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Characterization of N-acyl-D-biotinols by particle-beam liquid chromatography–mass spectrometry

An alternative to probe mass spectrometry for thermally labile samples

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ABSTRACT

In the course of preparing biotin-labeled nucleic acid probes, it was necessary to verify structures of intermediate N-acyl derivatives of biotinol. Characterization by mass spectrometry (MS) involved use of particle-beam liquid chromatography (LC)–mass spectrometry MS to supplement standard heated-solids probe techniques. The probe data for a sample of N-toluoylbiotinol indicated it to be a mixture of mono- and di-toluoylbiotinols which was inconsistent with other analytical information. Analysis of the same sample by LC–MS on a reversed-phase column with a water–acetonitrile gradient showed a single major peak with spectrum consistent with that for the monotoluoyl species. These results suggested that a thermal transacylation reaction might be occurring in the probe during heating prior to volatilization and ionization. This was confirmed by heating the sample to 200°C and then repeating the LC–MS analysis to find peaks now present for biotinol and ditoluoylbiotinol as well as the starting material. These results demonstrate the value of particle-beam LC–MS as a technique for obtaining electron-impact mass spectra of thermally sensitive compounds.

INTRODUCTION

The unusually strong and specific interaction between biotin and avidin has been used in a wide variety of applications in analytical biochemistry [1,2]. The basic approach involves labelling the analyte of interest with biotin and then allowing the biotinylated conjugate to bind to the corresponding target molecule. This divalent analyte–receptor complex can be detected by formation of a trivalent complex between the biotin ligand and an avidin derivative which has been conjugated to a signal-generating group such as an enzyme, antibody, fluorophore or chemiluminescent species.

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This approach has found great utility in nucleic acid probes [3,4]. Here biotin-labelled oligonucleotides are used to seek out specific target nucleic acid sequences within biological specimens. For example, the target may be derived from a bacterium or virus whose presence indicates the existence of an infectious disease etiological agent within the specimen. In the present work, biotinylated nucleid acid probes were prepared by the attachment of biotinol via the O-cyanoethyl phosphoramidite derivative to the 5′-terminus of synthetic oligonucleotides. During the course of this work, N-acyl biotinols were isolated as synthetic intermediates and characterized by several analytical techniques to verify the synthetic sequence. Standard heated-probe mass spectrometry (MS) results were found to be inconsistent with other analytical results and particle-beam liquid chromatography (LC)–MS was investigated as a supplemental method to verify purity.

EXPERIMENTAL

LC conditions

The LC system consisted of a multisolvent delivery system (Model 600-MS; Waters Assoc., Milford, MA, USA) and a Waters Model 484-MS absorbance detector. The separations were performed on a Waters Nova-Pak C_{18} (30 cm × 3.9 mm I.D.) reversed-phase column. The flow-rate of the water–acetonitrile mobile phase through the column was 0.6 ml/min. The initial gradient composition of 5% acetonitrile was linearly programmed to 95% in 30 min and then held at the latter condition for 20 min.

MS conditions

The LC–MS analyses were made on an ELQ-400 quadrupole mass spectrometer (Extrel Corp., Pittsburgh, PA, USA) equipped with a Thermabeam interface. A mass range of 70–700 a.m.u. was scanned at a rate of 500 a.m.u./s. The source temperature was 200°C. Helium was used for the nebulizing gas. The desolvation chamber (momentum separator) was held at 110°C and the nebulizer tip was at 150°C. High-resolution probe MS analyses were made on a ZAB-2F magnetic sector instrument (VG Analytical, Manchester, UK).

Chemicals and synthetic procedures

The N-acylbiotinols were prepared from biotin in several steps. D-Biotin (Sigma, St. Louis, MO, USA) was esterified with methanol in the presence of *p*-toluenesulfonic acid. The resulting ester was reduced with lithium aluminum hydride in tetrahydrofuran to D-biotinol [5]. The alcohol function was protected as the monomethoxytrityl ether during the acylation as described below.

N1-(p-Toluoyl)-D-biotinol. To a suspension of D-biotinol (0.860 g, 3.74 mmol) in anhydrous pyridine was added monomethoxytrityl chloride (1.501 g, 4.86 mmol) and the resulting mixture was stirred at room temperature for 16 h. Next, *p*-toluoyl chloride (0.751 g, 4.86 mmol) was added and stirring at room temperature continued for 24 h. After the pyridine was evaporated in vacuo, the residue was dissolved in ethyl acetate, extracted twice with saturated sodium bicarbonate solution and dried over magnesium sulfate. Evaporation of the ethyl acetate left a dark orange oil which was purified by flash chromatography on silica gel using gradient elution (ethyl ace-

tate–hexane, 1:4, v/v to 2:3, v/v and containing 2% triethylamine). The product was colorless foam (1.90 g, 82% yield). $C_{38}H_{24}N_2O_3S$ requires C, 73.52%; H, 6.49; N, 4.51. Found: C, 73.45; H, 6.55; N, 4.22.

