Journal of Chromatography, 476 (1989) 423–438
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1587

ESTER AND RELATED DERIVATIVES OF RING N-PENTAFLUOROBENZYLATED 5-HYDROXYMETHYLURACIL

HYDROLYTIC STABILITY, MASS SPECTRAL PROPERTIES, AND TRACE DETECTION BY GAS CHROMATOGRAPHY–ELECTRON-CAPTURE DETECTION, GAS CHROMATOGRAPHY–ELECTRON-CAPTURE NEGATIVE ION MASS SPECTROMETRY, AND MOVING-BELT LIQUID CHROMATOGRAPHY–ELECTRON-CAPTURE NEGATIVE ION MASS SPECTROMETRY

GERHARD M. KRESBACH, MOHAMED ITANI, MANASI SAHA and EUGENE J. ROGERS[a]
Barnett Institute of Chemical Analysis and Materials Science, Northeastern University, Boston, MA 02115 (U.S.A.)
PAUL VOUROS
Department of Chemistry, Northeastern University, Boston, MA 02115 (U.S.A.)
and
ROGER W. GIESE*
Department of Medicinal Chemistry, Northeastern University, Boston, MA 02115 (U.S.A.)

SUMMARY

One consequence of radiation damage to DNA is the conversion of thymine to 5-hydroxymethyluracil (HMU). In order to sensitively detect this DNA adduct by gas chromatography (GC) or high-performance liquid chromatography (HPLC) with electron-capture detection techniques, it is necessary to derivatize it. This study was designed to select an optimum ester derivative of the aliphatic hydroxyl group on HMU. N^1,N^3-Bis(pentafluorobenzyl)-HMU was formed as a parent derivative, and from this a series of esters. Also O-pentafluorobenzyl and O-tetrafluorobenzyl ether derivatives were prepared. Of the esters the pivalyl derivative was the best choice because it formed easily, was relatively stable to aqueous hydrolysis ($t_{1/2}$ = 9.8 days at pH 11.5, 24°C) and gave a response at fmol levels by GC and LC comparable to that of the ethers. Unanticipated was a good response as well for the parent derivative, a free hydroxyl compound, by GC and LC at this level. The work also demonstrates a high performance by LC–electron-capture negative ion mass spectrometry with a belt interface for the trace detection of derivatives of this type.

INTRODUCTION

We are developing mass spectrometric (MS) techniques for the determination of chemically and otherwise induced damage to human DNA, commonly called "DNA

[a] Present address: University of Lowell, Lowell, MA, U.S.A.

0021-9673/89/$03.50

adducts". Such trace level determinations are intended to help define the risks associated with human exposure to chemicals and radiation.

Some of these DNA adducts, especially those with alkyl and related modifications, can be obtained as free bases by acid hydrolysis[1] or oxidation[2] of DNA nucleosides. We have shown that it is attractive to derivatize alkyl nucleobases on their acidic ring NH sites with pentafluorobenzyl bromide (PFBBr) prior to the determination of such adducts by gas chromatography–electron-capture negative ion mass spectrometry (GC–ECNI-MS)[3], GC with electron-capture detection (GC–ECD)[4], or high-performance liquid chromatography (HPLC) with electron-capture negative ion mass spectrometry (LC–ECNI-MS) via a moving-belt interface[5].

In some cases a hydroxyalkyl group is present in a modified nucleobase, *e.g.* 5-hydroxymethyluracil (HMU), a consequence of damage to DNA by ionizing radiation. One strategy that we have employed to derivatize this hydroxy group is to conduct an alkylation reaction with PFBBr under phase transfer conditions[6]. While to date this latter reaction has been useful, we wanted to explore whether ester derivatives offer advantages in terms of reaction yield, ease of subsequent post-derivatization clean-up, or improved MS properties. In this paper we report the first stage of this work, in which several ester derivatives are evaluated.

MATERIALS AND METHODS

Reagents

5-Hydroxymethyluracil (Sigma grade) and Tris (Trizma, reagent grade) were purchased from Sigma (St. Louis, MO, U.S.A.). 2,3,5,6-Tetrafluorobenzyl bromide was from Lancaster Synthesis (Windham, NH, U.S.A.). α-Phenylcyclopentaneacetic acid, α-phenylcyclohexaneacetic acid, 4-dimethylaminopyridine (DMAP), acetic anhydride, benzoic anhydride, pivalic anhydride, butyric anhydride, thionyl chloride, pentafluorobenzyl bromide, tetrabutylammonium hydrogensulfate, and light petroleum (b.p. 35–60°C) (A.C.S. reagent) were from Aldrich (Milwaukee, WI, U.S.A.). Potassium carbonate, sodium bicarbonate, sodium sulfate, hydrochloric acid, and benzene were of Baker analytical reagent grade from J. T. Baker (Phillipsburg, NJ, U.S.A.). 1,1,1-Trichloro-3,3,3-trifluoroacetone was from Crescent (Hauppauge, NY, U.S.A.). Potassium di-hydrogenphosphate (HPLC grade), sodium borate (HPLC grade), and solvents (Burdick and Jackson) were from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Cinnamic anhydride was obtained as described[6].

Chromatography

Thin-layer chromatography (TLC) with silica gel GF was performed on Uniplates (Analtech, Newark, DE, U.S.A.) containing fluorescent indicator: 250-μm plates were used for monitoring reactions, and 1000-μm plates were used for preparative TLC. Flash chromatography, in which a moderate air pressure (*e.g.*, 10–20 p.s.i.) was used to establish a flow-rate of 1–2 ml/min, was performed with silica gel 60, 230–400 mesh (E. Merck, Darmstadt, F.R.G.) in a column 15 × 2.5 cm I.D. For off-line HPLC an Econosil C_8 silica reversed-phase column, 250 × 4.6 mm, 10-μm particle size (Alltech, Deerfield, IL, U.S.A.) was used. The mobile phase was acetonitrile–water, 80:20 (v/v) at 1 ml/min. Typically 20-μl injections were made containing 100 ng of compound. Detection of the derivative was performed at the

absorption maximum (264 nm) using a Spectromonitor III variable-wavelength detector (LDC-Milton Roy, Riviera Beach, FL, U.S.A.), and integration was done using an SP 4270 integrator (Spectra-Physics, San Jose, CA, U.S.A.). For the on-line HPLC–MS experiments, a Supelcosil LC-18 DB column, 150 × 4.6 mm, 3-μm particles (Supelco, Bellefonte, PA, U.S.A.) was used with 1.2 ml/min acetonitrile–water (70:30, v/v) as eluent.

