DMD Fast Forward. Published on December 19, 2018 as DOI: 10.1124/dmd.118.085597
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DMD # 85597

Short Communication

Gas-phase rearrangement of the *O*-glucuronide of Vildagliptin forms product ion fragments suggesting wrongly an *N*-glucuronide

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Running Title: Gas-phase rearrangement of O-glucuronide of Vildagliptin

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Number of text pages:	9
Number of Tables	1
Number of Figures:	2
Number of References:	20
Number of words in abstract	114
Number of words in introduction	156
Number of words in discussion	660

Abbreviations: CID, collision-induced dissociation; CI-MS, chemical ionization MS; COSY, correlation spectroscopy; DAD, diode array detection; DPP-4, dipeptidyl peptidase 4; HCD, higher energy CID; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence; PDA, Photodiode array detector; Q-TOF MS, quadrupole time of flight MS; ROESY, rotational frame nuclear Overhauser effect spectroscopy; TFA, trifluoroacetic acid;.

Abstract

The **O**-glucuronide of vildagliptin, a dipeptidyl peptidase 4 inhibitor (DPP-4), is a major metabolite in monkey and a minor metabolite in humans, rats and dogs. Its product ion spectrum shows fragments that can be only explained by an *N*-glucuronide. Biotransformation utilizing rat liver yielded mg amounts of the *O*-glucuronide and its structure was assigned unambiguously by NMR. The MS/MS spectrum of this compound was investigated in detail utilizing MS^n and accurate mass spectrometers and was identical to the animal metabolite. Thus, the MS/MS fragments suggesting an *N*-glucuronide had to be formed by gas-phase rearrangement. This gas-phase rearrangement can be observed on QTOF and ion trap mass instruments. The literature on gas-phase rearrangements is reviewed.

Introduction

Dipeptidyl peptidase 4 inhibitors, like vildagliptin (Galvus®) and sitagliptin (Januvia®) are widely used to treat diabetes II (Doupis & Veves 2008). The human and animal metabolism of vildagliptin have been investigated intensively (He et al. 2009a; He et al. 2009b). The major human metabolite was M20.7, where the cyano group was hydrolyzed to an acid. In rat the main circulating metabolites in blood plasma were M20.7 and, to a lesser extent, the *O*-glucuronide M20.2. The question, whether M20.2 was an O-glucuronide or an *N*-glucuronide, had to be addressed, even more as the O-glucuronide was presumed and later shown to be biologically active. Was it acceptable to do that based on relatively small MS/MS fragments ?

In the present study the MS^n product ion spectra of vildagliptin- O-glucuronide will be investigated in detail and it will be shown that some fragments had to be formed by gas-phase rearrangement. The mechanisms for these gas-phase rearrangements will be discussed.

Material and Methods

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The analytical liquid chromatograph consisted of a Waters UPLC Acquity (Waters, Milford, USA) equipped with a Waters Acquity PDA detector. Column: Acquity BEH C18, 1.7 µm; 1.0 x 150 mm (Waters); flow rate 0.1 ml / min.; eluent A: H_2O / TFA 100 : 0.02; eluent B: acetonitrile / TFA 100 : 0.02; gradient: 0 min. 2 % B; 15 min. 40 % B; 16 - 17 min. 95 % B; column temperature 40 °C; UV-detection: 210 nm, DAD from 205 - 350 nm, resolution 2.4 nm; injection volume 0.6 µl. The UPLC was controlled by Empower software (Version 3).

An ion trap mass spectrometer LTQ Velos Pro (Thermo Scientific, San Jose, CA, USA) equipped with heated electrospray interface was operated in the positive mode with Xcalibur software version 2.1 as follows: A sheath gas (nitrogen, >99.5 %) setting of 24 units and auxiliary gas of 5 units was used and a spray voltage of 3.5 kV applied. The heated metal capillary was maintained at 275 °C with a mass range of 300 to 600 Da. The system was optimized for *m/z* 549 $[M + H]^+$ of antimycin A1 in the positive ion mode. As antimycin A1 had a tendency for in-source fragmentation to *m/z* 265, our parameters reduced in-source fragmentation to a minimum. Typical parameters: S-Lens 62 %; multipole 00 offset – 1.8 V; multipole 0 offset – 7.5 V; front lens -9.1 V. MS/MS parameters: CID with isolation width 2.8 Da and without wide-band excitation activated; normalized collision energy 30 %; activation time 10 ms (default) or 2 ms and 200 ms and an activation Q value of 0.25. HCD with isolation width 2.8 Da and without wide-band excitation activated; normalized collision energy 25 %; activation time 10 ms (default) or 2 ms and 200 ms and activation Q value 0.08.

