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Short Communication

Gas-Phase Rearrangement of the *O*-Glucuronide of Vildagliptin Forms Product-Ion Fragments Suggesting Wrongly an *N*-Glucuronide

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ABSTRACT

The *O*-glucuronide of vildagliptin, a dipeptidyl peptidase 4 inhibitor, is a major metabolite in monkeys and a minor metabolite in humans, rats, and dogs. Its product ion spectrum shows fragments that can be explained only by an *N*-glucuronide. Biotransformation using rat liver yielded milligram amounts of the *O*-glucuronide, and its structure was assigned unambiguously by nuclear magnetic resonance. The tandem mass spectra (MS/MS) of this compound was investigated in detail using 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 quadrupole time-of-flight 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 type 2 (Doupis and Veves, 2008). The human and animal metabolism of vildagliptin have been investigated intensively (He et al., 2009a,b). The major human metabolite was M20.7, where the cyano group was hydrolyzed to an acid. In rats, the main circulating metabolites in blood plasma were M20.7 and, to a lesser extent, the *O*-glucuronide M20.2. The question of whether M20.2 was an *O*-glucuronide or an *N*-glucuronide had to be addressed, even more so 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, we investgated in detail the MS^n product ion spectra of vildagliptin-O-glucuronide and showed that some fragments had to be formed by gas-phase rearrangement. The mechanisms for these gas-phase rearrangements are discussed herein.

Material and Methods

The analytical liquid chromatograph consisted of a Waters UPLC Acquity (Waters, Milford, CT) equipped with a Waters Acquity photodiode array detector detector: column, Acquity BEH C18, 1.7 μm; 1.0 × 150 mm (Waters); flow rate, 0.1 ml/min; eluent A, H_2O/trifluoroacetic acid, 100:0.02; eluent B, acetonitrile/trifluoroacetic acid 100:0.02; gradient, 0 minute; 2% B; 15 minutes, 40% B; 16 to 17 minutes; 95% B, column temperature, 40°C; UV detection, 210 nm; diode array detection from 205 to 350 nm; resolution, 2.4 nm; injection volume, 0.6 μl. The UPLC was controlled by Empower Software (version 3 Waters).

An ion-trap MS LTQ Velos Pro (ThermoFisher Scientific, San Jose, CA) equipped with heated electrospray interface was operated in positive mode with Xcalibur Software version 2.1 as follows: a sheath gas (nitrogen >99.5%) setting of 24 U; auxiliary gas of 5 U was used, and a spray voltage of 3.5 kV was applied. The heated metal capillary was maintained at 275°C with a mass range of 300–600 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: collision-induced disocciation (CID) with isolation width, 2.8 Da and without wideband excitation activated; normalized collision energy 30%; activation time 10 (default) or 2 and 200 milliseconds, and an activation Q value of 0.25. Higher-energy CID (HCD) with isolation width 2.8 Da and without wideband excitation activated; normalized collision energy, 25%; activation time, 10 (default) or 2 and 200 milliseconds; and activation Q value, 0.08.

Other MS/MS spectra were obtained on an Xevo G2-XS quadrupole time-of-flight MS (Waters, Wimslow, UK) with electrospray ionization probe in the sensitivity mode and controlled by Unify Software (version 1.8.1; Waters). Important parameters: desolvation gas 800 liters/hour; desolvation temperature 350°C; spray voltage, 3 kV; cone gas, 50 liters/hour; 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.

For accurate mass measurements, we used an Orbitrap Fusion Lumos Tribrid Mass Spectrometer with 1 M resolution (Thermo Scientific). The compound was inserted by a syringe, ionized by electrospray ionization in the positive-ionization mode. Both MS and MS^2 were analyzed using the Oribrap analyzer at one million resolution using 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 and 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 in the patent, was based on ^1H and ^{13}C nuclear magnetic resonance using 2D homonuclear and heteronuclear spectra (correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC),

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ABBREVIATIONS: CID, collision-induced dissociation; HCD, higher-energy CID; MS, mass spectrometry; MS/MS, tandem mass spectrometry.

