

Supplementary information

Miniaturised 3D printed polypropylene reactor for online reaction analysis by mass spectrometry

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The 3D information for printing a miniaturised reactor is in the file “Polypropylene_microreactor.STL”. The layer thickness was 200 μm . Embedding a magnetic stir bar and a stainless steel nanospray capillary are the critical phases of the printing process. The nanospray needle is inserted by interrupting the 3D printer after 28 layers have been printed. After this, printing is resumed up to layer 54, after which printing is interrupted a second time and a PTFE coated stir bar is inserted. Finally, printing is resumed once again. The number of layers after which the printing should be interrupted to insert the stir bar depends on the size and shape of the stir bar, but it is generally not critical.

1.2 Building the measurement jig

With the three STL files provided one can 3D print parts of the measurement jig.

- Jig_top_part.STL – the top part of the jig (Fig. S2 A). The miniaturised polypropylene reactor is inserted inside this part.
- Jig_bottom_part.STL – the bottom part (Fig. S2 B). This part is attached to the top part with four M4 screws. The outer four holes are for the attachment of a 40-mm computer fan, and are designed for M3 screws.
- Jig_magnet_receptacle.STL – the magnet receptacle (Fig. S2 C). Into this part we insert two small magnets, and then glue it to the center of the 40-mm fan. This receptacle together with the magnets rotate inside the bottom part of the jig.

The two large holes in the top part should be threaded with an M6 tap, to accommodate the fluidic fittings. The holes in the bottom part should likewise be tapped with the corresponding taps – M4 for the inner holes, and M3 for the outer holes.

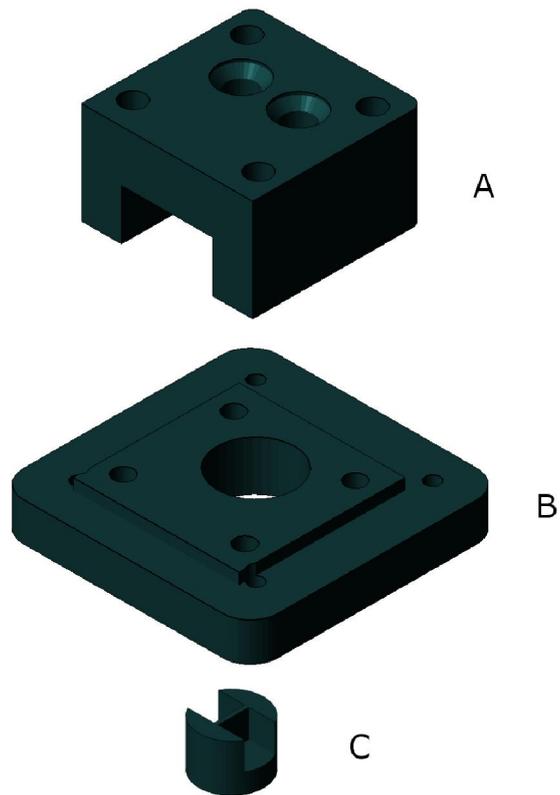


Fig. S2 Schematic illustration of the assembly of the measurement jig. A) The top part of the jig, B) the bottom part and C) the magnet receptacle, which rotates in the bottom of the jig.

2. MS measurement set-up

An XYZ-stage (Märzhauser Wetzlar GmbH & Co. KG) was used to position the jig with the chip in front of the mass spectrometer. An Iso-Tech DC power supply (IPS 603, RS Components) was used to supply power for the mixer fan. A copper wire that had one end wrapped around the nano-ESI capillary and the other end connected to a metal rod of the ion source frame provided the grounding for the nano-ESI capillary. A PHD 2000 syringe pump (Harvard Apparatus) and two 1-mL syringes (i.d. 4.6 mm, SGE Analytical Science) were utilised for infusing the reactant solutions. The syringes were connected to the miniaturised reactor in the jig by PEEK capillaries (i.d. 64 μm , Applied Research Europe GmbH), ferrules, nuts and sleeves (IDEX Health & Science LLC).

3. MS measurement conditions

Online and offline mass spectrometric measurements were mainly done using an Agilent 6330 ion trap mass spectrometer operated in positive ESI mode. Offline measurements with an Orbitrap Fusion mass spectrometer in positive ESI mode were used to measure accurate mass information for the ions observed with the ion trap mass spectrometer.

The Agilent 6330 ion trap was equipped with a capillary extension, towards which the nano-ESI capillary was directed. The capillary voltage was -2.5 kV, the drying gas flow rate was 6 L/min and the drying gas temperature was 200 °C. The m/z scan range used for the Diels-Alder reaction study was 200-500 and for the mixer's efficiency evaluation measurements the m/z scan range was 280-300. Mass spectra were typically recorded continuously for 60-80 min for the monitoring of the Diels-Alder

and subsequent retro Diels-Alder reactions. The presented mass spectra are averaged over one minute. The m/z scan range for the MS² and the MS³ experiments was 50-600. The precursor ion isolation width was 1.0 m/z for all the MS² and the MS³ experiments. The MS² and MS³ data were recorded for 30 s per precursor ion and the product ion mass spectra were averaged over this time. The m/z of the ion of interest for the extracted ion profiles was selected with one decimal's accuracy, and a mass window range of $\pm 0.5 m/z$ was used. The software DataAnalysis for 6300 series ion trap LC/MS version 3.4 (Build 192) (Agilent Technologies) was used to process the data. Additional MSⁿ experiments with the ion trap mass spectrometer were run offline to confirm the identity of some of the ions observed. The main differences to the online experiments were that the commercial ESI source of the ion trap was used and that the Diels-Alder and retro Diels-Alder reactions were allowed to proceed at least for one hour before the reaction mixture was directly infused and the measurements were performed.

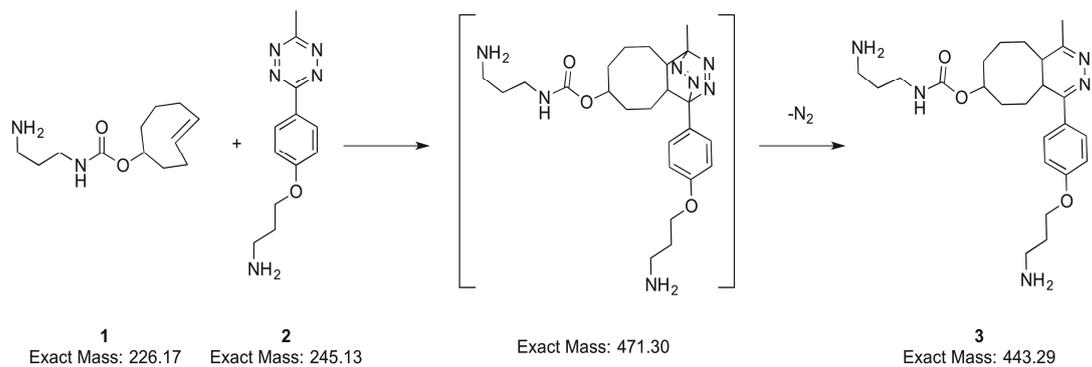
The accurate mass measurements were done offline with an Orbitrap Fusion mass spectrometer using direct infusion ESI in positive mode. The corresponding concentrations of reactants as used in the online experiments were used, but reactants were mixed offline and allowed to react more than 2 hours before diluting the sample ten times with acetonitrile:water 80:20 + 0.1 vol% formic acid for the direct infusion measurements (flow rate 5 mL/min). The ESI voltage was 3.3 kV and the ion transfer tube temperature 275 °C. The mass spectra were collected with the profile mode in the m/z scan range 50-600 and the mass resolution 120 000 FWHM (at m/z 200) was applied. The precursor ions were isolated with an isolation width of 1.0 m/z with the

quadrupole or ion trap mass analyser and fragmented with higher-energy collisional dissociation (HCD) with collision energy in the range of 10-35% of a normalised collision energy. The accurate mass data presented is taken from an average of at least 50 mass spectra. The software Xcalibur (Thermo Fisher Scientific Inc.) was utilised to process the acquired data, as well as for calculating the exact masses reported in Tables S2-3.

4. Reagents

Trans-Cyclooctene-amine hydrochloride salt (**1**, >95%) and methyltetrazine-propylamine hydrochloride salt (**2**, >95%) were obtained from Click Chemistry Tools. In the Orbitrap measurements *trans*-cyclooctene-amine hydrochloride salt (**1**, 99%) from Sigma-Aldrich was used. Acetonitrile (LC-MS Chromasolv grade), testosterone ($\geq 99\%$) and testosterone- d_3 (98%) were purchased from Sigma-Aldrich. Formic acid (98-100%) was purchased from Merck and water was obtained from a Milli-Q Plus (Millipore) purification system. For the online experiments, solutions of *trans*-cyclooctene-amine hydrochloride salt (35 $\mu\text{g/mL}$, 0.13 mM) and methyltetrazine-propylamine hydrochloride salt (70 $\mu\text{g/mL}$, 0.25 mM) were prepared in acetonitrile:water 80:20 + 0.1 vol% formic acid. Testosterone and testosterone- d_3 were diluted to 1 $\mu\text{g/mL}$ (3.5 μM of testosterone and 3.4 μM of testosterone- d_3) in acetonitrile:water 80:20 + 0.1 vol% formic acid.

5. The Diels-Alder and retro Diels-Alder reactions



Scheme S1 The Diels-Alder and retro Diels-Alder reactions as studied by the 3D printed reactor.

