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Matrix-Assisted Laser Desorption/Ionisation - High-Energy Collision-Induced Dissociation of Steroids: Analysis of Oxysterols in Rat Brain

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Abstract

Neutral steroids have traditionally been analysed by gas chromatography – mass spectrometry (GC-MS) after necessary derivatisation reactions. However, GC-MS is unsuitable for the analysis of many conjugated steroids and those with unsuspected functional groups. Here we describe an alternative analytical method specifically designed for the analysis of oxosteroids and those with a 3 β -hydroxy- Δ^5 or 5 α -hydrogen-3 β -hydroxy structure. Steroids were derivatised with Girard P (GP) hydrazine to give GP hydrazones which are charged species and readily analysed by matrix-assisted laser desorption/ionization mass spectrometry. The resulting [M]⁺ ions were then subjected to high-energy collision-induced dissociation on a tandem time-of-flight instrument. The product-ion spectra give structurally informative fragment-ion patterns. The sensitivity of the analytical method is such that steroids structures can be determined from low pg (low fmole) amounts of sample. The utility of the method has been demonstrated by the analysis of oxysterols extracted from rat brain.

Introduction

Neutral steroids have traditionally been analysed by gas chromatography – mass spectrometry (GC-MS)¹. However, the inability to directly analyse steroid conjugates by GC-MS, the requirement to derivatise many functional groups prior to GC, and the low sample capacity of GC columns has lead many researches to search for alternative mass spectrometric methods for the analysis of steroids. With respect to neutral steroids, the absence of either a basic or acidic group in their structure results in poor ion yields upon either electrospray (ES) or matrix-assisted laser desorption/ionisation (MALDI)^{2,3}, making direct analysis by conventional desorption/ionisation mass spectrometry relatively insensitive^{4,5}. Alternative ionisation techniques include atmospheric pressure chemical ionisation (APCI), and the recently introduced methods of atmospheric pressure

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Supporting Information

Supplementary material available:

photoionisation (APPI)⁶⁻⁸, and desorption ionisation on silicon (DIOS)⁹. APCI is currently gaining popularity for steroid analysis, but in the positive-ion mode tends to lead to the formation of dehydrated protonated molecules with an inherent loss in structural information¹⁰⁻¹³.

For the analysis of selected steroid classes, advantage can be taken of their specific chemistry. For example, steroids with an alcohol functionality can be reacted with pentafluorobenzyl bromide to give pentafluorobenzyl ethers which when subjected to electron capture atmospheric pressure chemical ionisation (ECAPCI) dissociates by loss of the pentafluorobenzyl radical to give an alkoxide ion, equivalent to the $[M-H]^-$ ion of the underivatised alcohol, in high abundance¹⁴. Alternatively, alcohols can be converted to sulphate esters which are readily ionised by ES to give $[M-H]^-$ ions^{15,16}, to mono-(dimethylaminoethyl) succinyl esters which give abundant $[M+H]^+$ ions upon ES ionisation¹⁷, or ferrocenecarbamate esters which give $[M]^+$ ions upon electrochemical oxidation in the ES capillary¹⁸. For the specific analysis of oxosteroids Higashi et al have derivatised oxosteroids with 2-nitro-4-trifluoromethylphenylhydrazine converting them to the corresponding hydrazones¹⁹, which when analysed by ECAPCI give $[M]^-$ ions. Shackleton and colleagues have also reacted oxosteroids with hydrazine reagents converting oxosteroids to Girard T (GT) hydrazones²⁰. GT hydrazones contain a quaternary nitrogen and are positively charged, hence easily analysed by ES mass spectrometry. We have used a similar derivative, the Girard P (GP) derivative, for the specific analysis of oxosteroids by ES mass spectrometry²¹, while others have used the Girard derivative to enhance the analysis of progesterone in high performance liquid chromatography (HPLC) – ES assays^{22,23}.

When subjected to collision induced-dissociation (CID) at low collision-energy (<200 eV, i.e. energies accessible to ion-trap, tandem quadrupole and quadrupole-time-of-flight (Q-TOF) instruments), steroids tend to fragment by the loss of small neutral molecules, and at one or two specific ring positions via charge-mediated reactions²⁻⁴. In contrast under high collision-energy CID conditions, steroids which possess, or have been derivatised to possess, a strongly acidic, strongly basic or pre-charged group, tend to undergo charge-remote fragmentation (CRF) reactions^{2,24}. Strongly-acidic, -basic or pre-charged groups have the effect of localising charge, (e.g. in a steroid sulphate analysed under negative-ion conditions the charge on the $[M-H]^-$ ion is localised on the sulphate ester group) and under high-energy CID conditions such precursor-ions can become sufficiently activated to fragment by reactions remote from the site of charge, and by mechanisms not directly involving the charge (i.e. by CRF). These reactions proceed through high-energy channels which are not normally populated under low collision-energy conditions²⁵. Tomer and Gross demonstrated the utility of CRF for steroid analysis in their seminal 1988 paper on the high-energy CID of steroid sulphates²⁴. In this paper they defined the classic CRF patterns of steroids which allow their structural determination, and demonstrated that CRF reactions occur through-out the steroid ring system and within the side-chain, and thereby provide a wealth of structural information. Traditionally, high collision-energy (>1000 eV) CID has been performed on magnetic sector instruments including three and four sector instruments, while magnetic sector - orthogonal-acceleration (OA) TOF hybrids have been used at intermediate collision-energy (400 – 800 eV) with heavy collision gas atoms (e.g. Xe) to generate CID spectra with high collision-energy characteristics². However, the comparative difficulty of interfacing sector instrument with either an ES or MALDI ion-sources, the large foot-print of sector machines, and their requirement of an experienced operator has led to the demise of magnetic sector instruments in biological research.

The advantages of high-energy CID are now available on a new class of instrument the MALDI-TOF/TOF, in which two TOF mass analysers are arranged in series separated by a

collision-cell²⁶⁻²⁸. The first TOF is for precursor-ion selection, high-energy CID proceeds in the collision-cell, and the second TOF mass (m/z) analyses the product-ions. The TOF/TOF instrument is interfaced with a MALDI ion-source, taking advantage of the pulsed nature of this ionisation mode, and its compatibility with automation. In this report we describe, for the first time, the MALDI-high-energy CID of neutral steroids which have been derivatised to both improve their ionisation properties and to undergo informative CID reactions. The high sensitivity of the method and its structurally informative nature is illustrated for the analysis of oxysterols in brain.

