Metal Carbonyl Labels for Oligonucleotide Analysis by Fourier Transform Infrared Spectroscopy

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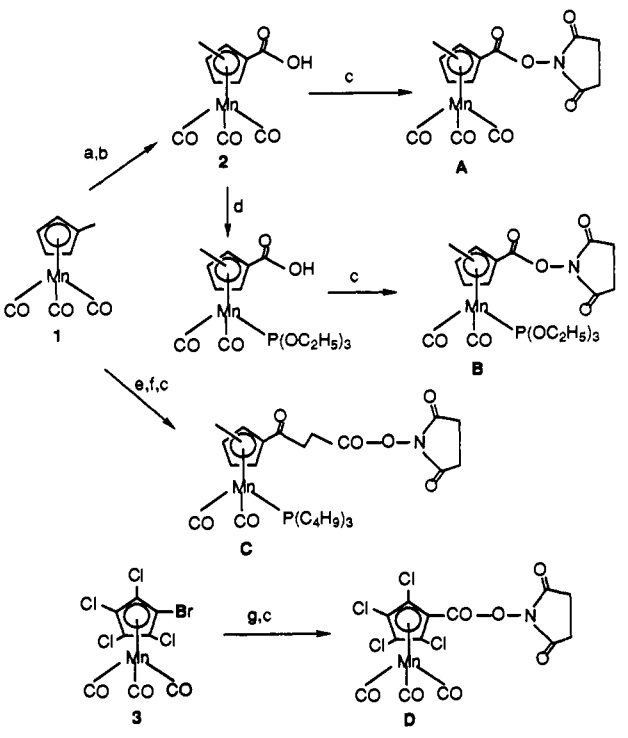
In many areas of DNA research a reagent is required to label the subject oligonucleotide. The most widely used labels presently are radioactive isotopes such as ^{32}P and ^{35}S,[1] but because of problems of licensing, safety hazards, and cost, there is a continuing need for new, sensitive, and nonradioactive labels. This has led to the development of fluorescent markers which have been employed successfully in automated DNA sequencers.[2] However, the utility of these methods is limited by overlapping absorptions and electrophoretic mobility changes associated with the labeled fragments. Recently, a novel labeling/detection tool, metal carbonyl Fourier transform infrared assay, has been reported for the study of biological systems and interactions.[3] This technique takes advantage of the intense, narrow (ca. 10 cm^{-1}), and unique IR absorption bands associated with transition metal carbonyl complexes (M–CO) which fall in a spectral window (1900–2200 cm^{-1}) where virtually all organic and biological molecules are transparent. In metal carbonyl FTIR analysis the substrate of interest is covalently modified with an appropriate metal carbonyl unit; detection of the labeled substrate or its interaction with larger biomolecules can be accomplished at picogram levels.[4] Important applications have included the study of steroid receptor binding (important in early breast cancer detection[5]), the detection of mycotoxins,[6] and use in immunoassay.[7]

The promise of the metal carbonyl FTIR tool has prompted us to seek metal carbonyl labeling agents appropriate for use in oligonucleotide analysis such as DNA hybridization, PCR product analysis, and potentially, automated DNA sequencing. We describe herein the preparation of four water stable cymantrene [(cyclopentadienyl)$Mn(CO)_2L$] based labeling agents which can be coupled to amino-linked DNA primers; the labeled primers are stable to polyacrylamide gel electrophoresis and DNA sequencing reaction conditions and are detectable by FTIR.

Although transition metal carbonyl complexes are notoriously unstable in air and water, trial experiments found the complex $(CO)_3Mn(C_5H_4COOH)$ (**2**) and several other (CpCOOH)Mn-$(CO)_2L$ derivatives to be stable in aqueous media for days and during polyacrylamide gel electrophoresis. The sensitivity of the positions of M–CO IR absorptions to the metal's electronic environment was exploited in the design of a set of four active ester labeling agents A–D with nonoverlapping M–CO absorptions (Scheme I). Succinimidyl ester A, displaying intense M–CO absorptions at 2019 and 1935 cm^{-1} (Table I, entries a), was prepared (as an isomeric mixture) by reaction of readily available acid **2** with disuccinimidyl carbonate.[8] Electron rich esters B and C (also as isomeric mixtures), having lower energy CO IR absorptions, were derived from acid **2** (for B) or from inexpensive **1** (for C) via photochemical ligand substitution and esterification. The fourth labeling agent, D, having the highest frequency CO absorptions, was produced from the electron deficient tetrachloro Cp derivative **3**.[9]

Labeling agents A–D were attached to an amino-linked M13 universal primer (eq 1) in aqueous DMSO or formamide (pH = 9, 20 °C, 12 h). After neutralization, ether extraction to remove unreacted and hydrolyzed labeling agent, and lyophilization, the presence of the metal carbonyl unit on the primer was established by FTIR analysis of the residue on a CaF_2 window using an

Scheme I[a]



[a] (a) $CH_3COCl/AlCl_3$; (b) I_2/KOH; (c) disuccinimidyl carbonate, pyr; (d) $h\nu$/THF; $P(OC_2H_5)_3$; (e) succinic anhydride/$AlCl_3$; (f) $h\nu$/THF; $P(C_4H_9)_3$; (g) C_4H_9Li; CO_2; H_3O^+.