Other MS/MS spectra were obtained on an Xevo G2-XS Q-TOF (Waters, Wimslow UK) with ESI probe in the sensitivity mode and controlled by Unify software (Version 1.8.1). Important parameters: desolvation gas 800 L/h; desolvation temperature 350 °C; spray voltage 3 kV; cone gas 50 L/h; cone voltage 40 V; source offset 20 V; sample cone 40 V; stepwave 2 offset 10 V; low energy collision offset 6 V; collision cell offset auf 150 V. For MS^E experiments the collision energy was ramped between 20 and 40 eV.

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For accurate mass measurements an Orbitrap Fusion Lumos Tribrid Mass Spectrometer with 1 M resolution (Thermo Scientific) was used. The compound was inserted by a syringe, ionized by ESI in the positive ionization mode. Both MS and MS^2 were analyzed with the Oribrap analyzer at one million resolution utilizing fluoranthere as an internal calibrant and standard parameters. MS/MS spectra were generated with HCD activation and normalized collision energy of 20 %.

Results

The preparation of the O-glucuronide by biotransformation is described in a patent (Hassiepen & Kittelmann 2009). Incubation of vildagliptin (340 mg) with mixed sex Sprague-Dawley rat liver homogenate in the presence of uridine 5'-diphosphoglucuronic acid trisodium salt yielded 150 mg of the O-glucuronide (66 % yield) after purification with reversed-phase chromatography. The structure elucidation, also described the patent, was based on ^{1}H and ^{13}C NMR utilizing 2D homo- and heteronuclear spectra (COSY, HSQC, HMBC, ROESY). An HMBC correlation from H-1' at the glucuronide moiety to carbon-3, the carbon where the hydroxyl function was attached at the adamantane ring, and ROESY correlations from H-1' to H-2 and H-4 confirmed the structure unambiguously. The preparation of the O-glucuronide by chemical synthesis has also been described (Lu et al. 2012).

The product ion spectra of biosynthetically prepared *O*-glucuronide are shown in Fig. 1 with CID (collision-induced dissociation) and HCD (higher-energy collisional dissociation) fragmentation. The accurate mass data of the fragments are compiled in Table 1. These data, as well as MS^3 experiments (Figure 2), fit to the proposed fragmentation mechanism (Scheme 1). Most fragments would fit to the *O*-glucuronide, but also to an *N*-glucuronide. The fragment at *m/z* 330 can be explained only by an *N*-glucuronide. It was formed with a relative intensity of 0.9 % upon CID activation, 6.8 % upon HCD fragmentation and 1.8 % in the QTOF MS^E spectrum (Supplemental Figure 1). The activation time had an influence on the relative intensity of that fragment in CID: From an activation time of 2 ms to 200 ms it increased by 72 % utilizing a normalized collision energy of 30 % (see Supplemental Table 1). With a lower normalized collision energy of 15 % the ions remained longer in the activated mass range giving

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more time for the rearrangement. The relative intensity of *m/z* 330 increased by a factor of 3.2 for the same activation time range. These findings indicate that the rearrangement takes place prior to CID fragmentation and partially directly in the ion trap. For HCD activation neither the normalized collision energy (15 or 30 %) nor the activation time had an influence. In an MS^3 experiment the ion *m/z* 330 fragmented further to *m/z* 196 corresponding to a loss of 134 Da and *m/z* 154 upon loss of glucuronic acid. The loss of 134 Da or $C_4H_6O_5$ in *N*-glucuronides has been discussed earlier by us (Fredenhagen et al 2017).

