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Electrophore Mass Tag Dideoxy DNA Sequencing

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Toward a goal of dideoxy sequencing DNA utilizing electrophore labels, we prepared four electrophore-labeled DNA oligonucleotide primers. Each primer has a different electrophore and DNA sequence but a common glycol keto (α,*β*-dihydroxyketo) release group. Cleavage of this latter group by either periodate oxidation or a thermal retroaldol reaction releases the electrophores for detection by mass spectrometry. Successful sequencing data with these primers was obtained by capillary electrophoresis on an ABI Model 310 after fluorescence dideoxy terminator cycle sequencing reactions were conducted. In a separate experiment, it was demonstrated that a cocktail of the four electrophore DNA primers could be detected as a dried sample spot by CO_2 laser desorption/capillary collection/gas chromatography electron capture mass spectrometry. These results establish some feasibility for our long-term goal of high-speed multiplex electrophore mass tag dideoxy DNA sequencing. Ultimately we plan to use a higher number of electrophore mass tags and to rely on direct detection of the desorbed electrophores by electron capture time-of-flight mass spectrometry.

DNA sequencing methodology is undergoing a transition to nonisotopic methods, a good example being fluorophore-labeled primers used for dideoxy polymerase reactions.[1−3] The resulting ladder of products is measured by electrophoresis with laser-induced-fluorescence detection. Automated commercial instruments are available for such methodology, e.g., from Applied Biosciences and Pharmacia Biotech. In the former system, four fluorescent dyes are employed, one for each of four primers (for A, G, C, and T base sites), and the subsequent electrophoretic separation is multiplexed in two ways: multiple lanes on a slab gel, and four dyes (providing one sequence) per lane.[4] In a related, also routine, approach, sequence tags are employed to give 40-multiplexing per lane on the electrophoresis gel.[5] These techniques can provide relatively high throughput at a moderate cost.

A number of alternative methods are emerging, or being studied, to sequence DNA even faster, while also reducing the cost. Most closely related to the current technology is the use of multiple, parallel, capillary electrophoresis columns instead of slab gel electrophoresis, while continuing to conduct four-dye sequencing reactions. Sequencing by hybridization on chips also is emerging,[6,7] and sequencing by mass spectrometry (MS) is being studied.[8−10] The latter can involve the detection (no electrophoretic separation) of dideoxy sequencing ladders by MS,[11−13] or the technique of fragmenting the target DNA (enzymatically[14,15]

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or via energetics in the MS[16,17]), followed by detection. Sequencing by MS also can involve the detection of "mass tag labels" such as sulfur isotopes[18] or metal ions[19] attached to DNA.

Here we present our initial progress toward a goal of sequencing DNA based on electrophore mass tag labels. An electrophore is a compound that readily ionizes in the gas phase by an event called "electron capture". Typically this takes place in an electron capture detector for gas chromatography (GC-ECD) or in the ion source of an electron capture mass spectrometer (typically GC/EC-MS). Several features of electrophore mass tags make them attractive for DNA sequencing. Electrophores can be easy to prepare, stable both chemically and physically, detectable in trace amounts, and available as numerous analogs for high multiplexing. In principle, the detection of electrophores by time-of-flight (TOF) EC-MS could be very sensitive and fast, helping to make it possible, with use of highly multiplexed electrophores, to sequence DNA quite rapidly. Some of this potential has been pointed out previously.[20−29]

EXPERIMENTAL SECTION

Materials. Organic solvents, such as THF, acetonitrile, methanol, ethyl ether, methylene chloride, and ethyl acetate were purchased from J. T. Baker (Phillipsburg, NJ). Sodium bicarbonate, sodium sulfate, sodium carbonate, sodium bisulfite, preparative TLC plates, and regular silica TLC plates were also purchased from J. T. Baker. PCR buffer was Sequitherm (Epicenter, Madison, WI), pH 9.3/50 mM Tris/2.5 mM $MgCl_2$. All other chemicals were purchased from Aldrich Chemicals Co., Inc. (Milwaukee, WI). All compounds (except the electrophore-labeled DNA oligomers) synthesized in our laboratories were confirmed by ^{1}H NMR and ^{13}C NMR. In some cases ^{19}F NMR also was used.

Equipment. A Zorbax RX-C18 (4.6 mm × 15 cm) reversed-phase column (Mac-Mod Analytical, Chadds Ford, PA) was employed for HPLC.

Model thermal release studies were conducted on a Varian 3300 gas chromatograph fitted with a thermal conductivity detector. An aluminum column ($^3/_{16}$ in. i.d., 4 ft long) was packed with 10% SP-2100 (Supelco, Bellefonte, PA) on Chromosorb W (Alltech, Deerfield, IL).

GC/EC-MS was performed using a Hewlett-Packard (HP) 5988A mass spectrometer coupled to a HP 5890 gas chromatograph. The instrument was controlled by a HP 59970C MS Chemstation computer for data acquisition in the single ion monitoring mode. Dwell time was set to be 50 ms with live screen display off when four ions were monitored. An Ultra-2 HP capillary column (25 m × 0.32 mm i.d., 0.17 μm film thickness) from Hewlett Packard was used. With helium as carrier gas, the column head pressure was set to 20 psi. The source pressure (methane) was set to 2.0 Torr at 250 °C. Injection was on-column at 120 °C, hold for 2 min, ramp column oven to 165 °C at 10 °C/min, then ramp to 290 °C at 70 °C/min, and hold for 3 min.

GC-ECD was performed with a Varian 3500 instrument (Walnut Creek, CA) fitted with a splitless glass insert ($^1/_4$ in. o.d. × 2 mm i.d. × 3 in. long) in a direct injector at 300 °C, and an Ultra-1 capillary column (10 m × 0.2 mm, 0.11 μm film thickness, HP). Detector temperature was 350 °C.