5.1 Averaged mass spectra from different time points

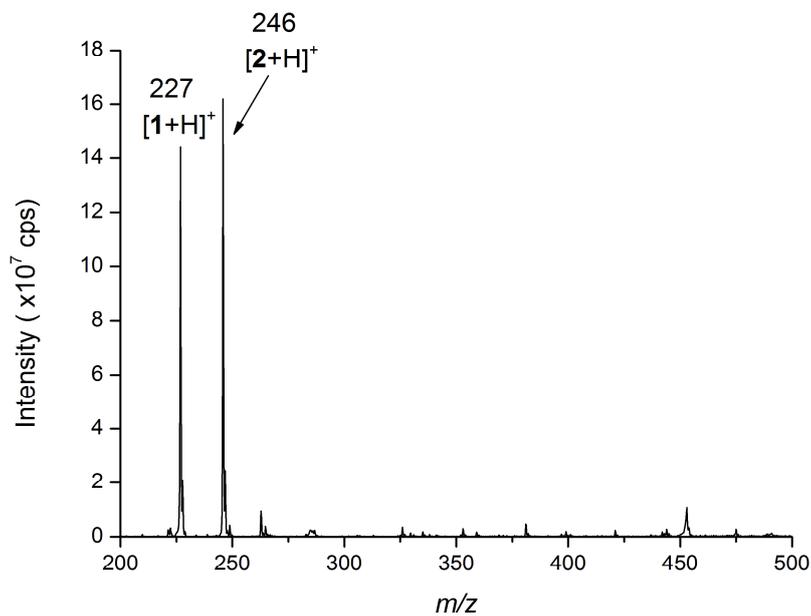


Fig. S3 A mass spectrum averaged over 2-3 minutes.

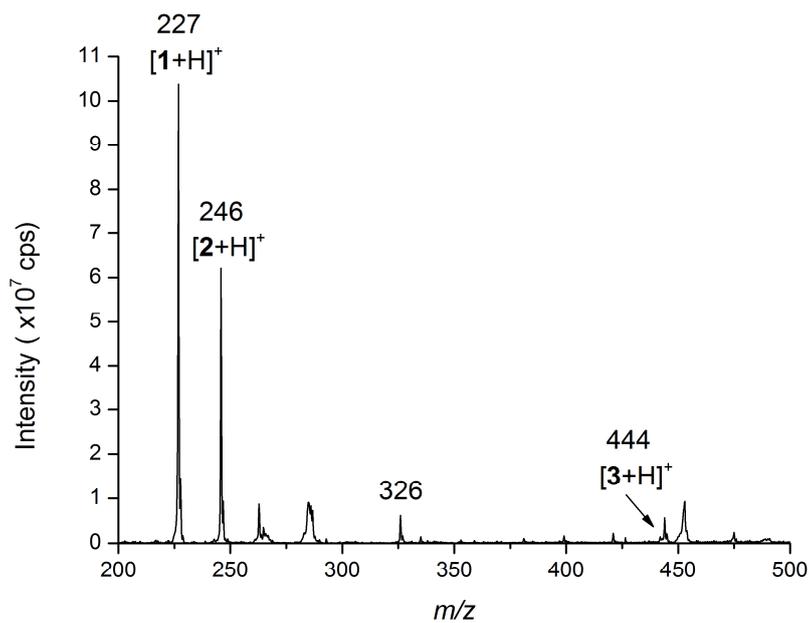


Fig. S4 A mass spectrum averaged over 5-6 minutes.

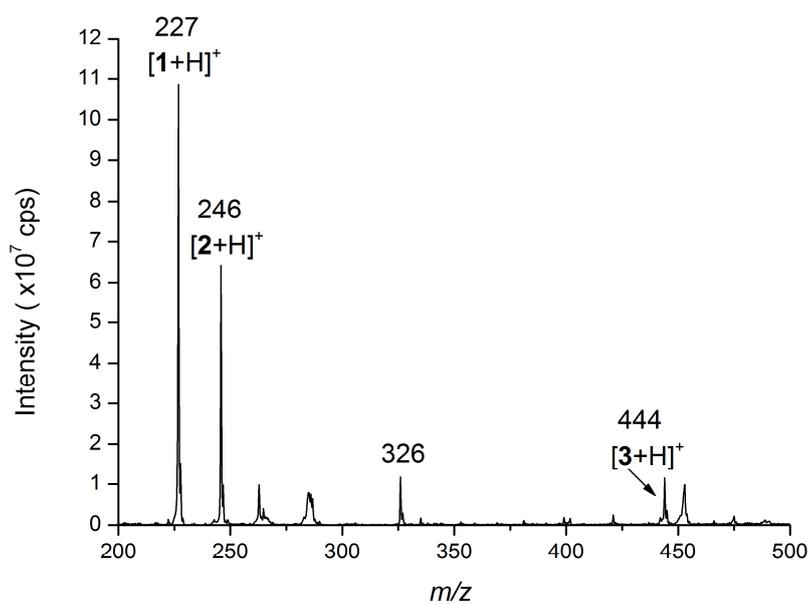


Fig. S5 A mass spectrum averaged over 10-11 minutes.

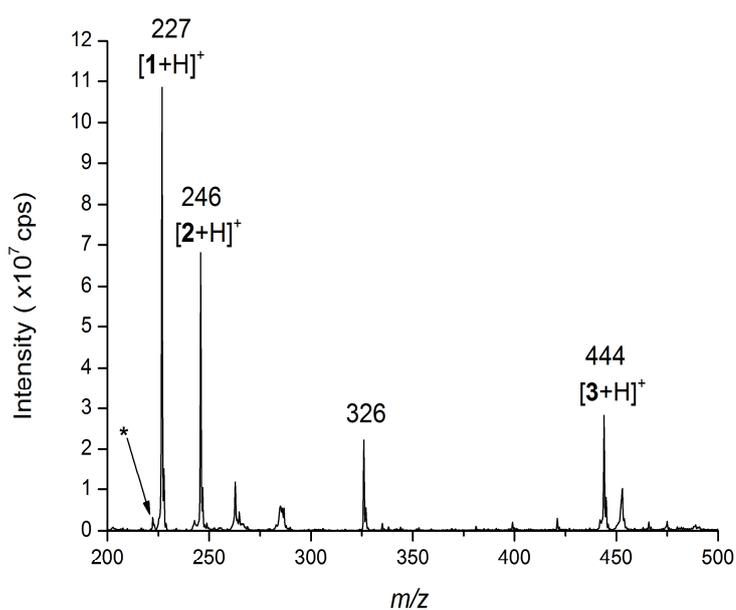


Fig. S6 A mass spectrum averaged over 20-21 minutes. *Doubly charged ion, $[3+2H]^{2+}$ at m/z 222.

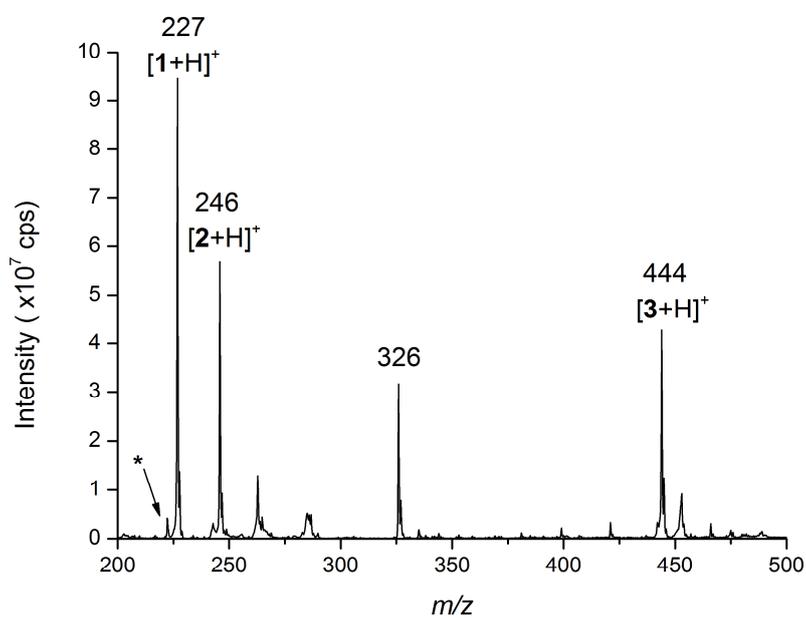


Fig. S7 A mass spectrum averaged over 30-31 minutes. *Doubly charged ion, $[3+2H]^{2+}$ at m/z 222.

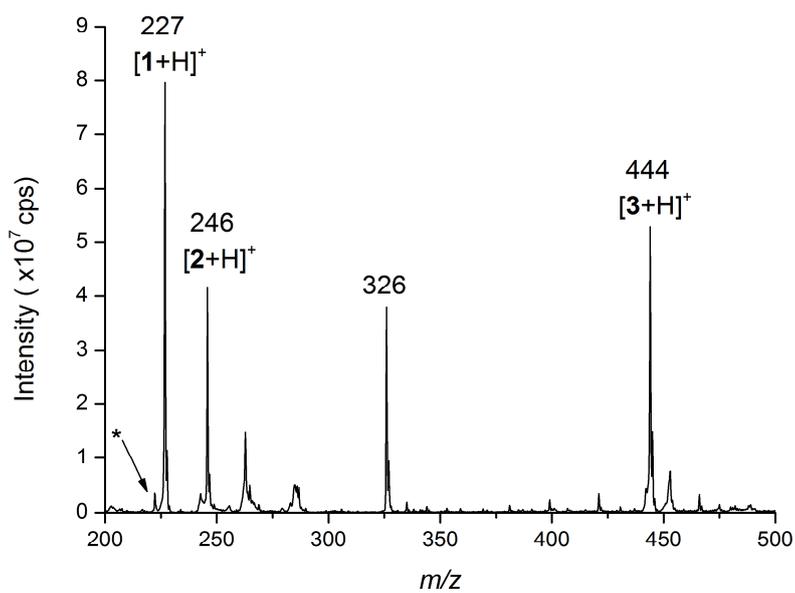


Fig. S8 A mass spectrum averaged over 40-41 minutes. *Doubly charged ion, [3+2H]²⁺ at *m/z* 222.

