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CINNAMOYLATION OF THE SUGAR HYDROXYLS OF 3-METHYLTHYMIDINE

FORMATION OF A RELATIVELY STABLE ESTER DERIVATIVE

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SUMMARY

A trace amount (51 ng) of 3-methylthymidine is derivatized with cinnamic anhydride in the presence of the acylation catalyst 4-dimethylaminopyridine. The yield of the diacylated product, 3′-5′-bis-(O-cinnamoyl)-3-methylthymidine, is 102 ± 3.6%. The product is stable at pH 4–8 in aqueous acetonitrile for 16 days and can be determined at the low pg level by gas chromatography with electron-capture detection. These results encourage the further development of α,β-unsaturated acylating reagents for use in this and related chromatographic analyses.

INTRODUCTION

Chemical derivatization of functional groups on analytes is widely employed in chromatographic and related types of analytical methodology[1,2]. The most common reasons for this are to enhance the chromatographic properties and detectability of the analyte. Ideally the derivatization is mild, rapid, quantitative, and yields a stable product.

While many analytes possess one or more hydroxyl functional groups that potentially can be derivatized, the reagents and conditions available for this purpose for some classes of compounds are limited. For example, silyl ether derivatives of nucleosides are easy to form but are susceptible to mild aqueous hydrolysis[3]. Derivatization with some sterically hindered trialkylsilyl reagents may reduce, but not eliminate, this problem[4]. O-Methyl derivatives are stable, but conditions required for permethylation tend to be severe[5–7]. Acetyl derivatives have been reported to be stable to water, but no detailed analytical measurements were given[8]. The trifluoroacetyl group has been used to derivatize the deoxyribose hydroxyls[9], but trifluoroacetyl esters generally have limited stability under aqueous conditions[2].

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Previously, we observed that an α,β-unsaturated ester was difficult to saponify[10]. This suggested that an α,β-unsaturated acylating reagent might form a stable derivative of hydroxyl compounds for use in chemical analyses such as gas chromatography (GC). Towards this goal, we report here the cinnamoylation of 3-methylthymidine as a model DNA adduct and the evaluation of both the aqueous stability and GC characteristics of the diacylated product.

EXPERIMENTAL

Reagents

Thymidine (Sigma grade) and TRIZMA (reagent grade) were from Sigma (St. Louis, MO, U.S.A.); cinnamoyl chloride (98%), methyl iodide (99%), HMDS (1,1,1,3,3,3-hexamethyldisilazane, 98%), and $Na_2HPO_4 \cdot 7H_2O$ were from Aldrich (Milwaukee, WI, U.S.A.); 4-dimethylaminopyridine was from Reilly Tar & Chemical (Indianapolis, IN, U.S.A.) and was recrystallized from toluene and toluene–hexane; K_2CO_3, Na_2HPO_4, and Na_2CO_3 were from Fisher Scientific (Fair Lawn, NJ); water (HPLC), KH_2PO_4, $NaHCO_3$, sodium hydroxide, acetic acid (HPLC), $CH_3COONa \cdot 3H_2O$ (HPLC), and phosphoric acid were from J. T. Baker (Phillipsburg, NJ, U.S.A.). All inorganic reagents were ACS grade except where noted, and organic solvents were of the highest purity available, suitable for GC–electron-capture detection (ECD) and/or high-performance liquid chromatography (HPLC) (Burdick & Jackson, Muskegon, MI, U.S.A.; Baker Resi-Analyzed, J. T. Baker).

Buffer solutions were prepared as follows: for pH 7 phosphate buffer, 9.1 g of KH_2PO_4 and 18.9 g of Na_2HPO_4 were dissolved in 1 l of water; pH 10 carbonate buffer had 6.5 g of $NaHCO_3$ and 13.2 g of Na_2CO_3 per l of water; pH 6 phosphate buffer had 23.2 g of KH_2PO_4 and 8.1 g of $Na_2HPO_4 \cdot 7H_2O$ per l; pH 4 acetate–phosphate buffer had 0.041 g of $CH_3COONa \cdot 3H_2O$ and 0.570 g of acetic acid in 100 ml of water with pH adjustment with phosphoric acid; and pH 8 Tris buffer contained 0.755 g of TRIZMA dissolved in 100 ml of water with pH adjustment with 6 *N* sodium hydroxide. As required, the buffers were extracted with hexane to minimize organic interferences detectable by GC–ECD.