Synthesis. (a) 4′-[(Pentafluorobenzyl)oxy]acetophenone, 1a′, and 2a′, 3a, and 4a. 4′-Hydroxyacetophenone (2.72 g, 20 mmol) and sodium hydroxide (0.90 g, 22.5 mmol) were dissolved in acetonitrile (50 mL) and water (30 mL). To this solution, α-bromo-2,3,4,5,6-pentafluorotoluene (3 mL, 19.9 mmol) was added. The reaction mixture was heated to reflux for 5 h and then cooled to room temperature. To this solution, a 5% sodium carbonate solution (150 mL) was added. The precipitate was collected by suction filtration, washed with water (200 mL × 2), and then recrystallized with methanol/water. Yield, 5.98 g (95%). The same method was used for the preparation of **2a′**, **3a**, and **4a**. For **3a** and **4a**, an equimolar amount of 3,5-bis(trifluoromethyl)benzyl bromide was used in place of α-bromo-2,3,4,5,6-pentafluorotoluene. The yield was 93% for **2a′**, 88% for **3a**, and 95% for **4a**.

(b) 4′-[(*p*-Methoxytetrafluorobenzyl)oxy]acetophenone, 1a, and 2a. 1a′ (5.98 g, 18 mmol) was dissolved in methanol (30 mL). To this solution, sodium methoxide (1.08 g, 20 mmol) was added. The reaction mixture was heated to reflux for 2 h and then evaporated to dryness. The resulting solid was washed with water (100 mL × 2) and air-dried. Yield, 6.1 g (99%). The same method was used for the preparation of **2a**. The yield was 89%.

(c) Ethyl *N*-[(Diethylphosphono)acetyl]isonipecotate (5). In a 50 mL round-bottom flask were placed 1 g (5.1 mmol) of diethylphosphonoacetic acid, 0.7 g (6 mmol) of *N*-hydroxysuccinimide, and 10 mL of dioxane that had been distilled from $LiAlH_4$. After cooling in an ice bath under nitrogen with stirring, 1.25 g (6.0 mmol) of *N*,*N*-dicyclohexylcarbodiimide in 5 mL of dioxane were added slowly. After stirring at room temperature overnight, 1.18 g (7.5 mmol) of ethyl isonipecotate in 5 mL of dioxane was added, followed by stirring at room temperature overnight. The white precipitate of dicyclohexylurea was filtered off, and the filtrate was concentrated on a rotary evaporator. The product could be purified by silica flash chromatography with ethyl acetate, giving 1.2 g (70%) of product based on diethylphosphonoacetic acid. In practice, the entire evaporated reaction mixture (a viscous oil) was used directly in the next step, when the amount of starting diethylphosphonoacetic acid was 1.15 mL (7.1 mmol).

(d) *N*-[3-[4′-[(*p*-Methoxytetrafluorobenzyl)oxy]phenyl]-crotonyl]isonipecotic Acid, 1b, and 2b−4b. The preceding viscous oil was dissolved in THF (50 mL). Sodium hydride (0.87 g, 21.75 mmol) was added followed by **1a** (1.61 g, 4.9 mmol). The reaction mixture was refluxed for 10 h, cooled to room temperature, and filtered. To the filtrate, a solution of KOH (1 g, 18 mmol) in water (20 mL) and acetonitrile (10 mL) was added. The reaction was stirred at room temperature for 24 h followed

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by evaporation of the organic solvents. The resulting aqueous solution was diluted with water (100 mL). Any insoluble material was removed by filtration. The filtrate was acidified with 5% HCl to pH ~4. The precipitated solid was collected by suction filtration, washed with water (100 mL × 2), and air-dried. Further purification was achieved by column chromatography (60−200 mesh silica gel; mobile phase, 2% methanol in methylene chloride). Yield, 1.2 g (51%). The same method was used for the preparation of **2b** (50%), **3b** (39%), and **4b** (53%). While **1b** was entirely the *E* isomer (based on analysis of the vinyl proton by ^{1}H NMR), the *E*/*Z* ratios for **3b** and 4**b** were 4:1 and 7:1, respectively. An assignment could not be made for **2b**.

(e) *N*-[3-[4′-[(*p*-Methoxytetrafluorobenzyl)oxy]phenyl]-3-methylglyceronyl]isonipecotic Acid, 1c, and 2c−4c. Compound **1b** (0.20 g, 0.42 mmol) was dissolved in *t*-BuOH (3 mL). To this solution, osmium tetraoxide (0.14 g, 0.55 mmol) was added followed by the addition of pyridine (0.3 mL). The reaction mixture was stirred at room temperature overnight. An aqueous 5% sodium bisufite solution (20 mL) was added. The resulting solution was diluted with water (100 mL) and acidified with 5% HCl to pH ~4. The precipitated solid was collected by suction filtration, washed with water (100 mL twice), and air-dried. The product was extracted into ether (50 mL). The ether extract was evaporated to dryness, and the residue was redissolved in 5% methanol in methylene chloride (100 mL). The solution was passed through a plug of silica gel and then evaporated to dryness to give a white solid. Yield, 0.13 g (61%). The same method was used for the preparation of **2c**, **3c**, and **4c**. The yield was 90% for **2c**, 47% for **3c**, and 80% for **4c**.

(f) *N*-[3-[4′-[(*p*-Methoxytetrafluorobenzyl)oxy]phenyl]-3-methylglyceronyl]isonipecotic Acid, *N*-Hydroxysuccinimide Ester, 1d, and 2d−4d. Compound **1c** (0.10 g, 0.194 mmol), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (0.045 g, 0.235 mmol), and *N*-hydroxysuccinimide (0.027 g, 0.235 mmol) were dissolved in acetonitrile (5 mL). The reaction mixture was stirred at room temperature overnight and then evaporated to dryness in vacuo. The residue was washed with water (50 mL twice) to yield a white solid which was collected by suction filtration and air-dried. Yield, 0.106 g (89%). The same method was used for the preparation of **2d** (80%), **3d** (85%), and **4d** (89%).

