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Analysis of derivatised steroids by matrix-assisted laser desorption/ionisation and post-source decay mass spectrometry

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ABSTRACT

Neutral steroids are difficult to analyse using desorption ionisation methods coupled with mass spectrometry (MS). However, steroids with an unhindered ketone group can readily be derivatised with the Girard P (GP) reagent to give GP hydrazones. Steroid GP hydrazones contain a quaternary nitrogen atom and are readily desorbed in the matrix-assisted laser desorption/ionisation (MALDI) process, giving an improvement in sensitivity of two orders of magnitude. Steroids without a ketone group, but with a 3β-hydroxy-Δ^5 function, can be readily converted to 3-oxo-Δ^4 steroids and subsequently derivatised to GP hydrazones for MALDI analysis. In addition to giving strong $[M]^+$ ions upon MALDI, steroid GP hydrazones give informative post-source decay (PSD) spectra. By using the accurate mass of the precursor-ion measured by MALDI-MS, in combination with the structural information encoded in its PSD spectrum, steroid structures can readily be determined.

1. Introduction

Since the early 1980s, biological mass spectrometry (MS) has undergone a paradigm shift. Pre-1981 biological mass spectrometry was largely concentrated on the analysis of thermally stable volatile molecules, or those that could be made so by simple derivatisation [1]. However, in 1981, Barber et al. published their seminal paper on fast atom bombardment (FAB) ionisation, which demonstrated the desorption ionisation of non-volatile, thermally labile and ionic biomolecules [2]. Following Barber's discoveries, biological mass spectrometry has changed out of all recognition. In 1984, Yamashita and Fenn described electrospray (ES)-MS [3], and in 1987, Karas et al. described matrix-assisted laser desorption/ionisation (MALDI)-MS [4]. Now, at the turn of the 20th century, mass spectrometry is no longer the preserve of the specialist, but is integrated into most biochemistry/biology institutions, and many of today's biological scientists have direct access to either a MALDI- or ES-MS instrument.

MALDI is most often coupled to time-of-flight (TOF) mass analysers, which can be used to obtain accurate mass data [5]. However, MALDI-TOF instruments fitted with a reflectron are also capable of post-source decay (PSD) fragment-ion analysis [6–8]. In PSD laser activated precursor-ions are analysed. PSD fragmentation occurs after ion acceleration, but before ions enter the electric field of the reflectron. The precursor-ion

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(along with its PSD fragments which reach the reflectron with the same velocity) is selected using a timed ion-selector (Bradbury Nielsen Gate) with a precursor-ion resolution (M/ΔM) of about 100, and fragment-ion spectra are sequentially recorded in several segments which are then stitched together.

While the MALDI and ES ionisation methods are well suited to the analysis of both acidic and basic biomolecules, they are not well suited to the analysis of neutral biomolecules [9]. This is a significant drawback in lipid analysis, where many important species are neutral. For example, steroid hormones, anabolic steroids, oxysterols and cholesterol itself are all neutral molecules and are ionised poorly by both ES and MALDI [10]. Atmospheric pressure chemical ionisation and atmospheric pressure photoionisation provide alternative methods of ionisation of neutral steroids, but these ionisation techniques often lead to the formation of dehydrated protonated molecules with the inherent loss in structural information [11,12]. One way of enhancing neutral steroid analysis by either ES- or MALDI-MS is to derivatise the neutral steroid to an acidic, basic or charged analogue. In the present communication, we described the derivatisation of neutral steroids to their charged Girard P (GP) hydrazone analogues, and the performance of these derivatives upon MALDI and PSD analysis.

2. Experimental

All solvents were of analytical grade. Water was from a Milli-Q water system (Millipore, Molsheim, France). The steroids androstenedione (A^4-3,17-dione), dehydroepisandrosterone (DHEA, A^5-3β-ol-17-one), testosterone (A^4-17β-ol-3-one), [19,19,19-$^2H_3$]testosterone, norgestrel (18-homo-E^4-17α-ethynyl-17β-ol-3-one), progesterone (P^4-3,20-dione), pregnanolone (5β-P-3α-ol-20-one), 7β-hydroxycholesterol (C^5-3β,7β-diol), 24S-hydroxycholesterol (C^5-3β,24S-diol), 7α,25-dihydroxycholesterol (C^5-3β,7α,25-triol), 7α,27-dihydroxycholesterol (C^5-3β,7α,27-triol) and [16,16,17(or 20),22,22,23,23-$^2H_7$]7α,27-dihydroxycholesterol were from previous studies in the Karolinska laboratory (Table 1).

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

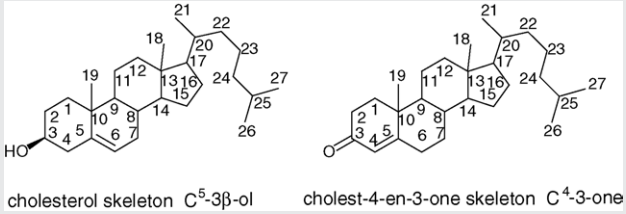
The 3β-hydroxy-Δ^5 steroids were oxidised with cholesterol oxidase essentially as described by Brooks et al. [13]. The 3β-hydroxy-Δ^5 steroids (1 μg) were dissolved in 50 μL of isopropanol, and 10 μL of cholesterol oxidase (1 mg/mL, 20 U/mg protein) in 1 mL of phosphate buffer (50 mM KH_2PO_4, pH 7) added, the mixture was incubated at room temperature (25 °C for up to 12 h). The reaction was stopped by the addition of 1 mL of methanol, and the solution was passed through a Sep-Pak C_{18} cartridge (Waters, Milford, MA) prepared in water. The effluent and 1 mL of water 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 1 mL of methanol.

2.2. *Derivatisation of 3-oxo-Δ^4 steroids*

The derivatisation of 3-oxo-Δ^4 steroids to GP hydrazones was carried out essentially as described by Wheeler [14]. Ten milligrams of GP hydrazine (Sigma–Aldrich) was added to a solution of oxosteroid (1 μg) in 70% aqueous methanol (1 mL) containing glacial acetic acid (200 μL), and warmed at 70 °C for 30 min. After cooling, the steroid GP hydrazone was dried under a stream of nitrogen gas and reconstituted in a solu-