GC–ECD experiments were performed on a Varian Model 3700 gas chromatograph (Varian, Palo Alto, CA, U.S.A.), equipped with a Varian on-column capillary injector (Model 03-908719-00) and a ^{63}Ni electron-capture detector. The GC was interfaced to an SP 4270 integrator with a memory module. GC conditions were: column, 25 m × 0.32 mm I.D. HP Ultra 2, coated with 5% phenylmethylsilicone, 0.17 μm film thickness (Hewlett-Packard, Avondale, PA, U.S.A.), 1.3 ml He/min (determined at 250°C), and a temperature gradient from 50 to 320°C with 25°C/min (oven and on-column injector). N_2 at a flow-rate of 29 ml/min was used as make-up gas for the electron-capture detector.

Mass spectrometry

Electron impact, positive chemical ionization, and electron-capture negative ion mass spectra were obtained by using a Finnigan 4021B quadrupole mass spectrometer with pulsed positive ion negative ion chemical ionization option, coupled to an HP 5890 gas chromatograph (Hewlett Packard, Waldbronn, F.R.G.). Data were acquired with an INCOS data system (Finnigan, San Jose, CA, U.S.A.).

GC conditions were: column, 10 m and 25 m × 0.32 mm I.D. HP Ultra 2, coated with 5% phenylmethylsilicone, 0.17 μm film thickness (Hewlett Packard), 15 p.s.i. head pressure, and a temperature gradient from 50 to 320° with 25°C/min, then 20 min isothermal. For the LC–MS experiments, a Finnigan moving-belt interface with Kapton (R) belts was used. Belt speed was adjusted to 2 cm/s. The HPLC eluent was deposited without split onto the belt, by using a direct electrically heated spray device[7].

Standard MS conditions were: ionizing energy 70 eV, filament current 0.5 mA, and source temperature 220°C. Methane chemical ionization (CI) gas pressure was maintained at an indicated range of between 0.22 and 0.25 Torr. Scan range was usually from 50 to 750 a.m.u. in 1 s unless indicated otherwise.

Glassware used for the trace GC–MS, GC–ECD and LC–MS detection steps was acid washed and gas phase silanized[4].

Synthesis

α-Phenylcyclohexaneacetyl chloride. α-Phenylcyclohexaneacetic acid (0.50 g, 2.4 mmol) was combined with 2 ml (24 mmol) of thionyl chloride and 15 ml of benzene. The resulting solution was refluxed with stirring under nitrogen for 2 h. The reaction mixture was evaporated four times with intermediate additions of benzene to fully remove the thionyl chloride. The resultant oily product was diluted with 10 ml of toluene and used as described below without further purification.

α-Phenylcyclohexaneacetic anhydride. Following a general procedure for synthesizing anhydrides[8], to 0.16 ml (1.2 mmol) of trichlorotrifluoroacetone in a 100-ml round bottom flask were added 0.022 ml (1.2 mmol) of water in a nitrogen atmosphere at room temperature. After 15 min, first 5 ml of toluene, then the above mentioned solution of acid chloride in toluene were added dropwise, followed by 0.1 ml of

TABLE I

^{1}H-NMR AND SELECTED IR DATA OF COMPOUNDS **1–10**

s = Singlet; d = doublet; t = triplet; m = multiplet; ch = cyclohexyl; cp = cyclopentyl; ph = phenyl.

Compound	*^{1}H-NMR chemical shift (ppm)*	*IR frequency (cm^{-1})*
1	7.36 (C^6-*H*, 1H, s), 5.23 (N^3-CH_2, 2H, s), 5.03 (N^1-CH_2, 2H, s), 4.40 (CH_2-OH, 2H, broad s)[a]	3000–3400[c]
2	7.50 (C^6-*H*, 1H, s), 5.12 (N^3-CH_2, 2H, s), 5.00 (N^1-CH_2, 2H, s), 4.83 (CH_2-OCO, 2H, s), 2.07 ($COCH_3$, 3H, s)[a]	1230[d]
3	7.47 (C^6-*H*, 1H, s), 5.18 (N^3-CH_2, 2H, s), 4.97 (N^1-CH_2, 2H, s), 4.83 (CH_2-OCO, 2H, s), 2.27 (OCO-CH_2-CH_2-CH_3, 2H, t), 1.95 (OCO-CH_2-CH_2-CH_3, 2H, m), 0.90 (OCO-CH_2-CH_3, 3H, t)[a]	1175[d]
4	7.42 (C^6-*H*, 1H, s), 5.17 (N^3-CH_2, 2H, s), 5.01 (N^1-CH_2, 2H, s), 4.83 (CH_2-OCO, 2H, s), 1.17 [$C(CH_3)_3$, 9H, s][a]	1140[d]
5	8.01 (CO-ph *ortho*, 2H, d), 7.68 (C^6-*H*, 1H, s), 7.63–7.40 (CO-ph *meta* and *para*, 3H, m), 5.23 (N^3-CH_2, 2H, s), 5.16 (N^1-CH_2, 2H, s), 5.04 (CH_2-OCO, 2H, s)[b]	1170[d]
6	7.82 (CH=CH-ph *ortho*, 2H, d), 7.63–7.13 (CH=CH-ph *meta*, *para* and C^6-*H*, 4H, m), 6.53 (CH=CH-ph, 1H, s), 6.27 (CH=CH-ph, 1H, s), 5.17 (N^3-CH_2, 2H, s), 5.10 (N^1-CH_2, 2H, s), 5.00 (CH_2-OCO, s)[a]	1170[d]
7	7.34 (C^6-*H*, 1H, s), 7.17 (CH_2-C_6F_4H, 1H, m), 5.22 (N^3-CH_2, 2H, s), 4.98 (N^1-CH_2, 2H, s), 4.67 (O-CH_2-C_6F_4H, 2H, s), 4.30 (CH_2-O, 2H, s)[a]	1120[e]
8	7.38 (C^6-*H*, 1H, s), 5.25 (N^3-CH_2, 2H, s), 5.02 (N^1-CH_2, 2H, s), 4.71 (O-CH_2-ph, 2H, s), 4.35 (CH_2-O, 2H, s)[b]	1125[e]
9	7.26 [C^6-*H* and CO-CH-(cp)ph 6H, m], 5.17 (N^3-CH_2, 2H, s), 4.84 (N^1-CH_2, 2H, s), 3.26 [CO-CH-(cp)ph, 1H, d], 2.6–0.8 (cp, 11H, m)	—[f]
10	7.39 (C^6-*H*, 1H, s), 7.20 [CO-CH-(ch)ph, 5H, s], 5.15 (N^3-CH_2, 2H, s), 4.86 (N^1-CH_2 and O-CH_2, 4H, s), 3.28 [CO-CH-(ch)ph, 1H, d], 2.2–0.3 (ch, 11H, m)[a]	—[f]