(g) *N*-Butyl-*N*′-[3-[4′-[(*p*-methoxytetrafluorobenzyl)oxy]-phenyl]-3-methylglyceronyl]isonipecotamide, 1e, and 2e. NHS ester **1d** (0.1 g, 0.163 mmol) was dissolved in methylene chloride (2 mL). A solution of butylamine (0.04 g, 0.55 mmol) in methylene chloride (1 mL) was added. The reaction mixture was stirred at room temperature for 2 h and evaporated to dryness in vacuo. Washing the residue with water (5 mL × 3), 5% aqueous potassium carbonate solution (5 mL), and water (10 mL × 5) gave a white solid, which was collected by suction filtration and then air-dried. Yield, 0.050g (54%). The same method was used for the preparation of **2e** (60%).

Thermal Stability of 1e in PCR Buffer. Amide **1e** (1 mg) was mixed with 700 μL of PCR buffer and 300 μL of acetonitrile in a vial. The solution was analyzed by HPLC before and after the vial was capped and heated in a 95 °C water bath for 30 min. In a related experiment, 20 mg of the amide **1e** was heated in 1 mL of acetonitrile plus 1 mL of PCR buffer for 1 h at 95 °C, followed by extraction into methylene chloride, drying, and analysis by NMR. This demonstrated that **1e** was fully stable to these conditions.

Thermal Release of Ketone 1a from Glycol Amide 1e. A packed aluminum column was installed in a gas chromatograph fitted with a thermal conductivity detector. The column and detector temperature were both set at 240 °C. Ketone **1a** was injected first with the injector temperature at 300 °C. At the same injector temperature, 0.67 mg of amide **1e** was injected. The experiment was repeated with the injector at 240 °C and after the column was removed and the injector was at 300 °C. For the latter injection, a vial was placed at the outlet of the injector to collect the volatile product(s), which was(were) analyzed by TLC.

Electrophore Labeling of Oligomers. DNA oligomers (Operon, Alameda, CA) NE-105 (5′-LTACCGGTCGTGATCTTGGCA), NE-106 (5′-LTGGTGATAACAGCGCTTGCG), NE-107 (5′-LCGATGTTTGTAGCGACGGTG), and NE-104 (5′-LTGGTACCGGTCATGACAACG) were labeled with **1d**, **2d**, **3d**, and **4d**, respectively, giving **1d**-NE-105, **2d**-NE-106, **3d**-NE-107 and **4d**-NE-104, respectively. L represents a C6 ($H_2N[CH_2]_6-$) amino linker.

Thermal Release of Ketones from Electrophore-Labeled Oligonucleotides. Four different electrophore-labeled oligonucleotides were combined using 100 μL of each in the following initial concentrations: **1d**-NE-105, 130 fmol/μL; **2d**-NE-106, 95 fmol/μL; **3d**-NE-107, 150 fmol/μL; **4d**-NE-104, 150 fmol/μL. The resulting sample was evaporated to dryness under nitrogen, redissolved in 50 μL of water, and 1 μL was injected into a GC-ECD. The temperature gradient started at 120 °C, was held for 1 min and increased to 300 °C at a rate of 50 °C/min followed by a 1 min hold.

Dideoxy Sequencing. About 0.5 pmol of each primer was used according to the manufacturer's protocols for the AmpliTaq DNA Polymerase FS, the Taq DyeDeoxy Terminator Cycle sequencing, the Centri-Sep spin column, the Template Suppression reagent, and the ABI 310 capillary electrophoresis at 7500 V for 130 min (Perkin-Elmer Applied Biosystems, Foster City, CA).

Laser Desorption Apparatus. The desorption device, which included an *x*−*z* translation stage (460A, Newport Irvine, CA), is shown schematically in Figure 1. The laser was a μ-TEA CO_2 laser (Laser Science, Inc., Newton, MA) with a pulse energy of 7 mJ and a pulse width of 500 ns at 1−20 Hz. A visible He−Ne laser beam was reflected partially by a ZnSe window and aimed collinearly with the CO_2 laser optical path, making it easier to align the latter, invisible radiation. Since the ZnSe window was AR coated at 10.6 μm, and the CO_2 laser was horizontally polarized, the loss due to reflection for CO_2 laser output was negligible. The collection capillary (~40 cm length × 0.32 mm i.d., Ultra 1, cross-linked methyl silicone bonded phase, HP) was of a type that is ordinarily used for gas chromatography. This capillary was mounted in a cutoff glass syringe barrel (10 μL syringe, No. 701, Hamilton Co., Reno, NE). A piece of flexible aluminum plate was cut (dumbbell shape) so that part of it could wrap around the end of the barrel, and part could be bent (135°) for tape-mounting a membrane target. The positions of the sample and the collection capillary were adjusted by relying on the He−Ne beam, and the distance between the capillary tip and the sample spot was 2 mm.

Laser Desorption of Electrophores. To a 0.5 μL droplet of water containing four glycol-release electrophore-labeled DNA oligonucleotides (100 fmol each) on a 50 μm thick, 0.4 × 2 cm^2 Kapton-type HN polyimide membrane (DuPont Co., Circleville, OH) was added 0.5 μL of 0.04 M sodium periodate. The membrane was kept at high humidity (in a small plastic box with a wet paper towel) for 15 min and then removed from the box to