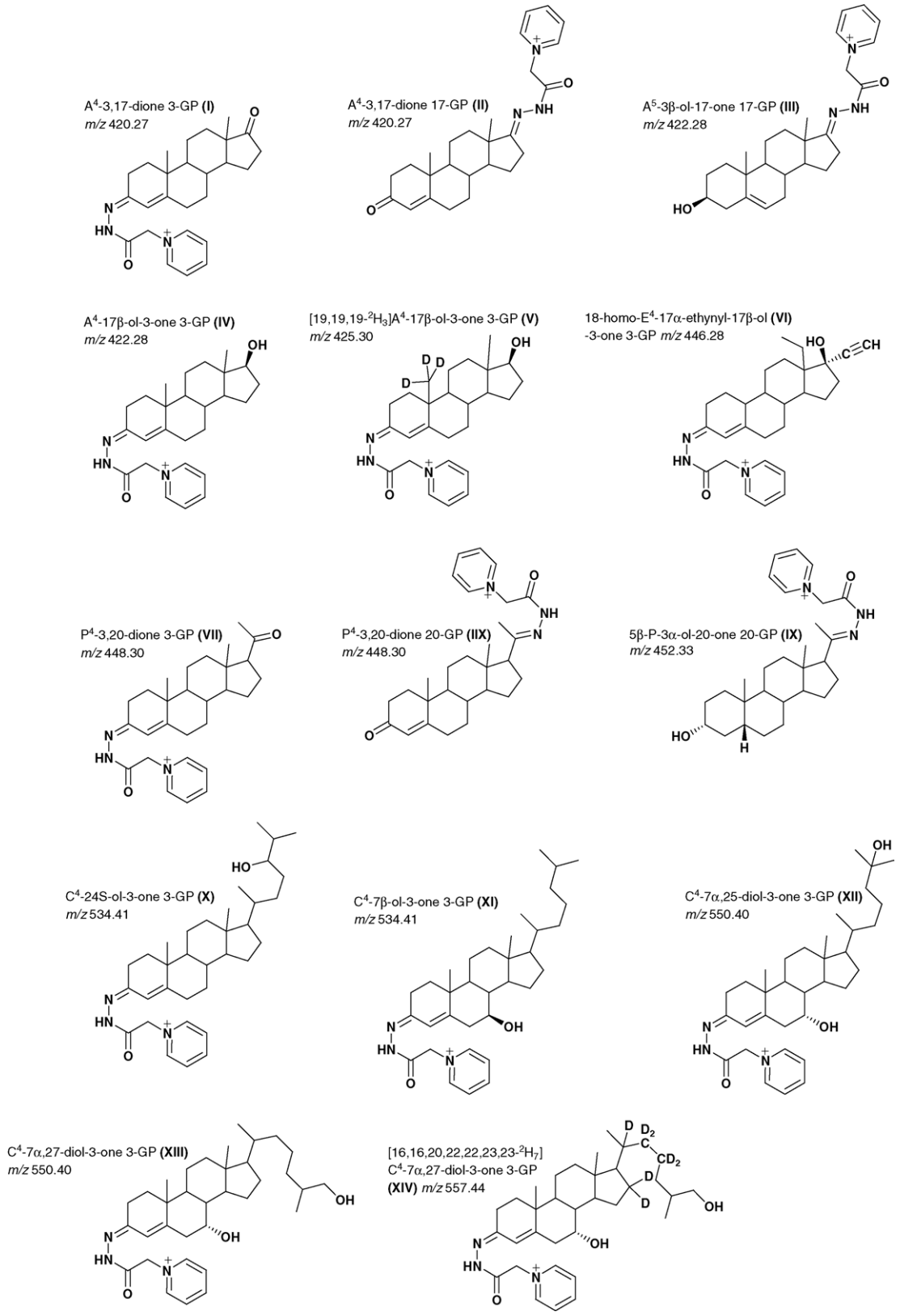
Table 1 – Oxysterols analysed by MALDI-PSD

Name	Abbreviation	Oxidation product[a]	Mass of GP	GP hydrazone
Androstenedione	A^4-3,17-dione	–	420	(I)/(II)
Dehydroepisandrosterone (DHEA)	A^5-3β-ol-17-one	–	422	(III)
Testosterone	A^4-17β-ol-3-one	–	422	(IV)
[19,19,19-$^2H_3$]testosterone	[19,19,19-$^2H_3$]A^4-17β-ol-3-one	–	425	(V)
Norgestrel	18-Homo-E^4-17α-ethynyl-17β-ol-3-one	–	446	(VI)
Progesterone	P^4-3,20-dione	–	448	(VII)/(IIX)
Pregnanolone	5β-P-3α-ol-20-one	–	452	(IX)
7β-Hydroxycholesterol	C^5-3β,7β-diol	C^4-7β-ol-3-one	534	(XI)
24S-Hydroxycholesterol	C^5-3β,24S-diol	C^4-24S-ol-3-one	534	(X)
7α,25-Dihydroxycholesterol	C^5-3β,7α,25-triol	C^4-7α,25-diol-3-one	550	(XII)
7α,27-Dihydroxycholesterol	C^5-3β,7α,27-triol	C^4-7α,27-diol-3-one	550	(XIII)
[16,16,17(or 20),22,22,23,23-$^2H_7$] 7α,27-dihydroxycholesterol	[16,16,17(or 20),22,22,23,23-$^2H_7$]C^5-3β,7α,27-triol	[16,16,17(or 20),22,22,23,23-$^2H_7$]C^4-7α,27-diol-3-one	557	(XIV)



cholesterol skeleton C^5-3β-ol cholest-4-en-3-one skeleton C^4-3-one

[a] Oxidation product upon treatment with cholesterol oxidase.



Scheme 1 – Structures of the steroid GP hydrazones analysed.

tion of 10% aqueous methanol (1 mL). The steroid GP hydrazone was separated from un-reacted GP reagent by extraction on a Sep-Pak C_{18} cartridge. After washing with 10% aqueous methanol (1 mL), the GP hydrazone was eluted from the cartridge with methanol (1 mL). The derivatisation protocol has been applied to mixtures of oxosteroids on the microgram–nanogram level [15] and is also suitable for the low level (picogram) derivatisation of neutral steroids extracted from tissue. For the derivatisation of oxosteroids with a labile 7-hydroxy-Δ^4-3-oxo structure, the amount of GP hydrazine reagent was increased from 10 mg to 100 mg and the reaction was carried out overnight at room temperature.

2.3. *MALDI spectra*

Steroid GP hydrazones (Scheme 1) in methanol were mixed with an equal volume of matrix solution (α-cyano-4-hydroxycinnamic acid, 10 g/L in 50% acetonitrile and 0.1% trifluoroacetic acid) and 1 μL aliquots of the mixture spotted on to the MALDI target plate. The samples were allowed to dry in air. All MALDI spectra were recorded on an Applied Biosystems (Framingham, MA, USA) Voyager TOF mass spectrometer equipped with a nitrogen laser (337 nm). All MALDI mass spectra were recorded in the positive-ion mode and were averages of 100 individual laser shots. The accelerating voltage was set at 20 kV, the focusing guide wire was at 0.075% and the extraction delay time was 200 ns. Spectra were externally, or internally, calibrated using steroid GP hydrazone standards.

In the PSD experiments, the precursor-ions were selected using the timed ion-selector. Fragment-ions were refocused onto the detector by stepping the voltage applied to the reflectron in appropriate increments to give ~10 segments. The individual segments obtained for each mirror ratio were stitched together. The laser intensity was adjusted for each segment to maximise the number of structurally significant fragments.

3. Results

Previous studies have shown that 3-oxo-Δ^4 steroids can be converted to their 3-GP hydrazone analogues in 30 min at 70 °C [15]. In the current study, it was found necessary to reduce the reaction temperature to 25 °C for the derivatisation of labile 7-hydroxy-Δ^4-3-oxo steroids to avoid thermal elimination of water. However, when the reaction was left overnight, quantitative conversions to 3-oxo-Δ^4 GP hydrazones were achieved (data not shown). Although 17- and 20-oxosteroids are not quantitatively converted to their GP hydrazones under the above conditions, when analysed by MALDI MS they were found to give ion-currents at least 100 times greater than the non-derivatised steroids.