[a] 60 MH_2 spectrum.
[b] 300 MH_2 spectrum.
[c] O-H-O hydrogen bonding.
[d] C-O stretching vibration of the ester.
[e] C-O stretching vibration of the ether bridge in position 5.
[f] Broad and weak signals.

pyridine. After stirring for 8 h at room temperature, 10 ml of ethyl acetate were added followed by an extraction with 10% hydrochloric acid, saturated sodium hydrogencarbonate, and saturated sodium chloride. The separated organic layer was filtered and dried over anhydrous sodium sulfate. Evaporation gave a white solid which was recrystallized from hexane to yield white crystals (67%). IR: 1820 and 1750 cm^{-1}.

α-Phenylcyclopentaneacetic anhydride. This compound was prepared in the same way as the preceding anhydride.

5-[(α-Phenylcyclohexane)acetoxymethyl]-N^1,N^3-bis(pentafluorobenzyl)uracil. To a stirred solution of 60 mg (0.12 mmol) of N^1,N^3-bis(pentafluorobenzyl)-5-hydroxymethyluracil in 0.1 ml of pyridine under nitrogen were added 15 mg of dimethylaminopyridine and 2 ml of dry (distilled and stored over molecular sieves) acetonitrile. After 10 min, 0.145 g (0.36 mmol) of α-phenylcyclohexaneacetic anhydride were added, and refluxing was done for 8 h (no starting material was detected at this point by silica TLC). Ethyl acetate (15 ml) was added and the solution was washed with 5% hydrochloric acid and saturated sodium hydrogencarbonate, then dried over anhydrous sodium sulfate. Evaporation gave an oil that was purified by flash chromatography (70% yield) using methylene chloride–ethyl acetate (1:1, v/v) and gave a single peak by HPLC.

Other esters. The corresponding product derived from α-phenylcyclopentaneacetic anhydride was prepared in the same way. The other esters were similarly prepared and characterized except that the acylation reaction was performed at room temperature and was complete after 6–12 h, depending on the anhydride. ^{1}H NMR and selected IR data are shown in Table I.

5-Hydroxymethyl-N^1,N^3-bis(pentafluorobenzyl)uracil. To a stirred suspension at room temperature of 485 mg (3.50 mmol) of potassium carbonate in acetone (10 ml) were added 100 mg (0.70 mmol) of 5-hydroxymethyluracil. Ten min later 485 mg (3.51 mmol) of PFBBr in 5 ml of acetone were added, followed by continued stirring for 24 h. Silica TLC using light petroleum–ethyl acetate (1:1, v/v) showed the product at R_F = 0.43. The potassium carbonate was removed by filtration and washed with acetone. The acetone solution was evaporated to a small volume and purified by flash chromatography using light petroleum–ethyl acetate (1:1, v/v) and 20-ml fractions were collected. Fractions 19–24 upon evaporation gave a clear colorless oil that turned white when light petroleum (10 ml) was added. After storage overnight at 4°C, the precipitate was collected on a filter, washed with light petroleum, and dried by suction and then in a vacuum desiccator over anhydrous calcium chloride giving 247 mg (70%) of white crystal (m.p. = 146–147°C).

5-Pentafluorobenzyloxymethyl-N^1,N^3-bis(pentafluorobenzyl)uracil. To a stirred solution of 170 mg (0.57 mmol) of tetrabutylammonium hydrogensulfate in 5 ml of 1 *M* potassium hydroxide were added 50 mg (0.10 mmol) of the above compound in a 50-ml round bottom flask. After stirring at room temperature for 20 min, PFBBr (0.15 ml, 1.1 mmol) in 3 ml of methylene chloride was added, and vigorous stirring was continued for 3 h. Silica TLC of the methylene chloride layer by using ethyl acetate–light petroleum (1:4, v/v) showed the product at R_F = 0.65. The separated aqueous layer was extracted with 2 × 10 ml of methylene chloride, and the combined organic fractions were washed with water, dried (sodium sulfate), filtered and concentrated to a yellow oil by rotary evaporation. Purification by preparative TLC on silica (half of the yield on each of two plates), by using ethyl acetate–light petroleum

(1:4, v/v), and extracting the product from the scraped silica in hot ethyl acetate gave a light yellow oil after rotary evaporation. Light petroleum was used to obtain, after vacuum drying, 42 mg (60%) of white crystals, m.p. 100–102°C.

5-(2,3,5,6-Tetrafluorobenzyloxymethyl)-N^1,N^3-bis(pentafluorobenzyl)uracil. The procedure for the preparation of the above product was used, except the product was purified by flash column chromatography using ethyl acetate–light petroleum (1:4, v/v) yielding 154 mg (56%) of white crystals, m.p. 109–111°C.

Hydrolysis

The compound was dissolved in the HPLC mobile phase and diluted with a 0.25 volume of buffer at time zero. (This buffer was 0.01 *M* borate pH 10; 0.381 g of $Na_2B_4O_7 \cdot 10H_2O$ were dissolved in 100 ml of water, and the pH was adjusted to 10.0 with 1.5 *M* KOH.) The initial apparent pH was 11.6, and the final pH ranged from 11.4 to 11.5 at the end of the experiment. The solutions were stored together at room temperature (24°C), and aliquots were analyzed by HPLC as a function of time up to two or more half-lives. A plot of $\ln(A_t/A_0 \times 100)$ *vs.* time was linear for each compound and used to calculate the half-life for ester hydrolysis, where A_0 = initial peak area for the ester, and A_t = area at the corresponding reaction time.