A partial solution of the above product (1.5 g, 2.4 mmol) in 100 ml of acetic acid–water (4:1, v/v) was held at 40°C for 1 h while being rotated on a rotary evaporator. The pale yellow solution was evaporated in vacuo and then coevaporated with toluene. The resulting gum was dissolved in 50 ml of methanol, diluted with an equal volume of diethyl ether and allowed to crystallize. A total of 0.669 g (80% yield) of colorless crystalline product was recovered in two crops. M.p. 166–68°C. $C_{18}H_{24}N_2O_3S$ requires C, 62.04; H, 6.94; N, 8.04. Found: C, 61.95; H, 7.01; N, 7.67.

^{1}H NMR ([$^2H_6$]dimethyl sulfoxide (DMSO-d_6)): 7.91 (s, 1H, NH), 7.38–7.16 (m, 4H, phenyl), 5.05 (m,1 H, H_{6a}), 4.36 (t, J=4.8 Hz, ex, OH), 4.21 (m, 1H, H_{3a}), 3.40 (m, 2H, H_{11}), 3.25 (m, 1H, H_4), 3.04 (dd, J=5.1, 12.3 Hz, 1H, H_6'), 2.88 (d, J=12.3 Hz, 1H, H_6''), 2.34 (s, 3H, CH_3), 1.68 (m, 2H, H_7), 1.6–1.3 (m, 6H, $H_{8,9,10}$).

^{13}C NMR (DMSO-d_6): 168.89, 155.39, 140.56. 132.55, 128.41, 127.74, 61.60, 60.56, 57.21, 54.86, 37.34, 32.24, 28.49, 28.10, 25.42, 20.98.

IR (KBr disc): 3400(br), 2930, 1738(s), 1658, 1392, 1339, 1253, 829, 752 cm^{-1}. High-resolution MS: $C_{18}H_{24}N_2O_3S$ requires 348.1508; found 348.1497.

*N1-(p-phenylbenzoyl)-*D*-biotinol.* This compound was prepared from D-biotinol by a sequence of reactions similar to those decribed above, but *p*-phenylbenzoyl chloride was used in place of *p*-toluoyl chloride. The product, which was obtained as a colorless foam, could not be induced to crystallize.

^{1}H NMR (DMSO-d_6): 7.99 (s, 1H, NH), 7.76–7.38 (m, 9H, *p*-biphenyl), 5.08 (m, 1H, H_{6a}), 4.38 (t, J=4.8 Hz, ex, OH), 4.22 (m, 1H, H_{3a}), 3.40–3.20 (m, 3H, H_{11} & H_4 with HDO peak), 3.05 (dd, J=5.1, 12.3 Hz, 1H, H_6'), 2.92 (d, J=12.3 Hz, 1H, H_6''), 1.70 (m, 2H, H_7), 1.6–1.3 (m, 6H, $H_{8,9,10}$). High-resolution MS: $C_{23}H_{26}N_2O_3S$ requires 410.1666; found 410.1661.

RESULTS AND DISCUSSION

The acylated biotinols were isolated and purified as intermediates in a synthetic route to biotinylated nucleic acid probes. It was necessary to confirm their structures to verify that the synthesis was proceeding as planned. There are three possible mono acylbiotinols (Fig. 1). Two of these are acylated at nitrogen (structures I and II) and one is acylated at the hydroxyl oxygen (III). Protection of the hydroxyl group as the monomethoxytrityl ether during acylation should have prevented formation of III. Steric considerations should favor the formation of I over II.

Mass spectral characterization of monoacylbiotinols by standard heated-probe methods was undertaken to supplement and verify the structure and purity as determined by other techniques such as nuclear magnetic resonance. The probe MS data obtained for a monotoluoylbiotinol (Ia) sample suggested that is was not pure, but was mixed with significant amounts of the ditoluoyl species (IVa). Fig. 2 shows single-ion plots for m/z 348 and 466, the corresponding molecular ions of Ia and IVa, respectively, generated from the probe MS data. As can be seen, the plots do not maximize at the same place and clearly indicate the presence of two separate species. This was inconsistent with other analytical information which indicated this sample to be relatively pure.

