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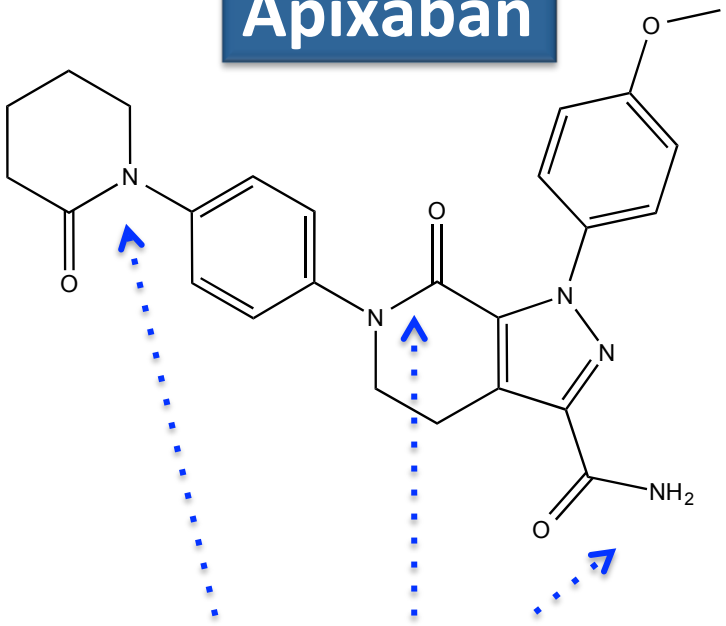
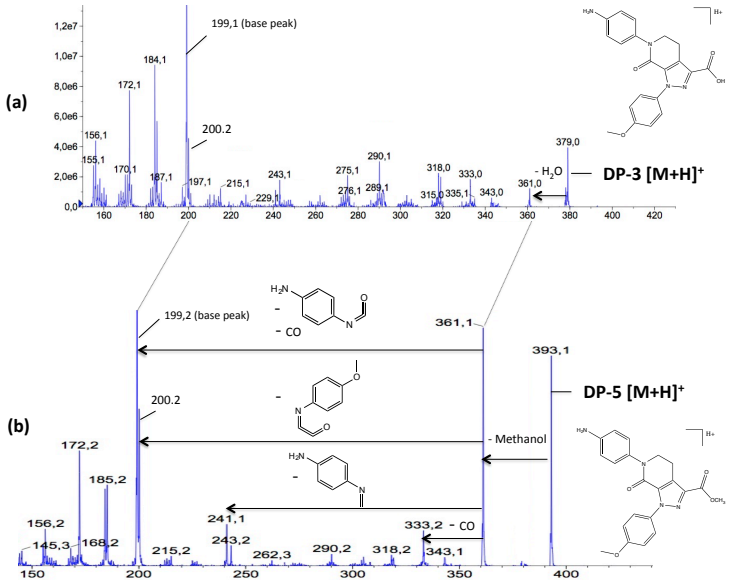
Degradation products

Apixaban



Structural elucidation

LC-MS^n and LC-HR-MS analysis



Stress conditions

COMPREHENSIVE STUDY OF APIXABAN'S DEGRADATION PATHWAYS UNDER STRESS CONDITIONS USING LIQUID CHROMATOGRAPHY COUPLED TO MULTISTAGE MASS SPECTROMETRY

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ABSTRACT

Apixaban is a novel anticoagulant drug acting as a direct, selective and reversible inhibitor of the coagulation factor Xa. Forced degradation under stress conditions were carried out in order to establish its stability profile. The drug was shown stable under photolytic, thermolytic and oxidative conditions, while under hydrolytic conditions, up to seven degradation products

were generated for about 15 % of drug degradation. The degradation products have been detected by linear gradient reversed phase high-performance liquid chromatography coupled with photo diode array and with electrospray ionization tandem mass spectrometry. Combination of multistage mass spectrometry and of high-resolution mass spectrometry (HR-MS) allowed the structural elucidation. The product ions of the degradation products were compared to those of apixaban protonated ion so to assign the most structures possible. This had required a study in depth of the drug's fragmentation pattern, which has not been reported so far. In view of the products formed, it appears that hydrolysis of the oxopiperidin moiety of apixaban occurred in acidic medium, whereas that of the tetrahydro-oxo-pyridine moiety would further happen under alkali conditions. Asides from characterization, LC method was shown stability indicating and validated as per the criteria described by the ICH guidelines.

INTRODUCTION

Apixaban (1-(4-methoxyphenyl)-7-oxo-6-[4-(2-oxopiperidin-1-yl)phenyl]-4,5-dihydropyrazolo[5,4-c]pyridine-3-carboxamide) is a novel anticoagulant drug acting as direct, selective and reversible inhibitor of the coagulation factor Xa[1-4]. It is prescribed as treatment of venous thromboembolism, which includes deep vein thrombosis and pulmonary embolism. A large scale randomized double blind study comparing the conventional therapy (subcutaneous enoxaparin, followed by warfarin) to apixaban, showed the non-inferiority of apixaban with significant less bleeding[5,6].

LC-MS/MS methods were developed to determine apixaban alone in plasma or in the presence of its major metabolites[7-11] to support clinical uses. Assay in tablets and simultaneous determination with other drugs using HPLC was also described[12,13]. A literature survey, however, did not reveal any further information about the stability profile of apixaban or about its potential degradation products likely to form in time and/or under stress

conditions. As drug may undergo degradations, leading potentially to activity loss or to occurrence of adverse effects associated with the appearance of degradation products, thorough knowledge of drug's stability profile is one of the key factors to prevent those risks during manufacturing, transportation and storage.

In this paper, we have focused on the identification and the characterization of degradation products generated in solution. Liquid chromatography combined with mass spectrometry has been well established and found to be a very useful technique for the identification and characterization of DPs[14-17]. That's why high performance liquid chromatography coupled with multistage mass spectrometry (HPLC-MS^n) was used. Different stress conditions were applied in order to simulate the degradation of active pharmaceutical substances, for which degradation can occur via many pathways such as basic and acidic hydrolysis, oxidation, photo-degradation or thermal degradation. The structures of observed degradation products were elucidated using multistage mass spectrometry and high-resolution mass spectrometry (HR-MS). A study in depth of apixaban fragmentation pattern was also achieved in order to help assign, by comparison, the structures of the major product ions coming from the degradation products ions. In addition, LC-UV method for quantitative determinations of apixaban in the presence of its degradation products has been validated as per ICH[18].

EXPERIMENTAL

Chemicals, reagents and stock standard solution

Apixaban (MW: 459. 4971g mol^{-1}) tablets (Eliquis®) are marketed by Bristol-Myers Squibb (Rueil Malmaison, France) / Pfizer (Paris, France). A stock standard solution of apixaban was prepared by extracting apixaban from crushed tablets using water/methanol 50/50 to get a final concentration of 250 µg mL^{-1}. Analytical grade acetonitrile came from Sigma-Aldrich (St

Quentin-Fallavier, France). Ultrapure water was produced by the Q-Pod Milli-Q system (Millipore, Molsheim, France). Hydrogen peroxide (H_2O_2) 30 % v/v was supplied by Carlo Erba SDS (Val de Reuil, France).

Instrumentation

LC-MS^n analyses were performed using a Dionex Ultimate 3000 system (DIONEX, Ulis, France) coupled to a triple quadrupole linear ion trap HybridQtrap 3200 MS (ABSciex Framingham, USA) system. LC-HR-MS was performed coupling this same LC system to an LTQ-Orbitrap Velos Pro system, composed of a double linear trap and an orbital trap (Thermo Fisher Scientific, CA, USA). LC system consisted of a quaternary pump, a degasser, a thermostated autosampler with a 100 µL - injection syringe and a thermostated column compartment. Separation was achieved using Zorbax® column SB-C18 (4.6 mm x 250 mm, i.d., 3.5 µm) kept at 25 °C in a thermostated compartment. The flow rate was set at 1.0 mL min^{-1}. A multi-stage gradient mobile phase (acetonitrile / water) was applied (Table 1).

Table 1. HPLC gradient program.

Detection and characterization were performed by mass spectrometry and high-resolution mass spectrometry. In both cases, an electro-spray ionisation (ESI) source operated in positive ion mode. In MS, the ionization conditions were set as following: ion spray-voltage was set at 5.5 kV, curtain gas (N_2) flow rate at 40 psi, nebulizer gas (air) flow rate at 30 psi and heater gas (air) flow rate at 50 psi. Temperature was set at 500 °C. Nitrogen was used as collision and damping gas. Acquisition in full scan mode over the mass range of 50-550 Da was performed for the detection of the degradation products. MS^n experiments for structural elucidation were carried out using 30 % (arbitrary units) collision energy level

(CEL). MS data were treated with Analyst® software version 1.5.2 and MS Manager® software version 12 (ACD Labs, Toronto, Canada). In HR-MS, the ionization conditions were set as follows: the source voltage was set at 3.4 kV and the temperatures were fixed at 53 °C (source) and 300 °C (capillary). S-Lens was set at 60%. Acquisition in full scan mode over the mass range of 300-550 Da was performed for the determination of the degradation products accurate masses. Data were treated with Xcalibur® software (version 2.2 SP 1.48). A Q-SUN XE-1 Xenon test chamber (LX 5080 Q-Lab Westlake, California, USA) was used for photo-degradation studies.