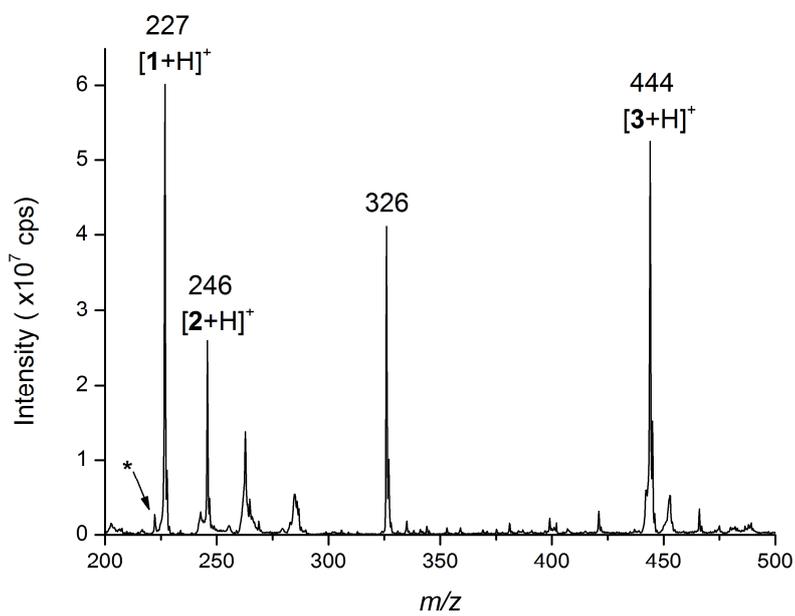


Fig. S9 A mass spectrum averaged over 50-51 minutes. *Doubly charged ion, [3+2H]²⁺ at *m/z* 222.

5.2 Extracted ion profiles of m/z 222, 326 and 444

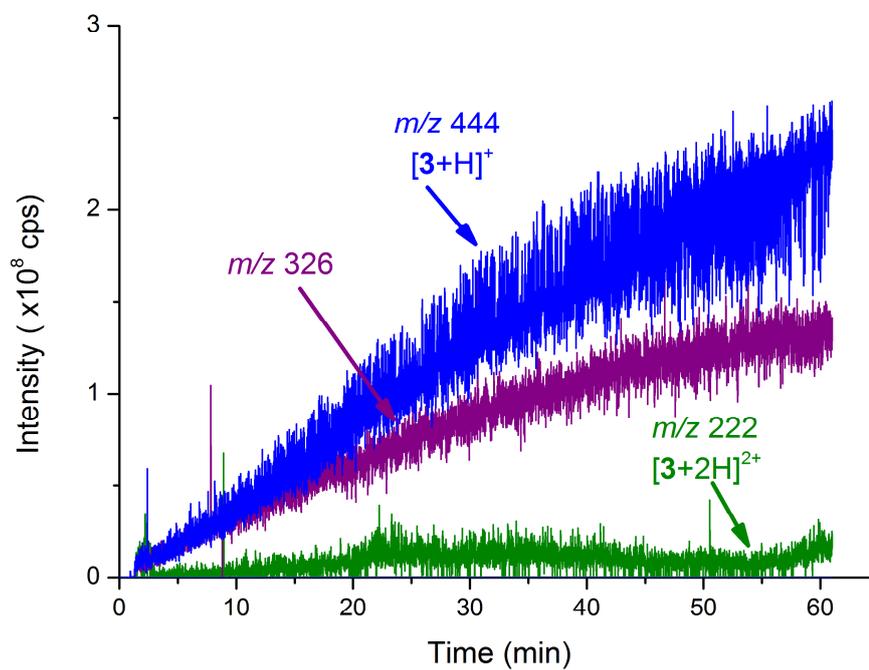


Fig. S10 Extracted ion profiles of the ions m/z 222 (green) [$3+2H$]²⁺, m/z 326 (purple) [$3+H-C_4H_{10}N_2O_2$]⁺, and m/z 444 (blue) [$3+H$]⁺.

5.3 Product ion mass spectra measured by the ion trap mass spectrometer

5.3.1 MS² spectra

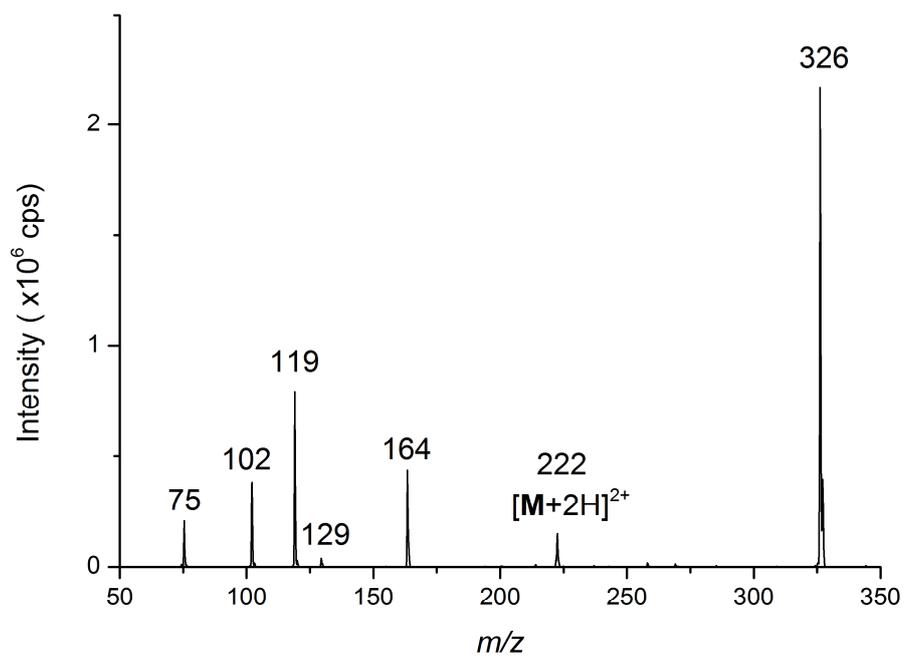


Fig. S11 The MS² spectrum of the precursor ion m/z 222. The fragmentation amplitude was 0.30 V. This MS² spectrum was recorded when the reaction was conducted offline.

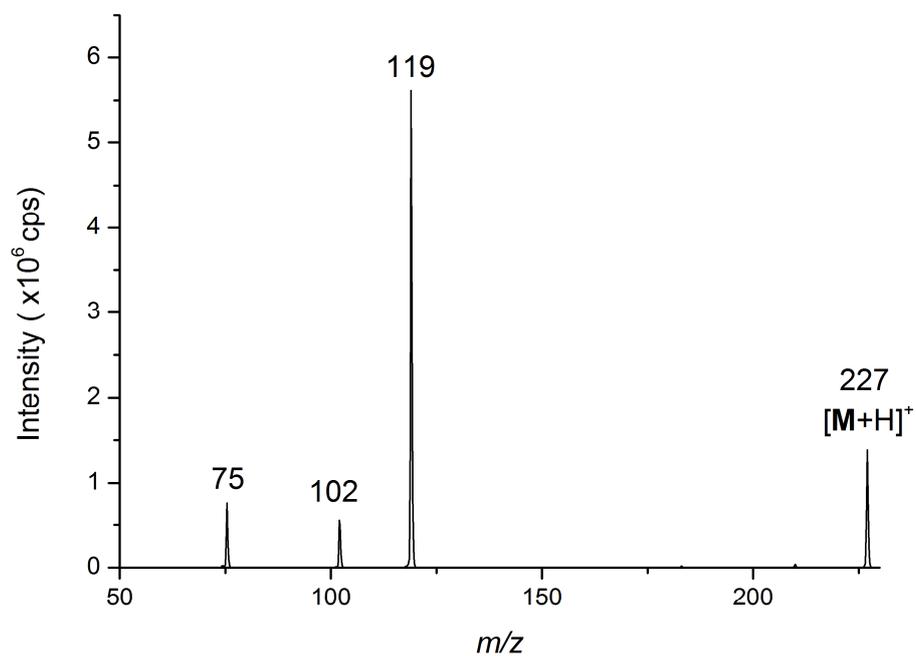


Fig. S12 The MS² spectrum of the precursor ion m/z 227. The fragmentation amplitude was 0.35 V. This MS² spectrum was recorded when the reaction was conducted online.