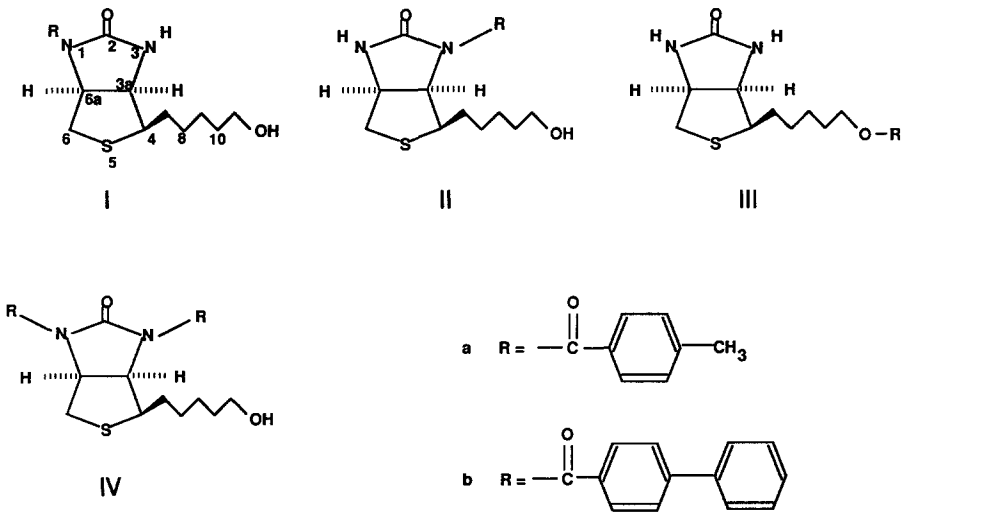

Fig. 1. Structures of acylbiotinols.

The sample was analyzed by particle-beam LC–MS to compare with the probe MS results. The reconstructed-ion chromatogram is shown in Fig. 3a. One major peak (C) was found, with two smaller impurity peaks (D and G) eluting later. The spectral data for peak C indicated a molecular weight of 348 with fragmentation consistent with that expected for Ia. Both D and G are toluoyl derivatives and have molecular weights 390 and 348, respectively. The molecular weight of the peak G species being the same as that of the main component suggests that it is an isomer.

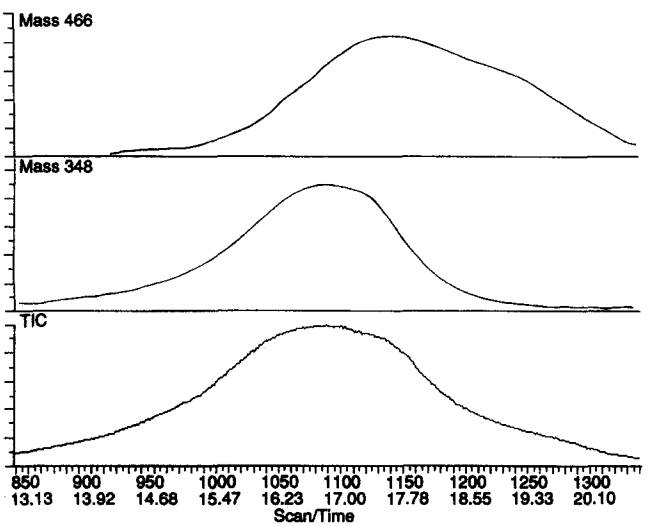

Fig. 2. Probe mass spectrum of mono-*p*-toluoylbiotinol (mol.wt. 348) showing thermal generation of di-*p*-toluoylbiotinol (mol.wt. 466). Time in min.

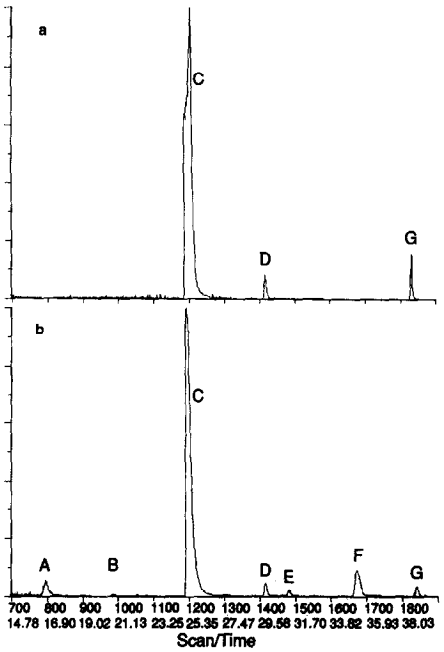

Fig. 3. Total-ion chromatograms of mono-*p*-toluoylbiotinol: (a) as received; (b) after heating to 200°C. Peaks: A = biotinol; B = sulfoxide of I_a (mol. wt. 364); C = I_a; D = acetyl derivative of I_a (mol.wt. 390); E = ring-opened form of peak D? (mol.wt. 392); F = IV_a; G = III_a. Time in min.