Experimental

All solvents were of analytical grade. Water was from a Milli-Q water system (Millipore, Molsheim, France). The steroids dehydroepiandrosterone (DHEA, A⁵-3 β -ol-17-one, **I**), testosterone (A⁴-17 β -ol-3-one, **II**), [19,19,19-²H₃]testosterone ([19,19,19-²H₃]A⁴-17 β -ol-3-one, **III**), nortestosterone (E⁴-17 β -ol-3-one, **IV**), norgestrel (18-homo-E⁴-17 α -ethynyl-17 β -ol-3-one, **V**), cholesterol (C⁵-3 β -ol, **VI**), cholest-4-en-3-one (C⁴-3-one, **VII**), 22-oxocholesterol (C⁵-3 β -ol-22-one, **VIII**), 27-hydroxycholesterol (C⁵-3 β ,27-diol, **IX**), 24-hydroxycholesterol (C⁵-3 β ,24-diol, **X**), 7 β -hydroxycholesterol (C⁵-3 β ,7 β -diol, **XI**), 7 α ,25-dihydroxycholesterol (C⁵-3 β ,7 α ,25-triol, **XII**), 7 β ,25-dihydroxycholesterol (C⁵-3 β ,7 β ,25-triol, **XIII**), 7 α ,27-dihydroxycholesterol (C⁵-3 β ,7 α ,27-triol, **XIV**), [16,16,17(or20),22,22,23,23-²H₇]7 α ,27-dihydroxycholesterol ([16,16,17(or20),22,22,23,23-²H₇]C⁵-3 β ,7 α ,27-triol, **XV**), 20R,22R-dihydroxycholesterol (C⁵-3 β ,20R,22R-triol, **XVI**) were from Sigma Aldrich (**I**, **II**, **VI**, **VII**) or from previous studies in the Karolinska laboratory (Scheme S-1a, See Table S-1 for the steroid numbering system).

Extraction of Oxysterols from brain

In brief, oxysterols were extracted from 100 mg of rat brain as follows: 100 mg of brain was homogenised in ethanol (1 mL), the ethanol extract was centrifuged and the supernatant retained. The precipitate was ultrasonicated in 1 mL of methanol/dichloromethane (1:1, v/v), centrifuged and the supernatant added to that retained previously. The combined supernatants were dried and re-dissolved in 2 mL of methanol/dichloromethane/water (7:2:1, v/v). This solution was added to a Lipidex DEAP (diethylaminohydroxypropyl Sephadex LH-20, PerkinElmer) anion-exchange column in its acetate form²⁹ (7 × 0.4 cm) prepared in methanol/dichloromethane/water (7:2:1, v/v). The column was washed with 2 mL of the same solvent, followed by 1 mL of methanol/dichloromethane/water (2:2:1, v/v), and the “flow-through” and “wash” combined, dried, and re-dissolved in 2 mL of hexane/dichloromethane (2:8, v/v). This “neutral fraction” was then applied to a Unisil column (8 × 0.8 cm, 200 - 325 mesh, activated silicic acid, Clarkson Chem) prepared in hexane, and washed with 20 mL of hexane/dichloromethane (2:8, v/v) prior to sample application. Following sample application the column was washed with 80 mL of hexane/dichloromethane (2:8, v/v), and eluted with 10 mL of ethylacetate. The eluate was dried and re-dissolved in 50 μ L isopropanol.

Oxidation of 3 β -hydroxy- Δ^5 steroids to 3-oxo- Δ^4 steroids

The 3 β -hydroxy- Δ^5 steroids were oxidised with cholesterol oxidase essentially as described by Brooks et al.³⁰ Recombinant, lyophilized, cholesterol oxidase from *Brevibacterium* was obtained from Roche Diagnostics GmbH, Mannheim, Germany, through the courtesy of Dr Ingo Preuss. The enzyme from *Brevibacterium* was selected since, in contrast to the common enzyme from *Nocardia*, it also catalyzes the oxidation of 3 β -hydroxy- Δ^5 steroids of the C₁₉ and C₂₁ series³¹. Steroids of the latter type are important intermediates in steroid hormone biosynthesis with biological functions, (e.g. as neurosteroids in brain²⁹). The 3 β -hydroxy- Δ^5 steroids (1 μ g) were dissolved in 50 μ L of isopropanol, and 10 μ L of

cholesterol oxidase (1 mg/mL, 20U/mg protein) in 1 mL of buffer (50 mM KH₂PO₄, pH 7) added, the mixture was incubated at room temperature (25 °C, 2 - 12 hr) and subsequently used as the starting solution for reaction with the GP reagent as described below. It is necessary to optimise the duration of incubation depending on the starting steroid. Steroids having additional substituents in the A-ring and steroids with no (C₁₉) or a short (C₂₁) side-chain react much slower than cholesterol (see ref. 31 for a Table of reaction rates for different steroids). We investigated the effect of an extended reaction time on the oxidation of cholesterol and found that minor products with additional oxygens were formed when the oxidation time exceeded 5 h, indicating that the cholest-4-en-3-one underwent further oxidation. In initial studies, designed to study the extent of the oxidation reaction, the enzyme reaction was stopped after incubation by the addition of 1 mL of methanol, and passed through a Sep-Pak C₁₈ cartridge (Waters, Milford, MA) prepared in water. The effluent and a 1 mL water-wash were combined and passed again through the same Sep-Pak cartridge. After washing with 1 mL of 20% methanol the 3-oxo- Δ^4 steroids were eluted with 2 $\times$ 1 mL of methanol (and 1 mL chloroform/methanol, 1:1 (v/v) if needed). The extent of the oxidation reaction was followed by monitoring the appearance of the [M+H]⁺ ion of the resulting oxosteroid by ES-MS. The activity of the enzyme was additionally evaluated with 3 β -hydroxycholest-5-en-27-oic and 3 β -hydroxychol-5-en-24-oic acids. Both of these acids will give [M-H]⁻ ions upon negative-ion ES ionisation, and hence their oxidation reactions can be monitored by a decrease in [M-H]⁻ ion current, and the complimentary increase in ion current of their oxidation products (2 Da lower in mass). Under the reaction conditions employed the oxidation reactions were found to proceed to completion.