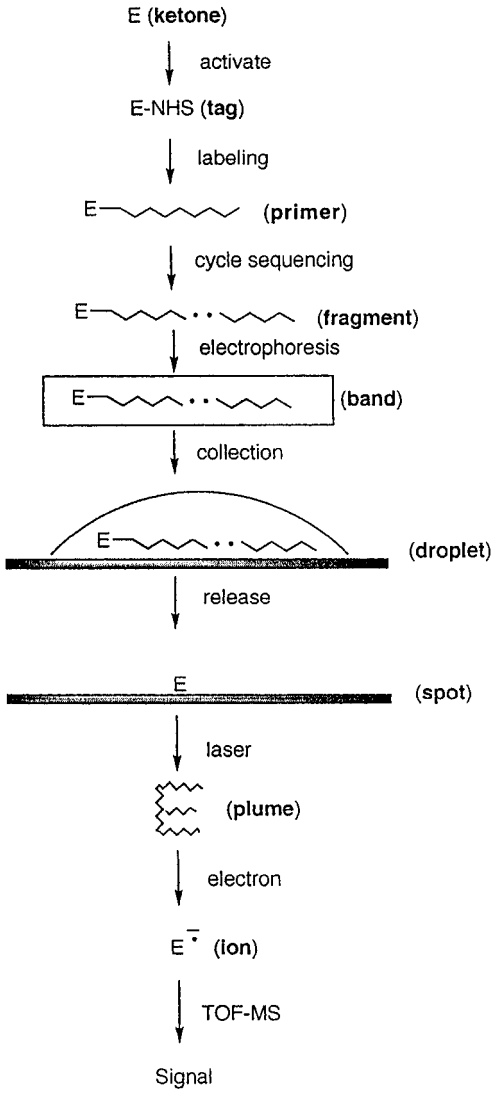

Figure 1. Scheme for electrophore DNA sequencing.

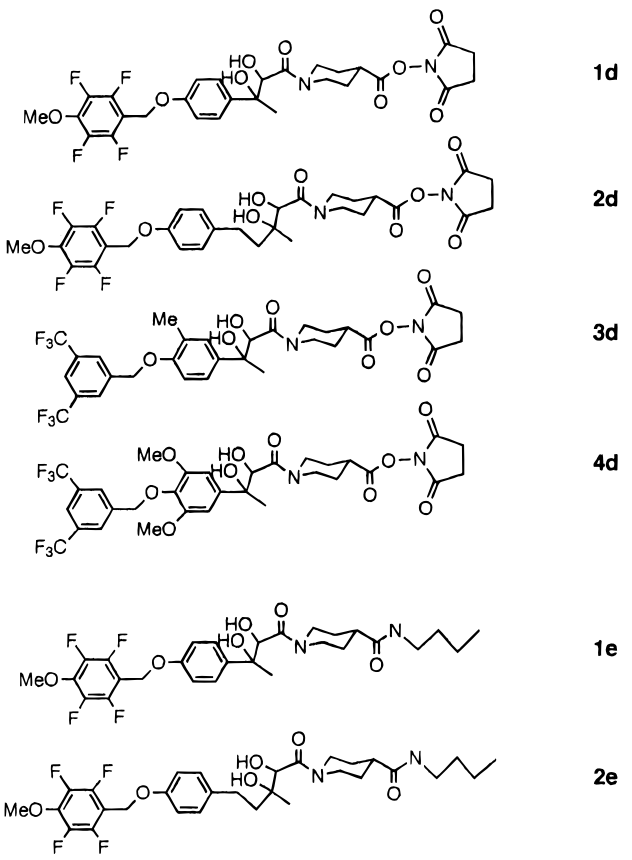

Figure 2. Structures of glycol keto electrophore *N*-hydroxysuccinimide esters (**1d**−**4d**) and the corresponding butylamides (**1e**−**2e**) for two of them.

dry. The membrane was mounted onto an aluminum plate (see above) with double-sided Scotch tape, so that the tape was not in the optical path. After rastering CO_2 laser pulses (approximately 150 × 200 μm in size) at ~1 Hz across the spot, trying to not hit the same zone twice, the collection capillary (under vacuum) was rinsed with 20 μL of ethyl acetate, and 1 μL of the sample was injected into a GC/EC-MS.

RESULTS AND DISCUSSION

Overall Scheme. Focusing on the key role of electrophore label, the scheme that we are developing for mass tag DNA sequencing is summarized in Figure 1. An electrophoric ketone is subjected to a series of synthetic reactions, leading to a glycol electrophore *N*-hydroxysuccinimide ester (E-NHS). This latter reagent, as a reactive tag, is attached to the 5′-amino group of a DNA primer obtained from a DNA synthesizer. This electrophore-labeled primer then is used in a cycle-sequencing reaction to generate a ladder of fragmentation products divided among four reactions (one for each DNA base). The resulting electrophore-labeled sequencing fragments are separated by capillary electrophoresis with sheath flow collection as droplets (e.g., we plan 2 nL droplets, 10−15 droplets/band) onto a solid surface such as a membrane. Electrophoric ketones (same structures as the starting ketones) are released from the spots of electrophore-DNA strands (in one of two ways as described below) and volatilized off the surface as a plume by heating with a pulse of energy from a laser. The gas-phase plume of electrophores then is ionized by electron capture and detected by TOF-MS.

Potentially a multiplicity of 400 or so can be reached for electrophore mass tags. This means that 400 different electrophores would be synthesized, where each one would yield a different, major ion by electron capture mass spectrometry. Once each was attached to a unique DNA primer, 400-multiplexing could be performed for some of the steps in DNA sequencing. This 100-fold boost in multiplexing relative to the use of four dyes could speed up and reduce the cost of some steps in DNA sequencing. In part this will depend on laser desorption EC-TOF-MS providing high sensitivity, since, at a constant total sample concentration, *n*-multiplexing requires each component of the sample to be diluted by *n*.

Glycol Electrophore NHS Esters. The structures of the four glycol electrophore NHS esters that we employed in this project are shown in Figure 2. They were synthesized according to the scheme shown in Figure 3, which summarizes details for one of them. As seen, the synthesis began with the pentafluorobenzylation of 4-hydroxyacetophenone, which gave ketone **1a′**. To avoid complications in the subsequent steps from the known lability of a *p*-fluorine substituent,[26] we replaced it with a methoxy group, forming **1a**.