RESULTS AND DISCUSSION

A modified nucleobase containing a hydroxyalkyl group can potentially be derivatized by alkylation of its acidic ring NH site(s) with pentafluorobenzyl bromide (PFBBr), followed by acylation of its hydroxyalkyl site(s) with an esterifying reagent such as acetyl chloride. In principle this approach is attractive because these types of reactions can proceed under mild conditions in good yields. Adequate sensitivity for ECNI-MS detection should be provided by the first step, which replaces each acidic hydrogen with a PFB group. Nevertheless, one concern is the susceptibility of an ester to aqueous hydrolysis, which could limit the usefulness of such a derivative for trace analysis. Accordingly, we evaluated a series of ester derivatives in terms of their relative ease of preparation, hydrolytic stability, and GC–MS as well as LC–MS properties.

For these studies we chose to esterify N^1,N^3-bis(pentafluorobenzyl)-5-hydroxymethyluracil (**1**, Fig. 1). The ester derivatives that we formed from this parent compound were compared relative to each other, and, as, appropriate, to the parent compound and its O-PFB and O-tetrafluorobenzyl (O-TFB) derivatives.

Synthesis

Based on TLC, each of the acylation reactions gave an essentially complete conversion of **1** to its corresponding ester. Consistent with this the preparative yields of the esters, working on a mg scale, were all above 65% with no effort to optimize this micropreparative yield. In this respect, these ester derivatives are more attractive than the ether derivative **8** since TLC shows some minor side products when **1** is converted to **8** by phase transfer alkylation. Within the acyl series of derivatives, products **2–6** could be formed at room temperature, whereas the formation of **9** and **10**, no doubt due to steric effects, required refluxing. Thus, derivatives **2–6** are most attractive from a synthetic standpoint, aside from parent compound **1**, which of course avoids the acylation reaction.

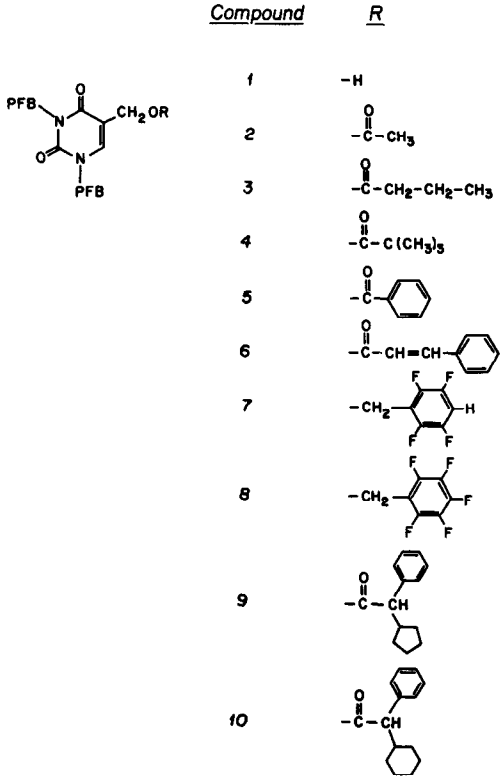


Fig. 1. Electrophoric derivatives of 5-hydroxymethyluracil (HMU).

Hydrolytic stability

The relative hydrolytic stabilities of the ester derivatives of **1** are shown in Table II. These data were obtained by dissolving each ester in 80% acetonitrile, and then diluting this solution at time zero with a 0.25 volume of 0.01 *M* borate, pH 10, giving an apparent pH of 11.6. The hydrolysis of each ester was then followed by HPLC. As seen in this table, the hydrolysis half-lives range from 1.4 to 11.6 days. Apparently the relative hydrolytic stabilities of the esters are largely determined by the steric properties of the acyl moiety. The most stable esters, **4**, **9**, and **10**, are those with the bulkiest side chains. Overall the best derivative up to this point, also taking into account the prior synthetic results, is the pivalyl derivative **4** since it forms easily and yet has a high hydrolytic stability ($t_{\frac{1}{2}}$ = 9.8 days under the conditions used).

An artifact was encountered early in our hydrolysis studies of the ester derivatives. When pivalyl ester **4**, the first compound that we studied, was dissolved in methanol–0.01 *M* phosphate, pH 7.8 (80:20, v/v), and subjected to reversed-phase silica HPLC, a second, earlier eluted peak formed slowly. It became equal in area to that of **4** after 12 d. This earlier eluted peak was not the anticipated hydrolysis product **1**. If **4** was dissolved instead in a corresponding buffer containing acetonitrile instead of methanol, this unknown product did not appear. The peak was collected and found by ECNI-MS to give an intense ion at *m/z* 451. Assuming that this corresponds to

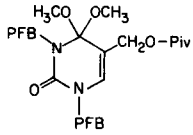
TABLE II

HPLC AND HYDROLYSIS PROPERTIES OF ESTER DERIVATIVES OF RING-PENTAFLUOROBENZYLATED 5-HYDROXYMETHYLURACIL

Apparent pH of hydrolysis solution was 11.6. See Materials and Methods for details.

Compound	*Substituent*	*Retention time (min) reversed-phase HPLC*	*Half-life (days) for hydrolysis*
1	H	2.9	–
2	Acetyl	3.2	1.4
3	Butyl	3.6	4.5
4	Pivalyl	4.0	9.8
5	Benzoyl	3.7	6.8
6	Cinnamoyl	3.9	5.8
7	Tetrafluorobenzyl		–
8	Pentafluorobenzyl		–
9	Phenyl-cyclopentaneacetyl	5.5	10.2
10	Phenyl-cyclohexaneacetyl	6.5	11.6

$[M-181]^-$ (see below), and taking into account the formation of the compound in aqueous methanol but not in aqueous acetonitrile, we concluded that it is a dimethylketal derivative of **4**, either at C2 or C4. The structure of the second option is as follows:



Mass spectrometry

The following issues were considered to be important in our evaluation of the utility of the derivatives **1–10** for trace analysis by GC–MS and LC–MS: (1) The presence of intense, structurally characteristic ions in the ECNI spectra for high sensitivity and reliable identification; (2) the influence of the side chain on the abundance of diagnostic ion(s); (3) the influence of the side chain on the GC and LC behavior; and (4) the amount of structural information which could be obtained from electron impact and positive chemical ionization mass spectra as a function of the moiety in position 5.

ECNI spectra. Fig. 2 shows the reconstructed ion chromatogram of 100 pmol each of compounds **1** and **3–10**, separated by GC and detected by ECNI-MS. Since compound **2** is not separated from **1**, the spectral data of the latter compound were obtained in a separate experiment. Symmetrical peaks are observed for all of the compounds, even for **1** containing a free hydroxyl group. The relative responses will be discussed later.