Although there are synthetic methods for preparing cinnamic anhydride[11,12], the compound was isolated from cinnamoyl chloride, as follows. A saturated solution of cinnamoyl chloride in ethyl acetate was treated with charcoal until the supernatant was nearly colorless. The supernatant was concentrated by rotary evaporation, and the concentrated liquid was evacuated under high vacuum for 16 h. The resulting crystals were collected, rinsed with cold ethyl acetate, and evacuated under high vacuum for 16 h. The anhydride (m.p. 133–134°C) was pure by silica gel thin-layer chromatography (TLC) using ethyl acetate–hexane (50:50) as mobile phase, and the identity was supported by NMR and IR. Prior to use, small quantities were dissolved in toluene and extracted with pH 10 buffer. The toluene was dried with granular sodium sulfate (previously heated at 280°C for 16 h), filtered, and rotary evaporated. The resulting white crystals were evacuated under high vacuum for 16 h.

TLC with silica gel GF was performed with Uniplates (Analtech, Newark, DE) containing a fluorescent indicator: 250-μm plates were used for monitoring reactions, and 500-μm plates were used for preparative TLC.

Flash column chromatography was performed with silica gel (60–200 mesh, J. T. Baker).

Equipment

HPLC was performed with a Series 4 liquid chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.). It was equipped with a Model 7125 injector and a 50-μl sample loop (Rheodyne, Cotati, CA, U.S.A.); a pre-column (Upchurch Scientific, Oak Harbor, WA, U.S.A.) packed with Vydac Reverse-Phase, C_{18} (Separations Group, Hesperia, CA, U.S.A.); and a LC-8 reversed-phase analytical column, 15 × 0.64 cm O.D., 5 μm particle size (Supelco, Bellefonte, PA, U.S.A.). Detection was at 254 nm with a Spectroflow 773 absorbance detector (Kratos, Ramsey, NJ, U.S.A.). Liquid chromatograms were recorded with a SP4270 integrator (Spectra-Physics, San Jose, CA, U.S.A.).

GC–ECD was performed with a Vista 6000 gas chromatograph equipped with a ^{63}Ni constant-current, variable frequency electron-capture detector; a Model 11095 non-vaporizing on-column capillary injector; and mass-flow controlled pneumatics (Varian, Sunnyvale, CA, U.S.A.). Gas chromatograms were recorded with a SP4270 integrator (Spectra-Physics). On-column injections were made with a 5-μl syringe equipped with a stainless-steel needle (Scientific Glass Engineering, Austin, TX, U.S.A.). The analytical column was a 15-m, 0.17 μm film thickness, 0.31-mm I.D. ULTRA crosslinked 5% phenylmethyl silicone fused-silica capillary column (Hewlett-Packard, Palo Alto, CA, U.S.A.). Helium carrier and nitrogen make-up gases were UHP (Granite State Oxygen, Nashua, NH, U.S.A.) and equipped with Oxiclear gas purifiers (Labclear, Oakland, CA, U.S.A.).

Trace-level reactions were performed in Reacti-Vials using a block-module heater (Pierce Chemical, Rockford, IL, U.S.A.). Evaporative concentration with a gentle stream of nitrogen was performed with high purity grade nitrogen (99%, Med-Tech, Medford, MA, U.S.A.) which had been passed through molecular sieve and activated charcoal traps (American Scientific Products, McGaw Park, IL, U.S.A.). PTFE liners for the vial caps were obtained from Arthur H. Thomas (Philadelphia, PA, U.S.A.). Vapor-phase silanization was performed in a vacuum oven (VWR Scientific, San Francisco, CA, U.S.A.).

Glassware

For analyses by HPLC, glassware was cleaned by soaking it in a hot (70°C) solution of 10% sulfuric acid in water for approximately 16 h. It was rinsed with tap water and soaked in a hot (70°C) solution of 2% Micro (a cleaning solution) (International Products, Trenton, NJ, U.S.A.) in water for approximately 4 h. After rinsing with tap water and draining, the glassware was heated at 280°C for approximately 16 h.

For analyses by GC–ECD, the glassware was similarly cleaned and then vapor-phase silanized. Silanization was performed by heating the glassware at 200°C under high vacuum in a vacuum oven for approximately 2 h. The vacuum was turned off, 1–2 ml of HMDS were added through a valve, and the glassware was silanized for 2 h. The vacuum was again applied, and the glassware was heated for an additional 2 h. After removing it from the vacuum oven, the glassware was heated at 280°C for approximately 16 h. A series of pieces were then serially rinsed with hexane, and the hexane was analyzed by GC–ECD to confirm that the glassware was clean.

