For molecules bearing reactive functional groups, traditionally the analyst has often turned to the selective chemical modification of the analytical target to form a product that is highly detectable. A classic example of this approach is the amino acid analyzer,[7] an instrument that is simply the combination of ion-exchange LC followed by postcolumn reaction detection, wherein the separated amino acids undergo transformation to a colored product.

Analysts have continued to devise numerous chemistries for the derivatization of many functional groups, with the overall goal usually being the enhancement of analyte detection. Many references to the original descriptions of such research can be found in a series of books devoted to sample preparation and derivatization techniques for use in LC.[8−14] In this regard, it appears that the vast majority of reagents developed for application in LC are intended to form a product **3** possessing efficient

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(1) Durkin, T. A.; Caliguri, E. J.; Mefford, I. N.; Lake, D. M.; Macdonald, I. A.; Sundstrom, E.; Jonsson, G. *Life Sci.* **1985**, *37*, 1803−1810.
(2) Caliguri, E. J.; Mefford, I. N. *Brain Res.* **1984**, *296*, 156−159.
(3) Matson, W. R.; Langlais, P.; Volicer, L.; Gamache, P. H.; Bird, E.; Mark, K. A. *Clin. Chem.* **1984**, *30*, 1477−1488.
(4) Takata, Y.; Taki, M.; Ito, M.; Murao, T. *J. Chromatogr.* **1985**, *332*, 117−125.
(5) Fenn, R. J.; Siggia, S.; Curran, D. J. *Anal. Chem.* **1978**, *50*, 1067−1073.
(6) Ehrenström, F. *Life Sci.* **1988**, *43*, 615−627.
(7) Spackman, D. H., Stein, W. H., Moore, S. *Anal. Chem.* **1958**, *30*, 1190−1205.
(8) *Chemical Derivatization in Analytical Chemistry, Volume 1: Chromatography*; Frei, R. W., Lawrence, J. F., Eds.; Plenum Press: New York, 1981.
(9) *Chemical Derivatization in Analytical Chemistry, Volume 2: Separation and Continuous Flow Techniques;* Frei, R. W., Lawrence, J. F., Eds.; Plenum Press: New York, 1982.
(10) *Reaction Detection in Liquid Chromatography*; Krull, I. S., Ed.; Marcel Decker: New York, 1986.
(11) *Detection-Oriented Derivatization Techniques in Liquid Chromatography;* Lingeman, H., Underberg, W. J. M., Eds.; Marcel Dekker: New York, 1990.
(12) *Handbook of Derivatives for Chromatography;* Blau, K., Halket, J., Eds.; John Wiley and Sons: Chichester, UK, 1993.
(13) *Selective Sample Handling and Detection in High-Performance Liquid Chromatography, Part A;* Frei, R. W., Zech, K., Eds.; Elsevier: Amsterdam, 1988.
(14) *Selective Sample Handling and Detection in High-Performance Liquid Chromatography, Part B*; Zech, K., Frei, R. W., Eds.; Elsevier: Amsterdam, 1989.

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fluorescence. As examples the following reagents: fluorescamine,[15] dansyl chloride (DNS),[16] 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F),[17] *o*-phthalaldehyde (OPA),[18−20] and naphthalene-2,3-dicarboxaldehyde (NDA),[21−23] all of which are useful for the derivatization of analytes possessing primary (DNS, NBD-F, OPA, and NDA applicable) or secondary (DNS and NBD-F applicable) amine functional groups, were all initially described in applications employing fluorescence detection.

In contrast the systematic development of derivatization reagents intended to impart electrochemical activity to analytes appears to have been much more modest. This is highlighted by the fact that after the introduction of OPA and NDA as primary amine specific fluorogenic reagents, other researchers subsequently reported the electrogenic nature of these reagents.[24−26] Thus, while perhaps not true in every case, it does appear that the majority of reagents available to convey EC activity upon an analyte were not purposefully designed for said purpose.

In using derivatization reactions for detection enhancement, one is frequently faced with situations where more than one functional group is kinetically competent to participate in the proposed derivatization reaction. When these situations are encountered, one can optimize the derivatization conditions to exhaustively label all available sites, thus overcoming the issue of forming multiple products; however, in the case of fluorescence detection there is the distinct possibility of interactions leading to fluorescence quenching and thus a substantially diminished detection sensitivity.[22,27] In contrast, investigations have clearly demonstrated that, in the case of electrochemical derivatization, chemical modification of multiple sites simply results in enhanced detectability.[28,29]

Current trends in bioanalytical chemistry are clearly mvoing toward miniaturization of sample size requirements and as a result the proportional down-sizing of the separation systems. As regards fluorescence detection, since this technique is optically based, these miniaturized separation systems naturally lead to the use of diminished optical path length detection cells, leading to a compromise in concentration detection limits. To compensate, one is often led to the use of laser-induced fluorescence (LIF); however, in most cases this is a situation where one has a miniature separation system that is burdened with a substantially larger detection system. Because electrochemical detection is a reaction−detection process dependent on analyte diffusion and because of the ease of electrode miniaturization, this technique actually benefits from down-sizing. There are now numerous examples where miniaturized LC systems[30−33] and capillary electrophoresis systems[34−42] have been successful when operated using EC detection. With regard to μ-chip scale instruments, several groups[43−46] have described the fabrication of separation−detection systems involving LIF detection, but recent publications are now describing the use of EC detection in association with a μ-chip/[μ-plate separation systems.[47,48] Thus, with the advent and continued progress in the development of these miniaturized separation systems, there is clearly the need for the development of reagents designed for use with EC detection.

On the basis of these considerations, the objective of the present research was the design and development of analytical derivatization reagents capable of imparting electrochemical activity upon analytes bearing primary or secondary amine functional groups. Because of documented detection sensitivity, ease of oxidation while maintaining stability in acid media, and oxidation−reduction reversibility, the hydroquinone (HQ) moiety was selected as the reagent substructure intended to convey EC activity to the analytical target. Herein we describe our initial strategies regarding reagent design and results obtained in the development of the candidate reagents intended to achieve this goal.

EXPERIMENTAL SECTION

Equipment, Solvents and Chemicals, Procedures, and Solutions. *Apparatus.* Isocratic liquid chromatography experiments were conducted on a modular HPLC system consisting of an ISCO LC5000 syringe pump (flow rate 1.5 mL/min), a Rheodyne model 7125 fixed loop (20 μL**)** injector, a Kratos