Derivatisation of 3-oxo- Δ^4 steroids

The derivatisation of oxosteroids to GP hydrazones was carried out essentially as described by Shackleton et al²⁰ and by Wheeler³² (Equation 1). The reaction mixture from the oxidation step above was used directly after incubation with enzyme, with out any further treatment. The oxidation mixture, 1 mL (~: 50 mM phosphate buffer, 5% isopropanol, 10 μ g enzyme and 1 μ g steroids) was diluted with 2 mL of methanol to give a ~ 70% methanol solution, and 150 mg of GP hydrazine (Sigma-Aldrich) and 150 μ L of glacial acetic acid were added. The mixture was left at room temperature overnight.

In initial studies, when the derivatisation reaction was applied to reference compounds, the next step was to dry the steroid GP hydrazone under a stream of nitrogen gas and reconstitute in a solution of 10% aqueous methanol (1 mL). The steroid GP hydrazone was then separated from excess reagent by extraction on a Sep-Pak C₁₈ cartridge. After washing with 10% aqueous methanol (2 mL), the GP hydrazone was eluted from the cartridge with methanol (1 mL). The efficiency of the derivatisation was monitored by using 3-oxocholest-4-en-27-oic acid and 3-oxochol-4-en-24-oic acids, which in their underivatised forms give [M-H]⁻ ions in ES spectra. The reaction was regarded to be complete when the signals for the underivatised acids fell to zero.

In order to avoid sample loss in the solvent exchange step prior to solid phase extraction using the C₁₈ cartridge, the extraction step above was modified for the analysis of oxysterols from brain. The modified procedure was then subsequently used in all further studies. Therefore, for brain samples (and for reference compounds) the GP reaction mixture (~ 3 mL 70% methanol) after overnight incubation was directly applied to a SepPak C₁₈ bed (1 cm $\times$ 0.8 cm in a glass column) followed by 1 mL of 70% methanol and 1 of mL 35% methanol. The combined effluent (now 5 mL) was diluted with 4 mL of water. The resulting mixture (now 9 mL in 35% methanol) was again applied to the column followed by a wash

with 1 mL of 17% methanol. To the combined effluent 9 mL of water was added. The sample was then in 19 mL of about 17.5% methanol. This was again applied to the column followed by a wash with 10 mL of 10% methanol. Now all the GP derivatives are extracted by the column. They were then eluted with two portions of 1 mL of methanol followed by 1 mL of chloroform/methanol, 1:1 (v/v). The three fractions were analysed separately by ES mass spectrometry. The GP derivatives were to be found predominantly in the second mL of methanol and the results of the analysis of this fraction are described below.

The derivatisation protocol has been applied to mixtures of oxosteroids on the $\mu\text{g-ng}$ level and is suitable for the low level (pg) derivatisation of neutral steroids extracted from tissue^{33,34}. This is illustrated in the current study where oxysterols in brain were oxidised and derivatised on the ng level.

Mass Spectrometry

MALDI—All MALDI spectra were recorded on an Applied Biosystems 4700 Proteomics Discovery System MALDI-TOF/TOF mass spectrometer. Derivatised steroids in methanol were mixed 1:1 (v:v) with a solution of matrix (α -cyano-4-hydroxycinnamic acid, 10 g/L in 50% acetonitrile 0.1% trifluoroacetic acid) and 1 μL aliquots of the mixture spotted on to the MALDI target plate. For the acquisition of CID spectra the collision-energy was 1 keV and air was used as the collision-gas at a pressure of 9×10^{-6} Torr. Spectra were calibrated using default calibration.

Electrospray

ES mass spectra were recorded on a Q-TOF Global mass spectrometer interfaced to a Cap-LC low flow-rate chromatography system (both Waters). Separation was achieved on a C₁₈ capillary column (PepMap C₁₈ column 180 $\mu\text{m} \times 150$ mm, 3 μm , 100 A, Dionex), at a flow-rate set at 1 $\mu\text{L}/\text{min}$. Mobile phase A was 50% methanol, 0.1% formic, and phase B was 95% methanol, 0.1% formic acid. Gradient elution was performed starting at 5% B and rising to 60% B over the first 10 min, increasing to 80% B during the next 5 min, and staying at 80% B for a final 10 min, before returning to 5% B.

Results

Enhancement of Ion Signal Upon Derivatisation

In a previous study using MALDI-MS we have shown that derivatisation of 3-oxo- Δ^4 steroids to GP hydrazones enhances their ionization by a factor of at least 10035. This was again evident in the current study, where e.g. the ionisation efficiency of testosterone (**II**) was increased by approximately two orders of magnitude following derivatisation with GP reagent (Fig. S-1).

Of the neutral steroids, those possessing a 3-oxo- Δ^4 group are found to be ionised more effectively than those possessing an unconjugated ketone group³⁵; and sterols, with only an alcohol function are ionised even less efficiently. For example in the MALDI mass spectrum of cholesterol (**VI**), there was no evidence for the formation of an $[\text{M}+\text{H}]^+$ ion at m/z 387, however, the dehydrated protonated molecule was found to give a peak at m/z 369 (Fig. S-1c). Oxidation of cholesterol to cholest-4-en-3-one (**VII**) enhances MALDI response (Fig. S-1d), and further gains in ion current are obtained upon derivatisation of cholest-4-en-3-one to its GP hydrazone (Fig. S-1e). An additional advantage of the incorporation of the GP hydrazone group into a steroid molecule is that the mass of the steroid ion is elevated by 133 Da moving it into a region of the MALDI mass spectrum less clustered by chemical noise.

Fragmentation of Underivatised Steroids

The fragmentation of underivatised steroids with a 3-oxo- Δ^4 structure has previously been investigated at both high and low collision-energy^{2,4}. The enhanced basicity of the 3-oxo group as a result of conjugation with the 4 - 5 double bond, leads to preferential protonation at this group in the $[M+H]^+$ ion, and the majority of fragment ions formed contain the 3-oxo group and a least part of the A-ring. This is illustrated in Fig. 1a, which shows the MALDI high-energy CID spectrum of the $[M+H]^+$ of testosterone (**II**). The ions at m/z 97, 109 and 123 are characteristic of 3-oxo- Δ^4 steroids and correspond to b_1' -12, b_3' -28 and b_2' fragments⁴ (Scheme 1). Additional fragment ions at m/z 253 and 271 correspond to $[M+H-36]^+$ and $[M+H-18]^+$ ions resulting from the loss of two and one water molecule from the $[M+H]^+$ ion respectively (Scheme 1).

Scheme 1. Fragmentation of $[M+H]^+$ ions of underivatised 3-oxo- Δ^4 steroids. A prime to the right of a fragment describing letter indicates that cleavage proceeds with the fragment ion containing the added proton.