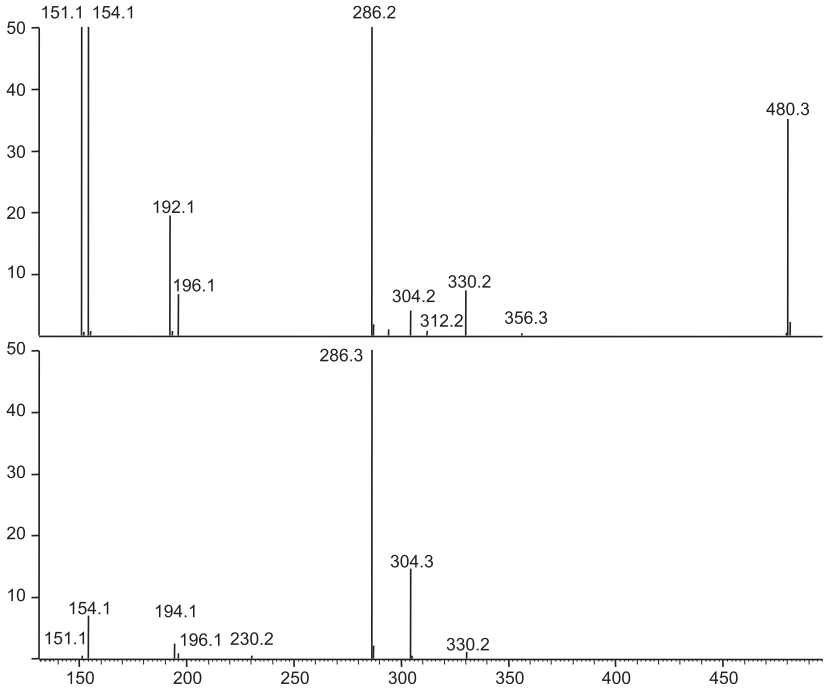


Fig. 1. MS^2 spectra of vildagliptin *O*-glucuronide upon HCD (upper) and CID (lower) activation.

heteronuclear multiple-bond correlation (HMBC), rotational frame nuclear Overhauser effect spectroscopy (ROESY)). A heteronuclear multiple-bond correlation correlation from H-1' at the glucuronide moiety to carbon-3, the carbon where the hydroxyl function was attached at the adamantane ring, and rotational frame nuclear Overhauser effect spectroscopy 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 and higher-energy collisional dissociation (HCD) fragmentation. The accurate mass data of the fragments are compiled in Table 1. These data, as well as MS^3 experiments (Fig. 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 Fig. 1). The activation time had an influence on the relative intensity of that fragment in CID: From an activation time of 2–200 milliseconds, it increased by 72% using 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 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 before 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. We have discussed earlier the loss of 134 Da or $C_4H_6O_5$ in *N*-glucuronides (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 nuclear magnetic resonance spectra. Additional evidence was the MS^3 spectrum of *m/z* 304 (Fig. 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: 1) loss of water from the adamantyl moiety and 2) loss of glucuronic acid from a (not present) *N*-glucuronide could be excluded.

Discussion

Gas-phase rearrangement was first observed in sugars using chemical ionization mass spectroscopy (McNeil, 1983). Warrack et al. (1998) observed the internal loss of fucose from the anthracycline-type natural product **2** (Scheme 2) with CID activation either underivatized or peracetylated. Although the mechanisms in sugars is still unknown, it

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

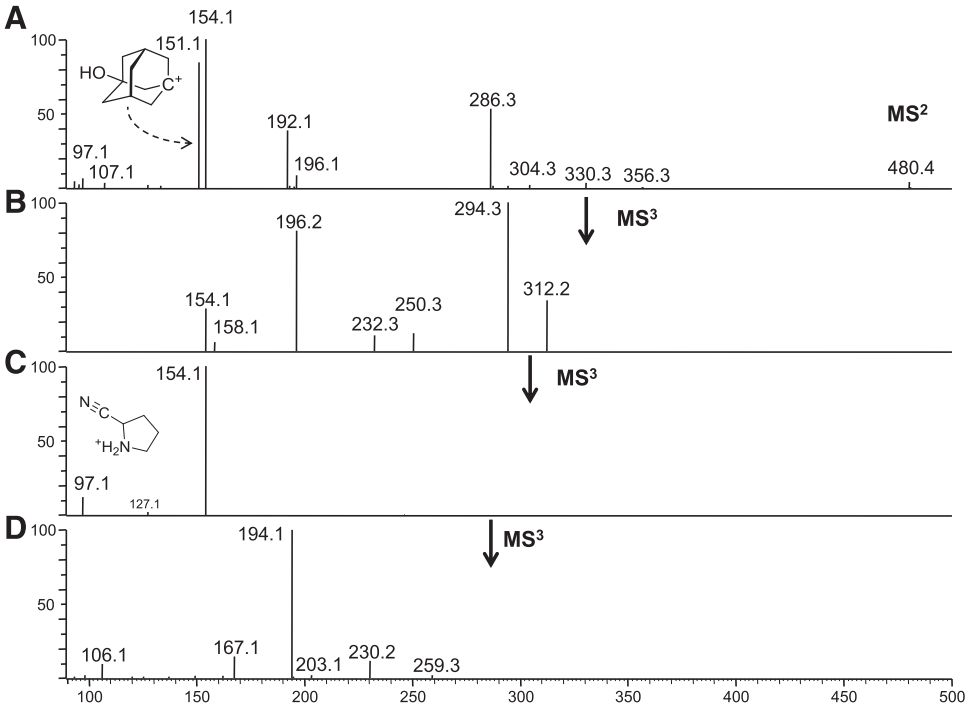
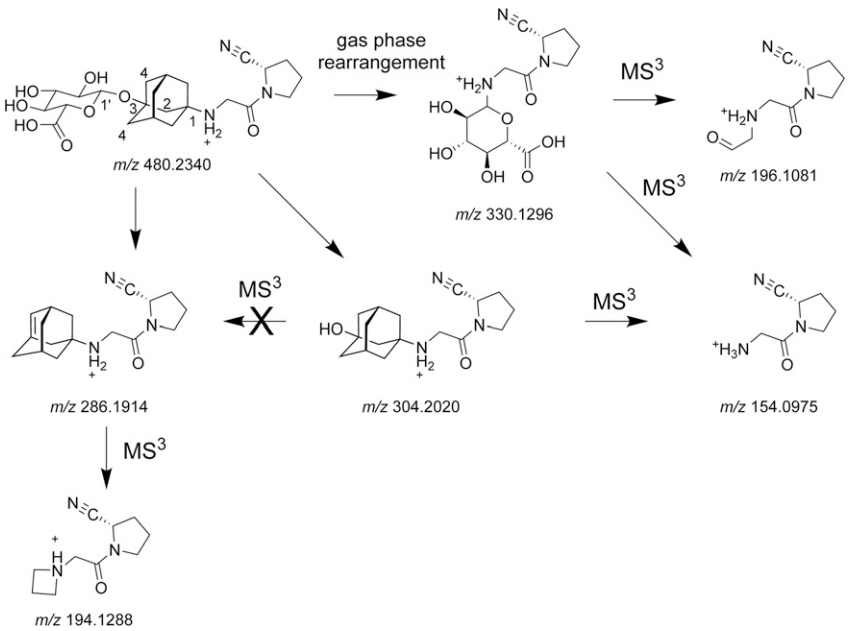