(15) Rubenstein, M.; Chen-Kiang, S.; Stein, S.; Udenfriend, S. *Anal. Biochem.* **1979**, *95*, 117−121.
(16) DeJong, C.; Hughes, G. J.; Van Wieringen, E.; Wilson, K. J. *J. Chromatogr.* **1982**, *241*, 345−359.
(17) Watanabe, Y.; Imai, K. *Anal. Biochem.* **1981**, *116*, 471−472.
(18) Hill, D. W.; Walters, F. H.; Wilson, T. D.; Stuart, J. D. *Anal. Chem.* **1979**, *51*, 1338−1341.
(19) Davis, T. P.; Gehrke, C. W.; Cunningham, T. D.; Kuo, K. C.; Gerhardt, K. O.; Johnson, H. D.; Williams, C. *H. J. Chromatogr.* **1979**, *162*, 293−310.
(20) Hogan, D. L.; Draemer, K. L.; Isenberg, J. I. *Anal. Biochem.* **1982**, *127*, 17−24.
(21) De Montingy, P.; Stobaugh, J. F.; Givens, R. S.; Carlson, R. G.; Srinivasachar, K.; Sternson, L. A.; Higuchi, T. *Anal. Chem.* **1987**, *59*, 1096−1101.
(22) Matuszewski, B. K.; Givens, R. S.; Srinivasachar, K.; Carlson, R. G.; Higuchi, T. *Anal. Chem.* **1987**, *59*, 1102−1105.
(23) Roach, M. C.; Harmony, M. D. *Anal. Chem.* **1987**, *59*, 411−415.
(24) Joseph, M. H. *Abstracts of Papers,* Third LCEC Symposium on the Biomedical Applications of LCEC and Voltammetry; Indianapolis, IN, 1982; Abstract #56.
(25) Allison, L. A.; Mayer, G. S.; Shoup, R. E. *Anal. Chem.* **1984**, *56*, 1089−1096.
(26) Oates, M. D.; Cooper, B. R.; Jorgenson, J. W. *Anal. Chem.* **1990**, *62*, 1573−1577.
(27) Kristjansson, F.; Thakur, A.; Stobaugh, J. F. *Anal. Chim. Acta.* **1992**, *262*, 209−215.
(28) Lunte, S. M.; Mohabbat, T.; Wong, O. S.; Kuwana, T. *Anal. Biochem.* **1989**, *178*, 202−207.
(29) Nussbaum, M. A.; Przedwiecki, J. E.; Staerk, D. U.; Lunte, S. M.; Riley, C. M. *Anal. Chem.* **1992**, *64*, 1259−1263.
(30) Slais, K. *J. Chromatogr. Sci.* **1986**, *24*, 321−329.
(31) Slais, K.; Krejcí, M. *J. Chromatogr.* **1982**, *235*, 21−29.
(32) Lada, S. H.; Kennedy, R. T. *J. Chromatogr. B* **1997**, *704*, 43−52.
(33) Hsieh, S.; Jorgenson, J. W.; Wightman, R. M. *Anal. Chem.* **1995**, *67*, 4514−4521.
(34) Ewing, A. G.; Mesaros, J. M.; Gavin, P. F. *Anal. Chem.* **1994**, *66*, 527A−537A.
(35) Wallingford, R. A.; Ewing, A. G. *Anal. Chem.* **1987**, *59*, 1762−1766.
(36) Park, S.; Lunte, C. E. *Anal. Chem.* **1995**, *67*, 4366−4370.
(37) Voegel, P. D.; Baldwin, R. P. *Electrophoresis* **1997**, *18*, 2267−2278.
(38) Holland, L. A.; Lunte, S. M, *Anal. Commun.* **1998**, *35*, 1H−4H.
(39) Lu, W.; Cassidy, R. M. *Anal. Chem.* **1994**, *66*, 200−204.
(40) Fermier, A. M.; Gostkowski, M. L.; Col6n, L. A. *Anal. Chem.* **1996**, *68*, 1661−1664.
(41) Zhong, M.; Lunte, S. M. *Anal. Commun.* **1998**, *35*, 209−212.
(42) Jacobson, S. C.; Hergenröder, R.; Koutny, L. B.; Ramsey, J. M. *Anal. Chem.* **1994**, *66*, 1114−1118.
(43) Jacobson, S. C.; Hergenröder, R.; Koutny, L. B.; Warmack, R. J.; Ramsey, J. M. *Anal. Chem.* **1994**, *66*, 1107−1113.
(44) Koutny, L. B.; Schmalzing, D.; Taylor, T. A.; Fuchs, M. *Anal. Chem.* **1996**, *68*, 18−22.
(45) Effenhauser, C. S.; Manz, A.; Widmer, H. M. *Anal. Chem.* **1993**, *65*, 2637−2642.
(46) Effenhauser, C. F.; Paulus, A.; Manz, A.; Widmer, H. M. *Anal. Chem.* **1994**, *66*, 2949−2953.
(47) Wooley, A. T.; Lao, K.; Glazer, A. N.; Mathies, R. A. *Anal. Chem.* **1998**, *70*, 684−688.
(48) Gavin, P. F.; Ewing, A. G. In *Handbook of Capillary Electrophoresis*, 2nd ed.; Landers, J. P., Ed.; CRC Press, Inc.: Boca Raton, FL, 1997; Chapter 26.

Spectroflow 783 programmable UV absorbance detector, and a Bioanalytical Systems, Inc. LC4B dual channel amperometric detector (carbon disk, Ag/AgCl reference([CIi] = 3 M), 0.002 in. gasket). Reversed-phase LC was conducted using an in-house packed column (150 mm × 4.6 mm i.d.; ODS Hypersil, 5-μm particles). Preparative LC experiments were performed using a Whatman Magnum 9 (Partisil 10, ODS-2), 50-cm semipreparative column. Mobile-phase pH was determined using an Accumet pH meter 910 that was calibrated using pH 4 and pH 7 buffer standards obtained from Fisher Scientific, Inc. A Houston Instruments Omniscribe strip chart recorder was used to record chromatogram peak heights. Derivatization reaction temperatures were maintained using a Pierce Reacti-Therm heating module. Derivatizations were carried out in both 4- and 1-mL vials with silicon rubber/Teflon septum to enable head space evacuation when applicable. Preparative normal phase chromatography was performed using a Fluid Metering Inc. pump (model IP-5Y) and an Isco Instrumentation Co. UV absorbance monitor (model 1840). ^{1}H NMR, IR, and mass spectra were obtained on a GE QE 300 Plus NMR, a Perkin Elmer 1420 ratio recording infrared spectrophotometer, and a Nermag R10-10 quadrupole GC/MS system with SPECTRAL 30 data system, respectively. Voltammetry experiments analysis were performed using a Cypress Systems, model CS-1087 potentiostat, version 6.1/2V equipped with a carbon disc electrode (4-mm diameter), Ag/AgCl reference electrode (RE-4) and a platinum counter electrode, all from Bioanalytical Systems Inc.

Chemicals and Solvents. The reagent chemicals, 2,5-dihydroxyphenylacetic acid (98%), 2,5- dihydroxyphenylacetic γ-lactone (97%), *p*-nitrophenol (99%), *N*-ethylbenzylamine (97%), 1-methylimidazole (99%), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl (+98%) were obtained from Aldrich Chemical Co. and were used as received. *N*-ethylbenzylamine was purified by vacuum distillation prior to use in trace analysis experiments. The dipeptide, Ile-Leu methyl ester was obtained from Bachem Biosciences Inc. and was used as received. Methylene chloride, hexane, ethyl acetate, and acetonitrile were HPLC grade solvents (99.9%) obtained from Fisher Scientific, Inc. Methylene chloride was distilled from calcium hydride and stored over 4 Å molecular sieves (Union Carbide Corp.) All other solvents were used as received. Water was purified with bulk carbon and mixed-bed deionization cartridges and then passed through a Millipore MILLI-Q water system to a resistance of 18 Ω/cm. The silica gel 60 used in preparative scale chromatography was obtained from EM Science Inc.