Stress-testing protocol

The forced degradation solutions were prepared by diluting apixaban stock standard solution in water or with reagent solutions as to obtain a final concentration of 125 µg mL^{-1} of apixaban in each of them (working solutions). Four stress conditions were tested: thermal, hydrolytic, photolytic and oxidative conditions. Each experiment was performed in triplicate and the working solutions were allocated in 5 mL hermetically sealed glass vials. Thermal stress was achieved at 80 °C up to 7 days. Hydrolysis was studied at room temperature over a period of 72 hours, using HCl 0.1 M or NaOH 0.1 M. Oxidation was tested in the presence of an equivalent of 3 % (*v/v*) H_2O_2, at room temperature for 72 h. Photo-degradation consisted in exposing working solutions to light for 36 h using a xenon test chamber (Q-SUN Xe-1). Emitted wavelengths ranged from 300 to 800 nm. The light intensity was delivered at 1.50 W/m².

Validation protocol

An RP-HPLC-UV with stability indicating capability was implemented and validated according to ICH Q2 (R1)[18] . Specificity was established based on good chromatographic separation

and UV detection at 220 nm. The system suitability tests were conducted throughout the validation studies by injecting 125 µg mL^{-1} of apixaban solution. Peak tailing as well as peak efficacy was systematically assessed. Linearity and accuracy were studied across concentration range 25 - 150 µg mL^{-1} of apixaban, through three series of samples independently prepared. Intermediate precision and repeatability were tested by injection of six individual solutions of 75 and of 125 µg mL^{-1} apixaban on three consecutive days. Limits of quantitation (LOQ) and detection (LOD) were determined considering the level of dilution leading to signal to noise ratios of 10:1 and 3:1, respectively.

RESULTS AND DISCUSSION

Optimised chromatographic conditions and method validation

Implementing a stability indicating method (SIAM) relies on its capacity to separate apixaban from its degradation products. After several optimization steps, the method described above was found suitable for the separation of apixaban from most of the other analytes, in gradient mode, using water/acetonitrile mobile phase (Table 2, Fig. 1). In such conditions, apixaban eluted at 13.3 min. The peak-tailing factor (*A*s) for apixaban was systematically inferior to 1.2 (average of six determinations = 1.15) and the theoretical plates (*N*) was at around 4500 (average of six determinations = 4498). A component detection algorithm (CODA) analysis also allowed examining the main peak purity, showing that it uniquely contained signals from apixaban regardless of the sample. Three of the degradation products eluted ahead of the drug and the rest came after. But the resolution factor (*R*s) between apixaban and one of the degradation products (DP-4) was below 1.5 (average of six determinations = 1.32). Mass balance (% assay + % total degradation products) of all the stressed samples of apixaban was obtained in the range of 98.2-99.5 %. The regression analysis using a linear model

expressing apixaban concentrations as a function of UV chromatogram signals within a range 25 - 150 µg.mL^{-1}, resulted in a determination coefficient R^2 of 0.9978 and a *y*-intercept of the linear equation which was statistically insignificant (p=0.205). The distribution of the residuals can well be approximated with a normal distribution according to the p-value of the Shapiro-Wilk normality test (p=0.198), so that it could be safely assumed that the calibration data fitted to a linear model. The LOD and LOQ were of 0.4 and 1.3 µg mL^{-1}, respectively. The repeatability verified by a six-fold analysis of the concentration level 75 µg mL^{-1} yielded a RSD inferior to 1.50 %, and the intermediate precision studied over three different days following the same protocol, led to a RSD equal to 2.09 %.

As a result, the method could be used for the assay determination with implementation of system suitability testing criteria, i.e. *R*s (apixaban/DP-4, ≥1.3), *A*s (≤ 1.2) and *N* (≈4500).

Same chromatographic conditions were applied for the characterization of the degradation products by LC-ESI-HR-MS^n (Table 1).

Degradation behaviour of apixaban

Under oxidation, thermal and photolytic stress conditions, neither loss of apixaban nor appearance of degradation products was detected, which was consistent with what has been reported recently[12]. Conversely, apixaban was very prone to degradation under hydrolytic stress. As shown in Table 2, loss of 15 % of apixaban was observed after 24 hours in acidic conditions, while 11 % was highlighted only after 3 hours in basic conditions.

In total, seven degradation products were detected in the solutions subjected to hydrolysis, when taken at a degradation rate still inferior to 15 %. Even if degradation continued beyond 15 %, we have limited the present study to that of the degradation products formed precociously in the stress conditions, insofar as the others, sometimes secondarily formed, can be considered as less likely with respect to real-storage conditions[19]. The studied degradation products are named “DPn”, where n accounts for the elution order. Base

hydrolysis resulted DP-1 and DP-2 with relative retention times of 0.65 and 0.73, whereas acidic degradation chromatograms showed DP-2, DP-3, DP-4, DP-5, DP-6 and DP-7 with relative retention times of 0.73, 0.96, 1.01, 1.07, 1.16 and 1.21 (Fig. 1, Table 2). Aside from these DPs, the acidic degradation chromatogram also highlighted the presence of two other but much less intense compounds, eluted about 10.9 min. But unlike the other ones, they were not detected in mass spectrometry and therefore, cannot be studied or structurally elucidated.

Fig. 1. Chromatograms of (a) basic stress sample and (b) acidic stress sample.

Table 2. Forced degradation outcome

Characterization of apixaban and DPs

ESI-MS^n and ESI-HR-MS^n fragmentation studies of apixaban

The drug was initially subjected to LC-MS^n and to HR-MS^n analyses to establish its complete fragmentation pattern, which has not been reported so far. Table 3 lists the precursor and product ions along with errors (ppm) and elemental compositions obtained from HR-Orbitrap-MS instrument.

Under positive ESI-MS conditions, $[M+H]^+$ and $[M+Na]^+$ ions were detected at m/z 460 and 482, respectively. Among the nitrogen functions apixaban is composed of, only carboxamide-amine function exhibits basic properties with a pKa of 13.1. It is therefore more amenable to protonation than are the others regarding the ionization process. The ESI-$HRMS^2$ spectrum of $(M+H)^+$ ion yielded 2 product ions with m/z of 461 and 443 (Table 3, Fig. 2a). The first ion would have been formed by hydrolysis affording a carboxylate derivative ($C_{25}H_{25}N_4O_5^+$), while the second would be derived from deamination. Acylium derivative $C_{25}H_{23}N_4O_4^+$ was also

shown to have been produced from m/z 461 ion by loss of H_2O when this one was taken as precursor during MS^3 study (data not shown). Subjected to the MS^3 process, $C_{25}H_{23}N_4O_4^+$ (m/z 443) gave rise to the formation of four major product ions with m/z of 415, 282, 241 and 199 (Table 3, Fig. 2b). The other product ions were detected at a much lower intensity and as discussed later, they turn out to have originated from the previous ones (Fig. 3).

Table 3. High-resolution multistage mass spectrometry data of apixaban.

Fig. 2. High-resolution MS^2 spectrum of (a) the protonated ion of apixaban and (b) high-resolution MS^3 spectrum of the product ion at m/z 443.

Transition 443➔415 may be related to CO departure by heterolytic cleavage of 3C and the acyl carbon bond. Carbocation $C_{24}H_{23}N_4O_3^+$ was formed. In turn, $C_{24}H_{23}N_4O_3^+$ only generated one significantly intense MS^4 product ion ($C_{23}H_{23}N_4O_2^+$) at m/z 387, by loss of CO (Table 3, Fig. 3a). According to the scheme (Fig. 4), it was proposed that such a neutral loss would come from the oxo-piperidin moiety through a rearrangements cascade, triggered by migration of a hydrogen atom from 9C to 3C through 1,4 H-transfer. The conformation of $C_{24}H_{23}N_4O_3^+$ is such that thereof would be quite stable by resonance.

Fig. 3. High-resolution MS^4 spectra of the product ions at (a) m/z 415, (b) m/z 282, (c) m/z 241 and (d) m/z 199.

Elimination of 2-(4-methoxyphenylimino)ethenone was proposed to explain the formation of m/z 282 ion (Fig. 4). The premise here was that another intermediate (2-(4-methoxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-2H-pyrazolo[3,4-c]pyridine-3-carbonyl ion) was formed through a rearrangement such as transamination, as illustrated in Fig. 4. From there, hydrogen migration from 8'C to the H-bond acceptor 1'N, through 1,6 H-transfer, would have led to the pyrazole ring opening by 1'N-2'N bond cleavage, to the

formation of a π bond between 2'N and 3'C, and to the switch of the adjacent double bond. Eventually, the 161 Da moiety would have been released by heterolytic rupture of 4'C-3'C bond. When taken as precursor for MS^4 study, $C_{16}H_{16}N_3O_2^+$ (m/z 282) yielded the product ions at m/z 264, 254 and 240 (Table 1, Fig. 3b), likely by dehydration, CO elimination and by loss of $H_2C=C=O$, respectively. Furthermore, as stated in the proposed fragmentation scheme of apixaban (Fig. 4), the product ion at m/z 227 would have been generated from m/z 254 ion by expulsion of HCN.

Fig. 4. Proposed fragmentation patterns of the protonated ions of apixaban, DP-2 and DP-6.