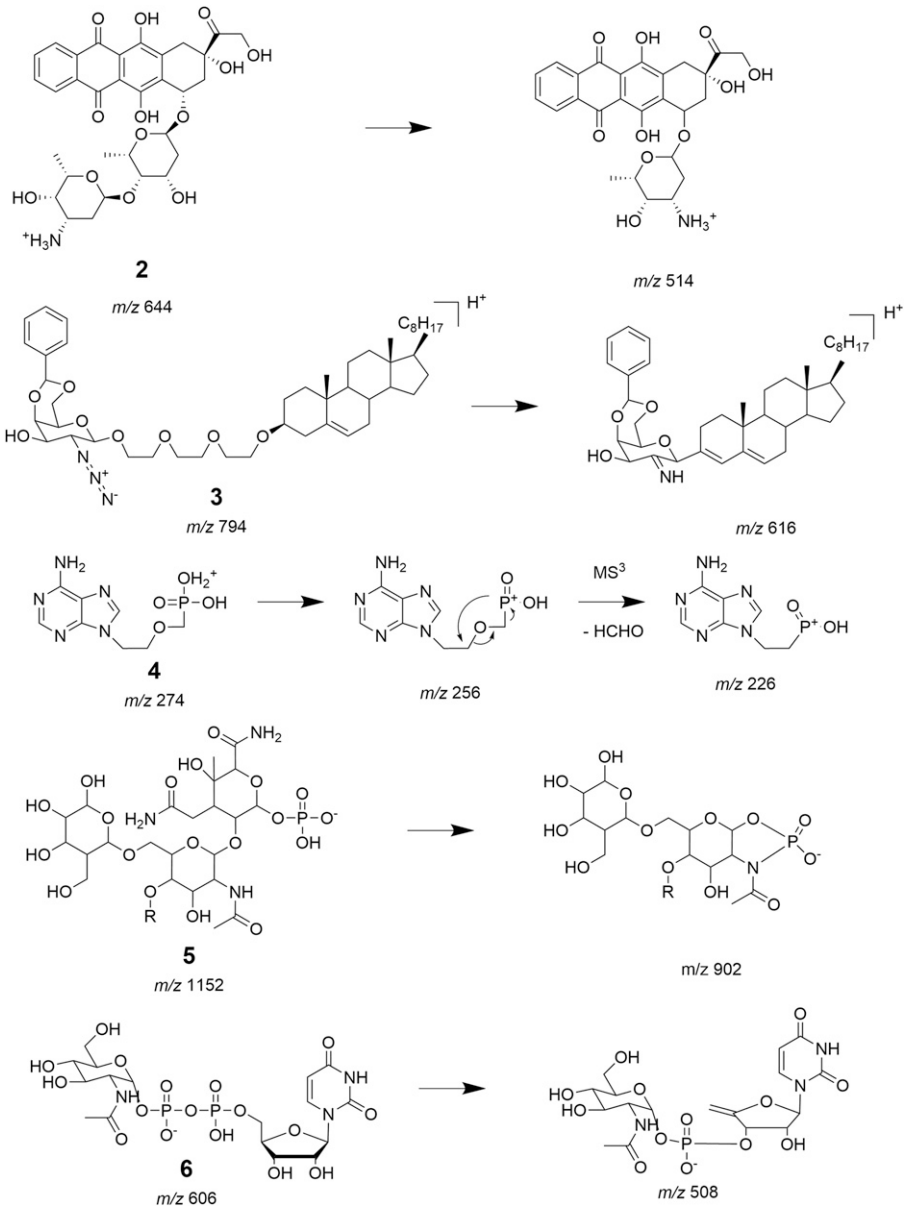
Fig. 2. Important MS^3 spectra of vildagliptin *O*-glucuronide using CID activation twice. (A) MS^2 spectrum of *m/z* 480.3 upon HCD fragmentation; (B) MS^3 spectrum *m/z* 480.3 > 330.3; (C) MS^3 spectrum *m/z* 480.3 > 304.3; (D) MS^3 spectrum *m/z* 480.3 283.3.