A noteworthy feature of the synthesis shown in Figure 3 is the novel reagent **5**, which takes **1a** a long way toward its destination (**1d**) in a single step (**1a** → **1b**, a Wittig-Horner

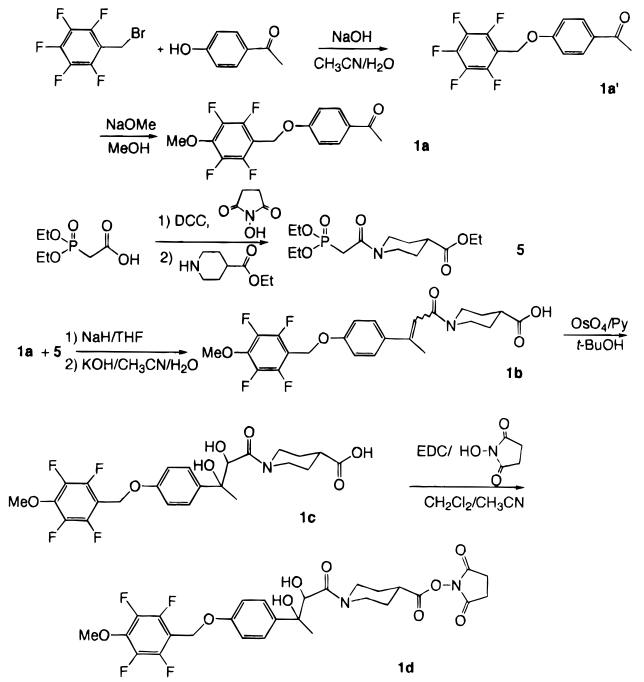

Figure 3. Synthesis of **1d** (**2d**−**4d** were synthesized similarly).

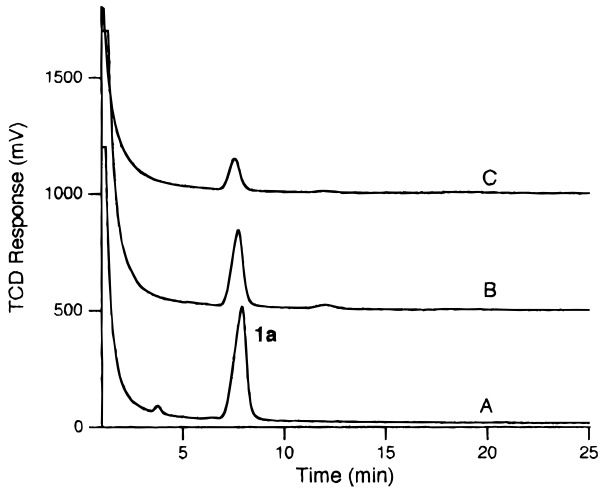

Figure 4. Detection of **1a** or **1e** by packed-column gas chromatography with thermal conductivity detection: (A) Ketone **1a** (0.5 mg injected) with injector temperature at 300 °C; (B) amide **1e** (0.67 mg) with thermal conversion of **1e** to **1a** in the injector; (C) amide **1e** (0.46 mg) with injector at 240 °C; 46% ketone **1a**. Both the column and detector temperatures were 240 °C.

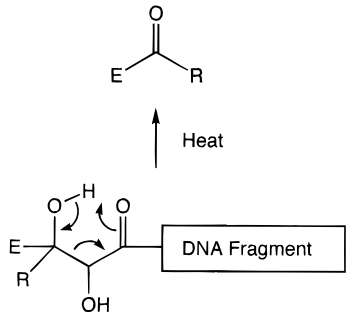

Figure 5. Mechanism for the release of a ketone electrophore from a glycol keto electrophore labeled-DNA fragment by a retrothermal aldol reaction.

reaction). As shown, **5** was synthesized from commercially available diethylphosphonoacetic acid, dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), and ethyl isonipecotate in a one-pot reaction. The crude reaction mixture (which contains 70% **5** and 30% NHS) could be used directly in the next step (the Wittig-Horner reaction), as long as extra NaH was employed to overcome the NHS. The latter was easily removed in the workup. The olefinic acid **1b** was oxidized to glycol **1c** with osmium tetraoxide. Finally **1c** was converted to the corresponding *N*-hydroxysuccinamide ester, **1d**, via a carbodiimide coupling reaction.

Glycol Electrophore Butyl Amide: Ketone Release. One way to release a ketone electrophore from a glycol electrophore is oxidation with aqueous periodate. Potentially, in our scheme of Figure 1, we might introduce periodate in the sheath flow solvent so that the ketone electrophores are released while the collected droplet evaporates. If the surface on which the droplet is deposited is nonpolar (e.g., polyimide membrane), then the released ketone electrophores could bind hydrophobically to the surface, allowing residual DNA and salts to be washed away with water prior to laser volatilization of the electrophores. The feasibility for this was demonstrated by evaporating a methanol solution of **1a′** onto polyimide, washing the spot under flowing water for 5 min, extracting the spot with methanol, and observing by GC/EC-MS a complete recovery of the compound in the methanol extract.

It is known that glycols are cleaved by aqueous periodate over a wide pH range.[30] An alkaline pH makes the most sense for our scheme, since the electrolyte for separating DNA ladders by electrophoresis typically is buffered at alkaline pH. Oxidation of **1e** by aqueous periodate in pH 8.7 Tris (a common buffer for DNA sequencing electrophoresis) was very slow, based on monitoring the disappearance of **1e** by reversed-phase HPLC. This was not totally surprising, since Tris potentially might complex with periodate. Switching to morpholine ($pK_a = 8.5$) as a buffer overcame this problem, and the peak for **1e** was observed to completely disappear faster than we could inject the sample into an HPLC column (15 s). We confirmed by GC/EC-MS that the desired ketone formed quantitatively in this reaction. Thus, periodate oxidation is attractive for releasing our ketone electrophores.