The ECNI mass spectra obtained from the peaks in Fig. 2 are summarized in Table III. Notable are the extremely low relative intensities of the molecular ion peaks. Consistent with previous observations made with other PFB derivatives of even less

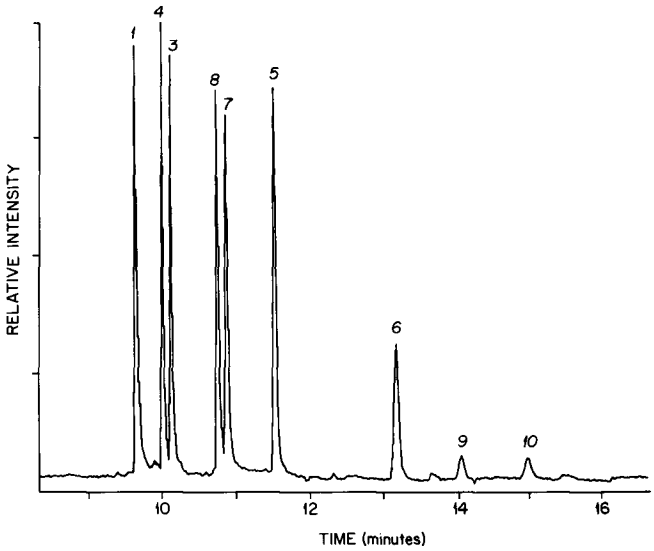


Fig. 2. GC–ECNI-MS reconstructed ion chromatogram of 100 pmol each of compounds **1**, **3–10**. Source temperature: 220°C; methane pressure: 0.25 Torr; scan mode: 10–750 a.m.u. in 1 s; column: 10 m HP Ultra 5% phenylmethylsilicone.

stable compounds[9–12], the base peak is a $[M-181]^-$ ion, produced in a dissociative reaction from loss of a pentafluorobenzyl radical. Typical of this type of ionization, the formation of the pentafluorobenzyl ion is energetically less favored and thus only of minor abundance[9,13].

Fig. 3 summarizes the fraction of the total ion current (TIC) carried by $[M-PFB]^-$ ion for the different derivatives determined by GC–ECNI-MS in the pmol range. As much as 60% of the TIC from an extreme scan range of 10 to 750 a.m.u. is concentrated in this ion. Most of the remaining intensity is concentrated in the intense

TABLE III

ECNI SPECTRA OF ELECTROPHORIC HMU DERIVATIVES

Source temperature: 220°C; 0.25 Torr methane, electron energy: 70 eV. Data in parentheses refer to % relative intensity. n.r. = Not recorded.

Compound	M^-	$[M-PFB]^-$	$RO-$	PFB^-	b_1	b_2	b_3	*Other*
1	502(–)	321(100)	n.r.	181(4)	305(16)	304(4)	125(4)	
2	544(1)	363(100)	n.r.	181(4)	305(20)	304(9)	125(4)	
3	572(1)	391(100)	87(2)	181(1)	305(4)	304(–)	125(2)	
4	586(2)	405(100)	101(6)	181(3)	305(19)	304(11)	125(4)	
5	606(4)	425(100)	121(56)	181(6)	305(47)	304(25)	125(10)	
6	632(–)	451(100)	147(25)	181(5)	305(37)	304(16)	125(5)	407(22)
7	664(–)	483(100)	179(–)	181(2)	305(9)	304(4)	125(2)	
8	682(–)	501(100)	197(1)	181(5)	305(15)	304(5)	125(4)	
9	688(1)	507(100)	203(50)	181(6)	305(45)	304(22)	125(5)	
10	702(–)	521(100)	217(58)	181(8)	305(48)	304(27)	125(6)	

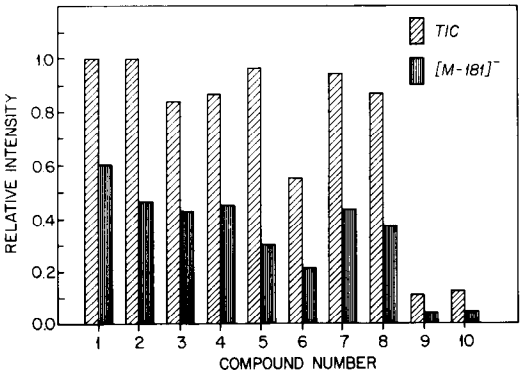


Fig. 3. Relative molar responses of 100 pmol each of electrophoric derivatives of HMU by GC–ECNI-MS; data obtained from the experiment of Fig. 2.

isotope cluster ion. The high absolute response of the $[M-PFB]^-$ ion makes it ideally suited for trace level determinations as well as for characterization purposes of this group of compounds by ECNI-MS.

It is apparent that optimal electron capturing properties are attained by the two pentafluorobenzyl groups as incorporated in compound **1**. Additional electrophoric or other types of groups do not enhance the sensitivity in terms of total ion current any further and, in some cases, are detrimental (see below) for GC–ECNI-MS analysis.

The structures of the most significant fragment ions in the GC–ECNI mass spectra of the derivatives are given in Fig. 4. In addition to the $[M-PFB]^-$ ion, this includes the ions *m/z* 304 (loss of PFB and RO groups) and *m/z* 305 (same as *m/z* 304, plus back-transfer of a hydrogen radical). Relative to these latter ions, the RO^- ion is

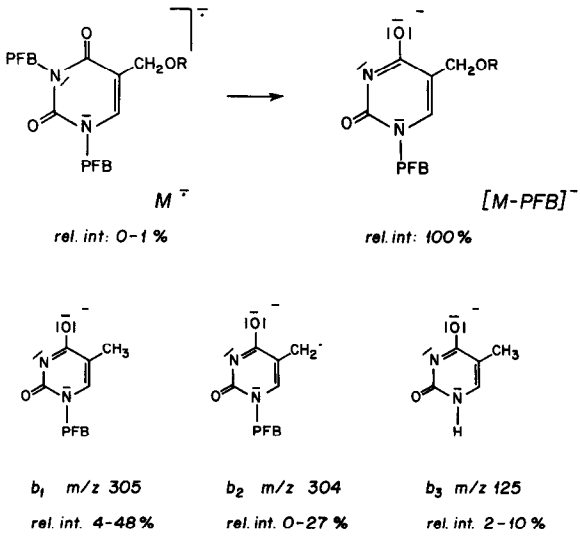


Fig. 4. Principal ions in the GC–ECNI mass spectra (see Table III) of electrophoric derivatives of HMU.

similarly abundant, indicating there is similar charge retention by the core and side chain for loss of RO. As expected, due to the higher strength of ether bonds, these ions are very weak for compounds **7** and **8**. Complete cleavage of PFB groups from the core leads to *m/z* 125. No further influence of the side chain on the extent of the formation of this ion is apparent.