The PTFE liners for the vial caps were boiled for approximately 10 min each in methanol, acetone, hexane, and pentane. They were then heated at 280°C for 16 h.

Synthesis

3-Methylthymidine. To a 10-ml vial was added 0.28 g (1.2 mmol) of thymidine, 0.94 g (6.8 mmol) of potassium carbonate, and 600 μl (9.7 mmol) of methyl iodide. Approximately 9 ml of acetone were added, the vial was capped, and the reaction mixture was stirred at room temperature for 26 h. The reaction mixture was filtered, the filtrate was concentrated, and the product was isolated by silica gel preparative TLC using ethyl acetate as mobile phase. The product was further purified by recrystallization from acetone–hexane and ethyl acetate–hexane (12% isolated yield). The identity of the product as 3-methylthymidine (m.p. 128.5–129.5°C) was supported by NMR and IR analysis. The product was shown to be pure by silica gel TLC using ethyl acetate as mobile phase and reversed-phase HPLC as described below.

3',5'-Bis-(O-cinnamoyl)-thymidine. To a 100-ml round bottom flask was added 0.66 g (2.7 mmol) of thymidine, 1.4 g (8.5 mmol) of cinnamoyl chloride, and 2.1 g (17 mmol) of 4-dimethylaminopyridine. Toluene (50 ml) was added; the reaction was stirred at 50°C for 1 day; 50 ml more toluene were added, and the reaction mixture was stirred at 50°C for 5 more days (84% yield by HPLC as described below). The reaction mixture was quantitatively transferred with ethyl acetate to a 250-ml round bottom flask, and the solvent was evaporated with a rotary evaporator. The product was purified by precipitating it with water from hot acetonitrile, then from hot acetone. The product was redissolved in hot acetone, filtered, and then precipitated by adding hexane. Residual solvents were removed from the collected precipitate by evacuation under high vacuum for approximately 16 h. The product (56% yield) gave a single spot by silica gel TLC developed with ethyl acetate and had a m.p. of 181–181.5°C. Its structure was supported by NMR, IR, and mass spectral analysis.

3',5'-Bis(O-cinnamoyl)-3-methylthymidine. The above product, 0.57 g (1.1 mmol), was dissolved in 20 ml of hot acetone in a 100-ml round bottom flask. To this was added 1.6 g (12 mmol) of potassium carbonate (previously heated at 280°C for 16 h) and 2 ml (32 mmol) of methyl iodide. The reaction mixture was stirred at 30°C for 24 h in the dark (100% yield by HPLC as described below.) The volatile reagents were removed by rotary evaporation, the sample was suspended again in acetone and filtered, and the filtrate was concentrated by rotary evaporation. Toluene was added, and the toluene-soluble residue was transferred to another flask and concentrated by rotary evaporation. The residue was dissolved in 16 ml of ethyl acetate–hexane (50:50), and the product was isolated by flash column chromatography with hexane–ethyl acetate (60:40) as mobile phase. The product (white foamy crystals, 81% yield) was pure based on silica gel TLC developed with ethyl acetate–hexane (50:50) as mobile phase and by reversed-phase HPLC as described below. Its structure was supported by NMR, IR, and mass spectral analysis.

Stability of 3',5'-bis-(O-cinnamoyl)-3-methylthymidine to hydrolysis

A volume of 10 μl of a 430 ng (830 pmol)/μl solution of 3',5'-bis-(O-cinnamoyl)-3-methylthymidine in acetonitrile were added to seven solutions containing 100 μl of acetonitrile and 150 μl of either 0.01 *N* sodium hydroxide, pH 10 carbonate buffer, pH 8 Tris buffer, pH 7 phosphate buffer, pH 6 phosphate buffer, pH 4 acetate buffer, or HPLC water in 0.3-ml clear Reacti-Vials. (All buffers were pre-extracted with hexane.) The vials were capped, and the solutions were vortexed. After analyzing

10 μl of the solutions by HPLC, as described below, the vials were sealed with PTFE tape and stored in the dark at room temperature. Periodically, 10 μl of each were analyzed by HPLC, as described below.