In the present study, hydroxycholesterol substrates have been converted quantitatively to 3-oxo-Δ^4 steroids using cholesterol oxidase. Using this enzyme, it is possible to oxidise steroids with a 3β-hydroxy-Δ^5 or 5α-hydrogen 3β-hydroxy function to 3-oxosteroids, and furthermore, the specific enzyme used (cholesterol oxidase, from Brevibacterium, recombinant, lyophilized, obtained from Roche Diagnostics GmbH, Mannheim, Germany, through the courtesy of Dr. Ingo Preuss) has catalytic activity towards steroids with shorter C-17 side-chains than cholesterol (data not shown).

3.1. *MALDI mass spectra*

Neutral steroids are difficult to analyse by MALDI with out derivatisation. Taking testosterone as an example of a 3-oxo-Δ^4 steroid, derivatisation to the GP hydrazone gave an enhancement in ion-current of at least 500. Furthermore, derivatisation of oxosteroids to their GP hydrazone analogues adds 134 Da to their measured mass and thereby moves the ion of interest to a region of reduced matrix-associated noise. Shown in Fig. 1 are MALDI mass spectra of mixtures of testosterone (**IV**) and pregnanolone (**IX**) GP hydrazones. In Fig. 1a, both steroids were present in the matrix solution at a concentration of 5 ng/μL, while in Fig. 1b the steroid concentration was 0.5 ng/μL. GP hydrazones contain a quaternary nitrogen and exist as ions in solution, and as salts in the crystalline form. In MALDI mass spectra GP hydrazone $[M]^+$ ions are observed which correspond to closed-shell even-electron species. It can be noted that preganolone GP hydrazone gives less abundant $[M]^+$ ions than testosterone GP hydrazone, this

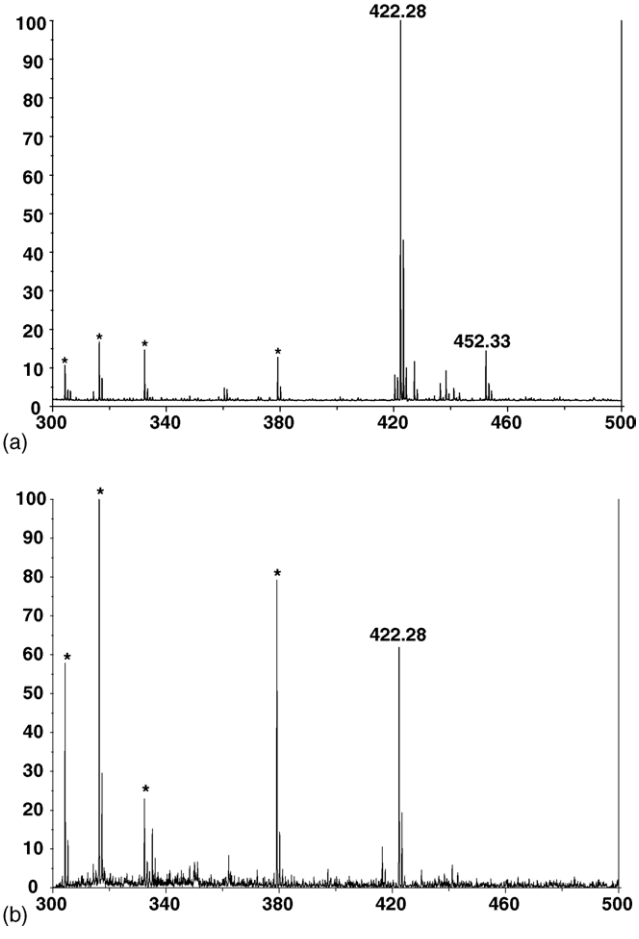


Fig. 1 – MALDI mass spectra of a mixture of GP hydrazones of testosterone (*m/z* 422.28) (IV) and pregnanolone (*m/z* 452.33) (IX). (a) 5 ng and (b) 0.5 ng of each steroid loaded on the MALDI target plate. Matrix peaks are indicated with an asterisk.

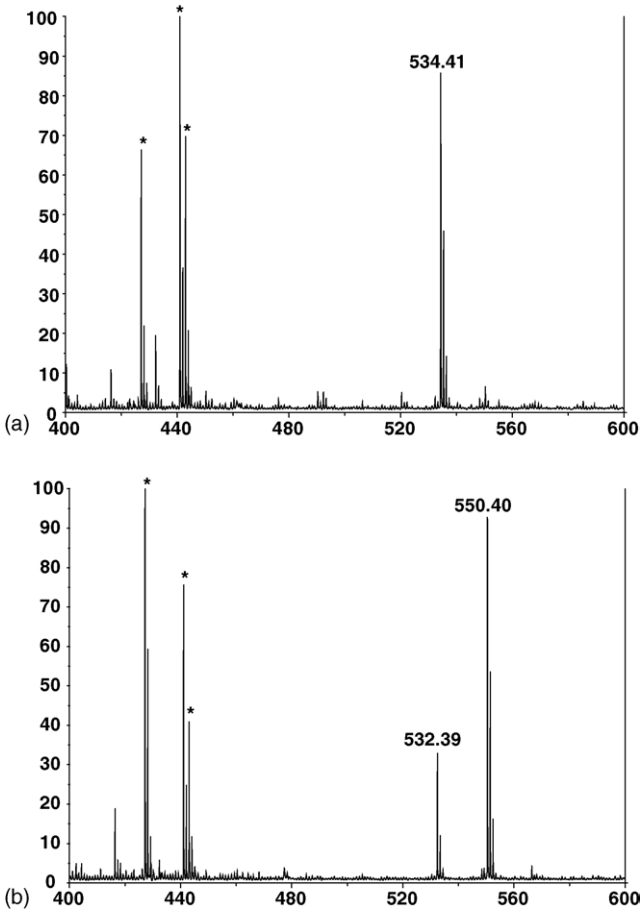
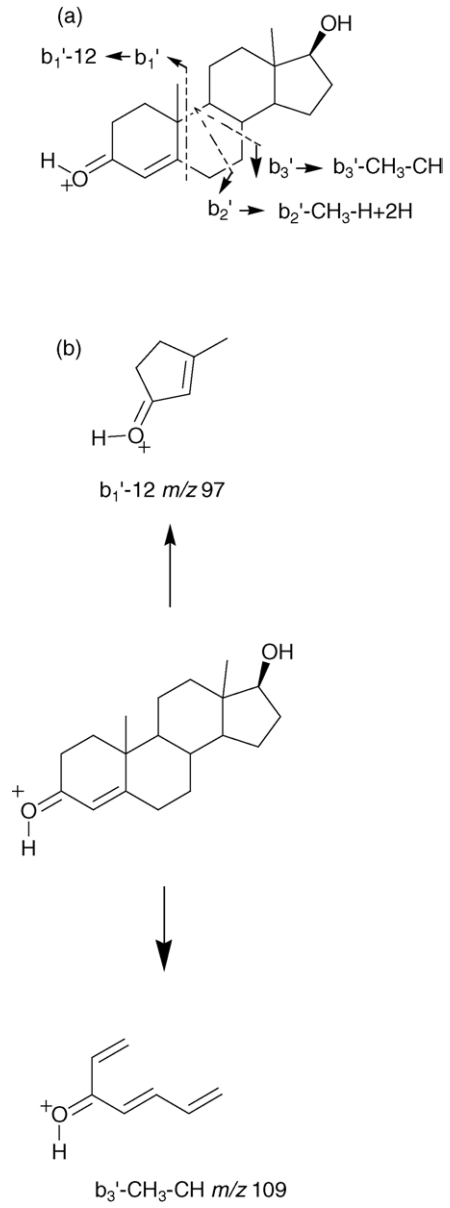