Fig. 1. MALDI-MS/MS spectra of (a) testosterone (**II**) $[M+H]^+$ m/z 289; (b) cholesterol (**VI**) $[M+H-H_2O]^+$ m/z 369.

Sterols containing just an alcohol group (e.g. cholesterol), tend to become dehydrated in the MALDI process and give dehydrated protonated molecules (e.g. $[M+H-H_2O]^+$ at 369 for cholesterol, Fig. S-1c). The CID spectra of these ions are complex (Fig. 1b), and their interpretation is not straight forward.

Fragmentation of Derivatised Steroids

Initial experiments were performed on derivatised steroids with a simple structure i.e. dehydroepiandrosterone (**I**), testosterone (**II**), [19,19,19-²H₃]testosterone (**III**) and nortestosterone (**IV**) (Table S-1 & Scheme S-1a). DHEA is a 17-oxo- Δ^5 steroid while testosterone and nortestosterone are both 3-oxo- Δ^4 steroids. High-energy CID of the GP derivatives of these steroids give a series of fragment ions characteristic of the derivatising group at m/z 80 (σ_1'), 93 (σ_2), 120 (σ_3), 135 (σ_4) and 137 (σ_4') (Figs. 2 & 3, Schemes 2 & 3). Additionally neutral losses to give fragment ions corresponding to $[M-79]^+$ and $[M-107]^+$ occur. When the GP group is located at the C-17 position 0B_3 (m/z 284) and 0C_3 (m/z 230) fragment ions are observed due to cleavage at the B/C and C/D ring junctions (Fig. 2, Scheme 2). These cleavages are characteristic of the high-energy CID of steroids when charge is localised at C-17 or on the C-17 side-chain². The high-energy CID spectrum of DHEA GP hydrazone also shows fragment ions at m/z 253 and 271 which correspond to carbonium ions of the triply unsaturated ABCD ring system and the doubly unsaturated monohydroxylated ABCD ring structure (Scheme 2, cf Fig. 1a & Scheme 1). The high-energy CID spectra of the 3-oxo- Δ^4 GP hydrazones also show structurally informative ring-fragment ions which are discussed below.

Scheme 2. Fragmentation of DHEA GP hydrazone. The fragment ions S_1 ($[M-79]^+$) and S_{1-28} ($[M-107]^+$) are drawn with the derivatising group in a linear form, alternative structures can be drawn with the derivatising group as part of a five- or four-membered ring attached to C-17.

Scheme 3. Fragmentation of 3-oxo- Δ^4 GP hydrazones. The fragment ions S_1 ($[M-79]^+$) and S_{1-28} ($[M-107]^+$) are drawn with the derivatising group in a cyclic form, alternative structures can be drawn with the derivatising group in a linear form. The inset shows fragmentation of the C-17 side-chain in C_{27} sterols. Fragment ions containing the complete GP derivatising group are indicated by a superscript zero e.g. 0d_1 , fragment ions derived

from the $[M-79]^+$ ion are indicated by an asterisk e.g. $*d_1$, and fragment ions derived from the $[M-107]^+$ ion by a superscript hatch e.g. $\#d_1$.

Fig. 2. MALDI-MS/MS of DHEA GP hydrazone m/z 422. A prime to the right of the fragment describing letter indicates that fragmentation proceeds with H-transfer to the ion, a prime to the left of the fragment describing letter indicates that fragmentation proceeds with H-transfer from the ion to the neutral leaving group.

Fig. 3. (a) MALDI-MS/MS of testosterone GP hydrazone m/z 422. (b) Expanded view of the m/z range 70 – 200.

3-Oxo- Δ^4 GP Hydrazones: Ring Fragmentation

It is of value to consider the fragmentation channels which are common to 3-oxo- Δ^4 GP hydrazones prior to a discussion of more complex steroids possessing this and additional functional moieties. 3-Oxo- Δ^4 GP hydrazones which do not possess any extra functional groups on the fundamental steroid ring structure give characteristic fragment ions at m/z 123 ($\#b_1-12$), 135 ($\#b_3-28$), 149 ($\#b_2$), 151 ($*b_1-12$), 163 ($*b_3-28$) and 177 ($*b_2$) (Scheme 3 & 4, Table S-1, Fig. 3a & 3b). This series of fragment ions has previously been observed in spectra recorded at intermediate and low collision-energy^{21,36}. The b_1-12 type fragments are suggested to consist of the A-ring plus the C-19 methyl group but minus C-5 (Scheme 3 & 4). The b_2 type fragments are proposed to consist of the A-ring plus the C-19 methyl group and the methylene group at C-6. Evidence from isotope labelling studies indicates that the b_3-28 type ions consists of the A-ring plus C-6 and C-7, but minus C-19 and C-10, additionally a H-atom is lost from C-6^{21,36}. The interpretation of these b-type fragmentations is supported in the current high-energy CID study by the spectra of [19,19,19- 2H_3]testosterone (**III**), nortestosterone (**IV**), and norgestrel (**V**) GP hydrazones (Figs. S-2 & S-3, Table S-1). The peak normally observed m/z 123 ($\#b_1-12$) in the spectra of 3-oxo- Δ^4 GP hydrazones is shifted to m/z 126 ($\#b_1-12$) in the spectrum of [19,19,19- 2H_3]testosterone GP indicating that this ion contains the C-19 methyl group; while in the spectrum of nortestosterone GP (and norgestrel GP) the peak is shifted to m/z 109 ($\#b_1-12$), on account of a H-atom being attached to C-10 rather than the C-19 methyl group. Similarly, the peak normally observed at m/z 151 ($*b_1-12$) in the spectra of 3-oxo- Δ^4 GP hydrazones is shifted to m/z 154 ($*b_1-12$) in the spectrum of [19,19,19- 2H_3]testosterone GP and m/z 137 ($*b_1-12$) in the spectrum of nortestosterone GP (and norgestrel GP). The peaks normally observed at m/z 135 ($\#b_3-28$) and 163 ($*b_3-28$) in the spectra of 3-oxo- Δ^4 GP hydrazones are not shifted in mass in the spectra of either [19,19,19- 2H_3]testosterone GP or nortestosterone GP (or norgestrel GP). This indicates that these fragments do not contain the C-19 methyl group. The $\#b_2$ and $*b_2$ fragments are expected to contain the C-19 methyl group or in the case of nortestosterone GP (and norgestrel GP) a 10β -hydrogen, hence their mass should be elevated by 3 Da in the spectra of [19,19,19- 2H_3]testosterone GP (i.e. $\#b_2$ m/z 149 $\rightarrow$ 152; $*b_2$ m/z 177 $\rightarrow$ 180), and decreased by 14 Da in nortestosterone GP (and norgestrel GP) (i.e. $\#b_2$ m/z 149 $\rightarrow$ 135; $*b_2$ 177 $\rightarrow$ 163).