This is probably the oxygen-acylated product IIIa which formed in small amounts in spite of the protective monomethoxytrityl group. The long retention time would be consistent with the reduced polarity of the esterified hydroxyl. The impurity at peak D may be a mixed diacylated species having one toluoyl group and one acetyl group. The acetyl group may have been incorporated from contact with ethyl acetate during the synthesis. The LC–MS data show no evidence for the ditoluoyl species detected by probe MS.

The presence of the ditoluoyl species during the probe analysis, but not during LC–MS, suggested that it might be forming by a thermal process during the heat-up of the probe prior to volatilization and ionization. To verify this, a portion of the sample was heated in a small vial to 200–210°C and then analyzed by LC–MS. The reconstructed-ion chromatogram is shown in Fig. 3b and and several new peaks are evident. The spectral data for peak A indicate it has molecular weight 230 and is unsubstituted biotinol. This is consistent with a transacylation reaction, since forming the diacyl species from the monoacyl species requires formation of an equal amount of non-acylated species. The required complement to peak A is peak F which shows apparent molecular weight 466 and has a spectrum consistent with that for the ditoluoyl species IVa. The LC–MS spectrum of this matches that from probe data in showing a fragment at 433 for loss of hydrosulfide from the molecular ion and also toluoyl- and biotinyl-derived fragments analogous to those in the spectrum of Ia. An N,O-ditoluoyl species is also possible, but was ruled out based on observed LC reten-

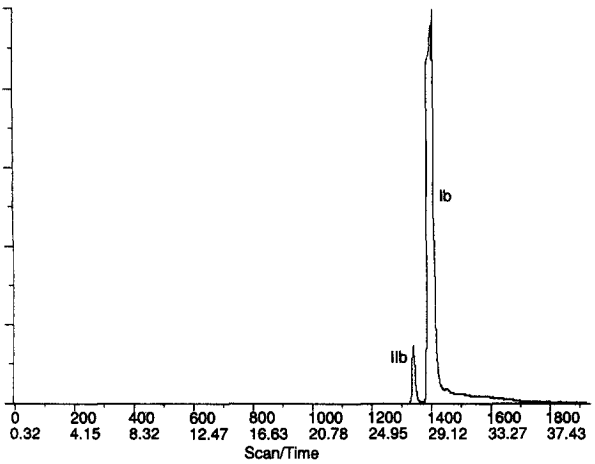

Fig. 4. Total-ion chromatogram of mono-*p*-phenylbenzoylbiotinol. Time in min.

tion time. If present, this species would be less polar than the mono-O-toluoyl compound IIIa (Peak G) and should elute after it on the reversed-phase column. The small peak at B has a molecular weight of 364 and is probably the sulfoxide derivative of Ia which formed during heating in the presence of air. Spectral data for peak E resemble that for peak D, but the molecular weight is 292. This is two units higher than D and may possibly indicate a ring-opened form of D.

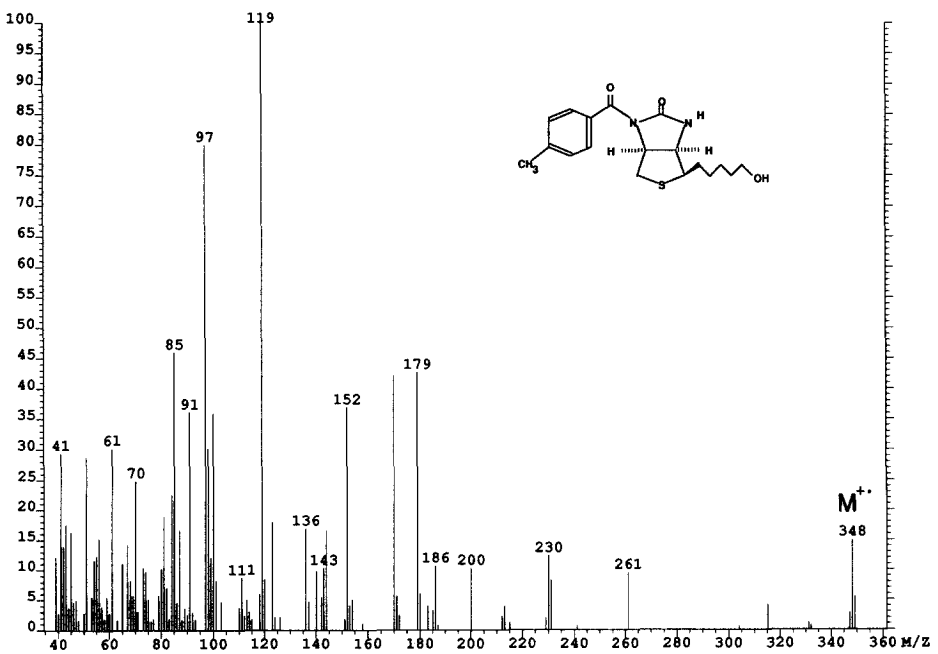

Fig. 5. Mass spectrum of mono-*p*-toluoylbiotinol.