Fig. 2 – MALDI mass spectra of 2.5 ng of GP hydrazones of: (a) C^4-24S-ol-3-one (*m/z* 534.41) (X) and (b) C^4-7α,27-diol-3-one (*m/z* 550.40) (XIII). Matrix peaks are indicated with an asterisk.



Scheme 2 – (a and b) Fragmentation of protonated testosterone. A prime to the right of a fragment-describing letter indicates the ion contains the added proton, e.g. b_1'-12.

may be a consequence of better desorption properties of 3-oxo-Δ^4 GP hydrazones than 20-oxo GP hydrazones, and/or incomplete derivatisation of the 20-oxo group in pregnanolone to its GP hydrazone. Each of the steroid GP hydrazones analysed were readily detected at the 2.5 ng/μL level, this is further illustrated in Fig. 2a and b which displays MALDI mass spectra of matrix solutions containing 2.5 ng/μL of GP hydrazones of C^4-24S-ol-3-one (**X**) and C^4-7α,27-diol-3-one (**XIII**). It can be noted in Fig. 2b that the presence of a hydroxyl group on the steroid ring system at position 7 results in the formation of $[M-18]^+$ ions in addition to $[M]^+$ ions.

3.2. *MALDI-PSD*

While accurate molecular weight information combined with chemical information can lead to the determination of a chemical formula [16,17], MALDI mass spectra provide little additional structural information. However, by application of the PSD method, a considerable amount of structural information can be obtained.

Initial PSD experiments were performed on the $[M+H]^+$ ion of underivatised testosterone. Using 100 ng of sample, the PSD spectrum shown in Fig. 3a was obtained. Fragment-ions are observed at *m/z* 97 (b_1'-12) and 109 (b_3'-CH_3–CH) [10,18,19] (Scheme 2). Although these ions are structurally informative, a sample requirement of at least 100 ng is a considerable drawback to the analytical method.

It has been demonstrated above that derivatisation of 3-oxo-Δ^4 steroids to GP hydrazones increases their "pseudo-molecular" ion current by a factor of 500; thus, PSD experiments were subsequently performed on derivatised testosterone and related steroids. Shown in Fig. 3b–e are MALDI-PSD spectra of 5 ng aliquots of GP hydrazones of: (b) testosterone (**IV**), (c) [19,19,19-$^2H_3$]testosterone (**V**), (d) norgestrel (**VI**) and (e) DHEA (**III**). Each of these spectra show fragment-

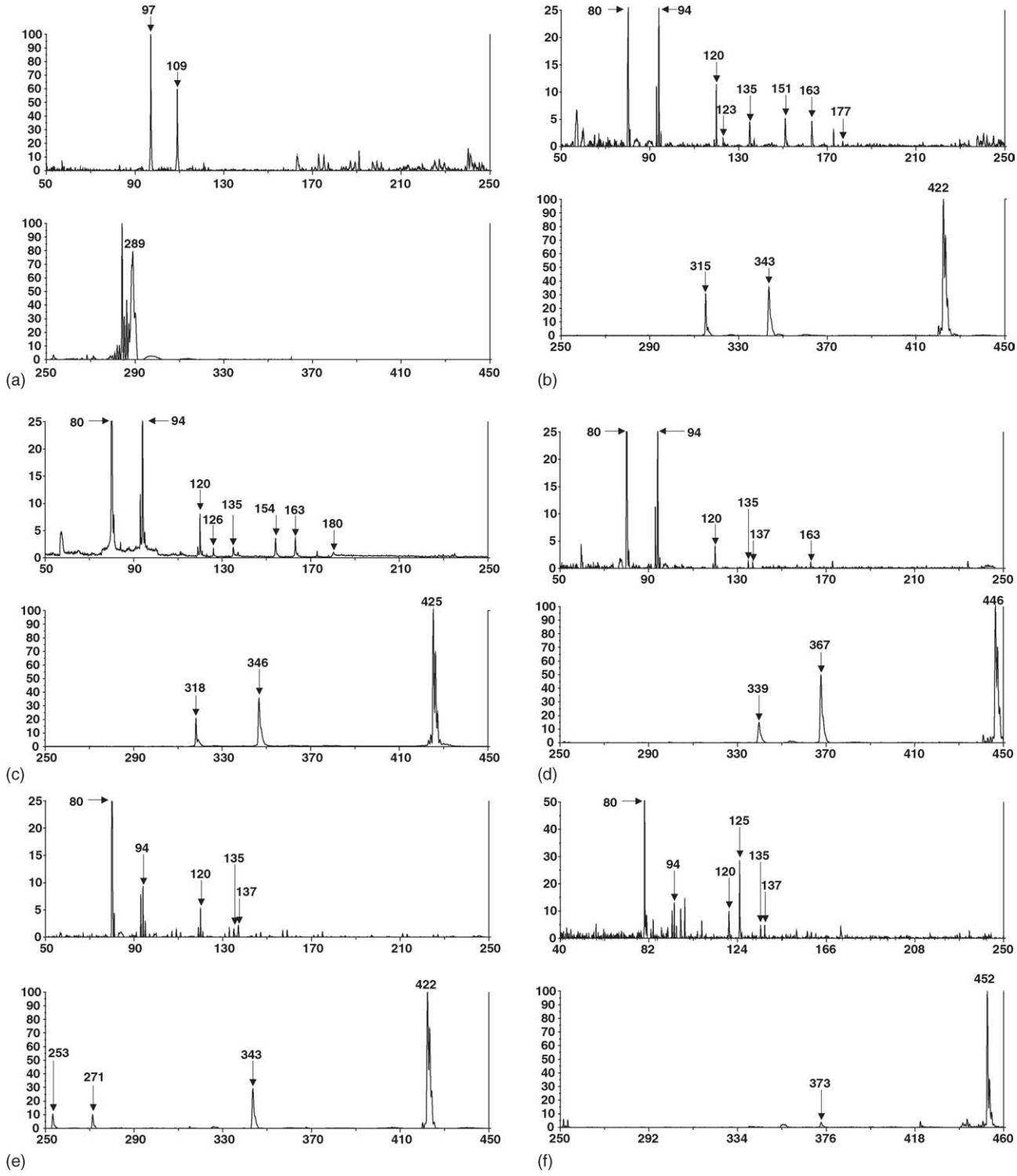


Fig. 3 – MALDI-PSD of the: (a) $[M+H]^+$ ion of testosterone (*m/z* 289); and the $[M]^+$ ions of GP hydrazones of—(b) testosterone $[M]^+$ (*m/z* 422) (IV), (c) [19,19,19-$^2H_3$]testosterone $[M]^+$ (*m/z* 425) (V), (d) norgestrel $[M]^+$ (*m/z* 446) (VI), (e) DHEA $[M]^+$ (*m/z* 422) (III) and (f) pregnanolone $[M]^+$ (*m/z* 452) (IX). One hundred nanograms of underivatised testosterone or 5 ng of derivatised steroid was loaded on the MALDI target plate. See Schemes 2–4 for a description of the fragment-ion nomenclature.