Scheme 4. Structures of $*b_1-12$, $*b_2-14$, $*b_3-28$ and $*b_2$ fragment ions from 3-oxo- Δ^4 GP hydrazones. The structures of the $\#b_1-12$, $\#b_2-14$, $\#b_3-28$ and $\#b_2$ fragment ions are similar to those shown but do not contain the CO group.

It should be noted that the peak at m/z 135 is a composite of σ_4 (135.05584) and $\#b_3-28$ (135.09222) fragments, this was confirmed by performing MS/MS experiments on 3-oxo- Δ^4 steroids derivatised with the GT hydrazine reagent (GT derivatives do not give the σ_4 fragment at m/z 135) and 17-oxo steroid GP hydrazones (do not give the $\#b_3-28$ fragment). However, the resolution of the current instrument was insufficient to resolve these fragment

(in Table S-1 fragments with one dominant component are in bold, those possibly containing more than one component in normal font).

In addition to the b-type fragment ions discussed above which have been reported to be present in CID spectra recorded at intermediate³⁶, and low collision-energy²¹, the CID spectra of 3-oxo- Δ^4 GP hydrazones recorded in the current study show fragment ions which are formed by cleavages characteristic of high-energy CID² (Table S-1, Fig. 4). Fragment ions formed by cleavage at the C/D ring junction give fragment ions at m/z 257 ($^{\#}d_1$), 285 (*d_1) and 364 (0d_1) (Scheme 3), these ions are elevated by 3 Da in the spectrum of [19,19,19- 2H_3]testosterone GP (Fig. S-2) and decreased by 14 Da in the spectrum of nortestosterone GP (Fig. S-3) (In norgestrel the presence of the 18-homo group i.e. an ethyl group attached to C-13; makes up for the absence of the C-19 methyl group and the d-type ions are not shifted in mass from steroids with the “fundamental” structure). A further fragmentation characteristic of the high-energy CID of steroids involves loss of the C-17 substituent with the concomitant loss of the C-13 substituent (i.e. C-18) to give e-15 type fragment ions^{2,24,25}. Ions of this type are observed at m/z 283 ($^{\#}e-15$), 311 ($^*e-15$) and 390 ($^0e-15$) (Fig. 4a, Table S-1, Scheme 3). Again these ions are elevated by 3 Da in the spectrum of [19,19,19- 2H_3]testosterone GP and decreased by 14 Da in the spectrum of nortestosterone GP.

Fig. 4. MALDI-MS/MS of C⁴-27-ol-3-one (**XVIII**) GP hydrazone m/z 534. (a) Full m/z range; (b) expanded view of m/z 69 – 200; (c) expanded view of m/z 200 – 410; (d) expanded view of m/z 410 – 525.

Fig. 5. MALDI-MS/MS of C⁴-7 β -ol-3-one (**XX**) GP hydrazone m/z 534. (a) Full m/z range; (b) expanded view of m/z 70 – 200; (c) expanded view of m/z 200 – 410; (d) expanded view of m/z 410 – 525.

The Effect of 7-Hydroxylation

Introduction of a hydroxyl group at C-7 changes the pattern of b-type fragments. Incorporation of a hydroxyl group at C-7 will shift the $^{\#}b_3-28$ and $^*b_3-28$ fragment ions from m/z 135 and 163 to m/z 151 and 179 respectively (cf. Fig. 4 & 5 Table S-1). The $^{\#}b_2$ and *b_2 at m/z 149 and 177 are not changed in mass, while the $^{\#}b_1-12$ (m/z 123) and $^*b_1-12$ (m/z 151) become the most abundant b-type ions. The elevated abundance of ions m/z 151 relative to those of m/z 163 is a signature of 7-hydroxylation. A similar change in the pattern of b-type fragment ions has also been observed at low collision-energy³⁷. It should be noted that the continued presence of ions at m/z 163 is due to a weak reaction channel leading to $^*b_2-14$ ions, and that a reaction channel giving $^{\#}b_2-14$ ions will give a peak at m/z 135, as will the $^{\sigma}_4$ fragment ions (Schemes 3 & 4).

Unlike spectra recorded at low collision-energy, d_1 and e-15 type fragments are observed in the spectra recorded at high collision-energy (Fig. 4). Incorporation of the 7-hydroxyl group into the steroid skeleton results in these ions being shifted up in mass by 16 Da, i.e. $^{\#}d_1$ to 273, *d_1 to 301, 0d_1 to 380; $^{\#}e-15$ to 299, $^*e-15$ to 327 and $^0e-15$ to 406 (Fig. 5, Table S-1).

Side-Chain Cleavage

Biologically important oxysterols may possess oxygen containing substituents on the C-17 side-chain, hence side-chain fragmentation may provide structurally important information.

Once it was established that the spectra recorded at 1000 eV in the present study showed features characteristic of high-energy CID, a search was made for ions formed by side-chain cleavage, remote from the site of charge, to give terminally unsaturated fragments (i.e. formed via classic 1,4- H_2 elimination mechanism²⁵). In 3-oxo- Δ^4 C₂₇ steroid GP

hydrazones fragment ions formed by side-chain cleavage of the C-17 – C-20 ($^{\circ}\text{e}$, m/z 404), C-20 – C-22, ($^{\circ}\text{f}$, m/z 432) and C-22 – C-23 ($^{\circ}\text{g}$, m/z 446) bonds are observed (Fig. 4, Table S-1, Scheme 3). The presence of an alcohol group in the steroid ring system will cause this series of fragment ions to be shifted up in mass by 16 Da (Fig. 5). Fragment ions derived from the $[\text{M}-79]^+$ and $[\text{M}-107]^+$ intermediates are also generated but unfortunately give some ambiguity in their interpretation, (e.g. for 3-oxo- Δ^4 GP hydrazones without further substitution in the steroid ring $^{\circ}\text{e}$ and $^{\circ}\text{f}$ both have a mass of 325 Da, while $^{\circ}\text{f}$ and $^{\circ}\text{h}$ fragments have a mass of 353 Da (Fig. 4, Table S-1)). The resolution of instrument is insufficient to resolve these closely lying components. While the presence of $^{\circ}\text{e}$ – $^{\circ}\text{g}$ type fragments should allow the localisation of substituents on side-chain carbons 20 – 22, differentiation of isomers differing by hydroxylation on C-23, C-24, C-25 or C-26 is more difficult (Table S-1).