Electrochemical Characterization of the Reagents, Intermediates, and Products. The cyclic voltammetry scans were obtained in 50:50 acetonitrile/phosphate buffer (0.1 M, pH 7.0). Cyclic scans were performed from −500 mV to +1000 mV to −500 mV (vs Ag/AgCl, [Cl^-] = 3 M) on the various compounds (1 mM concentrations) at a scan rate of 100 mV/s. The carbon electrode was polished using 5-μm alumina before each run. Standards of the derivatization product of *N*-ethylbenzylamine were generated from both the NDTE or HLTE intermediates (2 mM solutions in acetonitrile) in situ by exposure to an equivalent volume of phosphoric acid (pH 2.0). After acid cleavage of the THP protecting group(s), the solution was brought to pH 7.0 with 0.1 M NaOH for voltammetry measurements.

Stock Solutions. Reagent stock solutions were prepared by weighing the required mass of each reagent followed by dissolution in acetonitrile. The resulting concentration of the stock solutions was NDTE, 40 mM and HLTE, 200 mM. The acylation catalyst, 1-methylimidazole, was used in all cases with the appropriate amount being added to the reagent stock solutions by volumetric pipette. The catalyst concentrations in the NDTE and HLTE solutions were 40 and 100 mM, respectively. Amino acid and dipeptide stock solutions were prepared by measurement on a Cahn microbalance and then transfer into a solution of either acetonitrile or 50:50 acetonitrile/water. Subsequent dilutions for standard curves were prepared by dilution of the initial stock solution using class A volumetric pipettes and volumetric flasks. *N*-Ethylbenzylamine stock solutions were prepared volumetrically using density at 25 °C and dilution of this initial stock for standard curve construction. Molar concentrations quoted for limit of detection experiments reflect the concentration of analyte in solution during derivatization step 1.

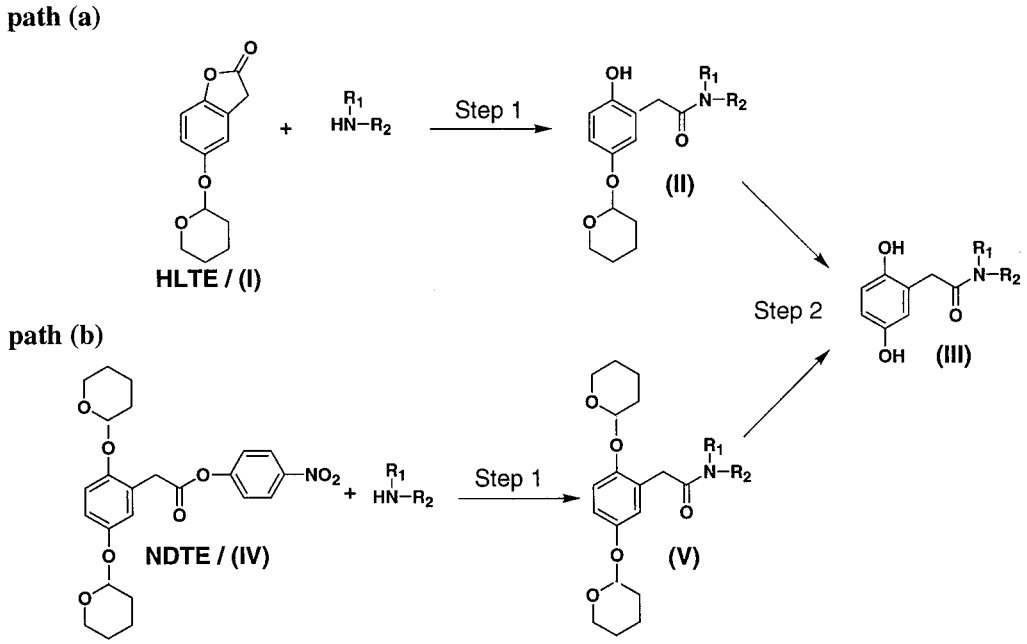
Derivatization Procedures. Reagent stock solutions were added to equivalent volumes of a known concentration of the amine to be derivatized in 50% acetonitrile and 50% water for NDTE derivatizations and 100% acetonitrile for HLTE derivatizations. Step 1 (Scheme 1) was allowed to proceed for a minimum of 30 min at 25 °C for NDTE, and 24 h at 50 °C for HLTE unless otherwise specified. HLTE derivatizations were performed under an argon head-space when noted. After step 1 (Scheme 1), the reaction mixture for both reagents was diluted 1:1 with aqueous phosphoric acid (pH 1.2, step 2) and allowed to sit at room temperature for a minimum of 1 h unless otherwise specified. The reaction mixtures were filtered and analyzed for derivatized amine by direct injection into the RPLC system.

HPLC Mobile Phases. Mobile phases for LC were prepared by mixing the appropriate ratio of acetonitrile with phosphate buffer (v/v) and then filtering under vacuum through a 0.2-μm Nylon 66 filter. The mobile phase was varied from 20 to 35% acetonitrile depending on the retention and resolution of the analytes involved. The phosphate buffer component was prepared by dissolution of Na_2HPO_4 (0.05 M) and dropwise titration to pH 7.0 with concentrated phosphoric acid.

Synthetic Preparations. *Homogentisic γ-Lactone Tetrahydropyranyl Ether (HLTE, **I**).* The precursor lactone, 2,5-dihydroxyphenylacetic acid γ-lactone (1033.7 mg, 7 mmol) and 3,4-dihyro-2H-pyran (883 mg, 10.5 mmol) were added to methylene chloride (50 mL) containing pyridinum *p*-toluenesulfonate (PPTS) catalyst (179.8 mg) prepared as described in a previously published method.[49] The reaction mixture was stirred for 24 h at ambient temperature. Upon completion (TLC) the solution was diluted with ethyl ether (50 mL) and washed with half-saturated NaCl solution (100 mL). The ether fraction was evaporated under reduced pressure (water aspirator) using a rotary evaporator with slight heating (40 °C) to afford a red oil containing the product and 2,5-dihydroxyphenylacetic acid γ-lactone as an impurity. Final purification was accomplished using low pressure preparatory chromatography (silica gel 60, particle size 63−200 μm.; glass column, 20 in. × 9/16 in.; solvent, 40:60 ethyl acetate/hexane; flow rate, 5.5 mL/min; Detection, UV at 254 nM). The product, a diastero-

(49) Miyashita, M.; Yoshikoshi, A.; Grieco, P. A. *J. Org. Chem.* **1983**, *48*, 3465−3471.

Scheme 1. Two General Pathways Examined for the Derivatization of Primary or Secondary Amines[a]



[a] Path a homogentisic γ-lactone tetrahydropyranyl ether (HLTE, **I**); path b *p*-nitrophenyl 2,5-dihydroxyphenylacetate, bis-tetrahydropyranyl ether (NDTE, **IV**).

meric mixture, was a faintly colored yellow oil which crystallized upon standing at room temperature to form similarly colored crystals (yield 77%, mp 64−65 °C. IR (neat) 2960, 1820, 1460, 1220, 1120, 1050, 970, 870, 815 and 640 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) 7.0 (s, 1H), 6.95 (d, 2H), 5.3 (t, 1H), 3.85 (t, 1H), 3.7 (s, 2H), 3.5 (m, 1H), 1.8 (d, 2H), 1.6 (m,4H). High resolution mass spectrum (FAB+), calculated MW: 234.0891, determined MW: 234.0887.