As for the MS^3 product ion detected at m/z 241 (Fig. 2b), it seemed to have been formed by loss of a 202 Da moiety, which could in all likelihood be attributed to 1-(4-(methyleneamino)phenyl)piperidin-2-one. As shown in Fig. 4, hydrogen atom migration from 8C to the H-bond acceptor 1N through 1,6 H-transfer, accompanied by a switch of 2N-3C single bond and of the adjacent double bonds, might have led in the first stage, to the opening of tetrahydropyridine ring. Next, a similar H-transfer process, that took place between 2''N and electron-deficient 8''C, would have ended up releasing the aforementioned neutral fragment by heterolytic cleavage of 8''C-9''C bond. Under the MS^4 conditions (Fig. 3c), $C_{25}H_{23}N_4O_4^+$ appeared to lose two CO to afford the product ion at m/z 185 ($C_{11}H_9N_2O^+$). The last other important fragmentation route of $C_{25}H_{23}N_4O_4^+$ (m/z 443) was represented by transition 443➔199. Thereof would be formed by loss of a 216 Da moiety and of CO. 1-(4-isocyanatophenyl)piperidin-2-one could possibly account for the so-called 216 Da moiety. Indeed, it was proposed that by tautomery, electron-deficient 5C had withdrawn a hydrogen atom to 9C, leading to the formation of a π bond between 4C and 9C. From there, switch of C9-C8 and 7N-8C single bonds would have allowed generating a metastable ion with m/z of 227, which in turn, would have undergone CO loss to afford m/z 199 ion (Fig. 4). When taken as precursor for MS^4 study, m/z 199 ion could notably produce m/z 172 ion by elimination of HCN (Fig. 3d).

Throughout this study, the product ions presented in Fig. 4 were all confirmed by accurate mass measurement.

Identification of the degradation products by LC-HR-MS and LC-MS^n

DP-1 gave a protonated ion with m/z of 478. Having 18 Da greater than that of apixaban, it could be considered as a hydrolysis product (Table 4). Indeed, its MS^2 spectrum also includes m/z 460 ion and some of the major product ions described above, i.e. m/z 227, 199, 184 and 172 ions, suggesting that the protonated ion of DP-1 could lose H_2O to afford apixaban $[M+H]^+$ ion, thus restoring the amide bond. Aside from this fragmentation path, the mass spectrum also exhibited abundant product ions at m/z 461, 417, 288, 271, 203 and 217 (Table 2, Fig. 5a). The ion at m/z 461 may be due to NH_3 expulsion revealing the presence of carboxamide function. Transition 461➔417 would correspond to CO_2 elimination caused by the presence of carboxylate function, demonstrating that DP-1 was well been formed by hydrolysis of an amide bond. In addition, opening of the oxopiperidin ring by hydrolysis of the amide bond could easily explain the formation of m/z 288 ion by loss of oxopiperidinylaniline group through N-dealkylation (Fig. 6). Such a characteristic product ion could in turn lose NH_3 to generate m/z 271 ion. The other abundant product ions would have been formed from the protonation of aniline-amine function as shown in Fig. 6. The ion at m/z 191 would be produced through N-dealkylation, while m/z 203 ion would be the result of the Mac-Lafferty rearrangement. As a result, DP-1 could be identified as 3-carbamoyl-1-(4-methoxyphenyl)-4-(2-(4-(2-oxopiperidin-1-yl)phenylamino)ethyl)-1*H*-pyrazole-5-carboxylic acid. This structure was also confirmed by the accurate mass measure of DP-1 (Table 4).

Table 4. Retention times, accurate masses with errors, elemental compositions and MS^2 relevant product ions of the degradation products.

Fig. 5. MS^2 spectra of the protonated ions of (a) DP-1, (b) DP-4 and (c) DP-7.

Fig. 6. Proposed fragmentation pattern of the protonated ion of DP-1.

MS/MS product ions of **DP-2 and DP-6** were almost all the same as that of apixaban. Only the protonated precursor ions were different (Table 4). Instead of transition 460➔443 corresponding to loss of NH_3, transitions 461➔443 and 475➔443 took place for DP-2 and DP-6, respectively. They might correspond to dehydration and loss of methanol (Fig. 4). As a result, DP-2 and DP-6 might correspond to 1-(4-methoxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1*H*-pyrazolo[3,4-*c*]pyridine-3-carboxylic acid and to methyl-1-(4-methoxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1*H*-pyrazolo[3,4-*c*]pyridine-3-carboxylate, respectively. These structures were also confirmed by the accurate mass measure of DP-2 and DP-6 (Table 4).

As DP-1, **DP-4** yielded a protonated ion with m/z of 478. Therefore, it could equally be considered as a hydrolysis product (Table 4). Its MS^2 spectrum includes common ions with that of apixaban as was already mentioned for DP-1. It also displays extra product ions with m/z of 432, 416, 404, 390, 378, 361, 333, 300 and 101 (Fig. 5b). The presence of some of them confirmed that the oxo-piperidin ring opening had occurred by hydrolysis of the amide bond. The ion at m/z 101 may be due to N-C bond heterolytic cleavage releasing n-pentanoic acid carbocation. The product ion at m/z 378 would have been formed through N-dealkylation after the protonation of aniline-amine function. The anilinium derivative could in turn successively lose NH_3 and CO to yield m/z 361 and m/z 333 ions, respectively. As shown in Fig. 7, existence of some of the product ions could be related to the C-C bonds rupture on the lateral chain. This seems to concern the product ions at m/z 432, 416 and 404. Aside from the fragmentation paths involving the lateral chain, elimination of 2-(4-methoxyphenylimino)ethenone that was already described for apixaban, would have generated m/z 300 ion, which in turn, would have lost a water molecule to afford m/z 282 ion. Similarly, loss of an isocyanatophenyl derivative along with CO would have formed m/z 199

ion. Therefore, such a fragmentation pattern is entirely consistent with the protonated ion of 5-(4-(3-carbamoyl-1-(4-methoxyphenyl)-7-oxo-4,5-dihydro-1*H*-pyrazolo[3,4-*c*]pyridin-6(7*H*)-yl)phenylamino)pentanoic acid, as precursor.

Fig. 7. Proposed fragmentation patterns of the protonated ions of DP-4 and DP-7.

DP-7 protonated ion was detected at m/z 492. Its accurate mass, measured by HR-MS, is consistent with $C_{30}H_{26}N_5O_5^+$ elemental formula (Table 4, Fig. 5c). Given a perfect parallelism between the fragmentation patterns of DP-4 and **DP-7** (Table 2, Fig. 5 and 7), DP-7 was identified as methyl 5-(4-(3-carbamoyl-1-(4-methoxyphenyl)-7-oxo-4,5-dihydro-1*H*-pyrazolo[3,4-*c*]pyridin-6(7*H*)-yl)phenylamino)pentanoate.

DP-3 and DP-5 were also found to include the characteristic fragments m/z 361 and m/z 333 within their ESI-MS/MS spectra. Moreover, by comparing the elemental formula of protonated DP-3 and of DP-5 to that of the drug (Table 4), it was easy to demonstrate that they corresponded to aniline derivatives having lost the oxo-piperidin group. In addition, it seems that DP-3 would carry a carboxylate group and DP-5 a carboxymethyl group instead of the initial carboxamide function (Table 4). These assumptions were further supported by the determination of their fragmentation pattern such described by Fig. 8. Loss of water and of methanol from protonated DP-3 and DP-5, respectively, was observed through the presence of m/z 361 ion on both MS^2 spectra. The protonated ion of DP-3 also corresponds to a product ion of the protonated ion of DP-5 after demethylation. As the degradation products structure had preserved the tetrahydropyridine-pyrazolo-methoxyphenyl core, it was logical that the transition involving loss of 2-(4-methoxyphenylimino)ethenone (361➔200) was one more time detected. Always from the product ion at m/z 361, m/z 241 and m/z 199 ions would have been produced according to the same mechanisms as those described for apixaban, as shown in Fig. 8. As a result, DP-3 might correspond to 6-(4-aminophenyl)-1-(4-methoxyphenyl)-7-oxo-4,5,6,7-tetrahydro-1*H*-pyrazolo[3,4-*c*]pyridine-3-carboxylic acid and

DP-5, to methyl-6-(4-aminophenyl)-1-(4-methoxyphenyl)-7-oxo-4,5,6,7-tetrahydro-1*H*-pyrazolo[3,4-*c*]pyridine-3-carboxylate.

Fig. 8. MS^2 mass spectrum of the protonated ions of DP-3 (a) and DP-5 (b) and their corresponding fragmentation patterns. Some common remarkable transitions are featured on the MS/MS spectrum of DP-5 (b).

Proposed degradation pathways of apixaban.

Given the outcome from the stress studies, it was clear that apixaban was more susceptible to acidic and alkali conditions than to the other tested conditions. It seems that the oxo-piperidin group was more amenable to acidic hydrolysis and that the oxo-tetrahydropyridine was more sensible to alkali conditions, according to the study conditions. Hydrolysis of the caboxamide function seemed to occur under one or the other condition. The carboxymethyl derivatives would have been formed in the presence of methanol, used for dissolution of apixaban API prior to being subjected to stress conditions. But under the alkali conditions, esterification should not occur or if it were the case, then the esters formed would have been saponified. The schematic representations of mechanism of formation of the degradation products under hydrolytic stress are depicted in Fig. 9.

Fig. 9. Proposed degradation patterns of apixaban under stress conditions.

Conclusion

The degradation behaviour of apixaban under hydrolytic (acid, base), oxidative, photolytic and thermal stress conditions was studied as per ICH guidelines. Degradation studies demonstrated that apixaban was more fragile with respect to hydrolysis conditions. Its MS^n

fragmentation scheme was studied in depth in order to help assign, by comparison, the structures of the product ions formed from the degradation products. A total of seven degradation products were highlighted in the samples exposed to hydrolysis conditions, having reached a degradation rate at maximum of 15 %. Based on their identification, it was possible to deduct major degradation mechanisms in the context of stress testing.