Table I. M–CO IR Absorptions (cm^{-1}) for Esters A–D and Labeled Oligonucleotides

	A	B	C	D
a[a]	2035(10)[b]	1960(18)	1928(17)	2054(9)
	1955(31)	1895(28)	1866(25)	1988(27)
b	2019(17)	1943(20)	1922(15)	2039(10)
	1935(31)	1871(25)	1861(22)	1967(20)
c	2022(10)	1947(20)	1925(15)	2042(10)
	1937(29)	1883(25)	1864(23)	1969(22)

[a] (a) A–D in chloroform solution; (b) thin film after reaction of A–D with amino primer and trituration with ether; (c) step b followed by gel electrophoresis and gel filtration. [b] Note: Figures in parentheses are peak widths measured at half peak height.

(1) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, 1989.

(2) (a) Prober, J. M.; Trainor, G. L.; Dam, R. J.; Hobbs, F. W.; Robertson, C. W.; Zagursky, R. L.; Cocuzza, A. J.; Jensen, M. A.; Baumeister, K. *Science* **1987**, *238*, 336. (b) Smith, L. M.; Sanders, J. Z.; Kaiser, R. J.; Hughes, P.; Dodd, C.; Connell, C. R.; Heiner, C.; Kent, S. B. H.; Hood, L. E. *Nature* **1986**, *321*, 674.

(3) (a) Jaouen, G.; Vessieres, A.; Top, S.; Ismail, A. A.; Butler, I. S. *C. R. Acad. Sci., Ser. 2* **1984**, *298*, 683. (b) Jaouen, G.; Vessieres, A.; Top, S.; Ismail, A. A.; Butler, I. S. *J. Am. Chem. Soc.* **1985**, *107*, 4778.

(4) (a) Le Borgne, F.; Beaucourt, J. P. *Tetrahedron Lett.* **1988**, *29*, 5649. (b) Salmain, M.; Vessieres, A.; Jaouen, G. *Anal. Chem.* **1991**, *63*, 2323. (c) Butler, I. S.; Vessieres, A.; Salmain, M.; Brossier, P.; Jaouen, G. *Appl. Spectrosc.* **1989**, *43*, 1497.

(5) (a) Jaouen, G.; Vessieres, A. *Pure Appl. Chem.* **1986**, *58*, 597. (b) Vessieres, A.; Top, S.; Vaillant, C.; Osella, D.; Mornon, J.-P.; Jaouen, G. *Angew. Chem., Int. Ed. Engl.* **1992**, *31*, 753.

(6) Gruselle, M.; Rossignol, J. L.; Vessieres, A.; Jaouen, G. *J. Organomet. Chem.* **1987**, *328*, C12.

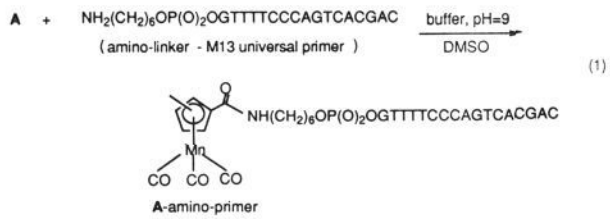
(7) (a) Ismail, A. A.; Jaouen, G.; Cheret, P.; Brossier, P. *Clin. Biochem.* **1989**, *22*, 287. (b) Salmain, M.; Vessieres, A.; Brossier, P.; Butler, I. S.; Jaouen, G. *J. Immunol. Methods* **1992**, *148*, 65.

(8) A–D were completely characterized spectroscopically and by elemental analysis. Preparative and characterizational data are available as supplementary materials.

(9) Reimer, K. J.; Shaver, A. *Inorg. Chem.* **1975**, *14*, 2707.

0002-7863/93/1515-4399$04.00/0

infrared microscope (Table I, entries b). Upon electrophoresis on a 20% polyacrylamide gel containing 7 M urea, the crude product was resolved into two bands (labeled and unlabeled primers) and visualized by UV. The entire labeled primer band was further purified by excision from the gel and extraction with OGEB buffer (500 mM NH_4Ac, 10 mM $MgAc_2$, 1 mM EDTA, 37 °C/12–16 h) and was desalted by being passed through a G-25 column (100 mM NH_4Ac eluant), and the oligonucleotide-containing fractions (monitored at 260 nm) were pooled, lyophilized, and their IR spectra obtained (Table I, entries c). The essentially unchanged IR absorptions of the metal carbonyl fragments following this process indicate that the labels are stable and remain attached to the amino-linked primer throughout the above purification procedure.