*N,N-Benzylethyl-2,5-dihydroxyphenylacetamide 5-tetrahydropyranyl ether (**VI**).* Homogentisic γ-lactone tetrahydropyranyl ether (**I**) (0.21 g, 0.0009 M) was added to *N*-ethylbenzylamine (0.6 g, 0.0045 mol) in 20 mL of dry methylene chloride. The reaction was stirred at 25 °C for 2 days. The product was isolated from starting materials using reverse phase semipreparatory chromatography (Partisil 10 ODS-2; column dimensions mm, 500 × 9 mm i.d.; mobile phase, 50:50 water/acetonitrile; flow rate, 4.0 mL/min; Detection, UV at 254 nM). The product, a mixture of two diastereomers, was a light yellow oil that solidified over 24 h. IR (neat) 2950, 2780, 1770, 1460, 1350, 1200, 1115, 865, and 810 cm^{-1}. ^1H NMR 7.3 (m, 5H), 6.9 (m, 3H), 5.2 (s, 2H), 4.65 (s, 2H) 1.95 (m, 2H), 1.8 (m, 2H),1.6 (m, 2H), 1.18 (q, 3H). Mass spectrum (CI mode, NH_3) *m*/*e* 370 (M + 1).

Methyl 2,5-Dihydroxyphenylacetate. The precursor carboxylic acid, 2,5-dihydroxyphenylacetic acid (1.10 g, 6.5 mmol) together with *p*-toluenesulphonic acid (200 mg, 1 mmol) was dissolved in methanol (150 mL) and refluxed for 4 h under argon. The methanol was evaporated under high vacuum using a rotary evaporator leaving a brownish solid (1400 mg) composed of 2,5-dihydroxyphenylacetic acid methyl ester and *p*-toluenesulphonic acid. A small amount of this solid was extracted into chloroform for identification. The chloroform was evaporated under high vacuum leaving a white solid consisting of the product. Isolated product mp 116−117 °C, ^1H NMR(acetone-d_6, 300 MHz) 7.82 (s, 1H), 7.7 (s, 1H), 6.6 (m, 3H), 3.5 (s, 2H), 3.56 (s, 3H).

2,5-Dihydroxyphenylacetic Acid, Bis-tetrahydropyranyl Ether. Crude 2,5-dihydroxyphenylacetic acid methyl ester (1200 mg, 7 mmol) containing *p*-toluenesulphonic acid as an impurity was added to degassed 3,4-dihydro-2H-pyran (150 mL) and stirred at room temperature for 4 h. The reaction mixture was then diluted with ethyl ether (150 mL) and washed with aqueous 1 N NaOH (2 × 100 mL). The organic layer was dried (Na_2SO_4) and evaporated under reduced pressure to afford a yellow oil (2500 mg), which was subsequently dissolved in methanol (160 mL) followed by the addition of water (40 mL). The pH (pH paper) of this mixture was raised to 14 with solid NaOH and allowed to stir overnight. After stirring, additional water (100 mL) was added and the mixture acidified to pH 4 by dropwise addition of concentrated phosphoric acid. The aqueous solution was extracted with chloroform (2 × 100 mL). The chloroform solution was dried (Na_2SO_4) and evaporated under reduced pressure to afford 2,5-dihydroxyphenylacetic acid bis-tetrahydropyranyl ether as a yellow semisolid. ^1H NMR ($CDCl_3$, 300 MHz) 7.0 (d, 1H), 6.88 (d, 2H), 5.27 (m, 4H), 4.9 (m, 4H), 3.9 (m, 2H), 2.2−1.8−1.5 (m, 12H).

*p-Nitrophenyl 2,5-Dihydroxyphenylacetate Bis-tetrahydropyranyl Ether (NDTE, **IV**).* 2,5-dihydroxyphenylacetic acid bis-tetrahydropyranyl ether (2.40 g, 7 mmol) and *p*-nitrophenol (1.390 g, 10 mmol) were dissolved in pyridine (15 mL) and cooled to ice temperature followed by the additional of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl (1.91 g, 10 mmol). The reaction was stirred under nitrogen for 36 h, slowly coming to room temperature over the first 12 h. Pyridine was evaporated under vacuum and the product was dissolved in ethyl acetate (100 mL). This was extracted with water (2 × 100 mL) and 1.0 M sodium bicarbonate (2 × 100 mL). The organic layer was dried and evaporated under vacuum to afford a red oil. This was dissolved in acetone (20 mL) and filtered through silica gel 60 to remove the red color. The gel was rinsed with additional acetone (60 mL) to elute any retained NDTE. The acetone was evaporated leaving

a light orange oil, which was a mixture of one major and three minor products separable by low pressure preparatory chromatography (silica gel 60; particle size, 63−200 μm; glass column, 20 × 9/16 in. i.d.; solvent, 40:60 ethyl acetate/hexane; flow rate, 5.5 mL/min; detection, UV at 254 nM). The major product peak was collected and the solvent evaporated to leave NDTE as a white solid (mp 114−117 °C). ^{1}H NMR ($CDCl_3$, 300 MHz) 8.2 (d, 2H), 7.25 (d, 2H), 7.05 (d, 1H), 7.0 (m, 2H), 5.3 (m, 2H), 3.85 (m, 4H), 3.6 (m, 2H), 1.9−1.85−1.6 (m, 12H).

*N,N-Benzylethyl-2,5-dihydroxyphenylacetamide Bis-tetrahydropyranyl Ether (**V**, $R_1 = C_2H_5$, $R_2 = CH_2C_6H_5$).* 2,5-Dihydroxyphenylacetic acid bis-tetrahydropyranyl ether (600 mg, 1.7 mmol) and *N*-benzylethylamine (675 mg, 5 mmol) were added to pyridine (15 mL). The solution was cooled to 0 °C and to this was added 1-(3-dimethylaminopropyl) 3-ethylcarbodiimide HCl (957 mg, 5 mmol). The reaction stirred under nitrogen for 24 h, slowly coming to room temperature over the first 12 h. The pyridine was evaporated under vacuum. The red product was dissolved in ethyl acetate (100 mL), washed with water (2 × 100 mL) and 1 N sodium bicarbonate (2 × 100 mL). The solvent was dried ($MgSO_4$) and removed using a rotary evaporator under high vacuum, leaving a brown red oil (178 mg, 0.4 mmol). The oil was purified using low pressure preparatory chromatography (silica gel 60; particle size, 63−200 μm; glass column, 20 in. × 9/16 in. i.d.; solvent, 40:60 ethyl acetate/hexane; flow rate, 5.5 mL/min; detection, UV at 254 nM). Further purification was accomplished using reverse phase preparatory chromatography (Partisil 10 ODS-2; 500 mm × 9 mm i.d.; mobile phase, 30:70 water/acetonitrile; flow rate, 4.0 mL/min; detection, UV at 254 nM). ^{1}H NMR ($CDCl_3$, 300 MHz) 7.25 (m, 5H), 7.14 (m, 1H), 7.04 (d, 1H), 7.0 (s, 1H), 5.27 (m, 2H), 4.0 (q, 2H), 3.9 (m, 4H), 3.75 (m, 2H), 3.55 (m, 2H), 1.6−1.8−1.9 (m, 12H), 1.1 (t, 3H). Mass spectrum (FAB+) *m*/*e* 453 (M).