A stability-indicating LC method was developed and it has shown suitable for the drug quantification as well as for the impurity determination.

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Tables

Table 1. HPLC gradient

Time (min)	A : Water (% v/v)	B : Acetonitrile (% v/v)
0➔2	95	5
2➔13	95➔40	5➔60
13➔19	40	60
19➔20	40➔95	60➔5

447 **Table 2.** Forced degradation outcome (n=3)

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Stress condition	Time	Average assay of API (% *w/w*, n=3)	Average total impurities (% *w/w*, n=3)	Average mass balance (assay + total impurities %, n=3)	Commentaries
Acid hydrolysis (0.1 M HCl)	24 hours	84.9	14.6	99.5	Degradation accompanied by appearance of DP-2, DP-3, DP-4, DP-5, DP-6 and DP-7
Base hydrolysis (0.1 M NaOH)	3 hours	88.1	10.8	98.9	Degradation accompanied by appearance of DP-1 and DP-2
Oxidation (3 % H_2O_2)	72 hours	99.3	N.D.	99.3	No degradation occurred
Thermal (80°C)	7 days	99.5	N.D.	99.5	No degradation occurred
Photolysis (UV light)	36 hours	98.6	N.D.	98.6	No degradation occurred

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Table 3. MS^n and high-resolution MS^n data of apixaban.

Product ions m/z	Origin	Best possible elemental formula	Theorical masses m/z	Accurate masses m/z	Error (ppm)
460	$[M+H]^+$	$C_{25}H_{26}N_5O_{4+}$	460.19793	460.19655	-3.00
461	MS^2 (460➔)	$C_{25}H_{25}N_4O_5^+$	461.18195	461.18054	-3.06
443	MS^2 (460➔)	$C_{25}H_{23}N_4O_4^+$	443,171382	443,17006	-2,98
415	MS^3 (460➔443➔)	$C_{24}H_{23}N_4O_3^+$	415,176467	415,17547	-2,40
387	MS^4 (460➔443➔415➔)	$C_{23}H_{23}N_4O_2^+$	387,18155	387,18073	-2,12
282	MS^3 (460➔443➔)	$C_{16}H_{16}N_3O_2^+$	282,123703	282,12279	-3,24
264	MS^4 (460➔443➔282➔)	$C_{16}H_{14}N_3O^+$	264,11370	264,1122	-5,68
254	MS^4 (460➔443➔282➔)	$C_{15}H_{16}N_3O^+$	254,128789	254,12791	-3,46
241	MS^3 (460➔443➔)	$C_{13}H_9N_2O_3^+$	241,06077	241,06004	-3,03
240	MS^4 (460➔443➔282➔)	$C_{14}H_{14}N_3O^+$	240,11314	240,11228	-3,58
227	MS^4 (460➔443➔282➔)	$C_{14}H_{15}N_2O^+$	227,11789	227,11711	-3,43
199	MS^3 (460➔443➔)	$C_{12}H_{11}N_2O^+$	199,086589	199,08620	-1,95
185	MS^4 (460➔443➔241➔)	$C_{11}H_9N_2O^+$	185,070939	185,07035	-3,18
172	MS^4 (460➔443➔199➔)	$C_{11}H_{10}NO^+$	172,07569	172,07516	-3,08

473 **Table 4.** Retention times, accurate masses with errors, elemental compositions and MS^2 relevant product ions of the degradation produc

Products	rT (min)	Conditions	$[M+H]^+$	Elemental compositions	Theoretical masses	Accurate masses with error in ppm	Relevant MS/MS product ions assigned (m/z)
DP-1	8,7	Basic	478	$C_{25}H_{28}N_5O_5^+$	478.20849	478.20755 (-1.97)	461 ; 460 ; 417 ; 288 ; 271 ; 244 ; 227 ; 203 ; 199 ; 191
DP-2	9,7	Acidic/Basic	461	$C_{25}H_{25}N_4O_5^+$	461.18195	461.18090 (-2.28)	443 ; 415 ; 387 ; 282 ; 254 ; 227 ; 199 ; 185 ; 172
DP-3	12,8	Acidic	379	$C_{20}H_{18}N_4O_4^+$	379.14008	379.13883 (-3.30)	361 ; 333 ; 303 ; 241 ; 200 ; 199 ; 185 ; 172
Apixaban	13,3	_	460	$C_{25}H_{26}N_5O_4^+$	460.19793	460.19674 (-2.59)	See Table 1
DP-4	13,6	Acidic	478	$C_{25}H_{28}N_5O_5^+$	478.20849	478.20702 (-3.07)	461 ; 460 ; 443 ; 432 ; 416 ; 404 ; 390 ; 378 ; 361 ; 333 ; 300 ; 282 ; 199 ; 185 ; 101
DP-5	14,6	Acidic	393	$C_{21}H_{21}N_4O_4^+$	393.15573	393.15436 (-3.48)	393 ; 379 ; 361 ; 333 ; 241 ; 200 ; 199 ; 185 ; 172 ; 156
DP-6	15,6	Acidic	475	$C_{26}H_{27}N_4O_5^+$	475.19760	475.19621 (-2.93)	475 ; 461 ; 443 ; 282 ; 254 ; 241 ; 227 ; 199 ; 185 ; 172 ; 156
DP-7	16,2	Acidic	492	$C_{26}H_{30}N_5O_5^+$	492.22415	492.22257 (-3.21)	475 ; 432 ; 416 ; 404 ; 390 ; 378 ; 377 ; 371 ; 333 ; 314 ; 282 ; 227 ; 199 ; 185 ; 115

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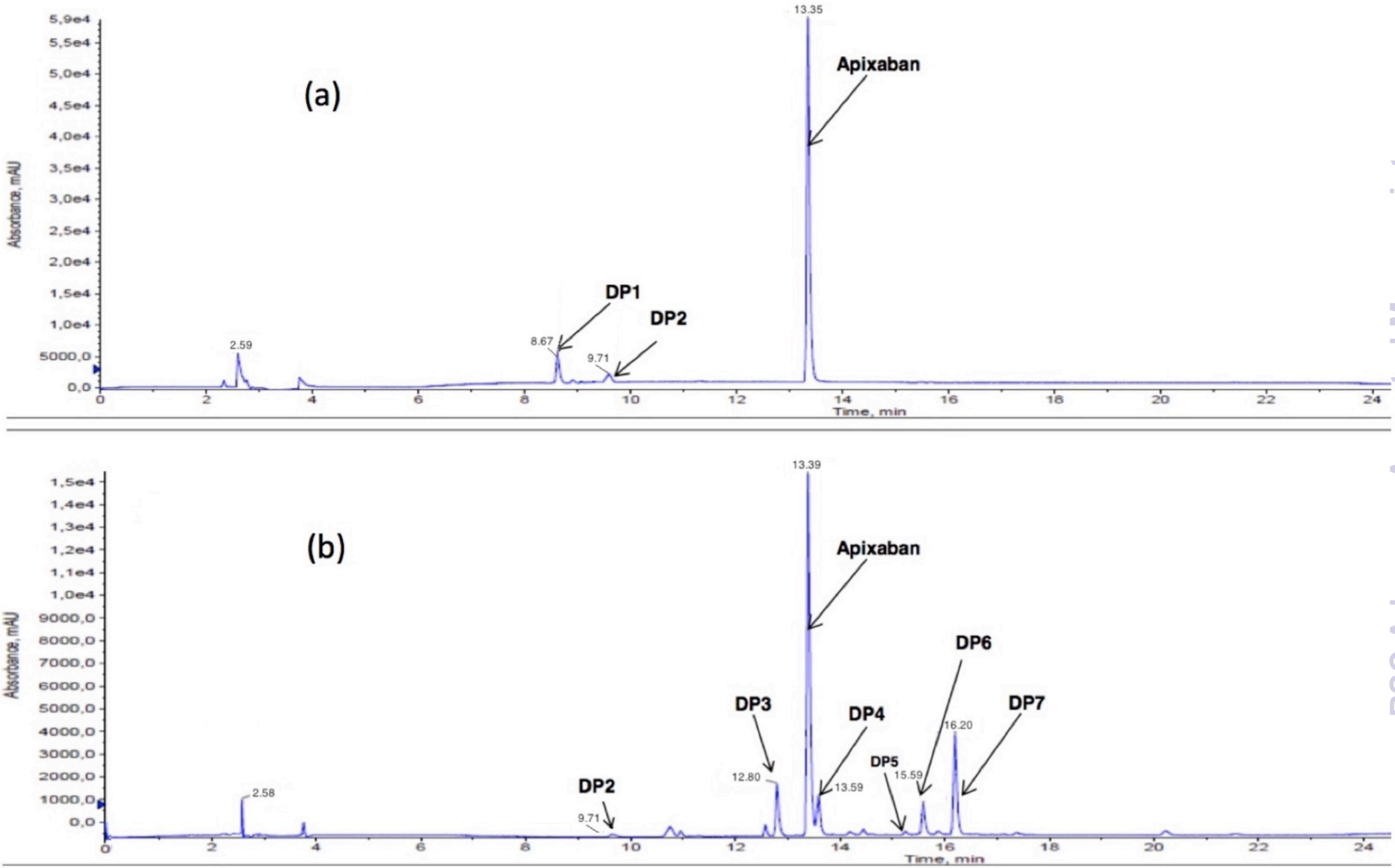