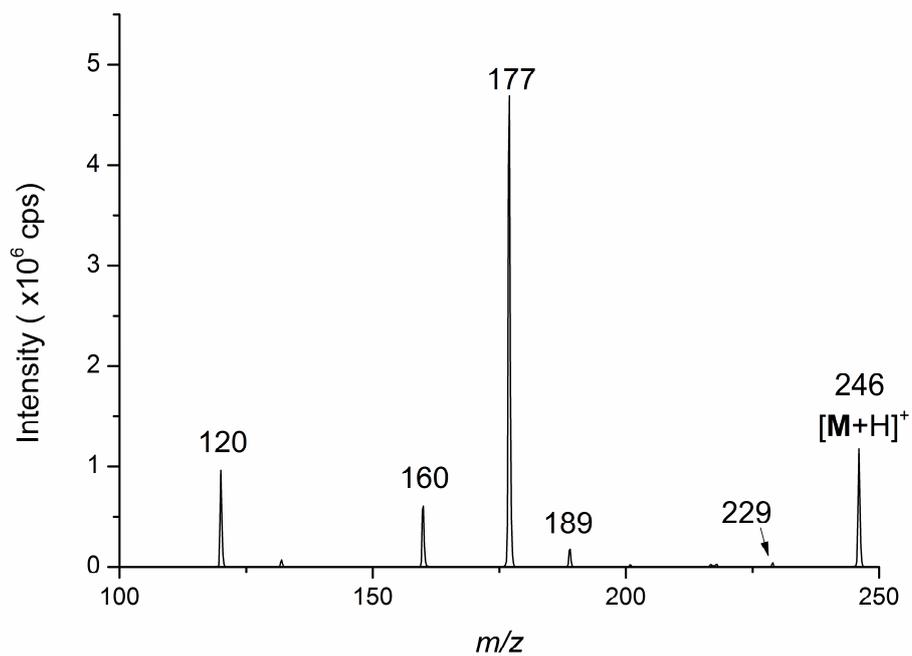


Fig. S13 The MS² spectrum of the precursor ion m/z 246. The fragmentation amplitude was 0.40 V. This MS² spectrum was recorded when the reaction was conducted online.

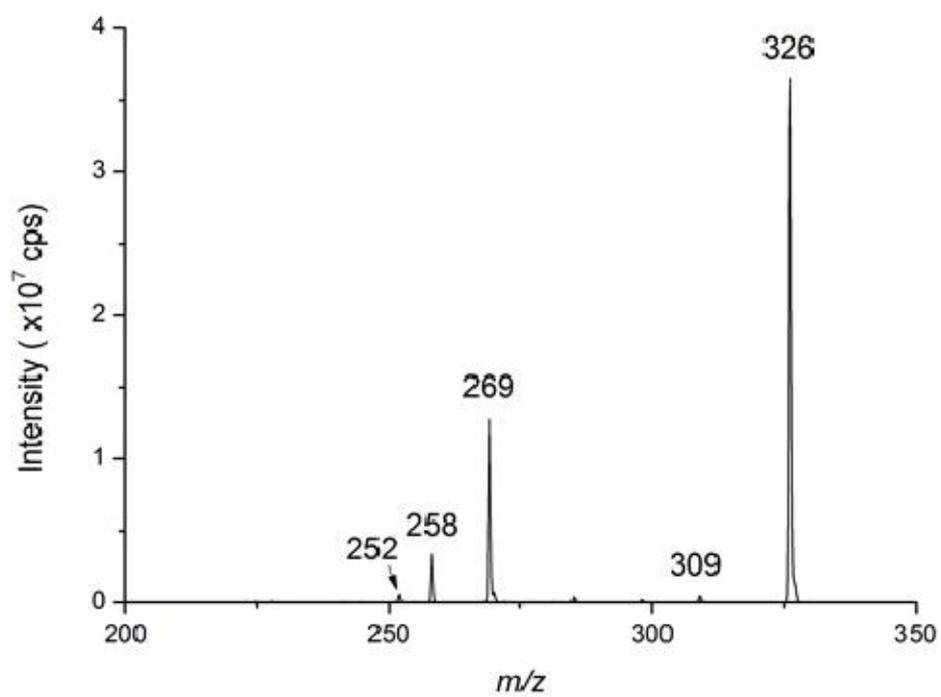


Fig. S14 The MS² spectrum of the precursor ion m/z 326. The fragmentation amplitude was 0.50 V. This MS² spectrum was recorded when the reaction was conducted online.

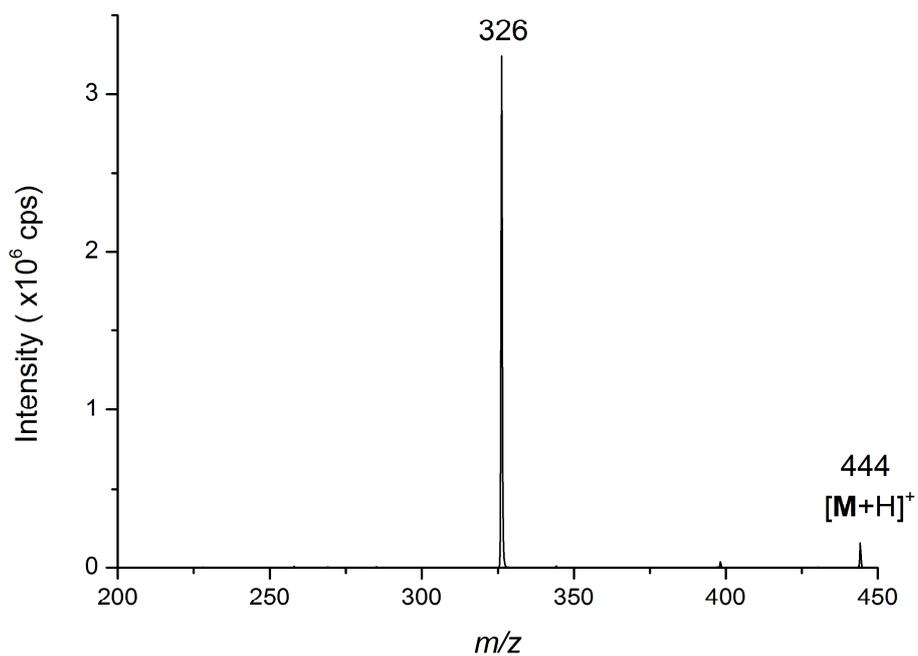


Fig. S15 The MS² spectrum of the precursor ion m/z 444. The fragmentation amplitude was 0.30 V. This MS² spectrum was recorded when the reaction was conducted online.

5.3.2 MS³ spectra

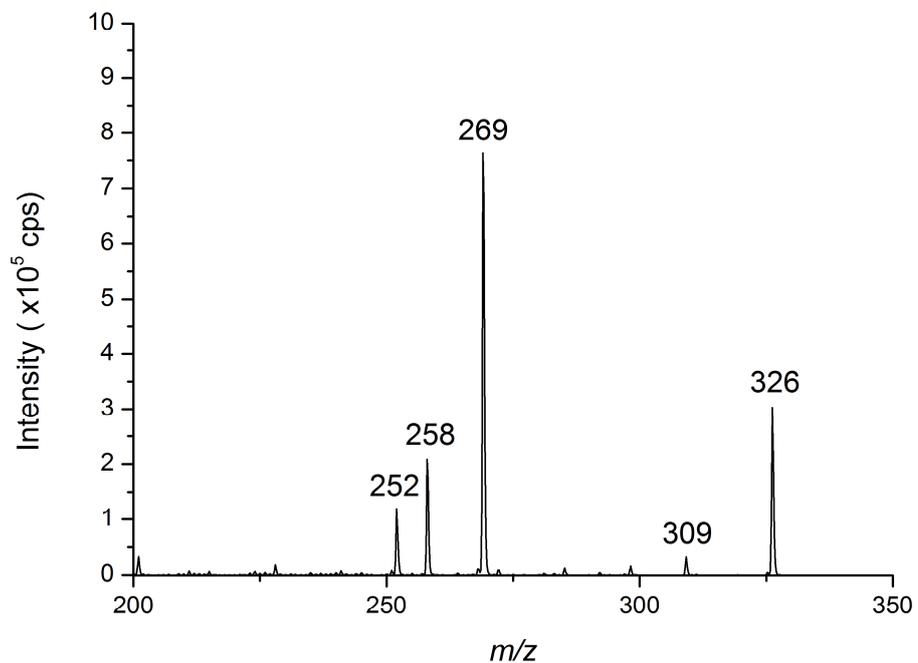


Fig. S16 The MS³ spectrum of the precursor ion m/z 326 resulting from isolating and fragmenting the ion m/z 326 (fragmentation amplitude 0.45 V) from the MS² measurement of the precursor ion m/z 222 (fragmentation amplitude 0.30 V). This MS³ spectrum was recorded when the reaction was conducted offline.

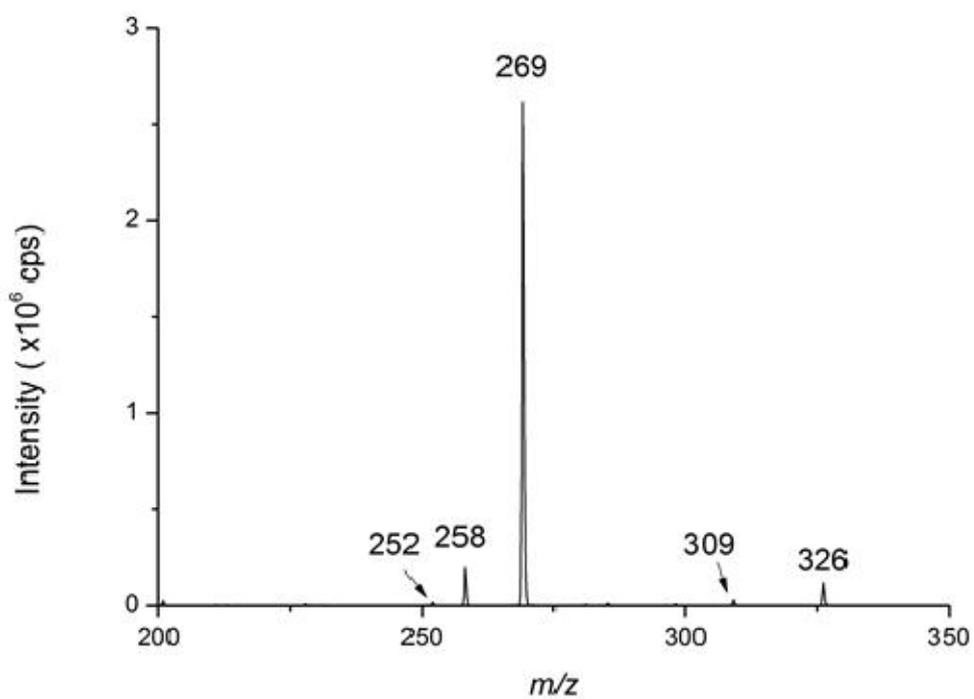


Fig. S17 The MS³ spectrum of the precursor ion m/z 326 resulting from isolating and fragmenting the ion m/z 326 (fragmentation amplitude 0.60 V) from the MS² measurement of the precursor ion m/z 444 (fragmentation amplitude 0.30 V). This MS³ spectrum was recorded when the reaction was conducted online.