The presence of two hydroxyl groups on the C-17 side chain as in C^4 -20R,22R-diol-3-one (**XXV**) GP hydrazone results in a quite specific fragmentation pattern (Fig. S-5). The $^{\circ}\text{e}$ fragment is not affected by the presence of the vicinal diol and appears at m/z 404, but the $^{\circ}\text{f}$ fragment is moved up in mass by 16 Da to m/z 448. A $^{\circ}\text{g}$ fragment can not be formed in a simple 1,4- H_2 elimination cleavage process due to the absence of a H-atom on C-20, but rather a $^{\circ}\text{g}-16$ (m/z 462) ion is formed. This triad of fragment ions provides a signature for the 20,22 diol, and is complimented by similar triads derived from $[\text{M}-79]^+$ and $[\text{M}-107]^+$ intermediates. The presence of the 20R,22R group also results in cleavage of the C-22 – C-23 bond but with charge residing on the C-23 containing fragment giving an ion derived from $[(\text{CH}_3)_2\text{CHCH}_2\text{CH}_2]^+$ at m/z 71. A ketone group at C-22 as in C^4 -3,22-dione (**XVII**) GP hydrazone also results in the formation of a fragment ion at m/z 71, due to cleavage of the C-22 – C-23 bond (Fig. S-8).

3-Oxo- Δ^4 dihydroxysteroid GP hydrazones with a 7-hydroxy group, and a second hydroxyl group on C-25 or C-27 undergo side-chain cleavage of the C-17 – C-20 ($^{\circ}\text{e}$, m/z 420), C-20 – C-22, ($^{\circ}\text{f}$, m/z 448), C-22 – C-23 ($^{\circ}\text{g}$, m/z 462) bonds; these fragments are 16 Da heavier than in equivalent sterols without 7-hydroxylation. (Fig. S-6, Table S-1). Fragment ions derived from the $[\text{M}-79]^+$ and $[\text{M}-107]^+$ intermediates are also generated, but again unfortunately give some ambiguity in their interpretation (e.g. $^{\circ}\text{e}$ and $^{\circ}\text{f}$ both have a mass of 341 Da, while $^{\circ}\text{f}$ and $^{\circ}\text{h}$ fragments have a mass of 369 Da (Fig. S-6)). While it is a straight forward matter to localise one hydroxyl group to C-7 (from the pattern of b-type fragment ions), and to conclude that the second hydroxyl group is on one of the side-chain carbons beyond C-22 (from the $^{\circ}\text{e}$ – $^{\circ}\text{g}$ fragments), determination of its exact location is more difficult, and requires spectra of the highest quality on account of the low abundance of the necessary structurally informative fragment ions i.e. $^{\circ}\text{h}$, $^{\circ}\text{i}$, $^{\circ}\text{j}_1$, $^{\circ}\text{j}_2$ (Scheme 3). This is evident in Fig. S-6, which shows the CID spectrum of 7 β ,25-dihydroxycholesterol (**XIII**) following cholesterol oxidase treatment and derivatisation to give C^4 -7 β ,25-diol-3-one (**XXII**) GP hydrazone. The fragment ions $^{\circ}\text{h}$ at m/z 476, and $^{\circ}\text{i}$ at m/z 490 indicate that the second hydroxyl group must be on C-25 or C-27, and its location to C-25 is given by $^{\circ}\text{j}_1$ and $^{\circ}\text{j}_2$ fragments both giving a peak at m/z 534. In the spectrum of the 7,27-diol the $^{\circ}\text{j}_1$ fragment appears to m/z 518, indicating that there is an hydroxyl group on C-27. In the spectrum of the [16,16,17(or 20),22,22,23,23- $^2\text{H}_7$] isotopomer of the 7,27-diol, the $^{\circ}\text{j}_1$ fragment appears at m/z 525 (Fig. S-7).

The observation above that oxidised and derivatised 27-hydroxysterols give a $^{\circ}\text{j}_1$ fragment corresponding to the loss of CH_4O (-32 Da), while other sterols give $^{\circ}\text{j}$ and $^{\circ}\text{j}'$ fragments corresponding to the loss of 16 or 14 Da (CH_4 or CH_2) allows the identification of 27-hydroxysterols (Table S-1).

Separation of Oxysterols by Liquid Chromatography

While the detailed analysis of MS/MS spectra goes a considerable distance towards the identification of derivatised oxysterols, the exact differentiation between closely related isomers can be difficult. Further, the occurrence of more than one isomer in a biological sample is possible making a separation step prior to mass spectrometry desirable. Commercial instrumentation is now available to collect low-volume fractions from a nano-LC column and spot them onto a MALDI target plate for subsequent mass spectrometric analysis³⁸. In this preliminary report we have not performed such studies, however we have developed a capillary-LC method that allows the separation of oxysterols and could be used in a future LC-MALDI study. Using a methanol/water/0.1% formic acid gradient the oxidised and derivatised monohydroxycholesterols products C⁴-24S-3-one (**XIX**) GP, C⁴-24R-3-one (**XIX**) GP, C⁴-27-ol-3-one (**XVIII**) GP and C⁴-7 β -ol-3-one (**XX**) GP were fully resolved (Fig. 6), while the dihydroxycholesterol derivatives were partially separated from one another.

Fig. 6. LC-MS separation of C⁴-7 β -ol-3-one (**XX**), C⁴-27-ol-3-one (**XVIII**), C⁴-24S-ol-3-one and C⁴-24R-3-one (**XIX**) GP hydrazones. RIC for m/z 534, C⁴-7 β -ol-3-one (**XX**) GP is shown in purple, C⁴-27-ol-3-one (**XVIII**) GP is in red, C⁴-24S-ol-3-one (**XIX**) GP and C⁴-24R-ol-3-one (**XIX**) GP are in green. C⁴-24S-ol-3-one GP elutes before C⁴-24R-ol-3-one GP. The RIC of m/z 534 from brain is shown in black. Separation was achieved on a C₁₈ capillary column (PepMap C₁₈ column 180 μ m $\times$ 150 mm, 3 μ m, 100 A, Dionex), at a flow-rate set to 1 μ L/min. Mobile phase A was 50% methanol, 0.1% formic acid, and B was 95% methanol, 0.1% formic acid. Gradient elution was performed starting at 5% B and rising to 60% B over the first 10 min, increasing to 80% B during the next 5 min, and staying at 80% B for a final 10 min, before returning to 5% B.