Fig 1. Chromatograms of (a) basic stress sample and (b) acidic stress sample

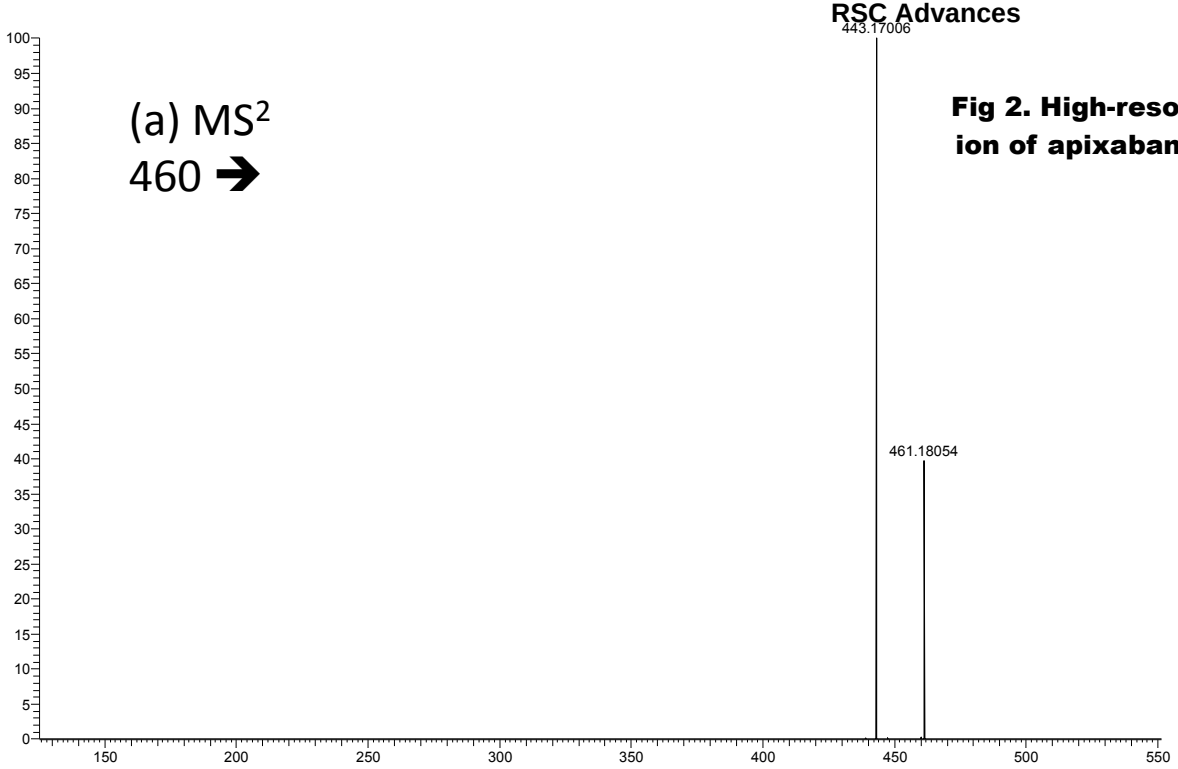
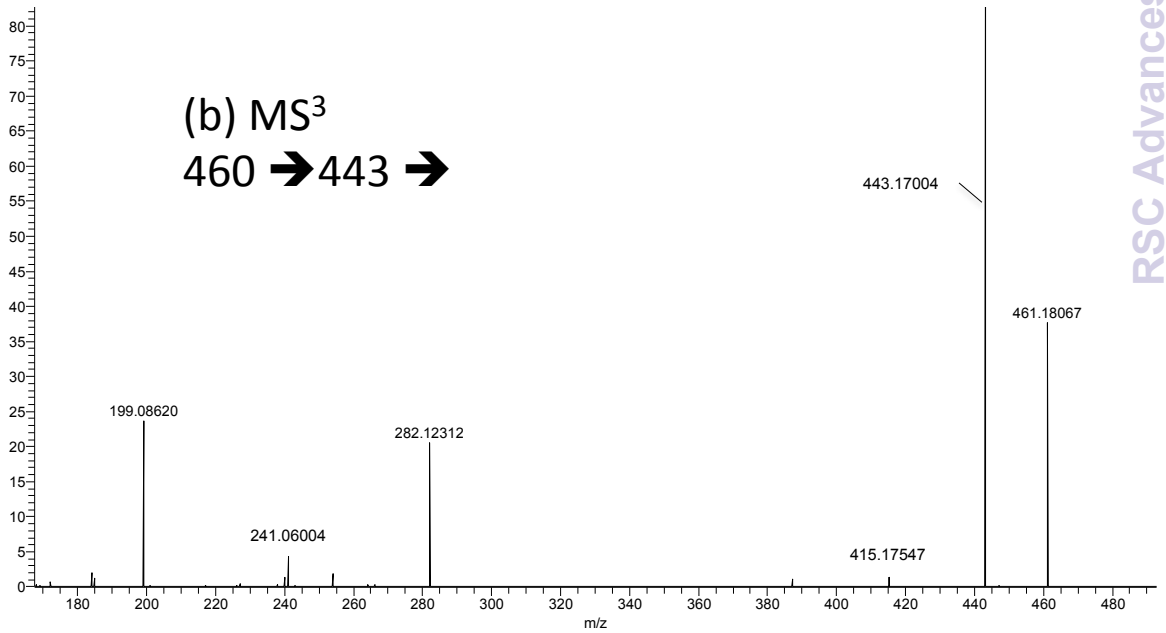
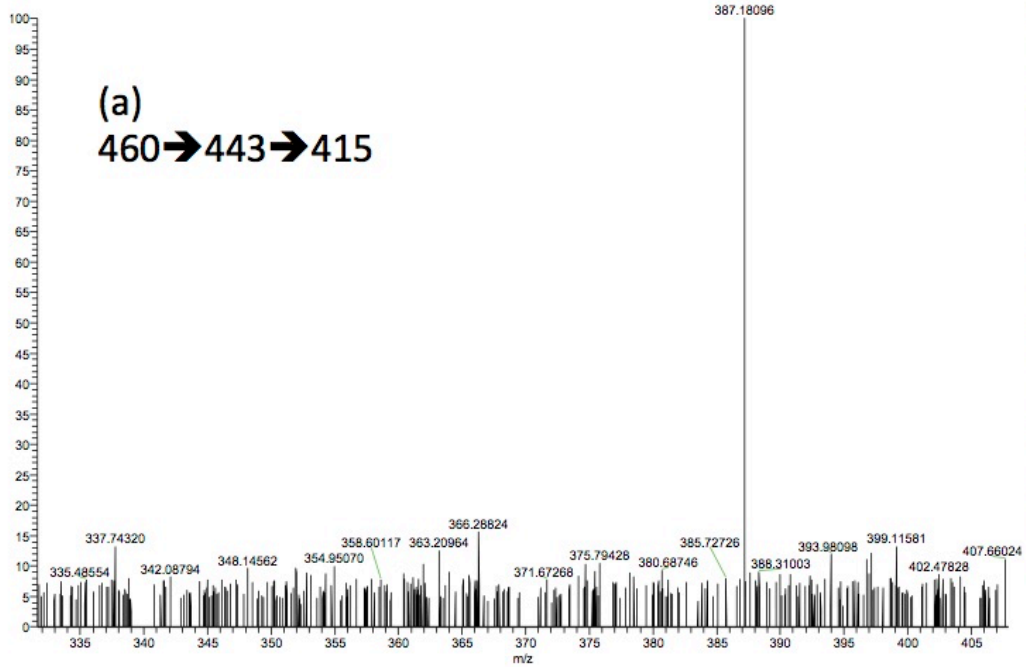
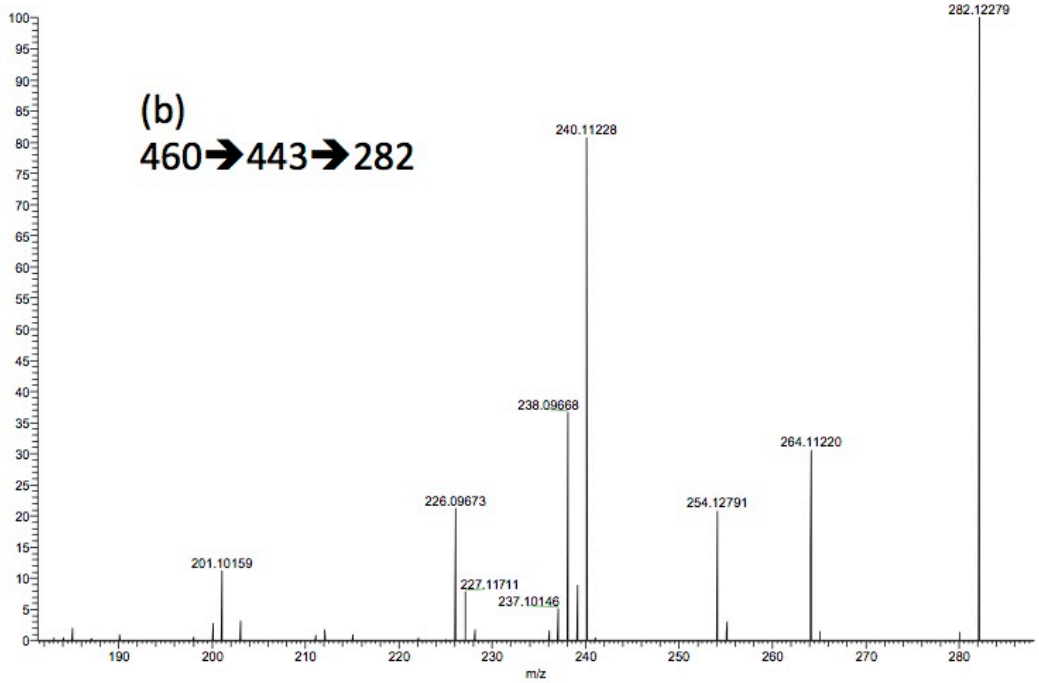
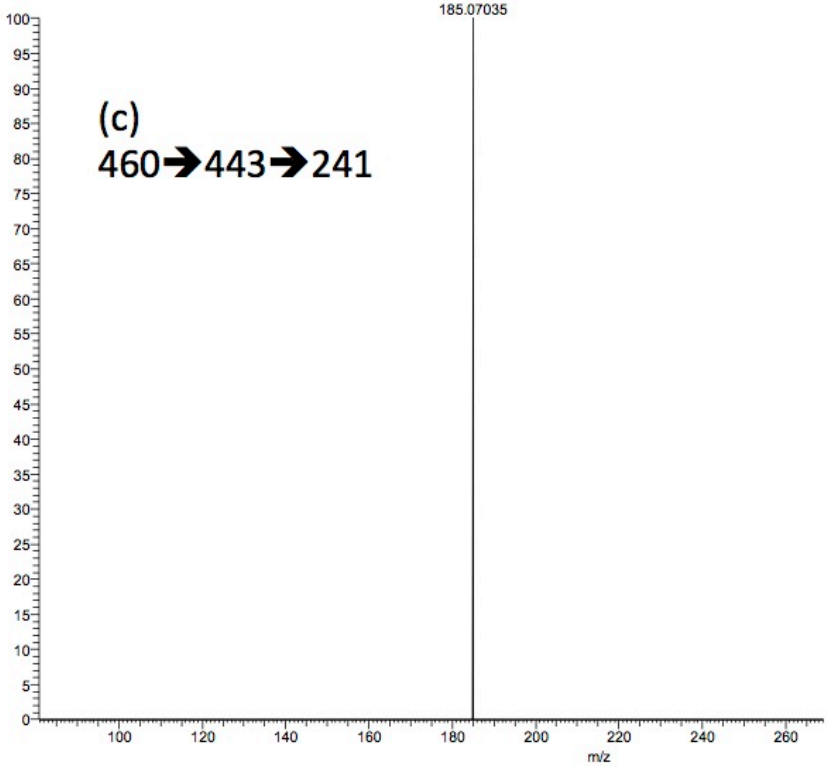