RESULTS AND DISCUSSION

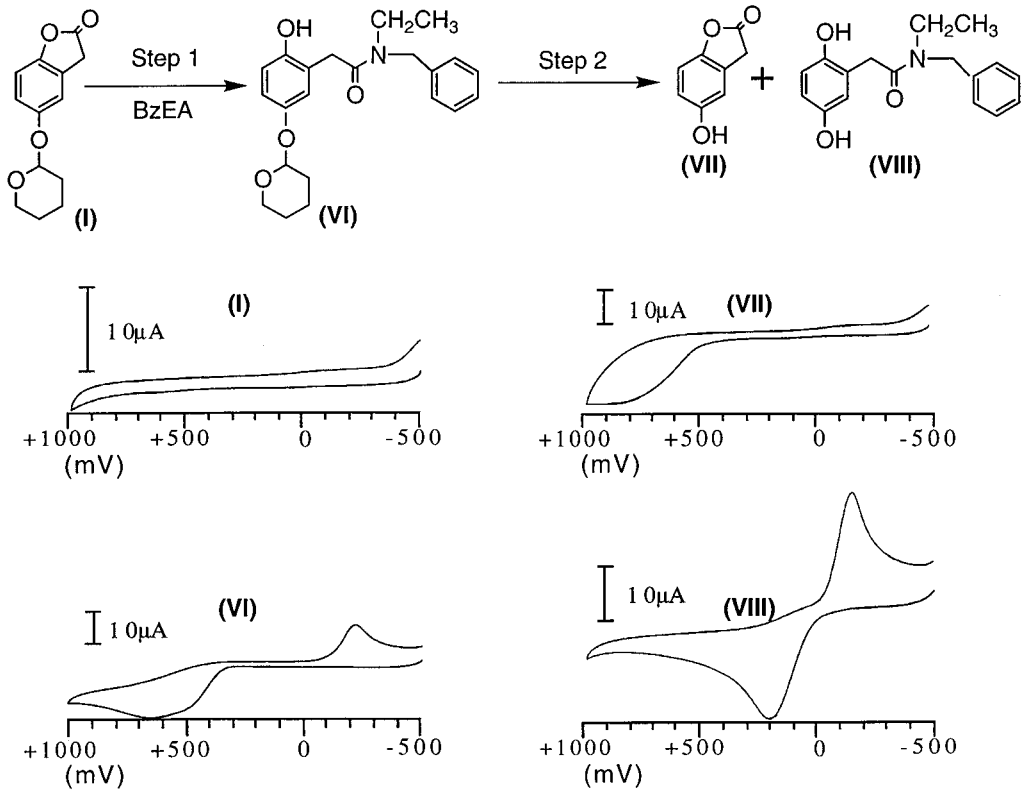
Reagent Design and Anticipated Properties. As noted earlier, the goal of this research was the design of reagents capable of conveying the HQ moiety to a primary or secondary amine-bearing analyte. This requires the introduction of a functional group that will ideally undergo rapid reaction with a primary/secondary amine to form a stable linkage between the reagent and analyte. From an operational standpoint, one has to conduct the reaction in mildly basic aqueous (organic-aqueous) media. Based on these criteria, one can narrow the derivatization process to a relatively few functional group transformations, with the following having been considered in the present research: isothiocyanate, activated carboxylic acid, and activated carbamate. Although precedence exists for the use of each of these derivative forming groups, after consideration of the overall goal in conjunction with availability of starting materials and ease of preparation, 2,5-dihydroxyphenylacetic acid (homogentisic acid, HGA) was selected as the template on which to base systematic reagent development. The structures presented in Scheme 1 (HLTE and NDTE) represent two differing approaches envisioned as modifications of the HGA template whereby the overall goal could be achieved.

The chemical pathways for amine transformation using HLTE (**I**) and NDTE (**IV**) are shown in Scheme 1. As shown, the reagents first acylate amine analytes in mildly basic media (step 1) to form amide intermediates (**II**, **V**). Acidification (step 2) causes hydrolytic cleavage of the tetrahydropyranyl (THP)-protecting group(s) to release the hydroquinone moiety (**III**) and thus achieve incorporation of the HGA template into the analytical product for EC detection. As shown by the reaction pathways, HLTE and NDTE differ in acyl-group activation, the intermediates formed, and native physico-chemical properties, but ultimately produce the same product. For both reagents, the following chemical aspects are of importance. Initially, the HQ moiety of each reagent is protected against autooxidation at the basic conditions required for amine acylation (step 1) due to the presence of either one (HLTE) or two (NDTE) THP-protecting **12** groups. Subsequently, acidification of the reaction mixture (step 2) serves to demask the HQ moiety in an acidic environment where liability to autooxidation is prevented prior to chromatography and detection.

EC Characterization of HLTE, Intermediates, and Products. HLTE, the first hydroquinone-labeling reagent studied in these experiments, demonstrates electrogenic properties when used as an amine derivatization reagent as it exhibits no significant electrochemical activity (Scheme 2, **I**) when examined by cyclic voltammetry (CV). Therefore, when used in LC applications with EC detection, excess HLTE would not appear as a chromatographic interference. Additionally, the cyclic voltammograms of Scheme 2 illustrate the redox properties of the isolated intermediate and final product of HLTE reaction with *N*-ethylbenzylamine (EBzA) as well as the major hydrolysis product (**VII**) obtained in acid media. The HLTE reagent shows no significant oxidative electrochemical activity in either its native (I) or deprotected form (**VII**) until approximately +550 mV. Both the phenolic intermediate (VI) and the de-protected HQ product (**VIII**) possess electrochemical activity at oxidation potentials lower than those observed for the unreacted reagent. The intermediate **(VI)** as shown in Scheme 3, is electrochemically active due to the EC-mediated cleavage of the THP group. The final product of the derivatization reaction (**VIII**) was shown by cyclic voltammetry to undergo a chemically reversible oxidation with a peak potential (E_{pa}) that is substantially lower (+0.200 V versus +0.600 V) than that observed for native hydroquinone analyzed under equivalent experimental conditions.

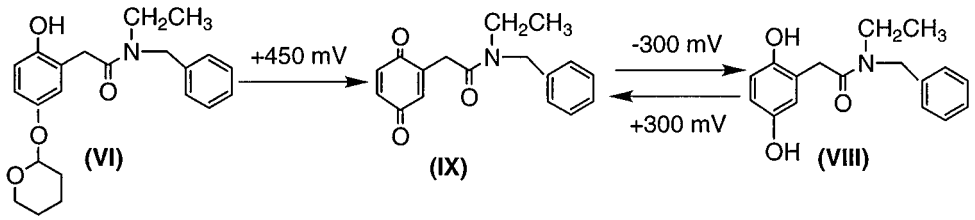
Electrochemical data for cyclic voltammetry performed on isolated standards of **VII** and **VIII** are summarized in Table 1 along with data for HGA and hydroquinone obtained under the same conditions. The high oxidative detection potential observed for **VII** at the end of the derivatization reaction is dependent on the lactone ring remaining closed. Though not observed to open at pH 7 during the 3-h time frame of the cyclic voltammetry experiments, in other experiments the lactone ring was observed to slowly open under hydrolytic .conditions. Ring opening was observed in 50:50 mixtures of acetonitrile/buffer at high pH (96% in 6 h at pH 8) and also at low pH (14% after 24 h at pH 1, 8.5% after 24 hours at pH 2). Due to the ring-opening reaction, the reagent loses some of its electrogenic characteristics as it is slowly hydrolyzed to HGA. Interestingly, as shown in Table 1, homogentisic acid, though more easily oxidized than the closed lactone, still possesses a peak oxidation potential which is approximately +150 mV greater than the HLTE-EBzA product (**VIII**). Even after deprotection (THP group removal) and hydrolysis of the lactone

Scheme 2. Components Present during the Derivatization of *N*-Ethylbenzylamine with HLTE and the Corresponding Cyclic Voltammograms at pH 7 of Each As Isolated 1 mM Standards[a]



[a] See Experimental Section for a detailed description and parameters.