It could be hypothesized that the fragment m/z 330 was due to a minor impurity. This could be excluded, as no impurities were observed in NMR spectra. Another evidence was the MS^3 spectrum of *m/z* 304 (Figure 2). A loss of water to *m/z* 286 was not observed, not even in traces. Therefore the formation of *m/z* 286 occurred in a single step and a mechanism in two steps, loss of water from the adamantyl moiety and loss of glucuronic acid from a (not present) N-glucuronide could be excluded.

Discussion

Gas-phase rearrangement was first observed in sugars utilizing CI-MS (McNeil 1983). Warrack et al. observed the internal loss of fucose from the anthracycline-type natural product **2** (Scheme 2) with CID activation either underivatized or peracetylated (1998). While the mechanisms in sugars is still unknown, it seems that a proton is necessary and that these internal residue losses occur neither with $[M + Na]^+$ nor $[M - H]^-$ as precursor ions (Brüll et al 1998; Wuhrer et al 2011). A somewhat similar case is the loss of a polyether spacer of **3** in positive ionization mode utilizing several types of instruments and for which a charge remote mechanism was proposed (Banoub et al. 2015).

Several examples can be found, where strong inorganic acids migrate: Internal loss of formaldehyde was found in the reverse transcriptase inhibitor adefovir **4** upon migration of the phosphonic acid in the positive ionization mode (Chen et al. 2004). The migration of negatively charged phosphate in the natural product moenomycin **5** was found upon negative ionization and resulted in the loss of a derivatized sugar

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(Zehl et al. 2006). One phosphate was also lost in the middle of uridine-diphosphate-N-acetylglucosamine **6** (Liu et al 2006). Palumbo and Reid observed that a transfer of phosphate groups in peptides to serine and threonine occurred in 45% of all peptides investigated (2008). They proposed a mechanism initiated by hydrogen-bonding interactions to arginine. Moreover they showed that a longer activation time in linear ion trap instruments (e.g. 2000 ms) resulted in more rearranged product, but even with an activation time of 2 ms the reaction could not be suppressed. Migration of sulfate has been proposed for a loss of galactose residue from a sulfatide **7** (Scheme 3) utilizing negative ionization mode and was observed on an ion trap instrument, but not on a triple quad MS (Hsu & Turk 2004).

Migrations where a carbon atom and not a heteroatom had to be reattached before the internal loss are less common. Migration of 4-(2-aminovinyl)benzene-1,2-diol has been observed in tunichrome **8**, a pentapeptide containing two such moieties and these ions were much larger with an ion trap instrument than with triple quandrupole (Taylor et al. 2003). Another example is migration of a methyl group from an aromatic methoxy group in **9** to a nitrogen in negative ionization mode (Tiller et al. 2001).

The examples of gas-phase rearrangements citied above suggest that positively or negatively charged moieties migrate more frequently and moreover there are many examples where sugars and other moieties with several oxygens rearrange. For the migration of a glucuronide observed by us, it could mean that a glucuronide can be regarded as a sugar derivative. Furthermore, and more importantly, coulomb attraction of a negatively charged carboxylic acid and a positively charged secondary nitrogen might play a key role in the migration.

The O-glucuronide is biologically active as a selective DDP-4 inhibitor with an IC_{50} value of 4 nM (Hassiepen & Kittelmann 2009), almost as active as the drug with 2.7 nM (Villhauer et al. 2003). Therefore, the correct structure of the glucuronide was important to assign the concentration of biologically active species in monkeys, where the *O*-glucuronide was an important metabolite (Lu et al 2012).

In conclusion our investigation showed, to the best of our knowledge for the first time, that glucuronide moieties are capable for gas phase rearrangements. This can lead to miss-assignments of glucuronides, if the MS/MS spectra were taken as the sole proof for its structure. We showed furthermore that on ion trap instruments these gas phase reactions can be investigated by shortening or prolonging the activation time. These experiments should be useful for other MS specialists to discriminate if fragment ions were formed by gas transfer rearrangement. Finally, for the compound in our hand, these undesired ions were considerably smaller on our ion trap instruments utilizing CID activation than on the same instrument with HCD activation or on a Q-TOF type instrument where fragmentation was induced by collision with argon.