Unique in the series of the derivatives of the ester type, the cinnamoyl ester **6** loses CO_2 via an intramolecular rearrangement as postulated in Fig. 5. According to the proposed mechanism, the information necessary for this transition state seems to be induced by the formation of a six-membered ring comprising positions 5 and 6 of the core, and further promoted by the loss of a neutral molecule. Upon loss of a styrene radical, the intermediate finally fragments to form the ion *m/z* 304. The importance of the ethylene group is further apparent when considering the mass spectrum of compound **5**. While the benzyl group is similarly capable of forming the complex, the shortened distance between base and aromatic center does not permit an overlap of the two aromatic rings, or introduces steric effects, so no similar fragment resulting from loss of CO_2 is observed.

Positive chemical ionization mass spectra. For these spectra (data not shown), obtained on GC peaks of the compounds, well defined protonated molecular ions were observed for compounds **1–8** and, in most cases, further confirmed by the occurrence of $[M + C_2H_5]^+$ adduct ions. A significant ion in every spectrum was *m/z* 485, resulting from cleavage of the side chain (loss of OR; see Fig. 1). Further loss of a PFB group and reprotonation yielded the fragments *m/z* 305 and *m/z* 307.

Favorable protonation of the PFB group was apparent judging from the intensity of the peaks at *m/z* 182 and 183, which far exceeded the intensity from calculated isotopic contribution. Loss of F$^{\cdot}$ (or HF) from these ions led to a prominent *m/z* 163 ion for all of the compounds.

In contrast to the results of the ECNI-MS experiments, direct structural information about the acyl substituents at the 5-hydroxymethyl group were obtained in the positive chemical ionization mode. Ions of the composition $[ROH + H]^+$ (protonated acids) were observed as base peaks in the spectra of the aliphatic and benzoic esters and were still very abundant in the spectra of the cinnamoyl and phenylcycloalkaneacetyl derivatives. In addition, the spectra of compounds **3**, **4**, and **5** showed significant R^+ ions.

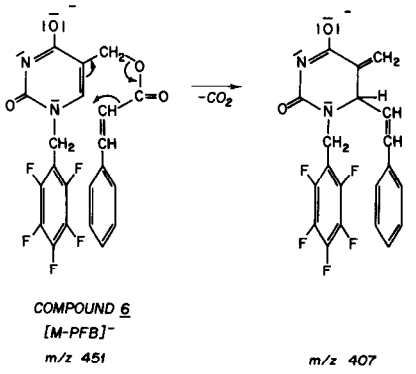


Fig. 5. Proposed fragmentation of the ion $[M-181]^-$ from compound **6**.

Electron impact mass spectra. For these spectra (data not shown), obtained on GC peaks of the compounds, most of the ion current was carried by the ion $[PFB]^-$ at *m/z* 181. Loss of HF from this ion yielded *m/z* 161. With the exception of the ethers **7** and **8**, ions derived from the core structure of the nucleobase were weak. Unique to the electron impact mass spectra was the ion *m/z* 262, which corresponded to $[C_6F_5N{=}CH{-}C(=CH_2){-}C{=}O]^+$. A loss of the RO side chain, followed by loss of pentafluorobenzylisocyanate, apparently led to this ion. A weak complementary ion at *m/z* 222 for $[C_6F_5CH{-}N{=}C{=}O]^+$ was also found. In compound **1**, which lacks a side chain, the same general pattern was observed with the additional presence of an ion *m/z* 278 due to retention of the hydroxyl oxygen atom. The formation of *m/z* 501 and a cluster 484 to 486 was very sensitive to the substitution at position 5 and was mirrored by the occurrence of the corresponding ions R^+ and $[R - CO]^+$.

Comparison of derivatives and detection techniques

In addition to hydrolytic stability and structural information by MS, another important criterion that we applied to the selection of a most suitable ester derivative of 5-hydroxymethyluracil was the chromatographic performance of the compounds, with special emphasis on sensitivity by GC–ECD, GC–ECNI-MS and LC–ECNI-MS.

Fig. 6 shows a GC–ECD chromatogram for a mixture containing 4 fmol each of compounds **1** and **3–10**. Compound **2**, which co-elutes with **1** under these conditions, is not included. The relative molar responses (peak areas) of compounds **1**, **3–5**, **7** and **8** are comparable (Table IV) whereas that for **6** is somewhat lower. Compounds **9** and **10**, which are also the least volatile, give only 1/10 of the response of the most sensitive compound, **8**. Since their lower response is independent of the absolute amounts determined, we suspect that **9** and **10** are inherently less sensitive in the ECD. However, it is difficult to rule out the alternate possibility that they undergo a low recovery in the chromatographic system. Although compounds **9** and **10** are the most hydrolytically stable of the ester derivatives, their more difficult synthesis and, as shown here,

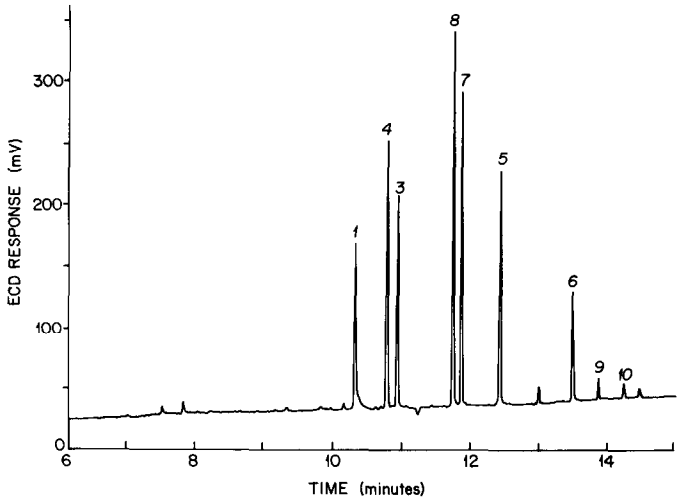


Fig. 6. GC–ECD chromatogram of 4 fmol each of derivatives **1**, **3–10**. 25 m HP Ultra 2, 5% phenylmethylsilicone column. See Materials and Methods for conditions.