Scheme 3. Nonreversible Removal of the HLTE Masking Group As Mediated by Oxidative Electrochemistry and Subsequent Reversible Reaction of the Liberated Hydroquinone[a]



[a] Example is specifically shown for HLTE reaction with *N*-ethylbenzylamine.

Table 1. Cyclic Voltammetry Parameters for the Intermediate (VI) and Final Product (VIII) in the Derivatization of *N*-Ethylbenzylamine Derivatized Using HLTE and for Homogentisic Acid and Hydroquinone Analyzed under Equivalent Conditions

analyte	E_{pa} (mV)	i_{pa} (μA)	E_{pc} (mV)	i_{pc} (μA)	$E^{o\prime}$ (mV)	ΔE (mV)
hydroquinone	+318	29.4	−240	23.1	+78	558
homogentisic acid	+361	21.4	−246	10.9	+115	607
VI	+639	15.1	−211	10.5	+428	850
VIII	+204	23.5	−148	18.9	+56	352

to the open form, the resulting HGA is still somewhat electrogenic since it is clearly more difficult to oxidize than the analytical derivative (**VIII**).

LC-EC of Isolated HLTE Intermediates/Products. The low oxidation potentials and chemical reversibility observed in free solution voltammetry for **VIII** are predictive of the low limits of detection that can potentially be obtained for this compound using reversed-phase (RP) LC with EC detection (RPLC-EC). Figure 1 shows an analysis by RPLC-EC using dual electrode detection of a pure synthetic standard of **VIII** at a high and a low concentration near the limit of detection. The chromatograms show the series electrochemical detector output for oxidation (upstream, +300 mV) of **VIII** to form the quinone (**IX**) and the subsequent reduction (downstream, −300 mV) of the quinone to the initial HQ-form (Scheme 3). Chromatogram (b), near the limit of detection, represents 1 pmol injected (20 μL injection, 50 nM solution). The mass limit of detection (MLOD) for this compound was found to be 100 fmol ($S/N = 4.5$ at +200 mV and $S/N = 3$ at +300 mV) at the upstream oxidative electrode (20 μL injection, 5 nM solution).

The low limit of detection observed for **VIII** demonstrates the usefulness of forming amine derivatives bearing the HQ moiety when determinations at trace concentrations are required. Figure 2 shows the hydrodynamic voltammogram for the oxidative detection of **VIII** using RPLC-EC at apparent pH 7. The current

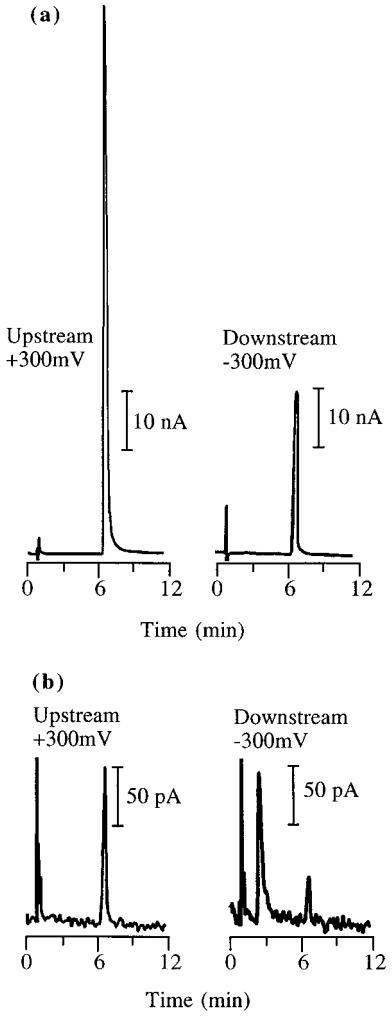


Figure 1. Isolated standard of the *N*-ethylbenzylamine derivative (**VIII**) analyzed using RP-LCEC in the dual detection mode (oxidation followed by reduction), (a) 5 nmol injected, (b) 1 pmol injected.

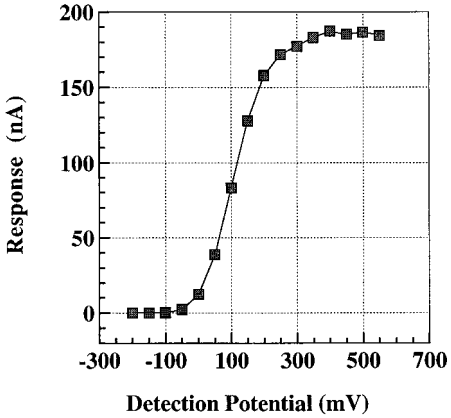


Figure 2. Hydrodynamic voltammogram for *N,N*-benzylethyl-2,5-dihydroxyphenylacetamide (**VIII**) generated by RPLC-EC; 20 μL injection of a 500 μM solution (see Experimental Section for details).

limiting plateau is reached at approximately +350 mV with less than an order of magnitude decrease in signal when the detection potential is lowered to +100 mV (175 nA → 75 nA). The low oxidation-potential observed for HGA-labeled analytes allows for selective detection against numerous compounds requiring higher potentials and the ease of the reverse reduction process provides additional selectivity for easily oxidized analyte interferences that do not participate in a similarly mild reduction. In addition, the mild detection potentials that could be used for the detection of hydroquinone-labeled analytes (+200 to +400 mV) offered significantly decreased electrode-fouling and decreased electrode equilibration time when compared to assays performed using higher potentials.

Table 2. Analytes and Concentration Ranges over Which Linear Calibration Plots Were Attained in the Derivatization of *N*-Ethylbenzylamine and the Dipeptide, Ile-Leu-OMe, with Each Reagent

derivatization reagent	analyte	number standards	concentration range (μM)	correlation (r^2)
HLTE	benzylethylamine	9	10 000−50	0.998
HLTE	benzylethylamine[a]	8	1000−5	0.994
HLTE	H-Ile-Leu-OMe[a]	9	1000−2	0.992
NDTE	benzylethylamine	11	25−0.025	1.000
NDTE	H-Ile-Leu-OMe	6	25−0.250	1.000

[a] Performed under an argon headspace.

HLTE Reactivity Issues. With the preceding data in hand, research efforts were next directed at the utilization of HLTE in an actual derivatization reaction, i.e., excess HLTE was used to derivatize aqueous solutions spiked with poorly detectable model amines. Table 2 lists the calibration curves generated for the HLTE derivatization and RPLC-EC determination of two model amines, EBzA and the dipeptide methyl ester, Ile-Leu-OMe. Sample chromatograms from the EBzA derivatization are shown in Figure 3. The lower limit of quantitation obtained for EBzA was a pre-derivatization concentration limit of detection (CLOD) of 5 μM (20 μL injection), representing a MLOD of 50 pmol. This is a 500-fold greater MLOD than that obtained for the pure standard of derivatized EBzA (**VIII**), which represents the lowest limit of detection that should be obtainable for a real sample in the limit of 100% derivatization efficiency and the generation of no chemical noise.