5.4 Fragmentation tables

The ions included in the fragmentation tables either had a relative intensity of $\geq 5\%$ of the intensity of the base peak or contributed significantly to the interpretation of the fragmentation of the precursor ions. The exact and accurate masses were obtained using the Xcalibur software (Thermo Fisher Scientific Inc.). Elements and their allowed numbers used in the elemental composition and exact mass calculations are presented in Table S1. The ion charge was set to one, except in the cases of the ions m/z 222.65, 163.61 and 129.58, when it was two. The nitrogen rule was not used. The mass tolerance was 140.00 mmu, and the ring double bond equivalents had the range of -1.0-100.0. Note that the difference between the measured accurate mass and the calculated exact mass was clearly below one millimass unit (mmu) for all the mass peaks reported in Tables S2 and S3. In addition, the elemental compositions presented in Tables S2 and S3 were all the time the first ones in the proposed elemental composition list provided by the Xcalibur software.

Table S1 Elements and their allowed number in the exact mass calculations.

Isotope	Min	Max
¹⁴ N	0	10
¹⁶ O	0	15
¹² C	0	30
¹ H	0	60

5.4.1 MS² experiments

Table S2 Precursor and product ion mass-to-charge ratios from the MS and MS² experiments, and the proposed elemental compositions. The mass-to-charge values obtained with the ion trap mass spectrometer are rounded to the closest integer value of the measured mass-to-charge ratio. The accurate mass values were measured with an Orbitrap mass spectrometer. The precursor ion accurate masses were obtained from a measured mass spectrum and the product ion accurate masses from the corresponding Orbitrap MS² experiment to those of the ion trap MS² measurements. All the Orbitrap measurements were done offline.

MS ² precursor* and product ions, <i>m/z</i>		Precursor* and product ions exact masses (calcd.)	Mass difference, mmu	Elemental composition (calculations with Xcalibur software & authors' interpretation)	Lost mass with integer value and the elements lost
<i>Ion trap</i> mass data (Fig. S11-S15)	Accurate mass data (<i>Orbitrap</i> MS)				
222	222.65203	222.65210	-0.07	C ₂₄ H ₃₉ N ₅ O ₃ , [3 +2H] ²⁺	
326	326.22239	326.22269	-0.30	C ₂₀ H ₂₈ N ₃ O, [3 +2H-C ₄ H ₁₁ N ₂ O ₂] ⁺	119, [C ₄ H ₁₁ N ₂ O ₂] ⁺
164	163.61495	163.61498	-0.03	C ₂₀ H ₂₉ N ₃ O, [3 +2H-C ₄ H ₁₀ N ₂ O ₂] ²⁺	118, C ₄ H ₁₀ N ₂ O ₂
129	129.58375	129.58368	-0.07	C ₁₅ H ₂₁ N ₃ O, [3 +2H-C ₄ H ₁₀ N ₂ O ₂ -C ₃ H ₈] ²⁺	186, C ₉ H ₁₈ N ₂ O ₂
119	119.08158	119.08150	0.08	C ₄ H ₁₁ N ₂ O ₂ , [3 +2H-C ₂₀ H ₂₈ N ₃ O] ⁺	326, C ₂₀ H ₂₈ N ₃ O
102	102.05511	102.05496	0.15	C ₄ H ₈ NO ₂ , [3 +2H-C ₂₀ H ₂₈ N ₃ O-NH ₃] ⁺	343, C ₂₀ H ₃₁ N ₄ O
75	75.09187	75.09167	0.20	C ₃ H ₁₁ N ₂ , [3 +2H-C ₂₀ H ₂₈ N ₃ O-CO ₂] ⁺	370, C ₂₁ H ₂₈ N ₃ O ₃

Table S2 (continued)

MS ² precursor* and product ions, <i>m/z</i>		Precursor* and product ions exact masses (calcd.)	Mass difference, mmu	Elemental composition (calculations with Xcalibur software & authors' interpretation)	Lost mass with integer value and the elements lost
<i>Ion trap</i> mass data (Fig. S11-S15)	Accurate mass data (<i>Orbitrap</i> MS)				
227	227.17525	227.17540	-0.15	C ₁₂ H ₂₃ N ₂ O ₂ , [1+H] ⁺	
119	119.08161	119.08150	0.11	C ₄ H ₁₁ N ₂ O ₂ , [1+H-C ₈ H ₁₂] ⁺	108, C ₈ H ₁₂
102	102.05512	102.05496	0.16	C ₄ H ₈ NO ₂ , [1+H-C ₈ H ₁₂ -NH ₃] ⁺	125, C ₈ H ₁₅ N
75	75.09189	75.09167	0.22	C ₃ H ₁₁ N ₂ , [1+H-C ₉ H ₁₂ O ₂] ⁺	152, C ₉ H ₁₂ O ₂
246	246.13483	246.13494	-0.11	C ₁₂ H ₁₆ N ₅ O, [2+H] ⁺	
229	229.10833	229.10839	-0.06	C ₁₂ H ₁₃ N ₄ O, [2+H-NH ₃] ⁺	17, NH ₃
189	189.07710	189.07709	0.01	C ₉ H ₉ N ₄ O, [2+H-C ₃ H ₇ N] ⁺	57, C ₃ H ₇ N
177	177.10224	177.10224	0.00	C ₁₀ H ₁₃ N ₂ O, [2+H-N ₂ -CH ₃ CN] ⁺	69, C ₂ H ₃ N ₃
160	160.07574	160.07569	0.05	C ₁₀ H ₁₀ NO, [2+H-NH ₃ -C ₂ H ₃ N ₃] ⁺	86, C ₂ H ₆ N ₄
120	120.04452	120.04439	0.13	C ₇ H ₆ NO, [2+H-NH ₃ -C ₂ H ₃ N ₃ -C ₃ H ₄] ⁺	126, C ₅ H ₁₀ N ₄
326	326.22247	326.22269	-0.22	C ₂₀ H ₂₈ N ₃ O, [3+H-C ₄ H ₁₀ N ₂ O ₂] ⁺	
309	309.19600	309.19614	-0.14	C ₂₀ H ₂₅ N ₂ O, [3+H-C ₄ H ₁₀ N ₂ O ₂ -NH ₃] ⁺	17, NH ₃
269	269.16473	269.16484	-0.11	C ₁₇ H ₂₁ N ₂ O, [3+H-C ₄ H ₁₀ N ₂ O ₂ -NH ₃ -C ₃ H ₄] ⁺	57, C ₃ H ₇ N
258	258.16003	258.16009	-0.06	C ₁₅ H ₂₀ N ₃ O, [3+H-C ₄ H ₁₀ N ₂ O ₂ -C ₅ H ₈] ⁺	68, C ₅ H ₈
252	252.13824	252.13829	-0.05	C ₁₇ H ₁₈ NO, [3+H-C ₄ H ₁₀ N ₂ O ₂ -C ₃ H ₁₀ N ₂] ⁺	74, C ₃ H ₁₀ N ₂
444	444.29668	444.29692	-0.24	C ₂₄ H ₃₈ N ₅ O ₃ , [3+H] ⁺	
326	326.22238	326.22269	-0.31	C ₂₀ H ₂₈ N ₃ O, [3+H-C ₄ H ₁₀ N ₂ O ₂] ⁺	118, C ₄ H ₁₀ N ₂ O ₂

*The precursor ions are marked in bold.