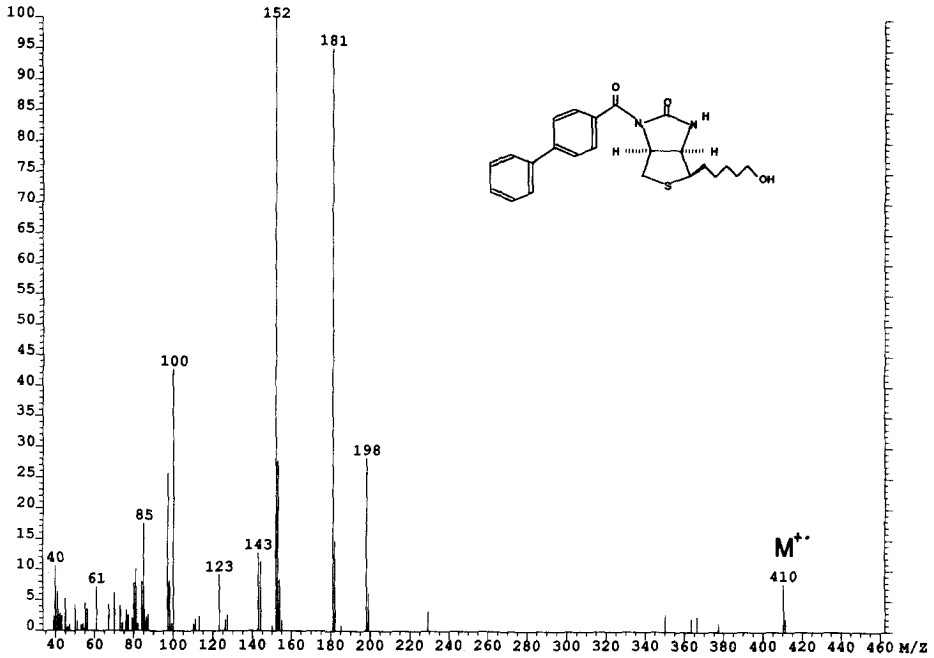

Fig. 6. Mass spectrum of mono-*p*-phenylbenzoylbiotinol.

The *p*-phenylbenzoyl derivative of biotinol (Ib) was also prepared and characterized. It was found to be far more thermally stable than the toluoyl derivative and no evidence for the corresponding diacyl species (IVb) was detected during probe MS analysis of samples of Ib. Fig. 4 shows the reconstructed-ion chromatogram from LC–MS analysis of a sample of Ib. The spectral data for the two peaks are virtually identical and indicate molecular weight 410. The larger peak is probably due to Ib and the smaller one to IIb. Having the less-hindered nitrogen uncovered should increase the polarity of IIb relative to Ib and would explain the shorter retention time.

Mass spectra of Ia and Ib from high-resolution solids probe data are shown in Fig.5 and 6, respectively. In both cases, the acyl ion and its fragments dominate the spectra. The molecular ions are detectable in both cases and lose 33 units (HS) to give their highest mass primary fragment. The lower mass region shows several major fragment peaks containing nitrogen or sulfur which may be stabilized as heterocyclic structures. Fragements such as *m/z* 179 ($C_9H_{11}N_2O_2$), 97 ($C_4H_5N_2O$) and 85 ($C_3H_5N_2O$) probably have imidazolidone-related structures, while 152 ($C_9H_{12}S$) and 100 (C_5H_8S) are thiophene derived. In the spectrum of Fig. 6, the *m/z* 152 peak would also be partly due to $C_{12}H_8$.

CONCLUSIONS

Particle-beam LC–MS has been successfully demonstrated to characterize acylbiotinols for purity and structure. A thermal transacylation reaction was suspected of occurring during probe MS of Ia and giving misleading results. This was verified by

obtaining similar results by heating the sample prior to LC–MS analysis. Particle-beam LC–MS offers a gentler method than probe MS for obtaining electron-impact mass spectra of thermally sensitive samples.

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