Keto compounds possessing a β-hydroxy group can undergo cleavage via a thermal retro-aldol reaction.[31] Such a reaction is illustrated in Figure 5. We observed this previously for a glycol keto (α,β-dihydroxyketo) electrophore.[27] Injection of **1e** into a GC fitted with a splitless injector set at 300 °C gave a peak for the corresponding ketone (95% yield) as shown in Figure 4. The minor, later-eluting peak apparently is derived from the other part of the molecule. Thus our glycol electrophores also can release a ketone electrophore via heating. Potentially this means that subjecting a glycol keto electrophore DNA sample deposited on a solid surface (see Figure 1) to a pulse of energy from a laser might directly form a plume of ketone electrophores (as we report here later). The concept for this shown in Figure 5.

Glycol Keto Electrophore DNA Oligomers. Four synthetic DNA oligomers possessing a 5′-aminoalkyl moiety were each labeled with a different glycol keto electrophore NHS ester. While

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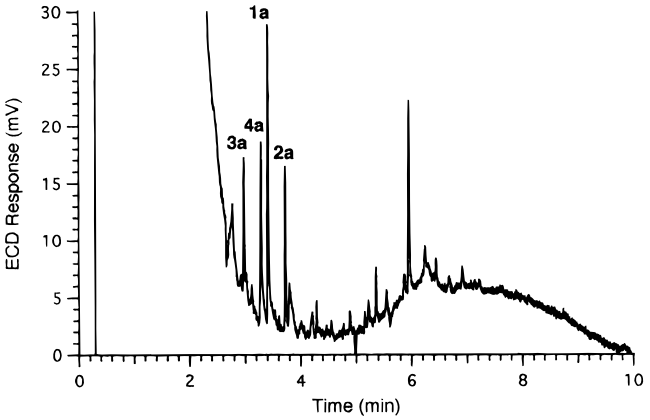

Figure 6. Detection of an aqueous cocktail of four electrophore-labeled DNA oligomers by injection into a GC-ECD fitted with a splitless injector at 300 °C. A 1 μL aliquot of water was injected containing 260, 190, 300, and 300 fmol each of **1d**-NE-105, **2d**-NE-106, **3d**-NE-107, and **4d**-NE-104, respectively. In turn, these yield **1a**, **2a**, **3a**, and **4a**, respectively, as thermally released ketone electrophore products.

we were unable to separate the labeled from unlabeled oligomers by reversed-phase HPLC on C18-silica, this separation was readily accomplished by electrophoresis. For the latter analysis, the oligomer samples were 3′-labeled with ^{32}P to facilitate their detection. The coupling yields for the electrophore labeling were observed by electrophoresis to be in the 80% range. Thermal release of the ketone electrophores from these electrophore-oligomers was demonstrated by injecting an aqueous mixture of them into a GC-ECD fitted with an injector set at 300 °C, an experiment similar to that of Figure 4. This gave the chromatogram shown in Figure 6, displaying peaks for the four ketone electrophores **1a**−**4a**.

Dideoxy Sequencing. We assessed the suitability of the electrophore-labeled DNA primers for DNA sequencing by comparing their performance against the precursor primers containing a 5′-aminoalkyl moiety. Cycle sequencing was done with commercially available reagents and equipment providing fluorescent, four-dye dideoxy sequencing reactions, capillary electrophoresis separation, and laser-induced fluorescence detection. Equivalent and valid sequencing results were obtained with each of the four primer pairs (labeled and unlabeled with the electrophore); representative data are shown for one of the pairs in Figure 7. The unlabeled primer gave the sequencing peaks shown in Figure 7A, while the corresponding electrophore-labeled primer gave the data shown in Figure 7B. Electrophoresing in the same capillary a mixture of the same sequencing reactions used in (A) and (B) allows one to see in (C) that the electrophore-labeled dideoxy-terminated products (upper case in Figure 7A−C) electrophorese almost exactly two nucleotides more slowly than the corresponding dideoxy extensions from the nonlabeled amino-link primer (lower case). In a separate but related experiment, the electrophore-labeled primers were enzymatically 3′-end-labeled using terminal deoxynucleotidyl transferase with α-[^{32}P]cordycepin triphosphate followed by electrophoresis in 20% polyacrylamide slab gel with 7 M urea. The electrophore-labeled primers, made visible after transfer to nylon by autoradiography, were observed to migrate more slowly than the corresponding 5′-amino-link precursors by ~10%, and corresponding dideoxy DNA sequencing bands were confirmed in a similar experiment to retain releasable electrophore by periodate oxidation GC/EC-MS (data not shown).