To assess the ability of the metal carbonyl labeled primers to serve as substrates in the dideoxy DNA sequencing reactions, they were tested using *Bacillus stearothermophilus* (*Bst*) DNA polymerase (Bio-Rad) under standard conditions as described previously.[10] The resulting DNA sequencing autoradiogram obtained after gel electrophoresis is shown in Figure 1. It can be seen that both the M13 universal unlabeled and labeled primers serve as substrates for these reactions, but with somewhat different results. The faint doublets observed with primer B and smearing with primer C may result from the isomeric labeling agents employed while primers A and D (from singly isomeric agent D) show none of these gel artifacts. In all instances the metal carbonyl-labeled primer bands lag behind the M13 primer-derived bands, as expected for molecules which are longer by virtue of the attached label.

The detection limit of the metal carbonyl FTIR technique with the presently available instrumentation (Bio-Rad FTS-40 with the UMA 300A IR microscope) was determined in both static and capillary electrophoresis configurations. In the former, 1 μL of 1.0×10^{-5} M A-labeled primer was evaporated onto a CaF_2 window forming a ring 1.44 mm in diameter (ca. 5 μm wide). After scanning a 12.5-μm-long section of the ring with the IR microscope (64 signal-averaged scans, S/N = 3:1), the primer could be clearly detected; it is estimated that ca. 30 fmol of A-labeled primer was present in the 62.5-μm^2 IR beam.[11] For capillary electrophoresis experiments, the IR beam was focused onto a fused silica capillary (50-μm i.d., 100-μm o.d.) with the aperture at 50 μm^2, where a window had been made by burning off the coatings. Various concentrations of A-labeled primer were then electrokinetically injected into the capillary,[12] and IR

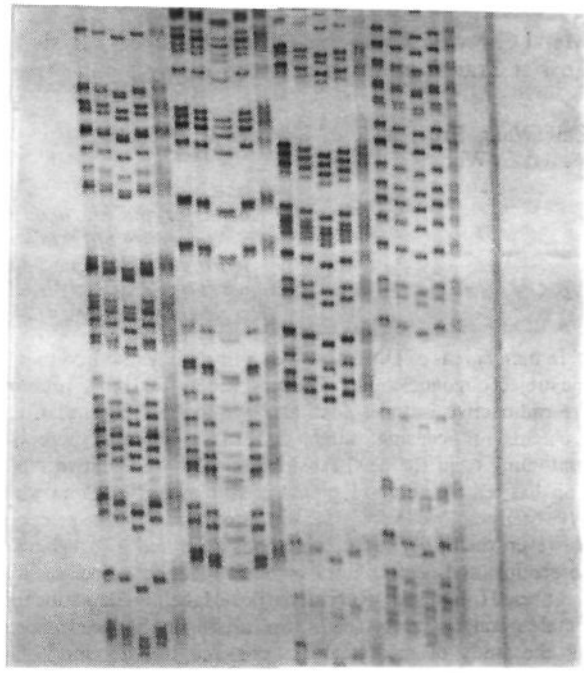


Figure 1. A 5% polyacrylamide, 9 M urea dideoxy DNA sequencing gel comparing the A–D labeled M13 universal primers with the M13 universal primer (GTTTTCCCAGTCACGAC) using *Bst* DNA polymerase.[10] A, C, G and T are the specific individual base sequencing reactions. Lane 1 was derived from B-labeled primer; lane 2, A-labeled primer; lane 3, unlabeled M13 primer; lane 4, D-labeled primer; and lane 5, C-labeled primer. The DNA template was single-stranded M13mp18 isolated as previously described.[10] The radioisotope used was [^{35}S]dATP.

data were collected as the samples migrated past the detector window. In this configuration, the detection limit was shown to be between 10^{-3} and 10^{-4} M (100 fmol/volume illuminated[13]). These results indicate that, although further improvements in detector and instrument design are needed to render the MC-FTIR technique practical for real time DNA sequencing, applications in diagnostic DNA PCR product analysis and DNA hybridization assay are feasible.

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Supplementary Material Available: Details of the preparation and characterization of A–D (6 pages). Ordering information is given on any current masthead page.

(10) Chissoe, S. L.; Wang, Y.; Clifton, S. W.; Ma, N.; Sun, H.; Lobsinger, J. S.; Kenton, S. M.; White, J. D.; Roe, B. A. *Methods: A Companion to Methods in Enzymology*; 1991; Vol. 3, p 55.

(11) Assuming that the primer was uniformly distributed along the ring, this gave the amount of primer detected as $(1.0 \times 10^{-6})(1.0 \times 10^{-5})(12.5)/(3.14)(1.44 \times 10^{3}) = 30$ fmol.

(12) CE conditions for electroendoosmosis: 1 × TBE buffer (16.2 g of Tris base, 2.75 g of boric acid, and 0.93 g of EDTA per liter); Bio-Rad HPE 100 capillary electrophoresis power supply; 50-cm untreated capillary; injection at (+) electrode, samples migrated from (+) to (–) electrode; 200 V/cm; 12–24 μA.

(13) The volume of the sample in the capillary scanned by the IR beam was $(3.14)(50\ \mu m^2)(50\ \mu m) = 1 \times 10^{-10}$ liter.