TABLE IV

COMPARISON OF THE RELATIVE MOLAR RESPONSE (PEAK AREA) UNDER DIFFERENT SEPARATION AND DETECTION CONDITIONS

FS = Full scan detection. SIM = Selected ion monitoring of ions $[M-181]^-$. RIC = Reconstructed ion chromatogram, ion traces $[M-181]^-$ extracted from the FS experiment. All areas are normalized relative to compound **8**. Relative standard deviation: usually smaller than 10%.

Method	*Compound*								
	1	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***
GC–ECNI-MS									
FS, n = 3, range: 4–20 pmol	1.0	1.1	1.1	1.0	0.7	1.0	1	0.2	0.2
RIC	1.0	1.0	0.9	0.6	0.4	1.0	1	0.1	0.1
GC–ECNI-MS									
SIM, n = 3, range: 10–40 fmol	0.5	0.5	0.6	0.4	0.2	0.9	1	–	–
GC–ECD, n = 6, range: 4 fmol	0.6	0.6	0.7	0.6	0.3	0.8	1	0.1	0.1
LC–ECNI-MS									
SIM, n = 1, range: 2 pmol	0.4	0.4	0.5	0.3	0.2	1.0	1	–	–
LC–ECNI-MS									
SIM, n = 3, range: 20–40 fmol	0.5	0.5	0.6	0.3	0.2	0.9	1	–	–

reduced response by GC–ECD, makes them unattractive as derivatives for the trace analysis of HMU. Compound **6** cannot be considered as a particularly useful derivative either. Not only is its response somewhat low, but also we previously encountered reproducibility problems when we similarly determined a cinnamoyl derivative of a nucleoside[14].

The chromatogram of a ten-fold concentrated sample (40 fmol each) of the above mentioned mixture, analyzed by GC–ECNI-MS in a selected ion mode, is shown in Fig. 7. In order to obtain a direct comparison of this and the prior GC–ECD data, the identical column as well as similar GC conditions were used. As seen in this figure, the relative responses of the compounds by GC–ECNI-MS are quite similar to those seen by GC–ECD, reflecting the similar nature of these two detection techniques. Consistent with their low response by GC–ECD, compounds **9** and **10** fail to give peaks at this fmol level by GC–ECNI-MS, although they were previously observed at the 100 pmol level by this technique (Fig. 2).

It is interesting to note the high relative response and relative symmetric shape for compound **1**, which contains a free hydroxyl group, both by GC–ECD (Fig. 6) and GC–ECNI-MS (Fig. 7) even at the fmol level. While bonded fused silica columns are well known to handle more polar solutes, the GC behavior of polar solutes can degrade when trace amounts are determined. (This is examined in more detail for this compound, and the esters, below.)

Taking into account the signal-to-noise ratios, the sensitivity was about 50-fold lower by GC–ECNI-MS than by GC–ECD (compare Figs. 6 and 7). It is likely that this simply reflects the conditions of the GC–ECNI-MS system when the experiment was

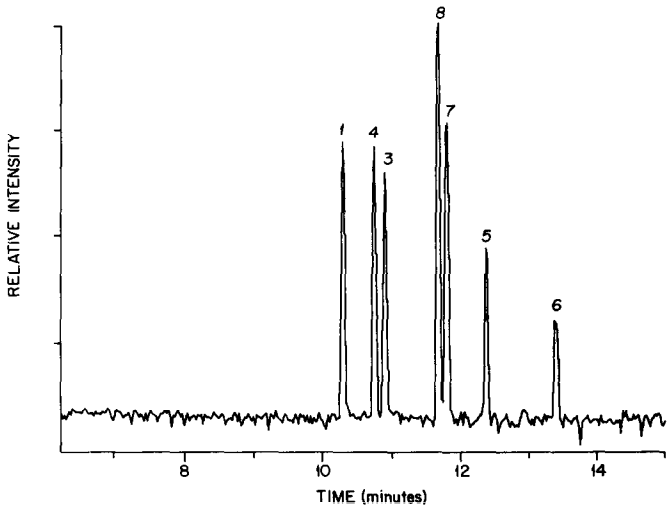


Fig. 7. GC–ECNI-MS reconstructed ion chromatogram of 40 fmol each of the derivatives **1**, **3–10**. Source temperature: 220°C; methane pressure: 0.25 Torr; selected ions: $[M-PFB]^-$, 0.1 s/ion. 25 m HP Ultra 2, 5% phenylmethylsilicone column.

conducted. In a number of cases we have observed that these two techniques give comparable responses for standards of strong electrophores, while GC–ECNI-MS is more useful for the analysis of "real" samples because of its greater discriminating power.

Table IV shows a comparison of the relative molar responses for the compounds as a function of their concentration and the mode of detection, summarizing data from two of the prior experiments (data from Figs. 6 and 7) and also additional studies especially by LC–ECNI-MS. The general pattern of relative responses is similar throughout all of these experiments, with the appearance of somewhat higher relative responses for compounds **1** and **3–6** at the 4–20 pmol level by GC–ECNI-MS. To investigate this latter variation in response in more detail, we measured the responses of representative compounds **1**, **4** and **8** as a function of concentration by GC–ECNI-MS as shown in Fig. 8a and b. As seen, there is a saturation of the response at levels $\geq$ 250 fmol, explaining the change in response for **1** and **4**, and therefore the other compounds, at lower levels relative to that of **8** when comparing fmol with pmol levels. In fact, this figure shows that the responses for **1**, **4** and **8** are all linear with concentration below 250 fmol. Fig. 8a shows this most clearly, in which the 1–100 fmol responses of Fig. 8b are plotted against linear axes. For this region of concentration, correlation coefficients greater than 0.9990 were obtained. Similarly, the same responses, within experimental error, are seen for these compounds and for the others by LC–ECNI-MS at 2 pmol and 20–40 fmol levels (Table IV; a representative chromatogram is presented in Fig. 9). It is interesting that, overall, the four compounds possessing an aromatic moiety in their ester group (**5**, **6**, **9**, and **10**) give the lowest responses.