5.4.2 MS³ experiment

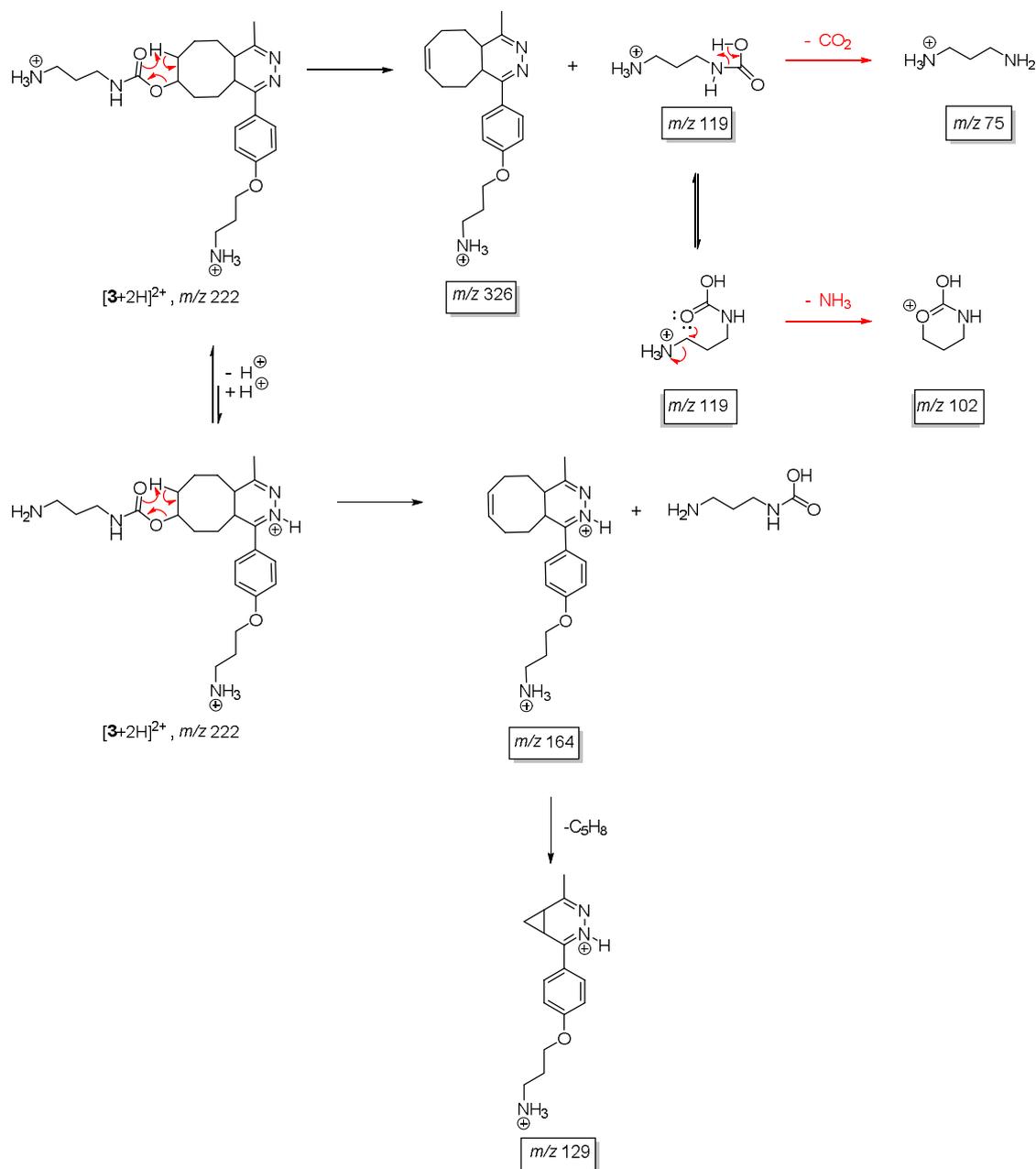
Table S3 Precursor and product ion mass-to-charge ratios from the MS³ experiments and the proposed elemental compositions. The mass-to-charge values obtained with the ion trap mass spectrometer are rounded to the closest integer value of the measured mass-to-charge ratio. The accurate mass values were obtained from the corresponding Orbitrap MS³ experiment to those of the ion trap MS³ measurements. All the Orbitrap measurements were done offline.

MS ³ precursor* and product ions, <i>m/z</i>		Precursor* and product ions exact masses (calcd.)	Mass difference, mmu	Elemental composition (calculations with Xcalibur software & authors' interpretation)	Lost mass with integer value and the elements lost
<i>Ion trap mass data (Fig. S16 and S17)</i>	Accurate mass data (<i>Orbitrap MS</i>)				
222 à 326	222.6 à 326.22226	326.22269	-0.43	C ₂₀ H ₂₈ N ₃ O, [3+H-C ₄ H ₁₀ N ₂ O ₂] ⁺	
309	309.19573	309.19614	-0.41	C ₂₀ H ₂₅ N ₂ O, [3+H-C ₄ H ₁₀ N ₂ O ₂ -NH ₃] ⁺	17, NH ₃
269	269.16451	269.16484	-0.33	C ₁₇ H ₂₁ N ₂ O, [3+H-C ₄ H ₁₀ N ₂ O ₂ -NH ₃ -C ₃ H ₄] ⁺	57, C ₃ H ₇ N
258	258.15976	258.16009	-0.33	C ₁₅ H ₂₀ N ₃ O, [3+H-C ₄ H ₁₀ N ₂ O ₂ -C ₅ H ₈] ⁺	68, C ₅ H ₈
252	252.13802	252.13829	-0.27	C ₁₇ H ₁₈ NO, [3+H-C ₄ H ₁₀ N ₂ O ₂ -C ₃ H ₁₀ N ₂] ⁺	74, C ₃ H ₁₀ N ₂
444 à 326	444.0 à 326.22237	326.22269	-0.32	C ₂₀ H ₂₈ N ₃ O, [3+H-C ₄ H ₁₀ N ₂ O ₂] ⁺	
309	309.19588	309.19614	-0.26	C ₂₀ H ₂₅ N ₂ O, [3+H-C ₄ H ₁₀ N ₂ O ₂ -NH ₃] ⁺	17, NH ₃
269	269.16461	269.16484	-0.23	C ₁₇ H ₂₁ N ₂ O, [3+H-C ₄ H ₁₀ N ₂ O ₂ -NH ₃ -C ₃ H ₄] ⁺	57, C ₃ H ₇ N
258	258.15988	258.16009	-0.21	C ₁₅ H ₂₀ N ₃ O, [3+H-C ₄ H ₁₀ N ₂ O ₂ -C ₅ H ₈] ⁺	68, C ₅ H ₈
252	252.13811	252.13829	-0.18	C ₁₇ H ₁₈ NO, [3+H-C ₄ H ₁₀ N ₂ O ₂ -C ₃ H ₁₀ N ₂] ⁺	74, C ₃ H ₁₀ N ₂

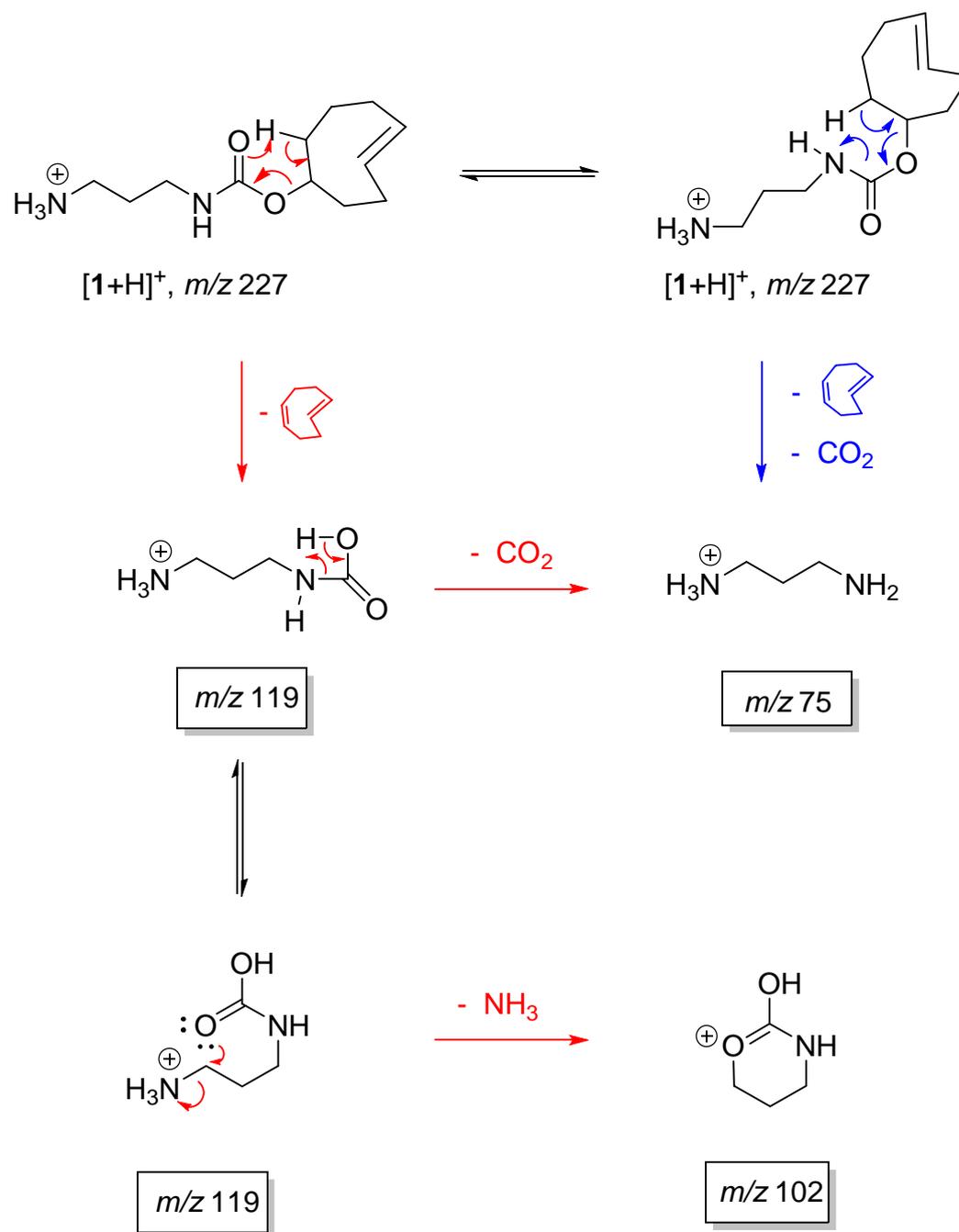
*The precursor ions are marked in bold.