The inability to reach these expected detection limits with HLTE reagent was due to a low yield of the product and the formation of unknown substances during the derivatization reaction, which were chromatographic interferences. Step 2 in the derivatization procedure was shown to proceed in a quantitative, side product free fashion in under 30 min at 25 °C when starting with the pure intermediate (**VI**). No additional side products were observed to form in this step due to presence of spiked excess HLTE reagent, its precursors, or excess amine. On the basis of these observations, step 1 was implicated as the cause of both the low product yield (2−5% observed for EBzA using the procedure detailed in the experimental section) and the generation of interfering side products. These data are highly suggestive that a major limitation of HLTE in the derivatization of amines is its poor acylation potential. This could be partially rectified using higher reaction temperatures and longer reaction times in step 1, but even with these aggressive measures quantitative yields of product were never fully achieved. As an example of the slow reactivity of HLTE, the yield for the derivatization of EBzA (1 mM) with a 100-fold excess of HLTE in acetonitrile was still incomplete after 48 h at 75 °C (80% yield). The high temperatures and extended reaction times required to generate adequate product

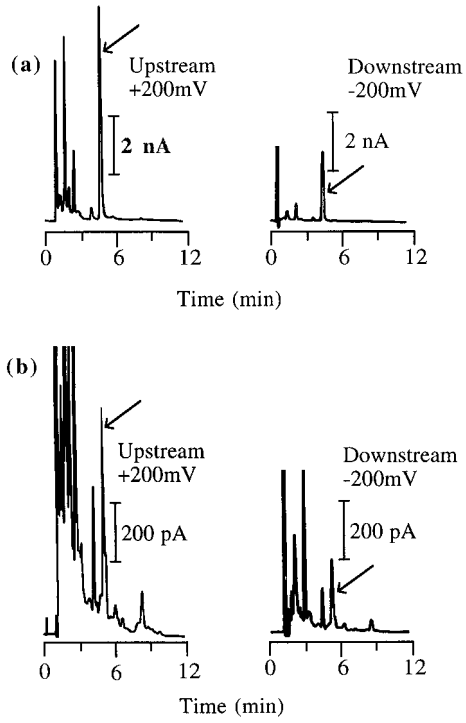


Figure 3. RP-LCEC determination using oxidative and reductive detection in series. The test solutions were as follows: (a) 250 μM solution of *N*-ethylbenzylamine in acetonitrile derivatized with HLTE, (b) 25 pM solution of *N*-ethylbenzylamine derivatized with HLTE. The injection volume was 20 μL in each case.

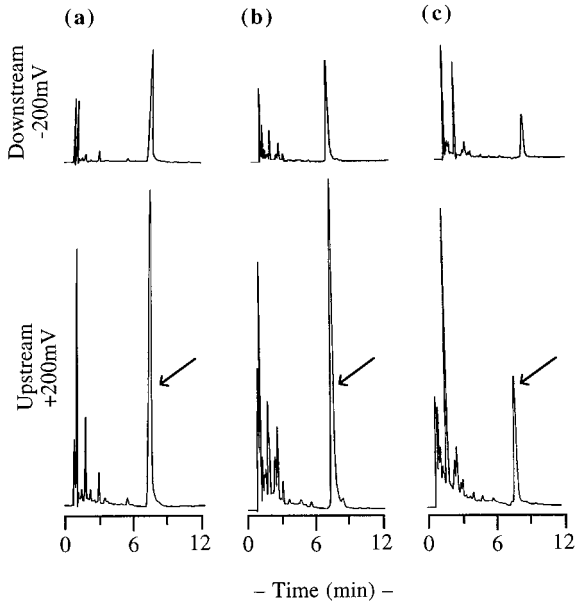


Figure 4. RPLC-EC chromatograms of identical HLTE derivatizations of 500 μM *N*-ethylbenzylamine solutions (2 mL) in the presence of variable headspace (2 mL) oxygen levels: (a) 100% argon headspace, (b) 21% oxygen headspace, (c) 100% oxygen headspace.

yields during step 1 are accompanied by a correspondingly high yield of what appear to be oxidative byproducts that both interfere with the derivatized analyte during trace quantitation and are also the source of electrode fouling. Figure 4 shows RPLC-EC chromatograms where identical concentrations of EBzA were reacted with HLTE in a sealed vial having a headspace consisting of 0% (argon), 21 (air), and 100% oxygen, Figure 4a, b, and c, respectively. Electrochemically detectable byproducts increase in relation to the presence of molecular oxygen (compare Figure 4a to b to c), and in the case of 100% oxygen headspace, there is a sharp decrease in derivatized product response due to electrode fouling from these oxidative byproducts (Figure 4c).

In summary, it was observed that the undesirable byproducts and associated electrode fouling were minimized by the exclusion of molecular oxygen from the reaction solution, but these byproducts were never fully eliminated. In any case, the use of reagents requiring the total exclusion of molecular oxygen are highly undesirable and impractical when large numbers of samples require derivatization. Thus, based on the information gleamed from these studies, it appeared that the next logical aspect of reagent design would be to enhance the reactivity (acylation potential) of the reagent so that a mild and rapid first step would be possible and this presumably would minimize the problem of oxygen related impurities that seemed to be associated with the aggressive conditions required in step 1 when HLTE was used as the derivatization reagent.

NDTE−HLTE Comparisons. NDTE (Scheme 1, **IV**), an alternate HGA-based reagent was developed in response to the limitations observed with HLTE. The rational for the design of the NDTE structure was the enhancement of acylation potential so that step 1 would be quantitative and rapidly proceed under relatively mild conditions, thus circumventing the formation of the unwanted byproducts previously observed with HLTE. Acylation ability was enhanced by the use of the *p*-nitrophenol active ester as an alternative to the lactone ring of HLTE. The remaining phenolic hydroxyl group was protected by forming an additional THP group, thus preventing any pre-oxidation from occurring in step 1 where it is necessary to use basic media.

A side by side comparison of the relative reactivity of NDTE and HLTE toward the model amine Ile-Leu-OMe is shown in Figure 5, along with separate derivatizations of blank solutions. Reaction time, temperature, and molar concentrations of both reagents and the analyte were the same for all experiments. NDTE is faster reacting and provides a 20−50-fold improvement in product yield in a significantly shorter time. For this direct comparison, step 1 acylation for both reagents was performed at 50 °C for 16 h, conditions required for the HLTE reagent to provide useful product yields. As will be seen in the following section, the NDTE reagent provides a similar yield of derivatized product to that shown in substantially less time; however, it was necessary to conduct the experiment in this fashion to appreciate the lack of side products formed with NDTE as compared to HLTE.