Fig 2. High-resolution MS/MS spectrum of (a) the protonated ion of apixaban and (b) high-resolution MS/MS/MS spectrum of the product ion at m/z 443









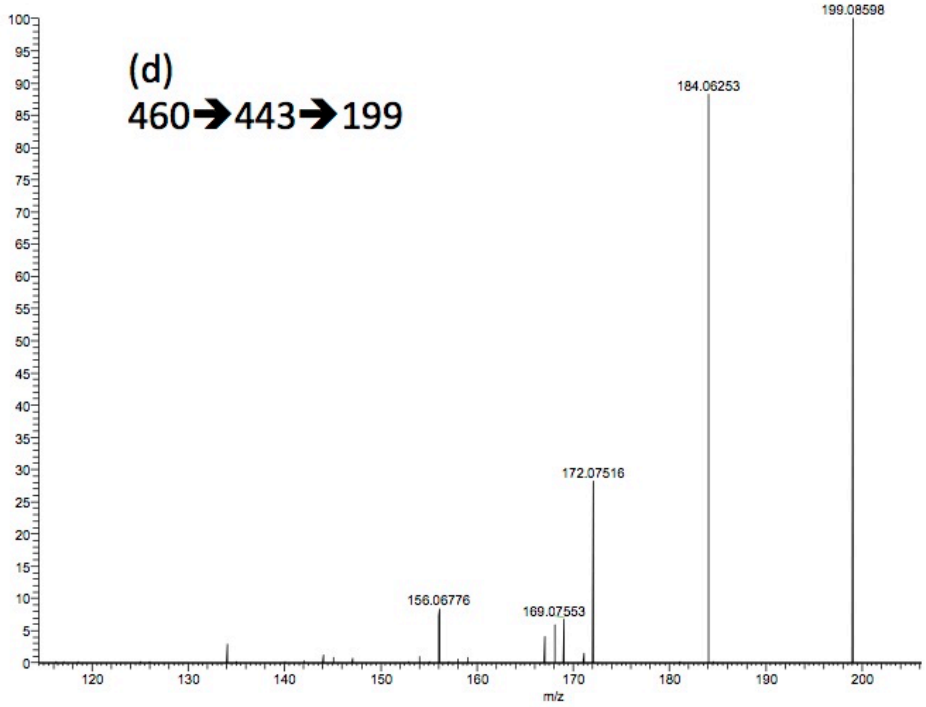


Fig 3 High-resolution MS4 spectra of the product ions at (a) m/z 415, (b) m/z 282, (c) m/z 241 and (d) m/z 199

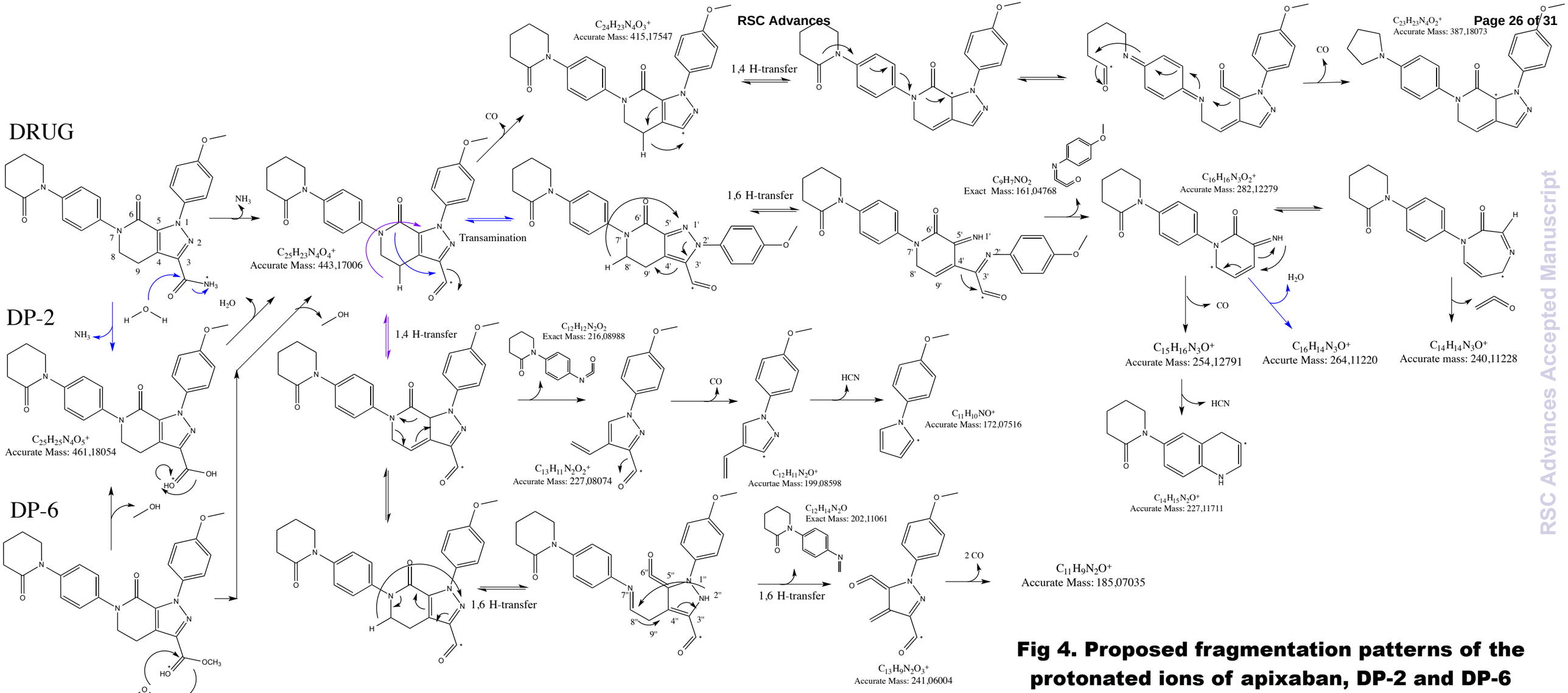


Fig 4. Proposed fragmentation patterns of the protonated ions of apixaban, DP-2 and DP-6