Acknowledgements

We thank Anton Kuhn, Aldo Meishammer and Dr. Georg Diehl for preparation of the material used in this study, to Alexandra Vargas for technical assistance and to Dr. Eric Francotte, and Dr. Oreste Ghisalba for the continuous support of this work. Thanks are also due to Dr. Reiner Aichholz for initial HR-MS measurements.

Authorship contributions

Participated in research design: Fredenhagen, Kittelmann

Conducted experiments: Fredenhagen, Kühnöl, Oberer

Performed data analysis: Fredenhagen, Kühnöl, Oberer

Wrote or contributed to the writing of the manuscript: Fredenhagen, Kittelmann

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Table 1: HR-MS/MS data of 1 of Selected Fragment Ions

Precursor ion (*m/z*)	**Fragment Ion Found (Da)**	**calc. Formula**	**calc. Fragment Ion** (Da)	**error (**ppm**)**
480.2 $[M + H]^+$	480.23391	$C_{23}H_{34}N_3O_8^+$	480.23404	0.27
	330.12946	$C_{13}H_{20}N_3O_7^+$	330.12958	0.36
	304.20172	$C_{17}H_{26}N_3O_2^+$	304.20195	0.76
	286.19110	$C_{17}H_{24}N_3O^+$	286.19139	1.01
	154.09755	$C_7H_{12}N_3O^+$	154.09749	0.39
	151.11180	$C_{10}H_{15}O^+$	151.11174	0.40
	194.12882	$C_{10}H_{16}N_3O^+$	194.12879	0.15
330.2	196.10810	$C_9H_{14}N_3O_2^+$	196.10805	0.25

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Legend for Figures / Schemes

Scheme 1: Proposed fragmentation mechanism of Vildagliptin *O*-glucuronide

Scheme 2: Examples of gas-phase rearrangements taken from the literature (part 1)

Scheme 3: Examples of gas-phase rearrangements taken from the literature (part 2)

Figure 1: MS^2 spectra of Vildagliptin *O*-glucuronide upon HCD (upper) and CID (lower) activation

Figure 2: Important MS^3 spectra of Vildagliptin *O*-glucuronide utilizing CID activation twice a) MS^2 spectrum of *m/z* 483.3 upon HCD fragmentation; b) MS^3 spectrum *m/z* 483.3 > 330.3; c) MS^3 spectrum *m/z* 483.3 > 304.3; d) MS^3 spectrum *m/z* 483.3 > 283.3.