Derivatization (trace-level) of 3-methylthymidine

Trace-level reactions (ng amounts of analyte) were performed by adding 10 μl of a 20 pmol/μl solution of 3-methylthymidine in acetonitrile to replicate 1-ml clear Reacti-Vials. The acetonitrile was gently evaporated under a stream of nitrogen. Either 10 or 20 μl of various concentrations of 4-dimethylaminopyridine (DMAP) in toluene and cinnamic anhydride or cinnamoyl chloride in toluene were added. The solutions were vortexed, and the vials were capped, sealed with PTFE tape, and heated at 50°C for either 2 or 4 h. To remove excess acylating reagent, 5 μl of methanol were added, and the vials were heated at 50°C for an additional 0.5 h. Hexane (400 μl) and pH 7 buffer (100 μl) were added, and the solutions were vortexed 10 times. The aqueous layer was removed with a 250-μl syringe and discarded. The organic layer was gently concentrated under a stream of nitrogen, and 50 μl of acetonitrile were added. The solutions were vortexed, and 25 μl (the sample loop was linear from 1-50 μl injected) were analyzed by HPLC as described below.

To determine procedural recovery, 10 μl of a 65 pmol/μl solution of $O^{3'}$,$O^{5'}$-dicinnamoyl-3-methylthymidine in acetonitrile were added to replicate 1-ml clear Reacti-Vials, and the procedure was continued as above.

As an analytical blank, 10 μl of acetonitrile were added to one clear Reacti-Vial, and the procedure was continued as above.

High-performance liquid chromatographic analyses

To check the purity of 3-methylthymidine, the following program was used: (1) the column was equilibrated for 7 min at a flow-rate of 1 ml min^{-1} with methanol–water (5:95); (2) upon injection, the solvent composition and flow-rate were linearly changed to methanol–water (90:10) and to 2 ml min^{-1}, respectively, over a period of 20 min; (3) the solvent composition was maintained for 5 min at methanol–water (90:10) at a flow-rate of 2 ml min^{-1}.

To check the purity of 3′,5′-bis-(O-cinnamoyl)-3-methylthymidine, the following program was used: (1) the column was equilibrated for 5 min with methanol–water (20:80) at a flow-rate of 1 ml min^{-1}; (2) upon injection, the solvent composition and flow-rate were linearly changed to methanol–water (90:10) and to 2 ml min^{-1}, respectively, over a period of 5 min; (3) the solvent composition was maintained for 5 min at methanol–water (90:10) at a flow-rate of 2 ml min^{-1}.

To monitor the reaction for synthesizing 3′,5′-bis-(O-cinnamoyl)-thymidine and the synthesis and stability of 3′,5′-bis-(O-cinnamoyl)-3-methylthymidine, the following conditions were used: (1) the column was equilibrated for 5 min with methanol–water (65:15) at a flow-rate of 1 ml min^{-1}; (2) upon injection, the solvent composition was linearly changed to methanol–water (90:10) over a period of 10 min as the flow-rate was changed to 2 ml min^{-1} using a 0.5 convex gradient.

To monitor the trace-level derivatization reactions, the following program was used: (1) the column was equilibrated for 5 min with methanol–water (50:50) at a flow-rate of 1 ml min^{-1}; (2) upon injection, the solvent composition was linearly changed to methanol–water (90:10) over a period of 15 min as the flow-rate was

changed to 2 ml min^{-1} using a 0.5 convex gradient; (3) the solvent composition was maintained at methanol–water (90:10) for 5 min at a flow-rate of 2 ml min^{-1}.

Gas chromatographic analyses

To determine 3′,5′-bis-(O-cinnamoyl)-3-methylthymidine by GC–ECD, the following conditions were used: the oven temperature was programmed from 90°C to 220°C at 15°C min^{-1} with a 1-min initial and 5.34 min final hold; the on-column injector was programmed from 30°C to 150°C at a setting of 180°C min^{-1} with a 14-min final hold; the detector temperature was 340°C; the helium carrier gas flow was 6 ml min^{-1} (purge is normally left open but this was measured with purge closed), and the nitrogen make-up gas flow was 24 ml min^{-1}. Chromatograms were recorded at an attenuation of 8 × 1. The molar response of the detector to 3′,5′-bis-(O-cinnamoyl)-3-methylthymidine relative to lindane was determined from a series of dilutions of analytical standards containing both compounds in toluene.

RESULTS AND DISCUSSION

As described in more detail below, reaction conditions were first established for efficiently converting thymidine at the milligram level to its dicinnamoyl derivative. Once this product was obtained and methylated, its aqueous stability at the nanogram level was investigated using HPLC for analysis. The milligram-level acylation conditions were then refined for derivatizing 3-methylthymidine at the nanogram level. Finally, the product, 3′,5′-bis-(O-cinnamoyl)-3-methylthymidine, abbreviated as $(Cin)_2$-3-MeThym, was then determined by GC–ECD.