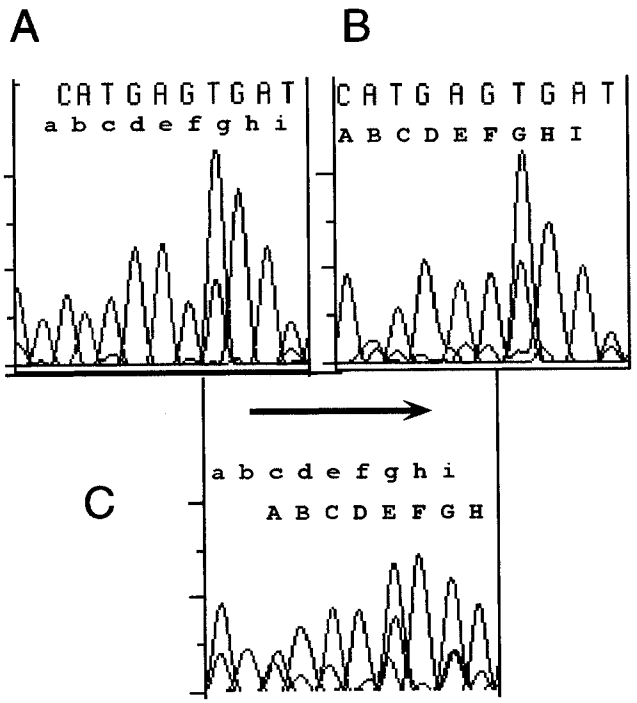

Figure 7. Sequencing data (products 32−43 bp in size) by capillary electrophoresis on an ABI Model 310 from fluorescent, four-dye dideoxy terminator cycle sequencing reactions. The primer is NE-105 in (A) and **1d**-NE-105 in (B). For (C), equal amounts of reactions A and B were combined before electrophoresis. Upper case lettering is used throughout for the data from the labeled primer, and lower case for the unlabeled primer. The arrow indicates the direction of increasing time and molecular weight along the electrophoretic (*X*) axis. The *Y* axis is in arbitrary fluorescence units of the ABI 310. Typical overall run time, 2 h.

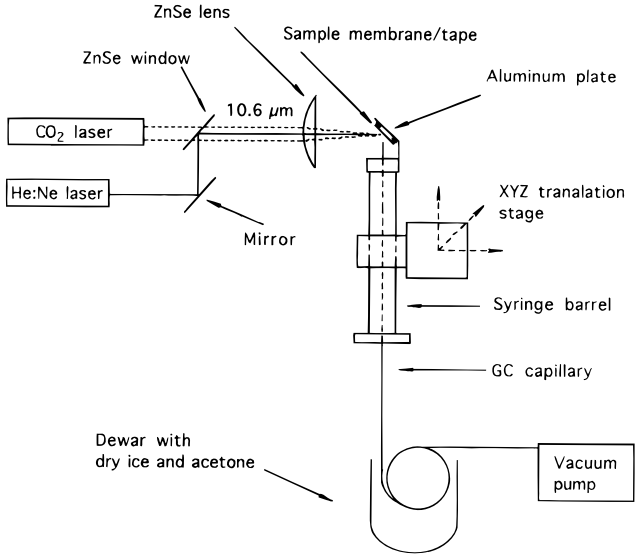

Figure 8. Apparatus for laser desorption/capillary collection of electrophore samples.

Laser Desorption: Electrophore-DNA. We proceeded to test the feasibility of the laser desorption step in our proposed overall method of Figure 1. This was done by employing the apparatus shown in Figure 8 and following the scheme shown at the top of Figure 9. An aqueous droplet containing periodate and 100 fmol each of the four electrophore DNA oligomers was placed on a polyimide membrane and allowed to evaporate. In order to give the periodate reaction adequate time, the evaporation was

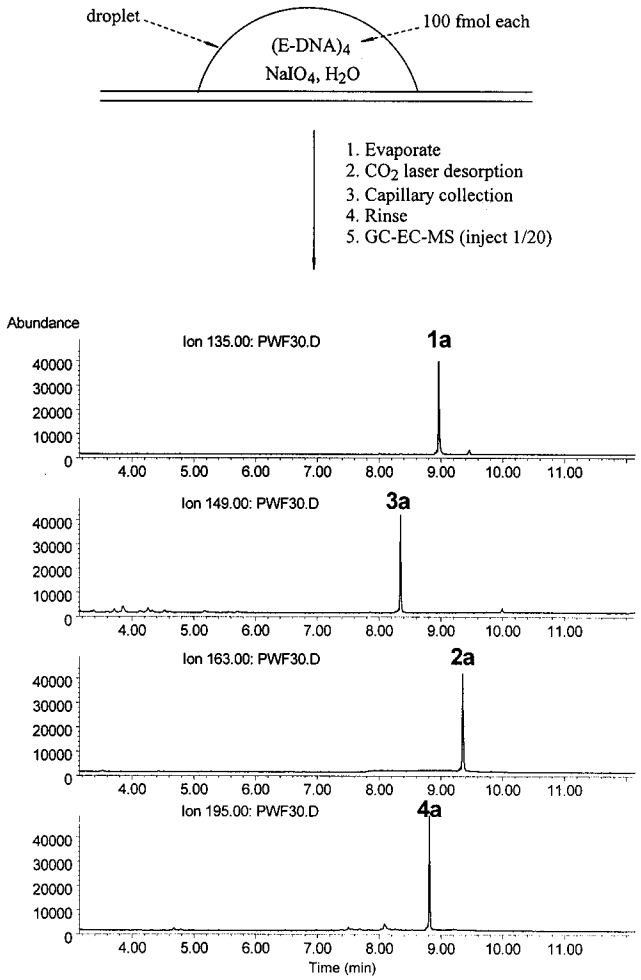

Figure 9. Scheme and detection of glycol keto electrophore-labeled DNA oligomers as a four-multiplex cocktail in water deposited onto a polyimide membrane.

conducted in a humidity chamber. (We could not assume a reaction half-life of 15 s as in our prior experiments, since the reaction conditions were different.) The dried spot was heated by manually rastering the beam from a CO_2 laser back and forth across it, making an effort to hit each part of the spot only once. Visually, this created a plume of "smoke", which appeared the same when blank polyimide was irradiated. At least some of the plume was collected by vacuum into a cooled capillary located near the spot. The capillary was rinsed with ethyl acetate, and injection of the latter solution into a GC/EC-MS set gave the single-ion mass chromatograms shown at the bottom of Figure 9. As seen, all four electrophore DNA oligomers were detected cleanly (released ketone electrophore signals). No peaks were observed when a corresponding sample of unlabeled DNA oligomers was tested in the same way. Apparently other desorbed products in the collected plume (of visual smoke) escaped detection since they lacked solubility in ethyl acetate, volatility in the GC column, capacity to capture electrons efficiently, or ability to produce ions at the m/z values monitored.