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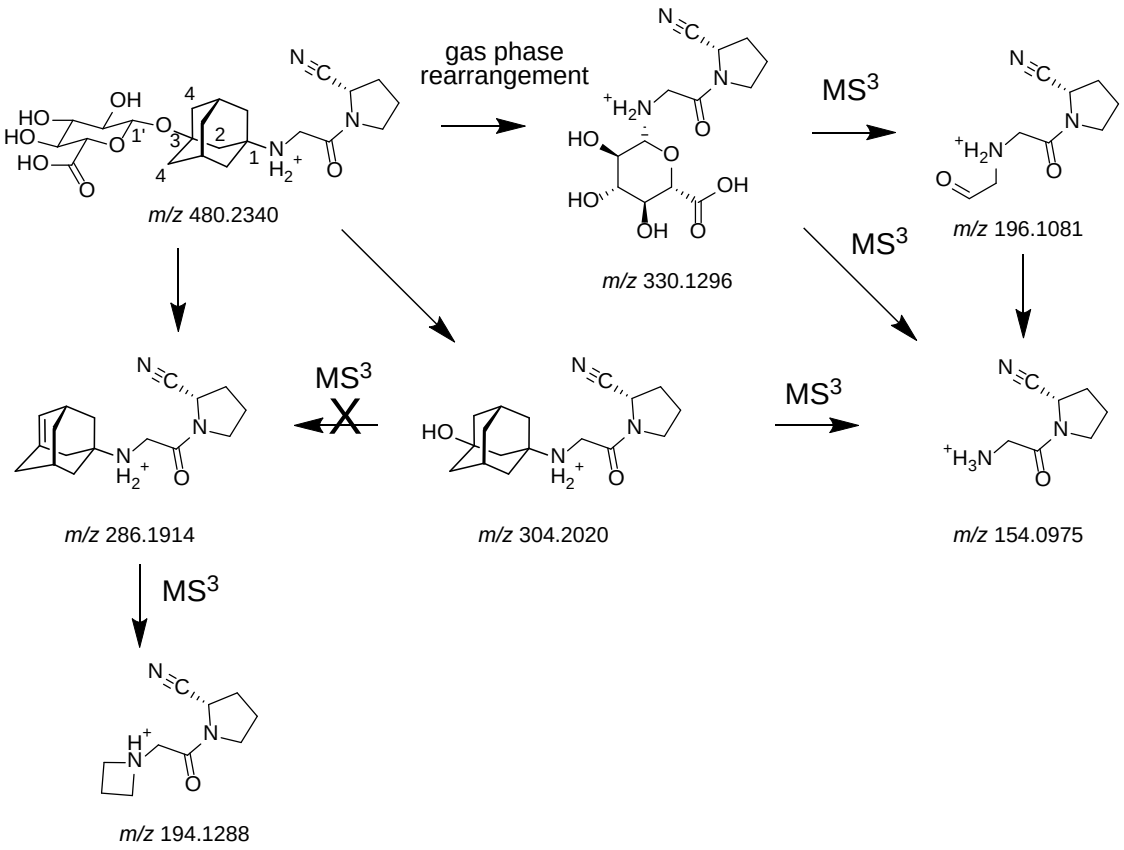

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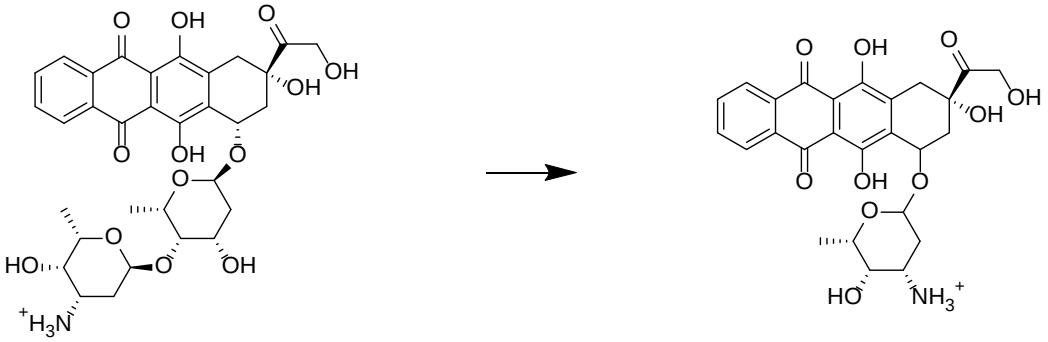

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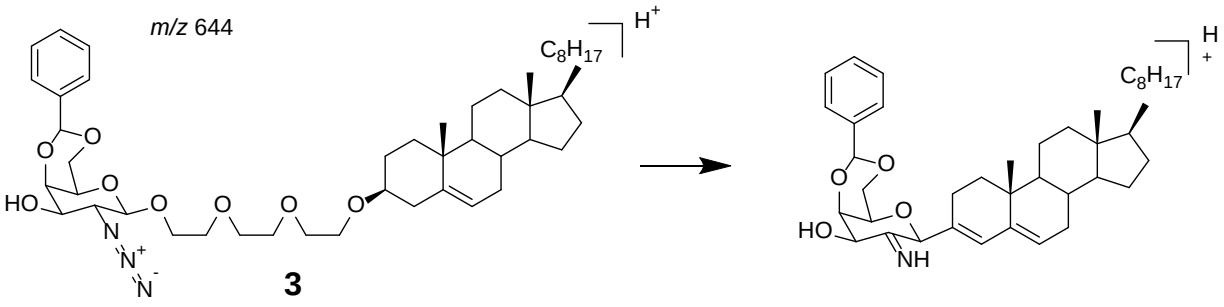
m/z 644