TABLE I

AQUEOUS STABILITY OF 3′,5′-BIS-(O-CINNAMOYL)-3-METHYLTHYMIDINE

Starting concentration is 33 pmol μl^{-1}. HPLC conditions are described in the Experimental section.

Aqueous conditions (room temperature in the dark)	*Time*	*Recovery of $(Cin)_2$-3-Methym (%)*
40% acetonitrile		
water	16 days	100
pH 4 acetate	16 days	100
pH 6 phosphate	16 days	100
pH 7 phosphate	16 days	100
pH 8 Tris	16 days	100
pH 10 carbonate	16 days	60
0.01 N sodium hydroxide	1.6 h	50*
50% methanol	16 days	50**

* The HPLC chromatogram showed only two peaks, one for remaining $(Cin)_2$-3-MeThym and one for the fully hydrolyzed product, 3-methylthymidine. No extra peaks were observed which would have represented a monodeacylated product.

** The HPLC chromatogram showed only two peaks, one for remaining $(Cin)_2$-MeThym and one with an intermediate retention between $(Cin)_2$-3-MeThym and 3-methylthymidine. The intermediate peak could have been the monoacylated $O^{3'}$-cinnamoyl-3-methylthymidine since the 5′-position is more readily deacylated (and acylated) than the 3′-hydroxy[18].

Cinnamoylation of thymidine

Several bases and solvents were tested initially for maximizing the acylation of thymidine with cinnamoyl chloride. Based on monitoring the reactions by TLC and HPLC, the best results were obtained with DMAP in toluene. For example, in the presence of a 3-fold molar excess of cinnamoyl chloride and a 6-fold excess of DMAP, thymidine was converted to a single diacylated product in an 84% HPLC yield.

This high yield of a single product contrasts with some other less successful reaction conditions. For example, neither triethylamine nor diisopropylethylamine in toluene gave a reaction. N-Methylmorpholine catalyzed the formation of a small amount of diacylated product but gave several side products. Neither cinnamic anhydride in toluene with pyridine as catalyst nor 1-*trans*-cinnamoylimidazole in toluene with DMAP as catalyst gave a reaction. Cinnamoyl chloride and DMAP in acetonitrile gave mostly the diacylated product along with a secondary product that was probably the monoacylated derivative.

As observed here, others similarly have found DMAP to be a useful catalyst for acylation reactions. DMAP has been used to catalyze the silylation of alcohols[13] and the acylation of ribonucleosides[14] and a variety of other compounds[15]. It also has been used to catalyze the acylation of *n*-propyl alcohol with either cinnamoyl chloride or cinnamic anhydride[16,17].

Aqueous stability of $(Cin)_2$-3-MeThym

Diacylated thymidine, prepared as above, was methylated in anticipation of its analysis by GC as described below. The aqueous stability of the resulting product, $(Cin)_2$-3-MeThym, was determined by HPLC analysis. As shown in Table I, the product is stable for 16 days in pH 4–8 aqueous buffers containing acetonitrile and has a half-life of 16 days in aqueous methanol. Even at pH 10 in aqueous acetonitrile, the half-life is 16 days. Thus, the overall aqueous stability of this derivative is good.

Trace cinnamoylation of 3-methylthymidine

Given these favorable results, the cinnamoylation reaction was optimized at the nanogram-level. Starting with 0.2 nmol of 3-methylthymidine and determining reaction yields by HPLC analysis, it was found that cinnamoylation with cinnamic anhydride occurred at a faster rate than cinnamoylation with cinnamoyl chloride. The use of the anhydride also prevented formation of a precipitate such as that formed when using cinnamoyl chloride: the precipitate was probably the chloride salt of the cinnamoyl–DMAP complex as observed by others[16,17]. Thus, starting with 51 ng (200 pmol) of 3-methylthymidine, a product yield of 102 $\pm$ 3.6% (mean $\pm$ S.D.) was obtained using 670 nmol of DMAP and 130 nmol of cinnamic anhydride in 40 μl of toluene. A representative HPLC chromatogram from this reaction is shown in Fig. 1.