NDTE−Reaction Kinetics with Model Analytes. Figure 6 shows the reaction kinetics at 25 °C for the derivatization of a trace concentration of EBzA using NDTE. Acylation (step 1) and deprotection (step 2) are each shown to reach maximal yield in under 30 minutes for a total derivatization time at 25 °C of 1 h. The reaction yield was 95.1% when compared with the pure standard (**VIII**). Based on reaction rate, yield, and the mild reaction conditions, NDTE is clearly a superior reagent for primary/secondary amine derivatizations as compared to HLTE. A negative aspect of NDTE is that it is nonelectrogenic as the excess reagent is converted to electrochemically active HGA during the derivatization procedure. From a practical standpoint,

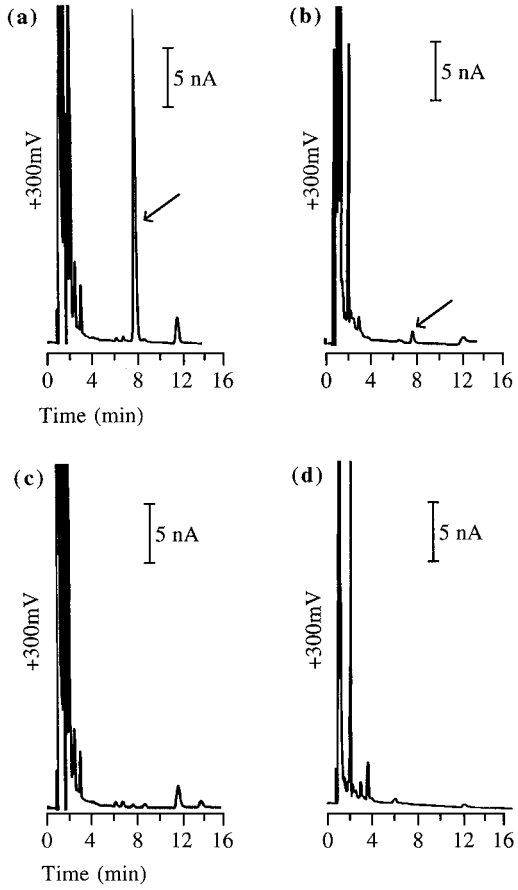


Figure 5. RPLC-EC chromatograms resulting from oxidative detection performed on (a) 50 pLM solution of isoleucine leucine methyl ester in acetonitrile derivatized with NDTE, (b) 50 μM solution of isoleucine leucine methyl ester in acetonitrile derivatized with HLTE; (c) blank of acetonitrile derivatized using NDTE, (d) a blank of acetonitrile derivatized using HLTE.

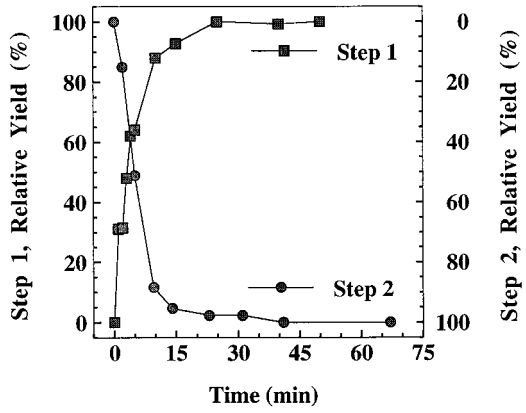


Figure 6. Time to maximal yield for step 1 (■) and step 2 (●) in the derivatization of *N*-ethylbenzylamine (50 μM) using NDTE reagent at 25 °C (see Experimental Section for detailed derivatization parameters).

the carboxylic acid group of HGA is fully ionized at pH 7, which results in minimal RPLC retention of the polar HGA. Accordingly in the determination of EBzA (Figure 7) the excess reagent was not a chromatographic interference.

For comparative purposes, NDTE was used for the determination of EBzA and Ile-Leu-OME as was previously done using HLTE. The resulting calibration data for each reagent is summarized in Table 2. Figure 7a shows the oxidative/reductive chromatograms for a single RPLC-EC determination of EBzA derivatized with NDTE at a prederivatization concentration of 62.5 nM or 625 fmol injected (1:1 acid dilution in step 2, 20 μL injection). The CLOD for EBzA was determined to be 25 nM (MLOD, 250 fmol injected, $S/N = 3/1$ at +300 mV). This MLOD is very similar to that obtained for the synthetically prepared standard of HGA-labeled EBzA (**VIII**), indicating that the derivatization procedure used and the performance of the reagent are now optimal. Figure 7b shows the results from a derivatization blank illustrating the minimal interferences resulting from the NDTE reagent.

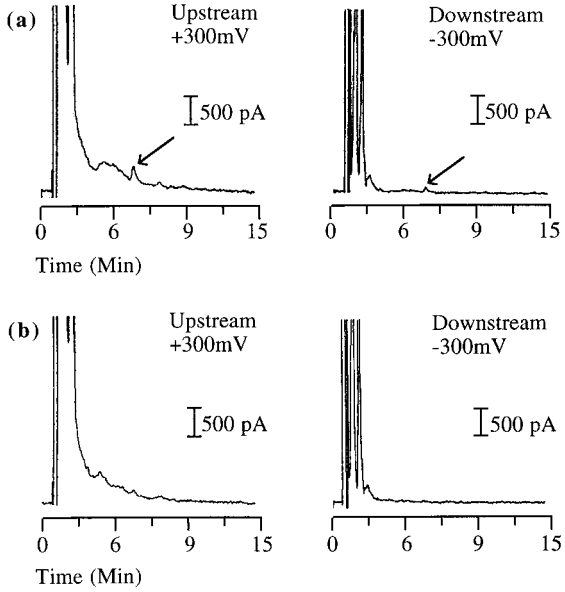


Figure 7. RPLC-EC determination using series oxidation and reduction performed on (a) a solution of 62.5 nM *N*-ethylbenzylamine in 75% acetonitrile and 25% water derivatized with NDTE, (b) a blank solution of 75% acetonitrile and 25% water derivatized with NDTE.

Experience gained in derivatizing various amines with NDTE using the herein described derivatization procedure have established CLODs usually falling between 25 and 250 nM, which is similar to the data presented in Table 2. The higher limit of detection for Ile-Leu-OMe is likely related to the fact that N-terminal isoleucine is slowly reactive to acylation type reactions,[50] and in data not presented (other amino acid and peptide analytes), it has been generally observed that detection limits similar to the 25 nM limit obtained with EBzA are normally attained.

CONCLUSIONS

The concepts that served as the basis for the design of HLTE and NDTE have proven to be valuable and represent significant steps toward the rational development of reagents intended to impart EC activity upon various amine analytes. The results of the present investigation will serve as a basis for the design of future reagents where one would seek to take advantage of the positive observations of the present work, i.e., the diminishment of EC activity that results from phenolic group lactonization and the need to have a mild acylation step to ensure high chemical yield and minimal reagent related byproducts. Of note, despite

(50) Rutter, W. J.; Santi, D. V. U.S. Patent 5,010,175, 1991.

the lack of electrogenicity of NDTE, this did not prove to be a substantial limitation primarily due to the highly polar nature of HGA which results in essentially void-volume elution in RPLC applications. Notwithstanding this result, an obvious next step in EC reagent development would be the incorporation of structural features that provide for a reagent exhibiting a high degree of electrogenicity. In this regard, perhaps one option would be the placement of a structural element that would have the effect of promoting lactonization of the reagent hydrolysis product (HGA or a related aromatic phenolic carboxylic acid). Such structural modifications have proven to serve this function in similar molecules.[51] Finally, it can be concluded that the development and application of reagents such as NDTE, and perhaps more advanced versions that possess electrogenicity, will become increasingly important as miniaturization in analytical chemistry continues to place limitations and demands on detection technologies.

(51) Hillary, P. S.; Cohen, L. A. *J. Org. Chem.* **1983,** *48,* 3465−3471.

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