m/z 514



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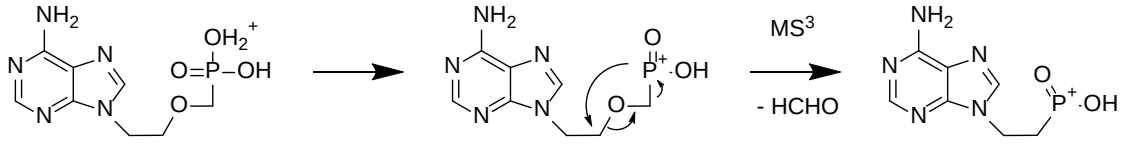
m/z 616



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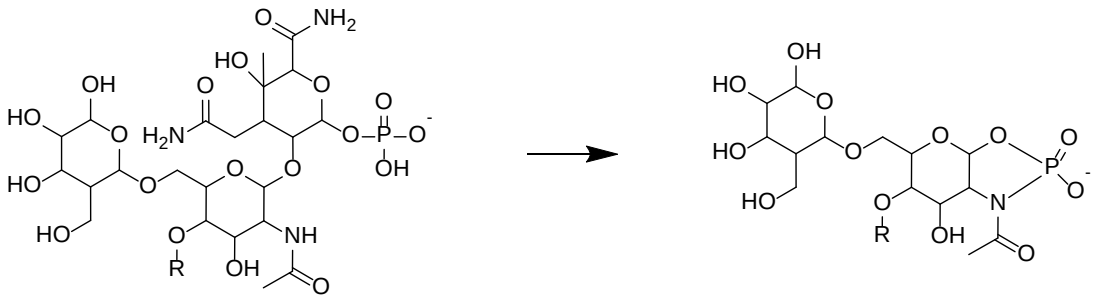
m/z 274

m/z 256

m/z 226



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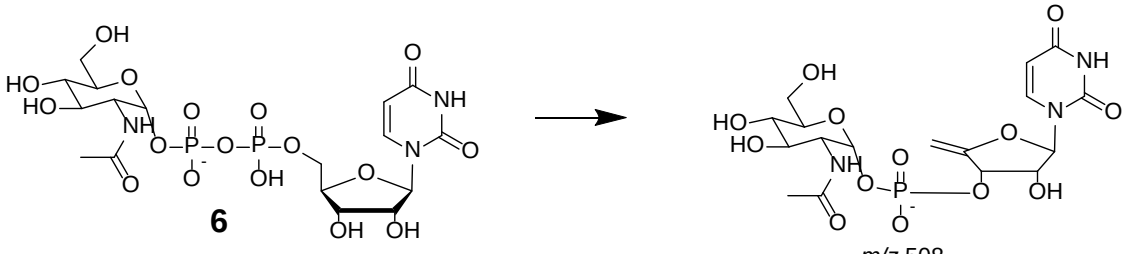
m/z 1152

m/z 902



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m/z 606

m/z 508

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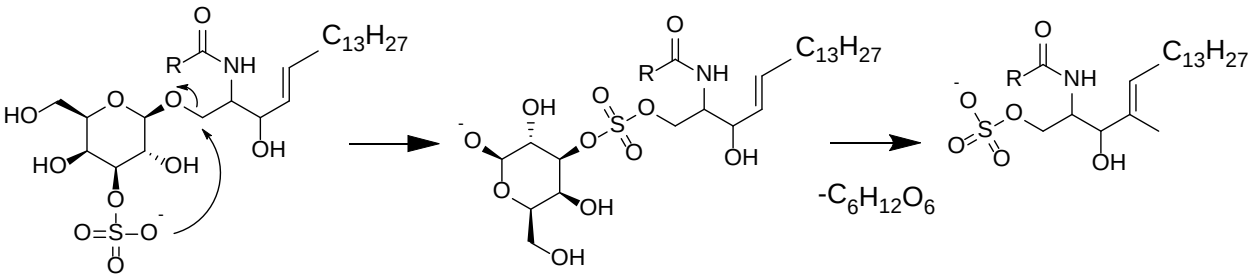