ions at 80 (σ'_1), 93 (σ_2), 94 (σ'_1), 120 ($'\sigma_3$), 135 ($'\sigma_4$) and 137 (σ'_4) which are characteristic of the GP hydrazone group (Table 2; Schemes 3 and 4). In addition, neutral losses of 79 and 107 Da from $[M]^+$ are observed, which are also characteristic of the GP hydrazone group. These patterns of fragment-ions allow the simple identification of GP hydrazones. In the PSD spectrum of testosterone GP hydrazone (**IV**), a triad of fragment-ions is observed at *m/z* 177, 163 and 151 (Fig. 3b).

Table 2 – Characteristic fragment-ions observed in the PSD spectra of oxosteroid mono-GP hydrazone $[M]^+$ ions

Precursor and fragment-ions	M	σ'_1	σ_2	σ'_2	$'\sigma_3$	$'\sigma_4$	σ'_4	$^{\#}b_1$-12	$^{\#}b_3$-CH_3–CH	$^{*}b_1$-12	$^{*}b_3$-CH_3–CH	$^{*}b_2$	M – 107	M – 79	Other major ions	
A^4-3,17-dione GP hydrazone (I)/(II)	420√	80√	93√	94√	120√	135[a]√	137√	123√	135[a]√	151–	163–	177–	313√	341√	269[h]√	
A^5-3β-ol-17-one GP hydrazone **(III)**	422√	80√	93√	94√	120√	135√	137√	–	–	–	–	–	315√	343√	253[i]√	271[k]√
A^4-17β-ol-3-one GP hydrazone **(IV)**	422√	80√	93√	94√	120√	135[a]√	137√	123√	135[a]√	151√	163√	177√	315√	343√		
[19,19,19-$^2H_3$]A^4-17β-ol-3-one GP hydrazone **(V)**	425√	80√	93√	94√	120√	135[b]√	137√	126√	135[b]√	154√	163[f]√	180√	318√	346√		
18-Homo-E^4-17α-ethynyl-17β-ol-3-one GP hydrazone **(VI)**	446√	80√	93√	94√	120√	135[c]√	137[d]√	109–	135[c]√	137[d]√	163[g]√	163[g]√	339√	367√		
P^4-3,20-dione GP hydrazone **(VII)**/**(IIX)**	448√	80√	93√	94√	120√	135[a]√	137√	123√	135[a]√	151√	163√	177–	341√	369√	125[j]√	
5β-P-3α-ol-20-one GP hydrazone **(IX)**	452√	80√	93√	94√	120√	135√	137√	–	–	–	–	–	–	373√	125[j]√	
C^4-24S-ol-3-one GP hydrazone **(X)**	534√	80√	93√	94√	120√	135[a]√	137√	123√	135[a]√	151√	163√	177–	427√	455√		
C^4-7β-ol-3-one GP hydrazone **(XI)**	534√	80√	93√	94√	120√	135√	137√	123√	151[e]√	151[e]√	179–	177–	427√	455√		
C^4-7α,25-diol-3-one GP hydrazone **(XII)**	550√	80√	93√	94√	120√	135√	137√	123√	151[e]√	151[e]√	179–	177–	443√	471√		
C^4-7α,27-diol-3-one GP hydrazone **(XIII)**	550√	80√	93√	94√	120√	135√	137√	123√	151[e]√	151[e]√	179–	177–	443√	471√		
[16,16,17(20),22,22,23,23-$^2H_7$]C^4-7α,26-diol-3-one GP hydrazone **(XIV)**	557√	80√	93√	94√	120√	135√	137√	123√	151[e]√	151[e]√	179–	177–	450√	478√		

See Schemes 2–4 for a description of the fragmentation nomenclature; '√' signifies the ion to be present, '–' indicates the ion to be absent.

[a] 135 corresponds to $'\sigma_4$ and $^{\#}b_3$-CH_3–CH.

[b] 135 corresponds to $'\sigma_4$ and $^{\#}b_3$-CD_3–CH.

[c] 135 corresponds to $'\sigma_4$ and $^{\#}b_3$-H–CH.

[d] 137 corresponds to σ'_4 and $^{*}b_1$-12.

[e] 151 corresponds to $^{\#}b_3$-CH_3–CH and $^{*}b_1$-12.

[f] 163 corresponds $^{*}b_3$-CD_3–CH.

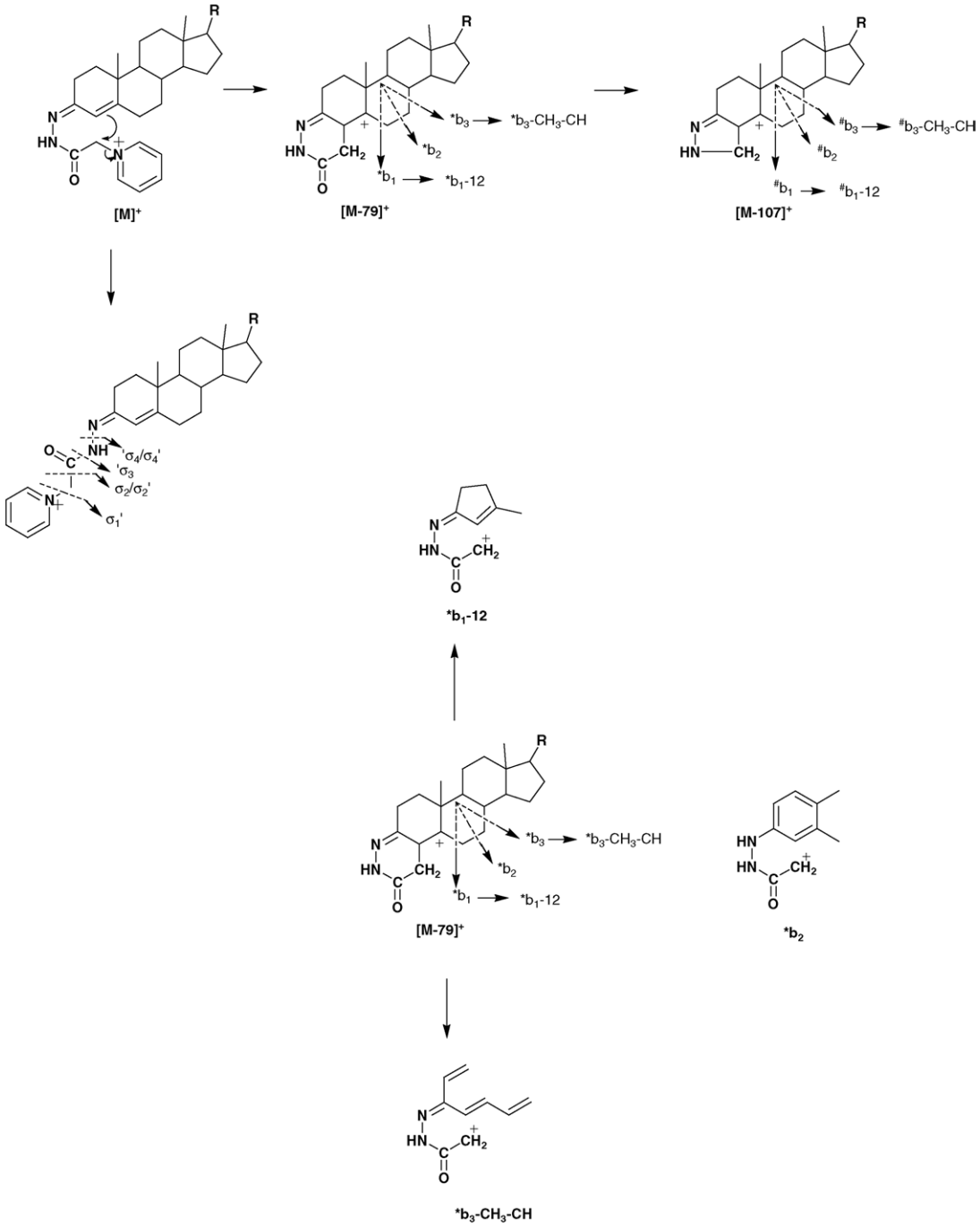
[g] 163 corresponds to $^{*}b_3$-H–CH and $^{*}b_2$.