Identification of Oxysterols in Rat Brain

Shown in Fig. 7 is the MALDI mass spectrum of the oxysterol fraction from rat brain, following oxidation with cholesterol oxidase and derivatisation with GP hydrazine. The spectrum was recorded from the equivalent of 100 μ g of brain loaded on the MALDI plate. By considering the exact mass of the peaks considerable information is immediately evident³⁹. Peaks at m/z 518.410, 534.407 and 550.401 correspond in mass to mono-GP derivatives of C⁴-3-one (**VII**) (calc 518.41104; Δ -1 mDa, -2 ppm), C⁴-ol-3-one (calc 534.40595, Δ 1 mDa, 2 ppm), and C⁴-diol-3-one (calc 550.40087, Δ 0.1 mDa, 0.2 ppm), and the peak at m/z 665.455 to the double derivative of a C⁴-dione (calc 665.45430, Δ 0.7 mDa, 1 ppm). To identify the structures of these ions MS/MS spectra were recorded and are shown in Fig. 8 and S-9. Fragment ions at m/z 80 (σ_1^+), 93 (σ_2^+) and 120 (σ_3^+) and neutral losses of 79 Da and 107 Da are indicative of a GP hydrazone, while the series of fragment ions at m/z 123 ($^{\#}b_1-12$), 135 ($^{\#}b_3-28$), 151 ($^*b_1-12$), 163 ($^*b_3-28$) and 177 (*b_2) are characteristic of a derivatised 3-oxo- Δ^4 steroid. These ions were observed in the MS/MS spectra of the four precursors (Fig. 8 and S-9). Additionally, ions at m/z 364 ($^o d_1$) and 390 ($^o e-15$) were present in the MS/MS spectra of the precursors at m/z 518 and 534 (Fig. S-9 & 8), indicating a steroid ring system without any further modifications. The comparatively high abundance of the precursor-ion of m/z 534 allowed the acquisition of a detailed MS/MS spectrum. The MS/MS spectrum of this ion from the brain extract is essentially identical to that from the reference standard of oxidised and derivatised 24-hydroxycholesterol (cf Fig. 8 & S-4, Table S-1). Fragments formed as a result of side-chain cleavages of the C-17 – C-20 ($^o e-15$, m/z 390), C-20 – C-22, ($^o f$, m/z 432), C-22 – C-23 ($^o g$, m/z 446) are weak but evident (Fig. 8, Table S-1). These, and ions at m/z 325 ($^* e$ and/or $^{\#} f$) and 353 ($^* f$ and/or $^{\#} h$) confirm the presence of a hydroxyl group on C-23, C-24, C-25 or C-27. The absence of a fragment corresponding to the loss of CH₄O (-32 Da) at m/z 502, but rather ions at m/z 520 and 518 corresponding to the loss of the terminal CH₂ group ($^o j^+$) and CH₄ group ($^o j$)

indicate that hydroxylation has occurred at C-24 or C-25. The final identification of this oxysterol as C⁴-24S-ol-3-one (**XIX**) GP, the product of oxidation and derivatisation of 24S-hydroxycholesterol (**X**) was made by comparison of its LC retention time with that of an authentic standard (Fig. 6).

Fig. 7. MALDI mass spectrum of oxysterol fraction from rat brain, following oxidation with cholesterol oxidase and derivatisation with GP hydrazine.

Fig. 8. MALDI-MS/MS spectrum of a monohydroxycholesterol isolated from rat brain, treated with cholesterol oxidase and derivatised with GP hydrazine to give an ion at m/z 534. (a) Full m/z range; (b) expanded view of m/z 70 - 200; (c) expanded view of m/z 200 - 410; (d) expanded view of 410 - 525.

The characterisation of the precursor ions at m/z 550 and 665 is more difficult (Figs S-10). LC-MS analysis of the brain sample showed the presence of many different compounds giving rise to a peak at m/z 550 (data not shown), and the spectrum shown in Fig. S-10 is a composite of at least five isobaric precursors. The presence of a weak peak at m/z 353 in the MS/MS spectrum suggests that at least one of these components possesses a 3-oxo- Δ^4 structures with two hydroxyl groups in the side-chain possibly at C-24 and C-25 (**XXVI**). The MS/MS spectrum of the precursor at m/z 665 is not of sufficient quality to interpret with certainty and it can only be concluded that a C⁴-3-one steroid is present with an additional ketone group.

It is important to note that endogenous 3-oxo- Δ^4 sterols may be present in brain. They should be analysed following extraction from brain and derivatisation with GP hydrazine directly, without treatment with cholesterol oxidase. In the current preliminary study, endogenous oxysterols with a 3-oxo- Δ^4 structure were not detected.

Discussion

In this study we have demonstrated the benefit of derivatisation of 3-oxo- Δ^4 steroids with GP hydrazine for MALDI analysis. The resulting GP hydrazones, which possess a quaternary nitrogen, give abundant $[M]^+$ ions in the MALDI process and an improvement in the sensitivity of analysis of about two orders of magnitude in comparison with the underivatised analogues. Additionally 3-oxo- Δ^4 GP hydrazones give structurally informative high-energy CID spectra.

The main focus of this work is towards the development of methods for the analysis of steroids in brain. While neurosteroids possessing a ketone group, (e.g. testosterone (**II**), DHEA (**I**), progesterone), will be readily derivatised with the GP reagent, others without this functionality will not. However, treatment of steroids possessing a 3 β -hydroxy- Δ^5 or 5 α -hydrogen-3 β -hydroxy structure with cholesterol oxidase from *Brevibacterium* will convert these steroids to 3-oxo- Δ^4 and 3-oxo steroids respectively, now suitable for derivatisation with the GP reagent. This methodology has been illustrated for the analysis of oxysterols in brain.