For the HPLC analyses, it was necessary to add methanol to remove excess anhydride and then partition the sample between hexane and aqueous buffer, as described in the Experimental section. This gave the relatively clean HPLC chromatogram shown in Fig. 1 and provided a procedural recovery of 91 $\pm$ 1.2% (mean $\pm$ S.D.). Methanol converts excess cinnamic anhydride to volatile methylcinnamate, while the partitioning step helps to remove excess DMAP. Without the partitioning step, an off-scale disturbance of the baseline develops after several samples are injected, apparently due to DMAP bleeding from the HPLC column.

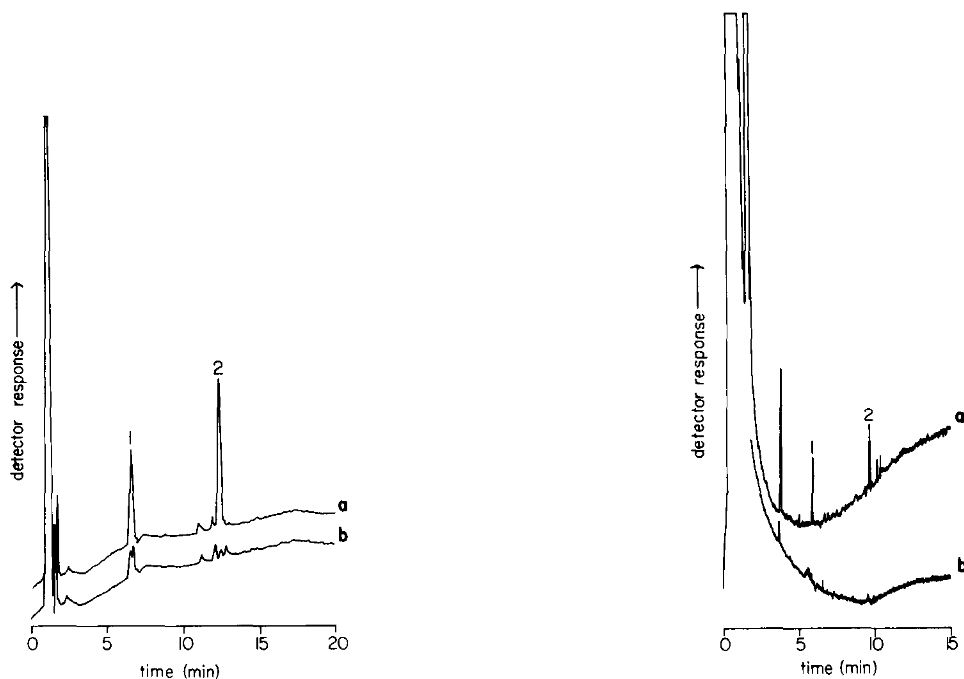

Fig. 1. HPLC chromatograms of a trace-level reaction starting with 200 pmol (51 ng) of 3-methylthymidine. (a) Reaction; (b) reaction blank. Peaks: 1 = methylcinnamate; 2 = 3′,5′-bis-(O-cinnamoyl)-3-methylthymidine.

Fig. 2. GC–ECD chromatograms of lindane (peak 1) and 3′,5′-bis-(O-cinnamoyl)-3-methylthymidine (peak 2). For a, 1 = 0.2 fmol (0.06 pg); 2 = 4 fmol (2 pg). Chromatogram b is a blank injection of toluene. Attenuation = 8 × 1.

Determination of $(Cin)_2$-3-MeThym by GC–ECD

The cinnamoyl group is expected to be a weak electrophore by GC–ECD, and the molar response of $(Cin)_2$-3-MeThym is only 2.3% that of lindane, a strong electrophore. Thus, the detection limit for this compound by GC–ECD is only in the low picogram range (Fig. 2). Nevertheless, $(Cin)_2$-MeThym exhibits good peak shape and retention characteristics by gas chromatography.

CONCLUSION

Cinnamoylation of 3-methylthymidine quantitatively yields a diacylated product at the nanogram level with DMAP as catalyst. The product is stable in aqueous acetonitrile and hydrolyzes only slowly in aqueous methanol. It exhibits good GC properties including detection at the low picogram level by GC–ECD. Thus, cinnamoylation can be useful for derivatizing hydroxyl compounds prior to GC analysis. These results encourage the development of more electrophoric derivatives of the cinnamoyl group, and of other α,β-unsaturated acylating reagents, to advance the usefulness of GC–ECD for providing highly sensitive determinations. One application of such reagents is the quantitation of DNA adducts by GC, as has been discussed[19,20].

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