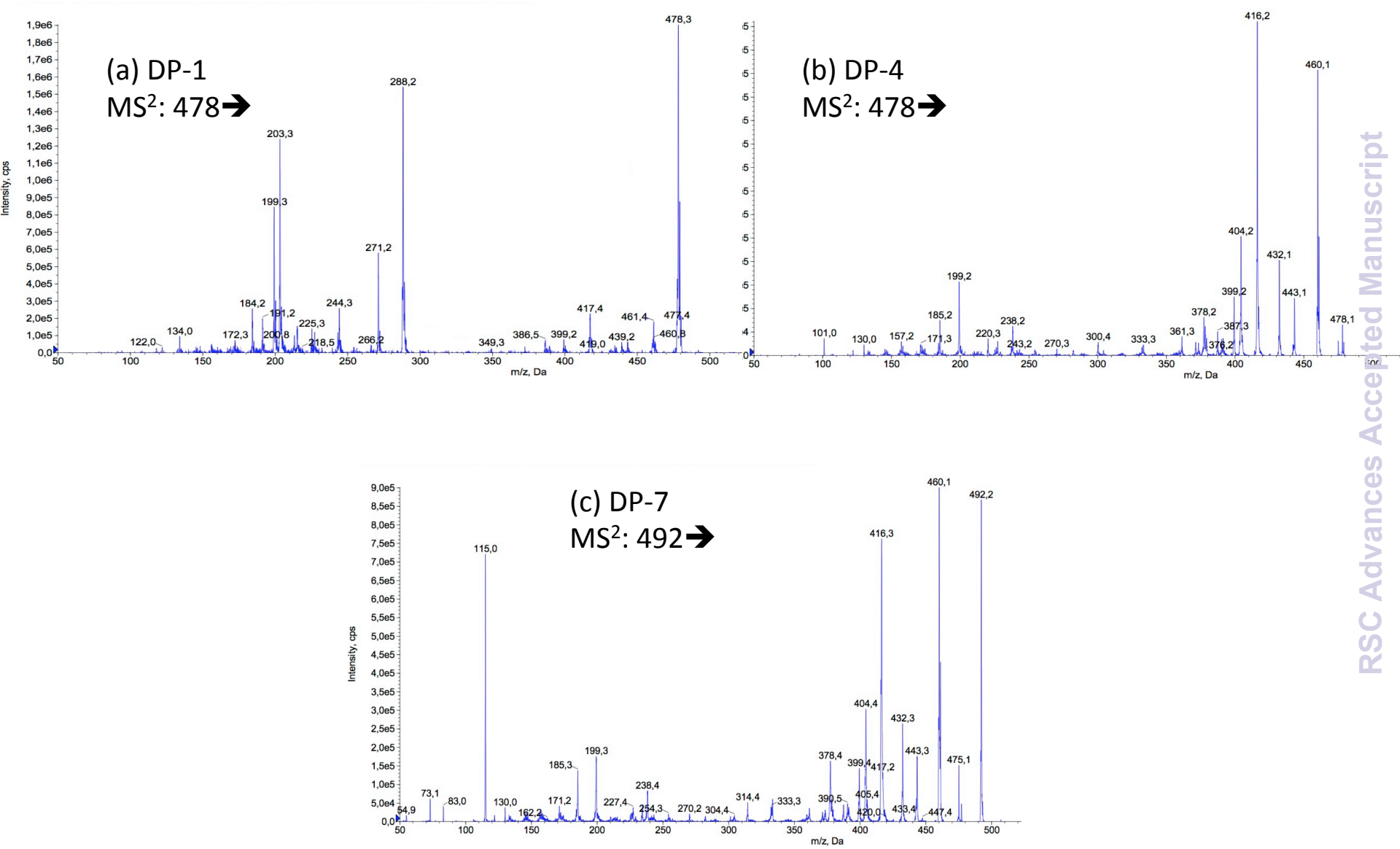
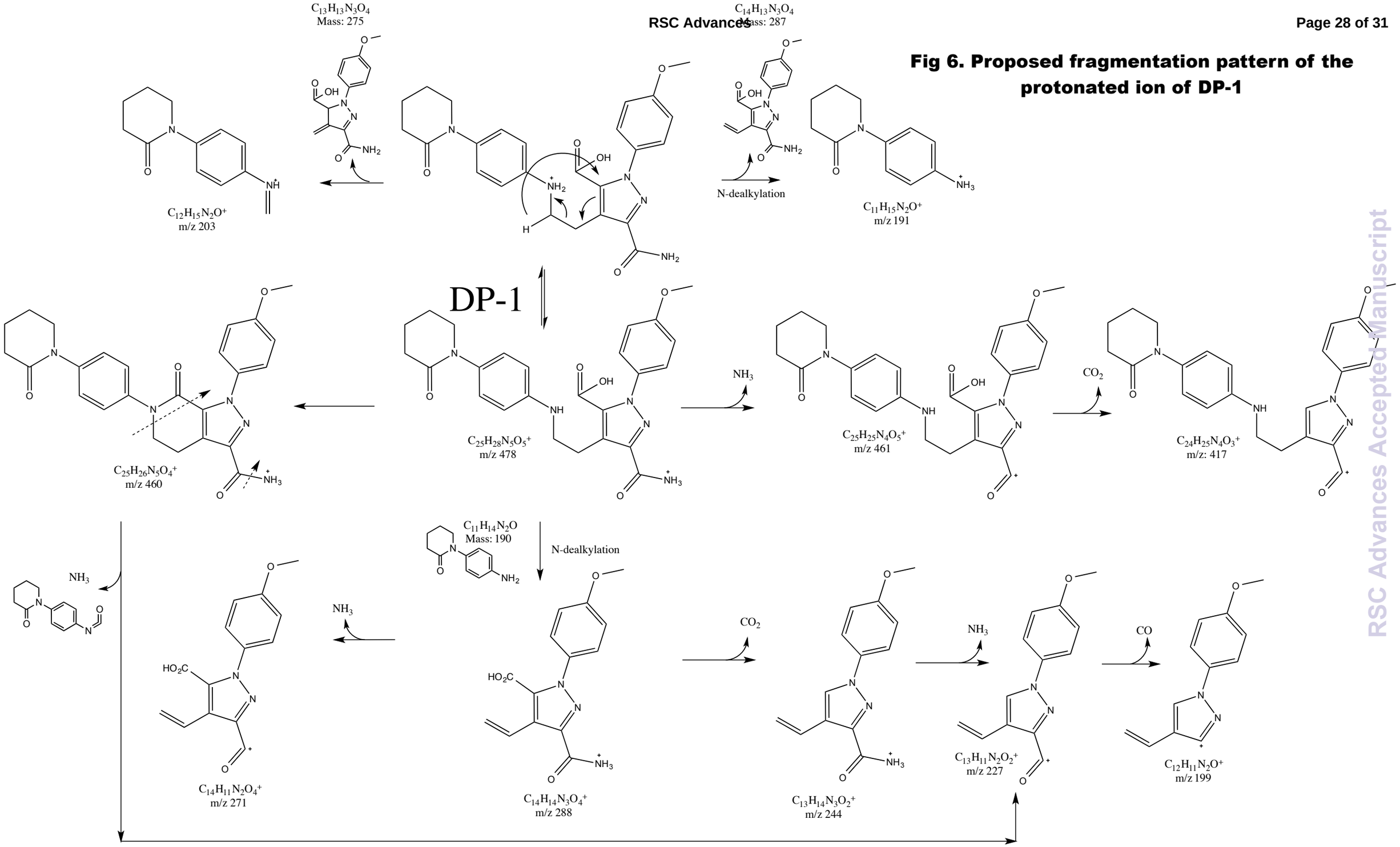