[h] 269 corresponds to $'e$.

[i] 253 corresponds to $'e$-18.

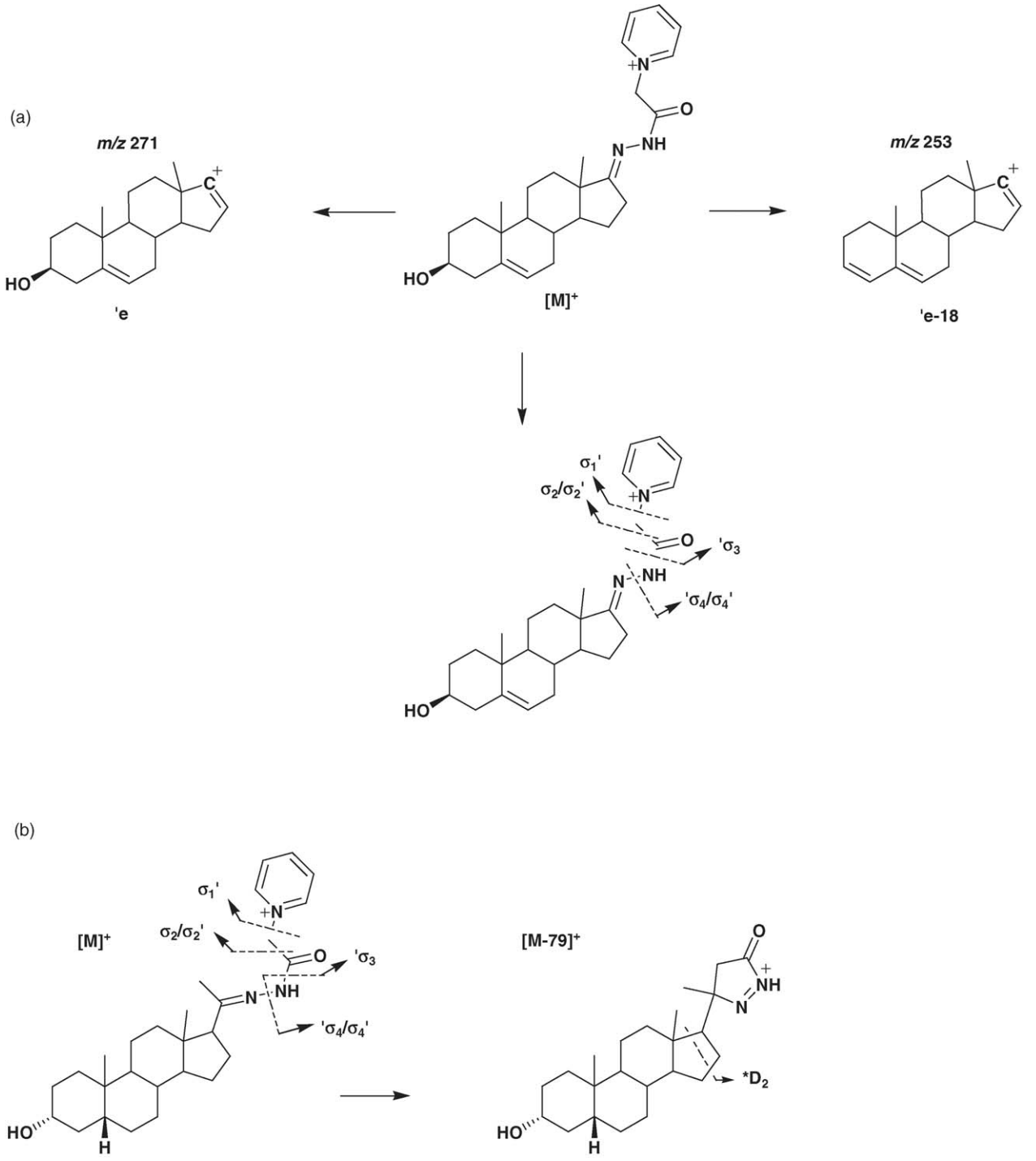
[j] 125 corresponds to $^{*}D_2$.

[k] 271 corresponds to $'e$.



Scheme 3 – Fragmentation of 3-oxo-Δ^4 steroid GP hydrazones. Fragment-ions derived from the $[M-79]^+$ intermediate ion are designated with an asterisk, e.g. $^*b_1$-12. Fragment-ions derived from the $[M-107]^+$ intermediate ion are designated with a superscript hatch, e.g. $^\#b_1$-12. A prime to the right of a fragment-describing letter indicates that cleavage proceeds with H-atom transfer from the neutral leaving-group to the fragment-ion, e.g. σ'_1. A prime to the left of the fragment-describing letter indicates that cleavage proceeds with H-atom transfer from the fragment-ion to the neutral leaving group, e.g. $'\sigma_3$. The $^*b_1$-12, $^*b_2$ and $^*b_3$-CH_3–CH fragment-ion structures are drawn with charge located on the side-chain, alternative structures could be envisaged with the side-chain forming part of a bicyclic ring.