Underivatised sterols are difficult to ionise in the MALDI process giving only low abundance $[M+H-H_2O]^+$ ions, which fragment by CID to give a plethora of fragment ions (Fig. 1b). Alternatively, sterols first oxidised with cholesterol oxidase and then derivatised with GP hydrazine give high abundance $[M]^+$ ions and informative CID spectra. Using this methodology we have been able to analyse oxysterols in rat brain. With the aid of LC-MS, 24S-hydroxycholesterol was confirmed as the most abundant oxysterol in brain⁴⁰, in addition two other oxysterols, one a dihydroxycholesterol with both hydroxyl groups in the side-chain, and the second a ketocholesterol were partially characterised. By using LC-MS/

MS and comparison with reference standards, the level of 24-hydroxycholesterol in brain was determined to be approximately 1 μg / 100 mg, and that of the dihydroxycholesterol to be 2 ng / 100 mg.

It is of value to point to the advantages provided by high-energy CID in this study. With the exception of the MALDI-TOF/TOF instrument few modern mass spectrometers employ high-energy CID. High-energy CID has advantages over its low-energy counterpart in metabolite, and particularly lipid analysis². While low-energy CID spectra are often dominated by charge-mediated fragmentation processes, high collision-energy provides access to additional fragmentation channels allowing CRF fragmentation^{2,25}. In the current study we observe the classical high-energy d_1 type ring fragmentation, the e-15 type fragment, and also other terminally unsaturated C-17 side-chain fragment ions in the MS/MS spectra of the 3-oxo- Δ^4 GP hydrazones (Scheme 3). These ions provide considerable extra structural information to compliment that provided by the b-type fragment ions. Fragment ions formed by cleavage of the side-chain are often weak, but are clearly discernable when spectra are plotted on an expanded scale (e.g. Fig. S-6). This is not unlike the situation for high-energy CID spectra recorded on magnetic sector instruments, where the fragment-ion abundance is often only a few percent of the precursor. Additionally the good mass accuracy of fragment ion measurement (~ 0.05 Da) provided by the TOF/TOF allows the differentiation of true fragments from contaminating matrix ions co-selected with the desired precursor.

In conclusion, analysis of steroids and sterols with a 3-oxo- Δ^4 or 5 α -hydrogen-3 β -hydroxy structure, including oxysterols is greatly enhanced by oxidation (where necessary) and derivatisation with GP hydrazine. High-energy CID of the resulting GP hydrazones allows structural determination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

Fragmentation of $[M+H]^+$ ions of underivatised 3-oxo- Δ^4 steroids. A prime to the right of a fragment describing letter indicates that cleavage proceeds with the fragment ion containing the added proton.

Scheme 2.

Fragmentation of DHEA GP hydrazone. The fragment ions S_1 ($[M-79]^+$) and S_{1-28} ($[M-107]^+$) are drawn with the derivatising group in a linear form, alternative structures can be drawn with the derivatising group as part of a five- or four-membered ring attached to C-17.

Scheme 3.

Fragmentation of 3-oxo- Δ^4 GP hydrazones. The fragment ions S_1 ($[M-79]^+$) and S_{1-28} ($[M-107]^+$) are drawn with the derivatising group in a cyclic form, alternative structures can be drawn with the derivatising group in a linear form. The inset shows fragmentation of the C-17 side-chain in C_{27} sterols. Fragment ions containing the complete GP derivatising group are indicated by a superscript zero e.g. $^{\circ}d_1$, fragment ions derived from the $[M-79]^+$ ion are indicated by an asterisk e.g. *d_1 , and fragment ions derived from the $[M-107]^+$ ion by a superscript hatch e.g. $\#d_1$.

Scheme 4.

Structures of *b₁-12, *b₂-14, *b₃-28 and *b₂ fragment ions from 3-oxo- Δ^4 GP hydrazones. The structures of the #b₁-12, #b₂-14, #b₃-28 and #b₂ fragment ions are similar to those shown but do not contain the CO group.

Fig. 1. MALDI-MS/MS spectra of (a) testosterone (II) $[M+H]^+$ m/z 289; (b) cholesterol (VI) $[M+H-H_2O]^+$ m/z 369.

Fig. 2. MALDI-MS/MS of DHEA GP hydrazone m/z 422. A prime to the right of the fragment describing letter indicates that fragmentation proceeds with H-transfer to the ion, a prime to the left of the fragment describing letter indicates that fragmentation proceeds with H-transfer from the ion to the neutral leaving group.

Fig. 3.

(a) MALDI-MS/MS of testosterone GP hydrazone m/z 422. (b) Expanded view of the m/z range 70 – 200.

Fig. 4. MALDI-MS/MS of C^4 -27-ol-3-one (XVIII) GP hydrazone m/z 534. (a) Full m/z range; (b) expanded view of m/z 69 – 200; (c) expanded view of m/z 200 – 410, (d) expanded view of m/z 410 – 525.

Fig. 5. MALDI-MS/MS of C^4 -7 β -ol-3-one (XX) GP hydrazone m/z 534. (a) Full m/z range; (b) expanded view of m/z 70 – 200; (c) expanded view of m/z 200 – 410; (d) expanded view of m/z 410 – 525.

Fig. 6. LC-MS separation of C⁴-7 β -ol-3-one (**XX**), C⁴-27-ol-3-one (**XVIII**), C⁴-24S-ol-3-one and C⁴-24R-3-one (**XIX**) GP hydrazones. RIC for m/z 534, C⁴-7 β -ol-3-one (**XX**) GP is shown in purple, C⁴-27-ol-3-one (**XVIII**) GP is in red, C⁴-24S-ol-3-one (**XIX**) GP and C⁴-24R-ol-3-one (**XIX**) GP are in green. C⁴-24S-ol-3-one GP elutes before C⁴-24R-ol-3-one GP. The RIC of m/z 534 from brain is shown in black. Separation was achieved on a C₁₈ capillary column (PepMap C₁₈ column 180 μ m $\times$ 150 mm, 3 μ m, 100 A, Dionex), at a flow-rate set to 1 μ L/min. Mobile phase A was 50% methanol, 0.1% formic acid, and B was 95% methanol, 0.1% formic acid. Gradient elution was performed starting at 5% B and rising to 60% B over the first 10 min, increasing to 80% B during the next 5 min, and staying at 80% B for a final 10 min, before returning to 5% B.

Fig. 7. MALDI mass spectrum of oxysterol fraction from rat brain, following oxidation with cholesterol oxidase and derivatisation with GP hydrazine.

Fig. 8. MALDI-MS/MS spectrum of a monohydroxycholesterol isolated from rat brain, treated with cholesterol oxidase and derivatised with GP hydrazine to give an ion at m/z 534. (a) Full m/z range; (b) expanded view of m/z 70 - 200; (c) expanded view of m/z 200 - 410; (d) expanded view of 410 - 525.