5.5 Fragmentation schemes

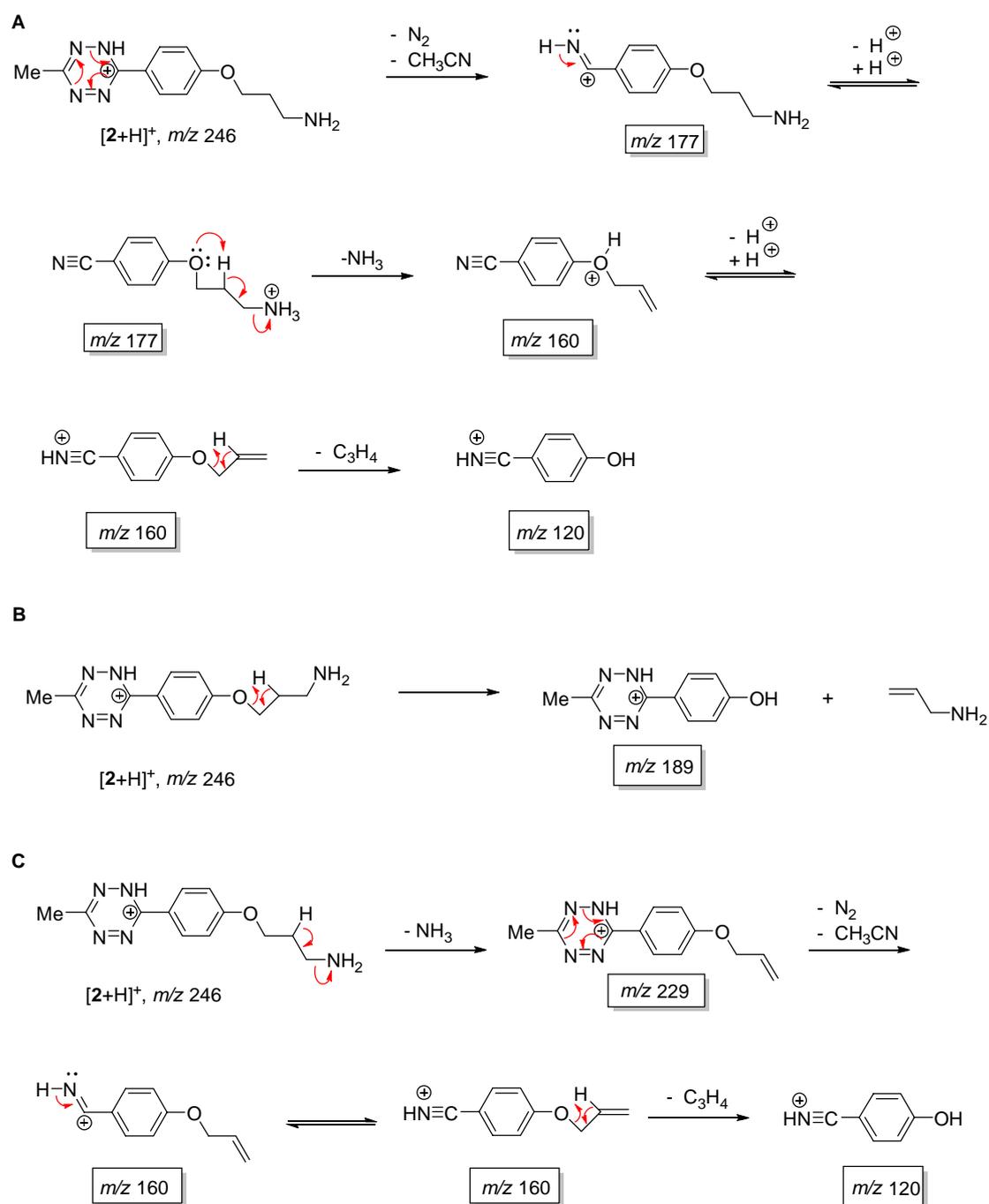
Note that the protonation sites of the ions below are indicative.



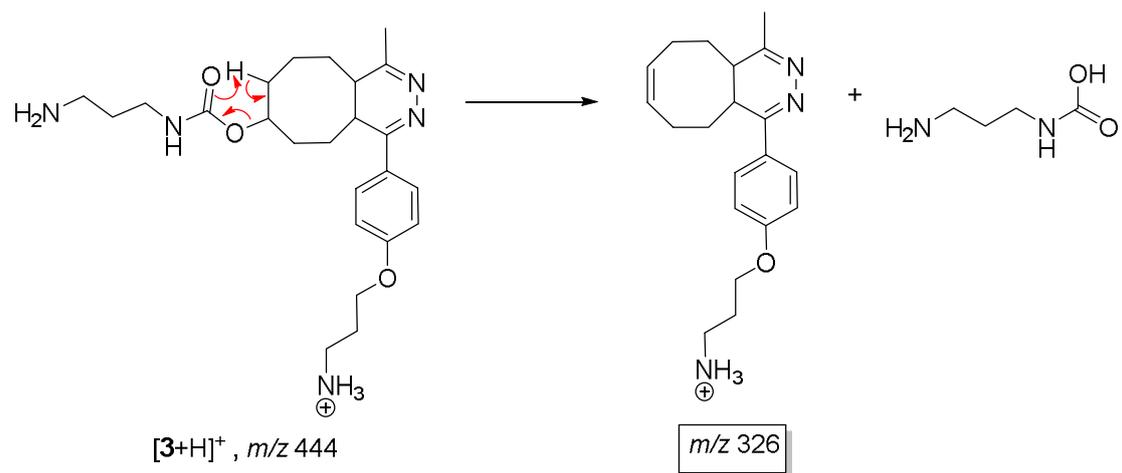
Scheme S2 The fragmentation scheme of the precursor ion m/z 222.



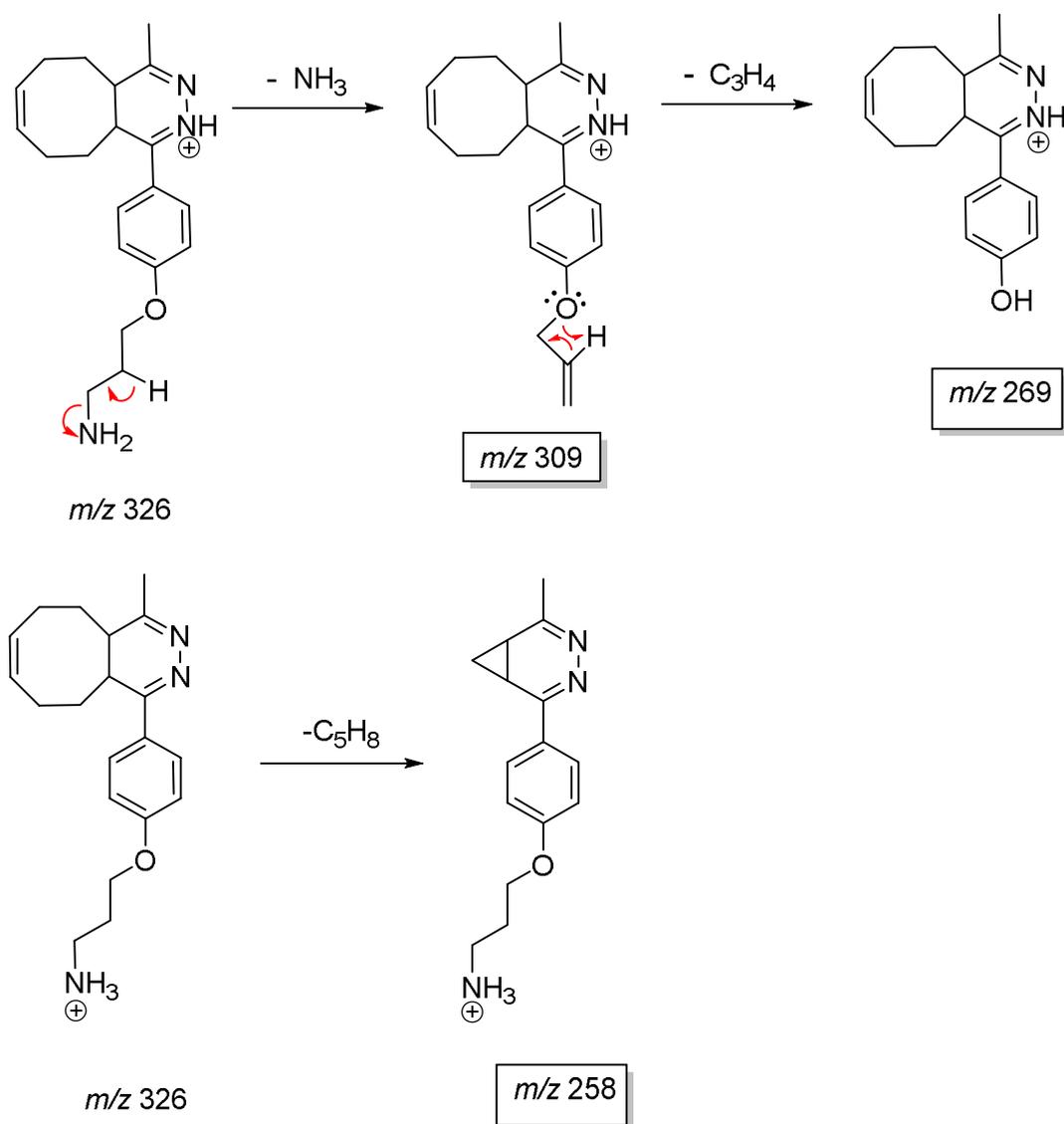
Scheme S3 The fragmentation scheme of the precursor ion m/z 227.



Scheme S4 The fragmentation scheme of the precursor ion m/z 246.



Scheme S5 The fragmentation scheme of the precursor ion m/z 444.



Scheme S6 The fragmentation scheme of the precursor ion m/z 326.