These ions correspond to the B-ring fragments $^*b_2$, $^*b_3$-28 ($^*b_3$-CH_3–CH) and $^*b_1$-12 (Scheme 3). Incorporation of three deuterium atoms on C-19 shifts the triad to *m*/*z* 180, 163 and 154 (Fig. 3c), while the absence of C-19 results in the elimination of ions at *m*/*z* 177/180 and 151/154, but the ion at *m*/*z* 163 remains (Fig. 3d). These results indicate that the $^*b_2$ ion corresponds to the A-ring plus C-19 (i.e. the C-10 β substituent) and C-6. The $^*b_3$-28 ion in testosterone GP hydrazone (**IV**) becomes a $^*b_3$-31 ion in its [19,19,19-$^2H_3$] analogue (**V**), and a $^*b_3$-14 ion in norgestrel GP hydrazone (**VI**). This indicates that in testos-



Scheme 4 – Formation of: (a) ′e-18 and ′e fragment-ions from DHEA 17-GP hydrazone (III) and (b) $^{*}D_2$ fragment-ions from pregnanolone 20-GP hydrazone (IX).

terone GP hydrazone, the $^{*}b_3$-28 fragment-ion consists of the A-ring plus C-6 and C-7, but has lost the C-19 methyl group (i.e. the C-10 β substituent), a C-atom possibly from C-10 and a H-atom, possibly from the A-ring, but or more likely from C-6 [10,18]. The $^{*}b_3$-28 fragment-ion can thus be described as $^{*}b_3$-CH_3–CH. The $^{*}b_1$-12 ion increases in mass by 3 Da in spectrum of [19,19,19-$^{2}H_3$] testosterone GP hydrazone as compared to the monoisotopic isotopomer, this suggests that the C-19 group is maintained in this ion which probably corresponds to A-ring plus C-19 but minus C-5. In norgestrel GP hydrazone, the absence of the C-19 group results in the $^{*}b_1$-12 ion being shifted to 137 Da. A satellite series of ions are observed in the spectra of 3-oxo-Δ^4 steroid GP hydrazones displaced by 28 Da from the *b-ion series. The satellite series ($^{\#}$b-ion series) differ from the main series in that a CO group has been additionally eliminated (Scheme 3). In DHEA GP hydrazone (**III**) (Fig. 3e), the

absence of a 3-oxo-Δ^4 GP function results in the exclusion of the 177, 163 and 151 triad of ions from the PSD spectrum. However, additional ions are observed at *m/z* 271 and 253 which correspond to the steroid- and dehydrated steroid ring system, respectively (Scheme 4).

Elongation of the C-17 side-chain, as in the oxysterol derivative C^4-24S-ol-3-one GP hydrazone (**X**), does not cause a major change in the pattern of fragment-ions characteristic of 3-oxo-Δ^4 steroid GP hydrazones, other than attenuation of the $^*b_2$ fragment-ion (Fig. 4a; Table 2). However, incorporation of a hydroxyl group at C-7 changes the pattern of fragment-ions observed (cf. Fig. 4a with Fig. 4b and c; Table 2). As in the PSD spectra of other 3-oxo-Δ^4 steroid GP hydrazones (with a C-19 methyl group), the $^*b_1$-12 ion is observed at *m/z* 151 in the spectra of 7-hydroxy-Δ^4-3-oxo steroid GP hydrazones, but fragment-ions corresponding to $^*b_3$-CH_3–CH which would be expected to be shifted in *m/z* from 163 to 179 due to the incorporation of a hydroxyl group on C-7 are not observed. This can be explained by the propensity of the C-7 hydroxyl group to be eliminated as water, and thereby generate a double bond between C-6 and C-7 which will inhibit $^*b_3$-CH_3–CH ion formation.

In Fig. 3, it is evident that 17-oxosteroid GP hydrazones fragment in a different manner to 3-oxo-Δ^4 GP hydrazones. In the PSD spectrum of DHEA (A^5-3β-ol-17-one) 17-GP hydrazone (**III**) (Fig. 3e) intense fragment-ions are observed at *m/z* 271 (′e) and 253 (′e-18) and these ions correspond to the steroid ring system, and the dehydrated steroid ring system, respectively (Scheme 4a; Table 2). The PSD spectrum of androstenedione (A^4-3,17-dione) mono-GP hydrazone (**I/II**) also shows an intense ion corresponding to the steroid ring system, but in this case at *m/z* 269 (′e) (Table 2). When the GP hydrazone is at C-20, as in pregnanolone (5β-P-3α-ol-20-one) GP hydrazone (**IX**), the PSD spectrum shows an intense fragment-ion at *m/z* 125, this corresponds to the $^*D_2$ fragment and is characteristic of C-20 oxosteroid GP hydrazones (Fig. 3f; Table 2; Scheme 4b).

The value of the derivatisation approach in a biological application is illustrated in Fig. 4d, which shows the PSD spectrum of a hydroxycholesterol, isolated from the equivalent of 0.1 mg of rat brain, following treatment with cholesterol