Fig 5. MS/MS spectra of the protonated ions of (a) DP-1, (b) DP-4 and (c) DP-7

Fig 6. Proposed fragmentation pattern of the protonated ion of DP-1



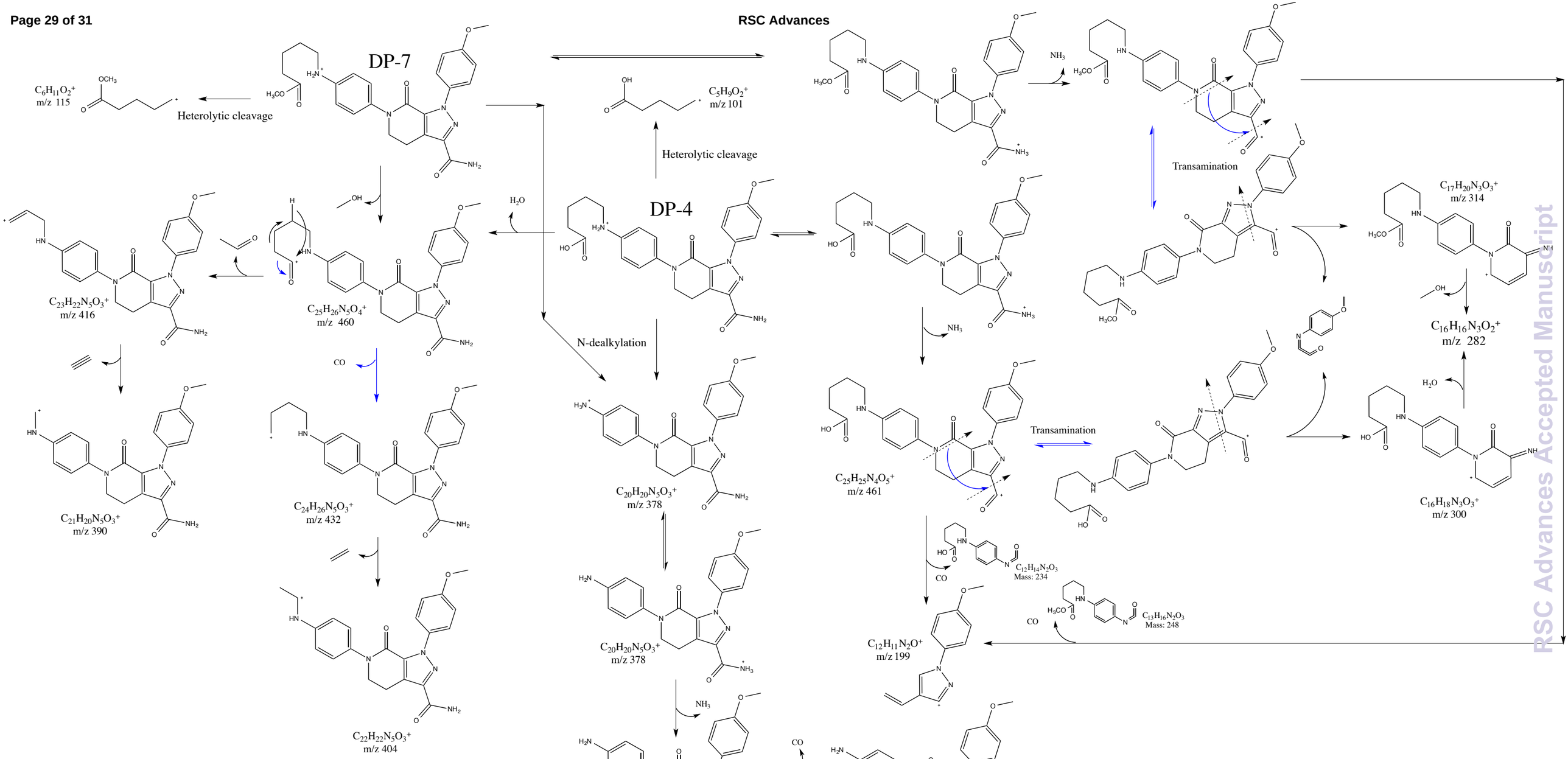


Fig 7. Proposed fragmentation patterns of the protonated ions of DP-4 and DP-7

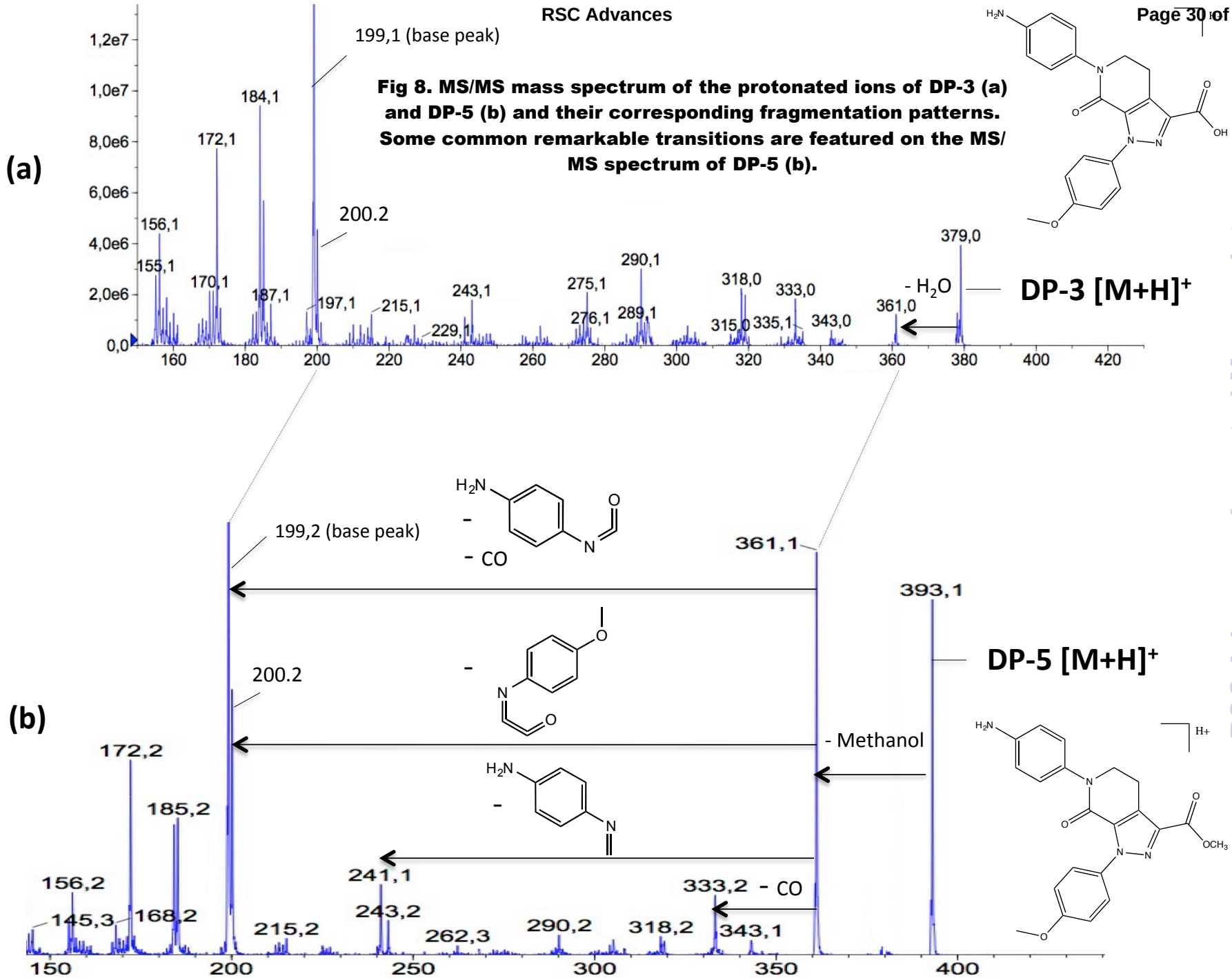


Fig 8. MS/MS mass spectrum of the protonated ions of DP-3 (a) and DP-5 (b) and their corresponding fragmentation patterns. Some common remarkable transitions are featured on the MS/MS spectrum of DP-5 (b).

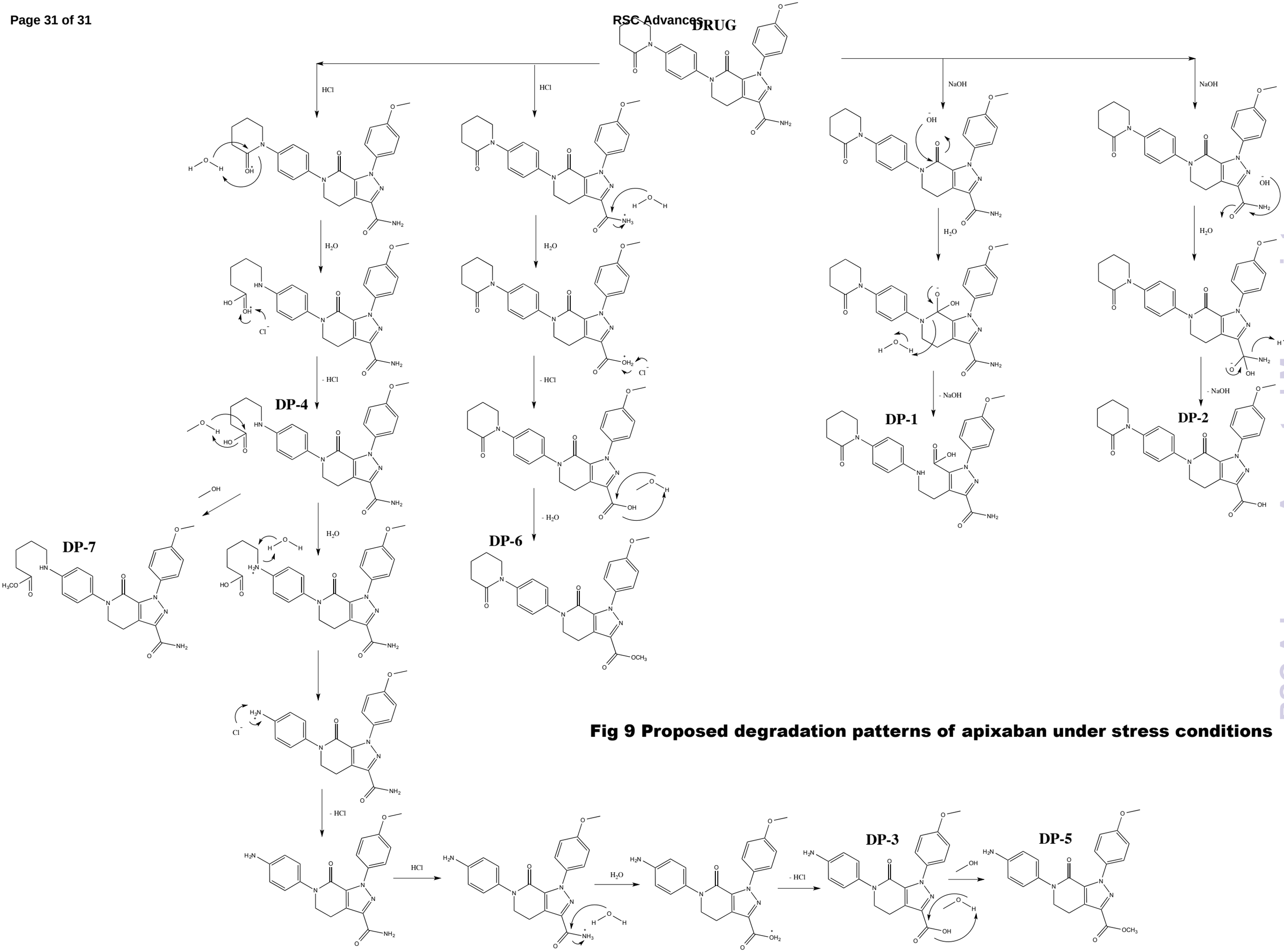


Fig 9 Proposed degradation patterns of apixaban under stress conditions