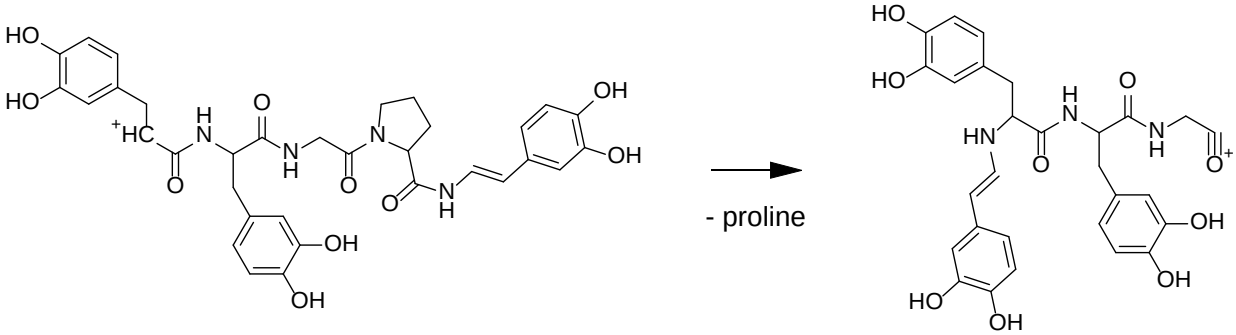
7

m/z 904

m/z 904

$-C_6H_{12}O_6$

m/z 742

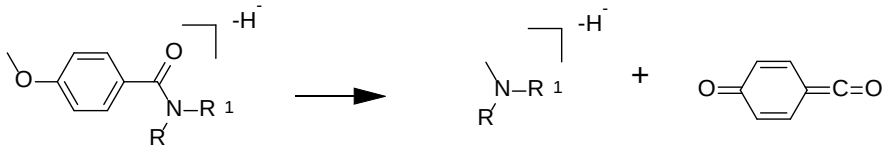


8

m/z 606

- proline

m/z 550



9

m/z 454

m/z 334

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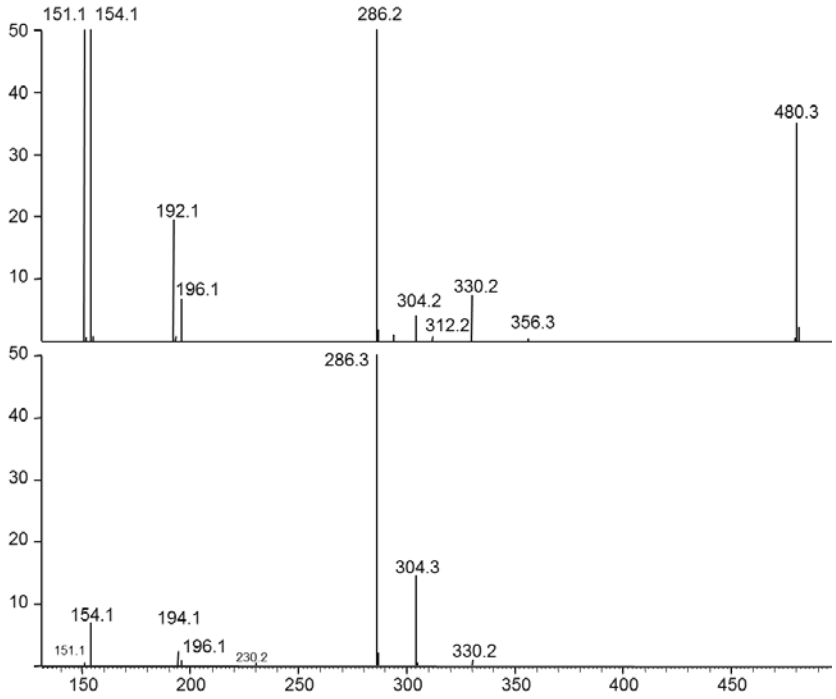

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