Ultimately the desorbed plume of ketone electrophores is intended to more directly undergo electron capture followed by TOF-MS. In this case co-desorbed products besides the ketone electrophores would be more likely to interfere. There are three ways in which potential interferences arising in this step might be overcome. In the first, the power density of the laser could be minimized. Calculations[32] indicate that the surface temperature

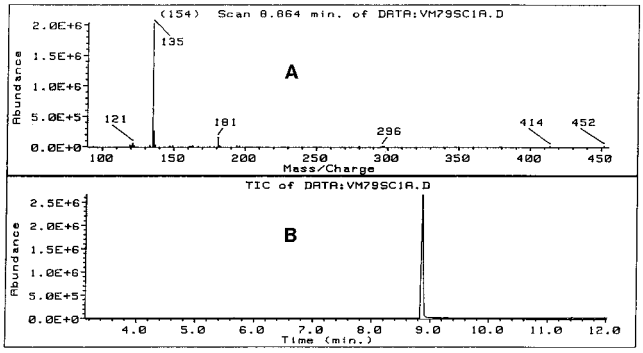

Figure 10. Mass spectrum (A) and corresponding mass chromatogram (B) for **1a′** utilizing GC/EC-MS; detection in (B) was by total ion current (TIC).

of the polyimide in the laser impact zones reached ~2340 K in the experiment in Figure 9, which means that the smoke basically must have contained pyrolysis products from the polyimide. We already know (as about to be discussed) that the electrophores can be desorbed off a membrane of this type at a much lower temperature. Second, the electrophore-DNA spot on the "front side" of the polyimide could be heated by applying CO_2 laser radiation instead from the back side (polyimide is only partly opaque to 10.6 μm photons), so any smoke would not enter the electron capture zone. In fact, when the above experiment was repeated in this way, clean chromatograms also were obtained (data not shown). Third, a more inert surface might be employed instead of polyimide. Direct detection by electron capture mass spectrometry of 270 amol of an electrophore deposited on polyimide and then released thermally has been demonstrated.[33]

Restricted Ion Electrophores. In order for a large number (e.g., 400) of different electrophores to be detected as a cocktail by mass spectrometry, the number of ions from each must be limited, including "fragmentation noise" (minor ions even down to a relative abundance of 0.025%). We began to test the potential difficulty of general electrophore fragmentation noise by injecting HPLC-purified ketone **1a′** (M_r = 316) into a GC/EC-MS under scanning conditions, giving the mass spectrum shown in Figure 10A. This spectrum belongs to the peak in the GC total ion current chromatogram shown in Figure 10B. The base peak in Figure 10A, as anticipated, arises from dissociative electron capture to yield an acetophenolate anion (135 u). The peak at 181 u (7.6%) arises from alternative cleavage of the same bond, yielding a pentafluorobenzyl anion. If we ultimately build up multiplicity based on pentafluorobenzyl species like **1a′**, 181 u will certainly be a signal mass that we must avoid, since every electrophore would thereby deposit significant secondary signal at this position.

The mass at 121 u (2.7%) seems to arise from a CH_2 loss from 135 u, since all of our hydroxyacetophenone-based electrophores give a corresponding ion (base peak − 14). Nevertheless, this generic methylene-loss ion will be at a unique position for each signal electrophore of this type and, therefore, will not be a problem since it will not accumulate at a given mass signal position. Thus, its contribution can be corrected for as needed (along with the isotopic peak at 136 u, and other analyte-specific, small secondary masses) during data handling. This is true as

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well for the ion at 296 u (loss of HF from the molecular ion). The other masses (414 and 452) highlighted by the computer in Figure 10A are from background substances in the GC/EC-MS; their presence is independent of analyte in the system. The unlabeled peaks apparent in Figure 10A (between 121 and 200 u), have intensities ranging from 0.09 to 0.15%. The two most intense ones (148 and 175 u) were studied further and both were found to be background peaks in the system. The remaining of these latter peaks were not tested further; instead we conducted the experiments described next.

Unfortunately, this experiment did not truly define whether significant, general "ion grass" might be present at or above the 0.025% level, since, under scanning conditions, abundances at this level could not quite be recorded in our system. Thus, we shifted to selected ion monitoring of 10 ions with the injection of 454 pg of **1a′**, which allowed quantitative measurement of abundances at least down to the 0.025% level for the ions selected. This was done four times, and each time 135 u was monitored (to define 100% abundance), along with some new masses. In one case, we repeated some masses that we had evaluated before under scanning conditions and obtained similar abundances. Arbitrary masses (e.g., 205, 215, 225...370, 380, 390) were selected. Aside from the ions already discussed above, since they were apparent in Figure 10A, 31 additional ions were monitored, and of these 52% had 0% (no measurable, i.e., <0.01% in this experiment) abundance, 35% had an abundance less than 0.1%, and 13% had an abundance less than 0.15%. From these data we anticipate that electrophore fragmentation noise will not be an important problem for our method. More definitive and realistic testing of such noise, and other sources of noise in our method, will be undertaken once an EC-TOF-MS instrument becomes available.

CONCLUSION

Some feasibility has been demonstrated here for the use of glycol keto release electrophore mass tags in DNA sequencing. Model tags have been synthesized conveniently in high yield. DNA oligomers have been labeled with these tags and employed successfully in a dideoxy sequencing procedure involving capillary electrophoresis and fluorescence detection with an ABI Model 310 instrument. A four-multiplexed electrophore-DNA spot on a polyimide membrane was detected by CO_2 laser desorption release of the electrophores followed by collection into a capillary and injection into a GC/EC-MS.

High-speed, low-cost DNA sequencing, the long-term goal of this technology, is needed not only to elucidate unknown genomes but also to provide molecular diagnostics testing, e.g., for mutations in known sequences causing genetic diseases, and for the presence of infectious disease agents. While hybridization assays are important in molecular diagnostics, DNA sequencing is regarded as the gold standard.

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