In all of the experiments shown in Table IV which were conducted at fmol levels, the response of pivalyl ester **4** is at least slightly higher than that for any of the other

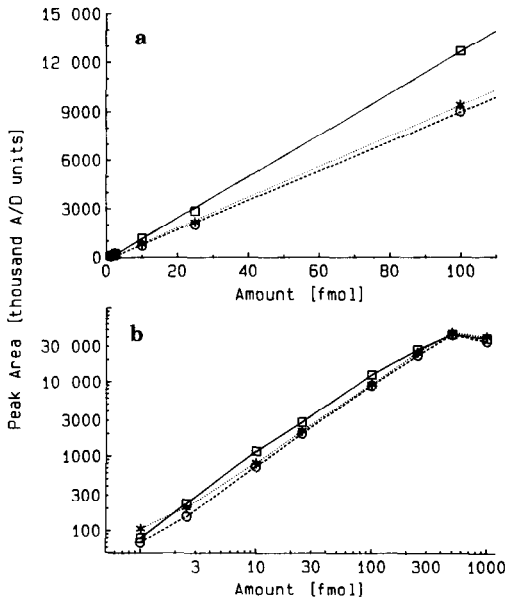


Fig. 8. Response by GC–ECNI-MS for compounds **1** (*), **4** (○) and **8** (□) as a function of concentration; conditions the same as in Fig. 7. In (a), the 1–100-fmol data of (b) are plotted against linear axes.

esters, and the free hydroxyl compound **1** consistently gives a high relative response as well. Thus, taking into account this information plus earlier considerations about the various esters, the pivalyl derivative emerges overall as the best one, but the free hydroxyl compound needs to be considered as well.

The GC–ECNI-MS (Fig. 7) and LC–ECNI-MS (Fig. 9) chromatograms can be

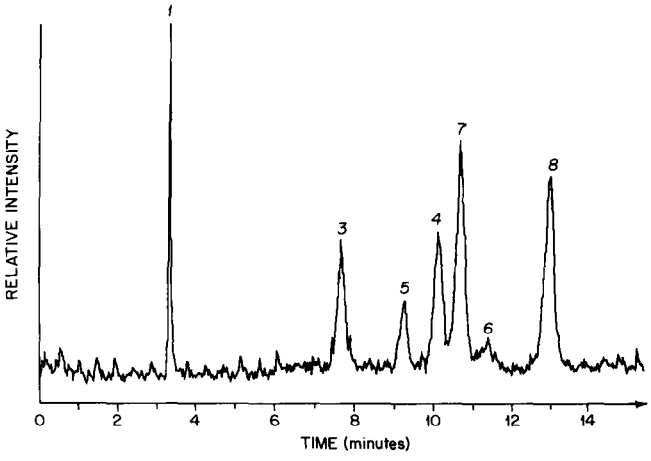


Fig. 9. Moving-belt LC–ECNI-MS reconstructed ion chromatogram of 40 fmol each of the derivatives **1**, **3–10**. Source temperature: 250°C; methane pressure: 0.20 Torr; selected ions: $[M-PFB]^-$, 0.1 s/ion. HPLC column: Supelcosil LC-18DB, 150 × 4.6 mm, 3 μm particles, 1.2 ml/min, 70% acetonitrile; belt speed: 2 cm/s; vaporizer temperature: 330°C.

compared directly as they were determined in the same mass spectrometer. The relative molar responses of the compounds at the 40 fmol level are essentially the same, and nearly identical detection limits are achieved both by GC–ECNI-MS and LC–ECNI-MS. This indicates that no significant loss of material occurs during the eluent deposition and solvent evaporation steps on the Kapton (R) belt, an issue that we have addressed previously[7]. Analogous to the results in the GC–MS experiment, compounds **9** and **10** were also not detected by LC–MS. Even under LC–MS conditions, compound **1** behaves favorably. It is important to point out this result, since LC–ECNI-MS has the important advantage over GC–ECNI-MS that larger sample volumes are more easily introduced into the MS system.

CONCLUSIONS

The goal of selecting an optimum ester derivative for the aliphatic hydroxyl moiety on ring-pentafluorobenzylated HMU was reached: pivalyl is the best choice. Unanticipated was a good performance at trace levels of the parent derivative, in which the hydroxyl group is left underivatized. Thus, four derivatives of HMU now deserve further study for its trace analysis by GC or LC with electron capture detection techniques; namely, the aforementioned two compounds and the two others (pentafluorobenzyl and tetrafluorobenzyl).

For electrophoric derivatives of HMU, LC–ECNI-MS with a belt interface produces similar sensitivity and identical spectral data as GC–ECNI-MS using the same MS equipment.

The structural information that can be obtained about the various derivatives by MS is comparable.

ACKNOWLEDGEMENTS

Financial support for this work was provided by Grant CA 35843 from the National Cancer Institute, and Grant CR 812740 from the Reproductive Effects Assessment Group of the United States Environmental Protection Agency (EPA). G.M.K. acknowledges additional support by a Gustel Giessen Award from the Barnett Institute. This is contribution Number 367 from the Barnett Institute for Chemical Analysis and Materials Science.

REFERENCES

1 O. Minnetian, M. Saha and R. W. Giese, *J. Chromatogr.*, 410 (1987) 453.
2 B. Singer and D. Grunberger, *Molecular Biology of Mutagens and Carcinogens*, Plenum Press, New York, 1983.
3 G. B. Mohamed, A. Nazareth, M. J. Hayes, R. W. Giese and P. Vouros, *J. Chromatogr.*, 314 (1984) 211.
4 J. Adams, M. David and R. W. Giese, *Anal. Chem.*, 58 (1986) 345.
5 R. S. Annan, G. M. Kresbach, R. W. Giese and P. Vouros, *J. Chromatogr.*, in press.
6 J. Adams and R. W. Giese, *J. Chromatogr.*, 347 (1985) 99.
7 G. M. Kresbach, T. R. Baker, R. J. Nelson, J. Wronka, B. L. Karger and P. Vouros, *J. Chromatogr.*, 394 (1987) 89.
8 S. Abdel-Baky and R. W. Giese, *J. Org. Chem.*, 51 (1986) 3390.
9 T. M. Trainor and P. Vouros, *Anal. Chem.*, 59 (1987) 601.
10 A. Martineau and P. Falardeau, *J. Chromatogr.*, 417 (1987) 1.
11 J. S. Hadley, A. Fradin and R. C. Murphy, *Biomed. Mass Spectrom.*, 15 (1988) 175.
12 H. Schweer, H. W. Seyberth, C. O. Meese and O. Furst, *Biomed. Mass Spectrom.*, 15 (1988) 143.
13 R. J. Strife and R. C. Murphy, *J. Chromatogr.*, 305 (1984) 3.
14 T. M. Trainor, R. W. Giese and P. Vouros, *J. Chromatogr.*, 452 (1988) 369.