5.6 Identification of the doubly charged ion

An additional interesting ion seen in the mass spectrum in Fig. 3a and also in the mass spectra of S6-S9 is the ion m/z 222, which was suspected to be a doubly charged ion. It was observed as m/z 222.3 or 222.5 with the ion trap, but only integer value, m/z 222, is used in discussion with the ion trap data. However, it was observed at m/z 222.65 when using the Orbitrap. The intensity of this ion was observed to vary a lot from experiment to experiment. Fig. S10 shows the intensity of the ion m/z 222 to increase over time, a behavior that is similar to that of the protonated product **3** (m/z 444) and its fragment ion m/z 326. Interestingly, the product ion mass spectrum of m/z 222 gave the ion m/z 326 as the major product (Fig. S11). Its MS³ measurement (m/z 222 \rightarrow m/z 326 \rightarrow products, Fig. S16) produced a product ion mass spectrum that is very similar to the product ion mass spectra presented in Fig. S14 and S17, i.e. MS² mass spectrum of m/z 326 selected directly from the mass spectrum and MS³ mass spectrum of the ion m/z 326 formed in the MS² measurement of the [**3**+H]⁺ precursor ion (m/z 444). In summary, all these MSⁿ observations show that the identity of the ion m/z 222 is the doubly charged product, [**3**+2H]²⁺. In addition, the accurate mass of the ion m/z 222.65 measured with the Orbitrap mass spectrometer agrees with this interpretation.

6. Mixer experiments

The mixing experiments were conducted as follows (Fig. S18-S20): The acquisition of mass spectra was started at time zero ($t = 0$ min), simultaneously as the infusion of testosterone and testosterone- d_3 solutions started at the flow rate of 125 $\mu\text{L}/\text{min}$ per analyte via separate syringes and separate inlets into the miniaturised reactor. In total 125 μL of each analyte solution was infused, resulting in the complete filling of the reaction chamber. When the filling was completed ($t = 1$ min), the mixer was activated in the experiments where it was in use. Mass spectra were recorded continuously during the whole procedure.

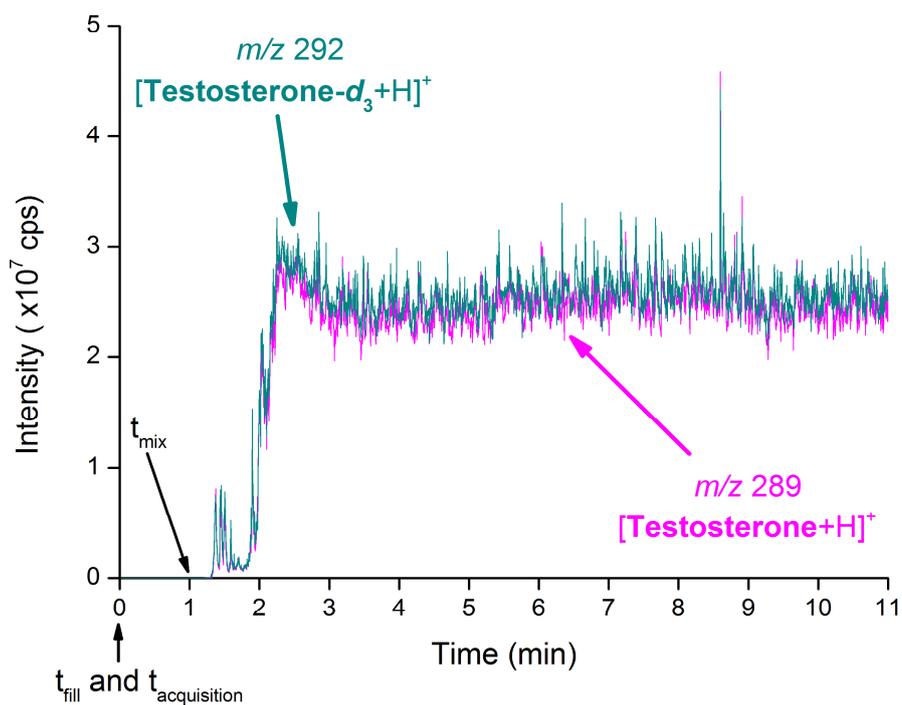


Fig. S18 The extracted ion profiles of m/z 289 ([testosterone+H]⁺, magenta) and m/z 292 ([testosterone- d_3 +H]⁺, turquoise) plotted against acquisition time. t_{mix} = time when the mixing was activated (1 min). t_{fill} and $t_{acquisition}$ times (0 min) when the infusion of testosterone and testosterone- d_3 and the acquisition of mass spectra started.

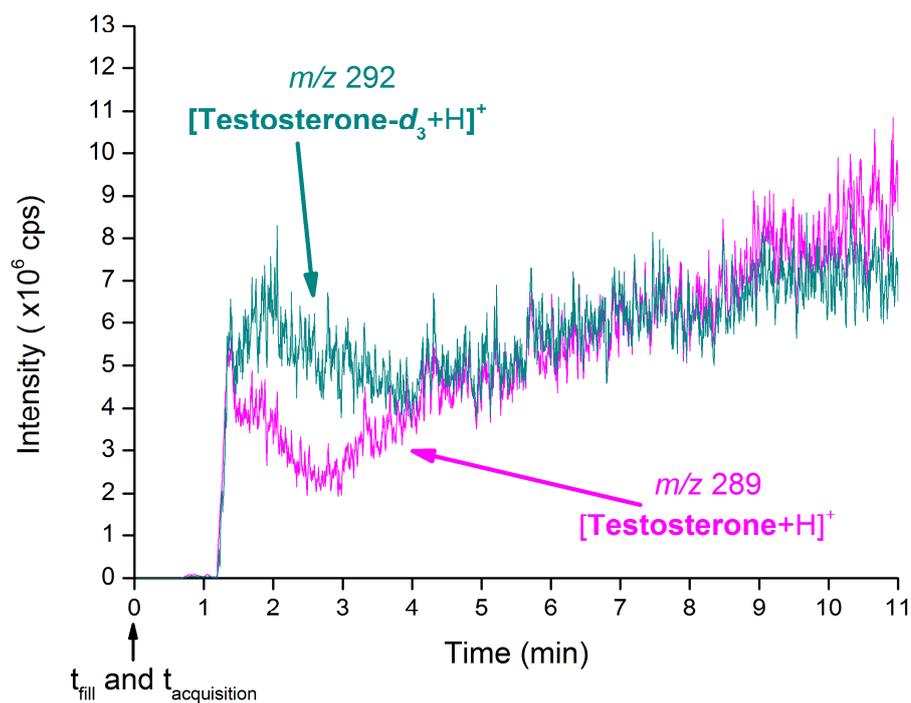


Fig. S19 The extracted ion profiles of m/z 289 ([testosterone+H]⁺, magenta) and m/z 292 ([testosterone- d_3 +H]⁺, turquoise) plotted against acquisition time. t_{fill} and $t_{acquisition}$ times (0 min) when the infusion of testosterone and testosterone- d_3 and the acquisition of mass spectra started. Mixing was not activated.

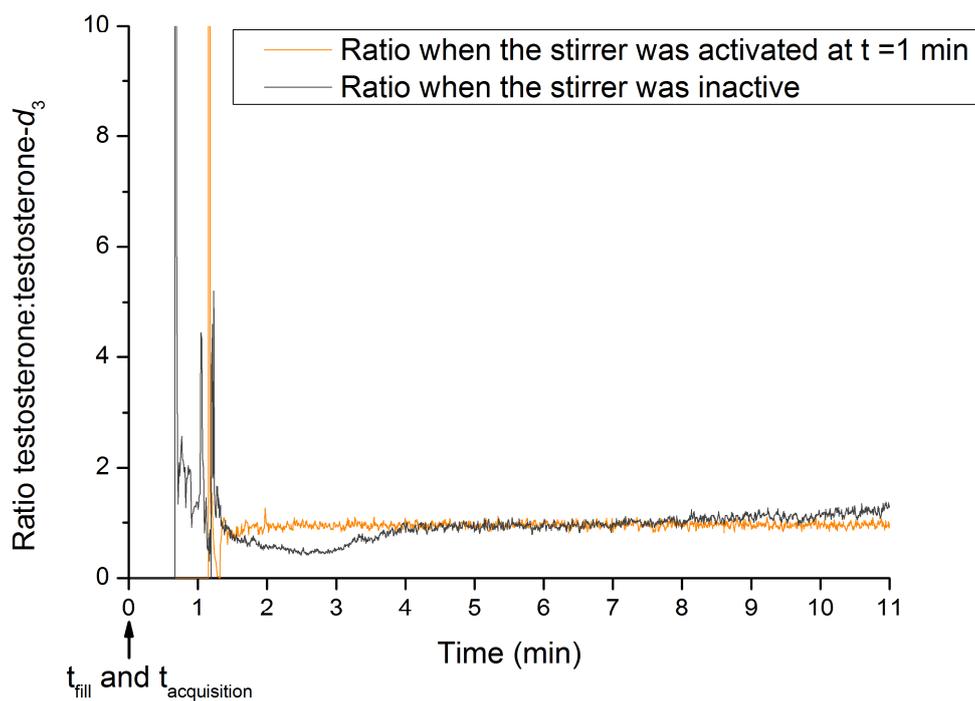


Fig. S20 The ratio of the ions $[\text{testosterone}+\text{H}]^+:[\text{testosterone-}d_3+\text{H}]^+$ plotted against the time when the stir bar was activated at $t = 1$ min (orange), and also when the stir bar was inactive (grey) (data from Fig. S18 and S19, respectively). The high peaks, i.e. the very high signal ratios, at the beginning of the graph are explained by testosterone alone reaching the nanospray needle.