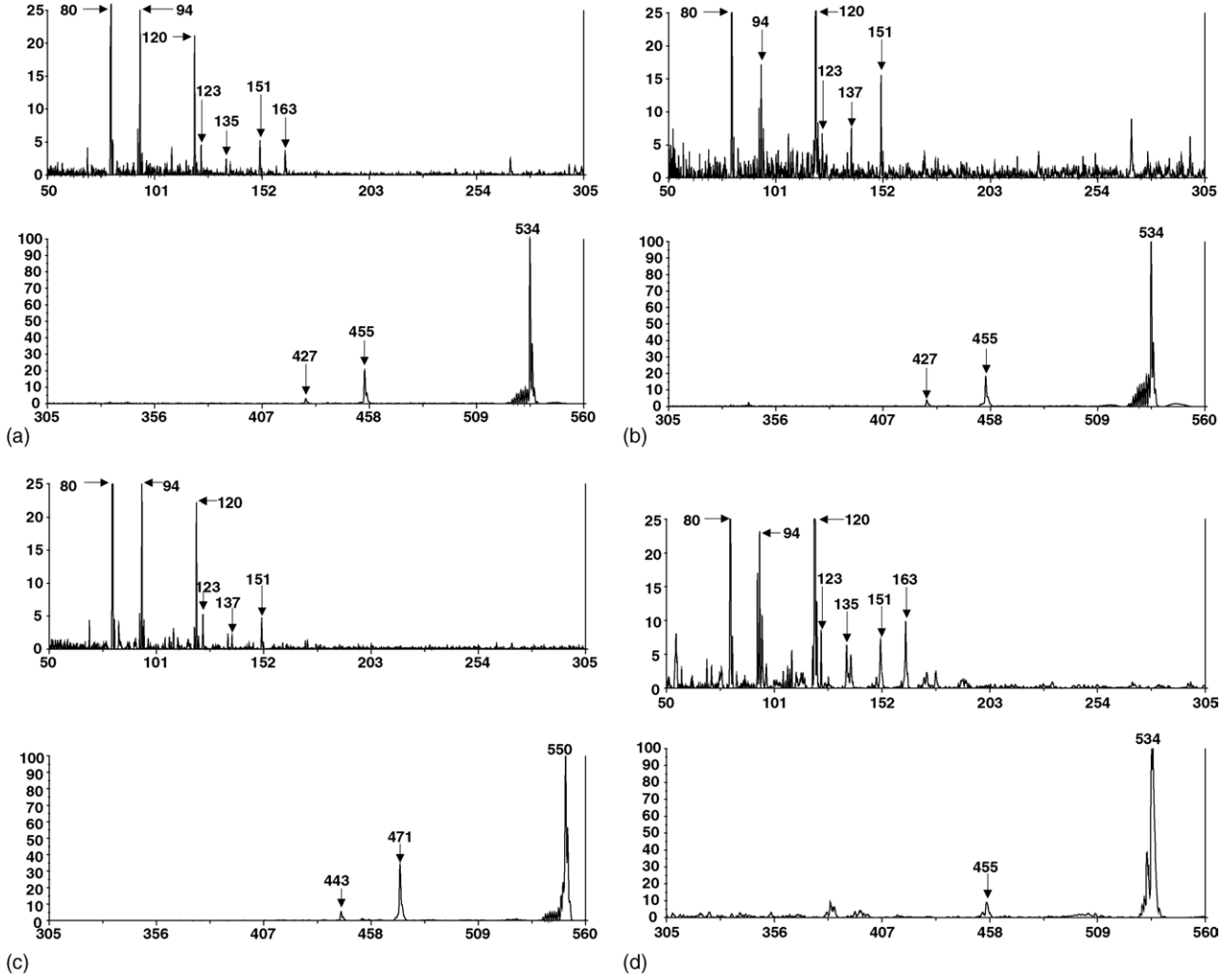


Fig. 4 – MALDI-PSD spectra of GP hydrazones of: (a) C^4-24S-ol-3-one $[M]^+$ (*m/z* 534) (X), (b) C^4-7β-ol-3-one $[M]^+$ (*m/z* 534) (XI), (c) C^4-7α,25-diol-3-one $[M]^+$ (*m/z* 550) (XII) (5 ng of each steroid was loaded on the MALDI target plate) and (d) 24S-hydroxycholesterol isolated from rat brain, oxidised with cholesterol oxidase and derivatised with GP reagent. See Scheme 3 for a description of the fragment-ion nomenclature.

oxidase and GP hydrazine. Accurate mass measurement performed in an initial MALDI spectrum of the GP hydrazone gave an elemental formula $C_{34}H_{52}N_3O_2$, this and the PSD spectrum (Fig. 4d) are consistent with the brain steroid being predominantly 24-hydroxycholesterol. 24*S*-Hydroxycholesterol is known to be the major oxysterol in brain [20].

4. Discussion

3-Oxo-Δ^4, 17-oxo and 20-oxo steroids can be derivatised to GP hydrazones. The derivatisation reaction is simple, and even if the reaction does not go to completion, gives an enhancement in MALDI precursor-ion current of at least a factor of 100. By performing derivatisation at room temperature, the reaction is applicable to labile structures such as 7-hydroxy-Δ^4-3-oxo. Further, conversion of 3β-hydroxy-Δ^5 steroids to their 3-oxo-Δ^4 analogues makes them amenable to derivatisation with the GP reagent, and subsequent MALDI analysis. In this way, many steroids of biological interest such as oxysterols, and cholesterol itself, can be analysed by MALDI at high sensitivity to give molecular weight information, and by MALDI-PSD to give structural information. This is in contrast to the untreated steroids which are poorly ionised by MALDI and often do not give $[M+H]^+$ ions. This is illustrated in Fig. 4d which shows the MALDI-PSD spectrum of 24*S*-hydroxycholesterol isolated from rat brain, oxidised with cholesterol oxidase and derivatised with GP reagent.

MALDI-MS has previously been used in lipid analysis, particularly in the area of phospholipids [21–24]. However, few previous studies have been made using MALDI for steroid analysis. Schiller et al. [25,26] have analysed cholesterol in bronchoalveolar lavage fluid, spermatozoa and seminal plasma using MALDI-TOF, while Rujoi et al. [27] have analysed cholesterol in lipid rafts. Cholesterol was found to give an ion at *m*/*z* 369 corresponding to the $[M+H-H_2O]^+$ moiety rather than the $[M+H]^+$ ion. In previous studies, Griffiths et al. [15,28,29] have shown that cholesterol can be converted to its 3-oxo-Δ^4 analogue, derivatised to its 3-GP hydrazone and then ionised by MALDI. Griffiths' studies were performed on a MALDI-quadrupole-TOF instrument and their success inspired the present work.

In the current study, we have shown that MALDI mass spectra of steroid GP hydrazones can be obtained from 500 pg of steroid loaded onto the MALDI target plate (Fig. 1b), and that structurally informative MALDI-PSD spectra can be obtained from 5 ng loaded onto the plate (Figs. 3 and 4; Table 2). 3-Oxo-Δ^4 steroids give a characteristic pair of fragment-ions $^*b_1$-12 (*m*/*z* 151) and $^*b_3$-CH_3–CH (*m*/*z* 163), but the presence of a 7-hydroxy group results in attenuation of the $^*b_3$-CH_3–CH ion (now shifted to *m*/*z* 179), and relative enhancement of the $^*b_1$-12 ion. 17-Oxo steroid GP hydrazones give a characteristic ′e ion corresponding to the steroid ring system (Scheme 4a), e.g. at *m*/*z* 271 in A^5-3β-ol-17-one 17-GP hydrazone (**III**), and 269 in A^4-3,17-dione mono-GP hydrazone (**I/II**), while 20-oxo steroid GP hydrazones give a characteristic fragment-ion corresponding to $^*D_2$ at *m*/*z* 125 (Scheme 4b).

There is some debate as to the structure of the ion at *m*/*z* 163 in the spectra of 3-oxo-Δ^4 steroid GP hydrazones. Here, we describe the ion as a $^*b_3$-CH_3–CH fragment, which consists of the A-ring plus C-6 and C-7 but minus the C-19 methyl group, the C-10 carbon and a H-atom from C-6 (Scheme 3). This structure is analogous to that proposed by Williams et al. for the fragment-ion at *m*/*z* 109 in the CID spectrum of protonated testosterone (Scheme 2) (cf. Fig. 3a) [18]. We have previously described the ion at *m*/*z* 163 as $^*b_2$-CH_3–H + 2H, and proposed it to consist of the A-ring plus C-6, minus the C-19 methyl group and the 6β-hydrogen atom, and with two hydrogen atoms transferred to the fragment-ion from the neutral leaving group [15]. With data from the present study, it is impossible to differentiate between $^*b_3$-CH_3–CH and $^*b_2$-CH_3–H + 2H fragment-ions, and either could be the structure of the ion at *m*/*z* 163. It should be noted that for 7-hydroxy-Δ^4-3-oxo steroid GP hydrazones, a $^*b_3$-CH_3–CH ion should appear at *m*/*z* 179, while a $^*b_2$-CH_3–H + 2H ion should appear at *m*/*z* 163; unfortunately, intense peaks were not observed at either of these *m*/*z* values in the PSD spectra of the 7-hydroxy-Δ^4-3-oxo steroid GP hydrazones studied.

In conclusion, with the growing interest in metabolomics and lipidomics [30], methods to improve the ionisation properties of neutral thermally labile molecules are desirable. Derivatisation of ketone or aldehyde groups to Girard hydrazones [31] will provide one such solution; in the current study, we have demonstrated the utility of such a derivatisation method for the MALDI analysis of oxosteroids.

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