seems 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 using 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 (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 (2008) observed that a transfer of phosphate groups in peptides to serine and threonine occurred in 45% of all peptides investigated. 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 milliseconds) resulted in more rearranged product, but even with an activation time of 2 milliseconds, the reaction could not be suppressed. Migration of sulfate has been proposed for a loss of galactose residue from a sulfatide **7** (Scheme 3) using negative-ionization mode and was observed on an ion trap instrument, but not on a tripl-quadrupole MS (Hsu and Turk, 2004).



Scheme 1. Proposed fragmentation mechanism of vildagliptin *O*-glucuronide.



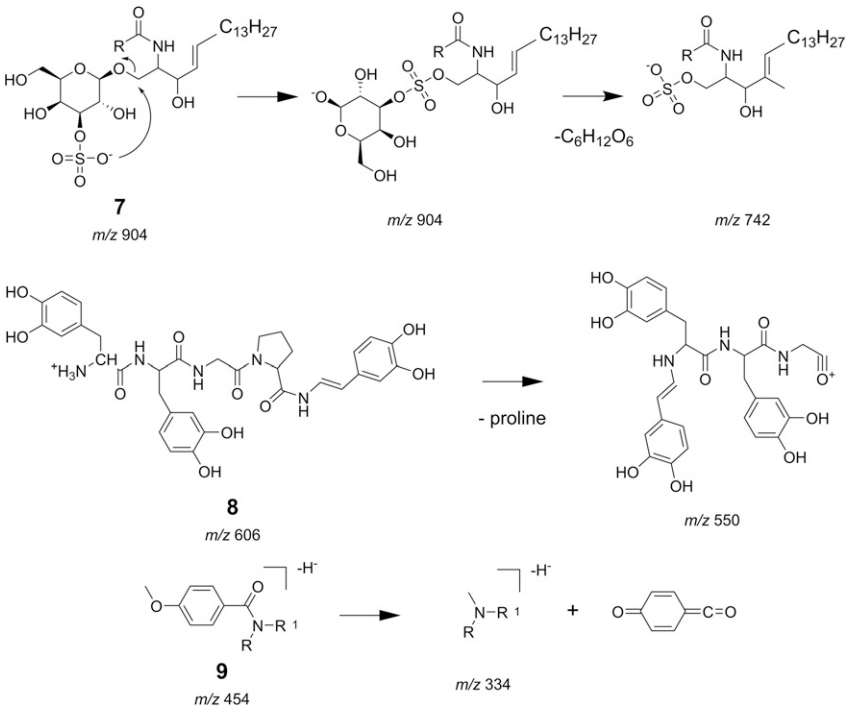
Scheme 2. Examples of gas-phase rearrangements taken from the literature (part 1).

Migrations where a carbon atom and not a heteroatom had to be reattached before the internal losses 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; 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 and 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 is 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 determine whether 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 using CID activation than on the same instrument with HCD activation or on a quadrupole time-of-flight mass spectroscopy type of instrument, where fragmentation was induced by collision with argon.



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

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Novartis Institutes for BioMedical Research, Global Discovery Chemistry, Basel, Switzerland

Andreas Fredenhagen
Jürgen Kühnöl
Matthias Kittelmann
Lukas Oberer

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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Address correspondence to: Dr. Andreas Fredenhagen, Novartis Institutes for BioMedical Research, Global Discovery Chemistry, WKL-122.P.37, CH-4002 Basel, Switzerland. E-mail: andreas.fredenhagen@novartis.com