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Identification by LC/MS^n of degradates of a novel carbapenem antibiotic in an aqueous matrix

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Abstract

Increased drug resistance in Staphylcocci and Enterococci to currently available antibiotics has significantly limited therapeutic options. Recently, a novel carbapenem antibiotic (Compound **A**) with a releasable side chain adjacent to the carbapenem was investigated to combat methicillin- and vancomycin-resistant Staphylococci and vancomycin-resistant Enterococci. The major advantage of Compound **A** over existing antibiotics can be attributed to the fact that cleavage of the side chain upon β-lactam ring opening retained anti-bacterial activity while expelling the immunodominant epitope of the presumed β-lactam hapten. In this work, LC/MS methods were developed to identify degradates of Compound **A** in an aqueous matrix utilized in assessing product safety and supporting analytical method and formulation development. A total of eight significant degradates were observed in this Compound **A** sample by LC/MS^n and other techniques. Detailed structural analysis of degradates based upon LC/MS^n data and other supporting results will be described in this work. Proposed molecular structures were confirmed by synthesis and use of authentic standards for several degradates. Degradates **1** and **4** were identified as degradates formed through the reversal of Michael reaction from Degradate **3** that is apparently formed by hydrolysis. Degradates **2** and **8** were found to be Hofmann elimination degradates. Degradates **5** and **6** are believed to be formed through dimerization of two parent molecules followed by the reversal of Michael reaction. Finally, Degradate **7** is attributed to a displacement reaction. Potential degradation pathways based upon these preliminary studies will also be discussed.

Keywords: LC/MS^n; Degradate identification; Carbapenem antibiotic; Degradation pathways

1. Introduction

Chemical purity assessment of drug substances and resulting formulations is critical to establishing the safety/efficacy for a pharmaceutical product. The current industry standard is to obtain molecular characterization and identification of any impurities in a drug substance at the level of $\geq 0.1\%$ (or $\geq 0.05\%$) depending on dose of the pharmaceutical product [1]. Furthermore, degradates that form in the product over time are also characterized when observed above threshold levels depending upon the drug product daily intake [2]. Fast and sensitive LC/MS techniques have

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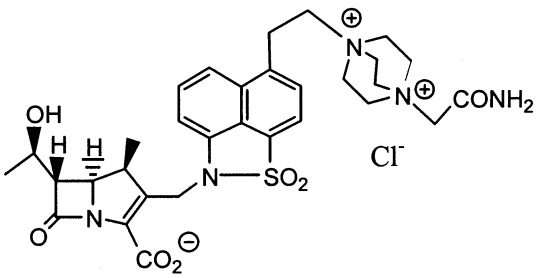
played an increasingly important role in this endeavor [3–10]. Recently, a novel carbapenem antibiotic with a releasable side chain adjacent to the carbapenem (Compound **A**) was developed for the treatment of methicillin-resistant *Staphylococcus aureus*, a severe, life-threatening infection [11–15]. Compound **A** is a chloride salt of 1β-methyl-2-(naphthosultamyl)methyl carbapenem bearing a dicationic, *N*,*N*′-dialkyl-1,4-diaza[2.2.2] bicyclooctane (DABCO) substituent, the structure of which is shown below. Coupled β-lactam ring opening with a chemical fragmentation results in the release of the naphthosultamyl moiety.



Release of this moiety is of importance since it is believed to be the immunodominant epitope of the hapten formed following acylation of proteins. Attachment of the DABCO moiety to the naphthosultamyl group improves water solubility, pharmacokinetics, and chemical stability. In this work, identification of degradates observed in a Compound **A** sample in an aqueous matrix by LC/MS^n and other techniques will be described. Molecular structures along with their formation pathways will also be proposed.

2. Experimental

2.1. Chemicals and reagents

Compound **A** (purity: 94.0%) and its related authentic standard of Degradate **4** (purity: 99.0%, UV area) were obtained from Merck Research Laboratories (Rahway, NJ). Phosphoric acid (HPLC grade) and potassium phosphate dibasic (reagent grade) were purchased from Aldrich (Milwaukee, WI). Ammonium acetate (ACS grade) and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Philadelphia, PA). Sucrose (USP), sodium chloride (powder, USP), citric acid anhydrous (USP) and sodium citrate dihydrate (USP) were purchased from Mallinckrodt Laboratory Chemicals (Phillipsburg, NJ).

2.2. Sample preparation

The Compound **A** sample in the aqueous matrix was made by dissolving the bulk drug (45 mg/ml) in an aqueous solution of sucrose (65.7 mM) and sodium chloride (80.4 mM) buffered by citric acid (0.15 mM) and sodium citrate (2.1 mM) at pH 5.8. The resulting solution was incubated for 6 h at 30 °C to induce about 4% degradation, then sterile filtered and stored at −70 °C. This stress condition was employed because it was most reflective of the degradation profile relevant to the pharmaceutical use of this compound. The target of 4% degradation was chosen to cover drug degradate qualification in the formulated product. Sample solutions of Compound **A** at a concentration of 1.0 mg/ml for the LC/MS^n analysis were prepared by diluting the Compound **A** sample in the aqueous matrix stored at 5 °C for 6 days with a diluent of 1/9 (v/v) acetonitrile/water, resulting in total degradate levels approaching 20%. Related authentic standards in the concentration of 0.01–0.1 mg/ml were made by dissolving them directly in acetonitrile and water (1/9, v/v). All the samples were then analyzed directly by LC/MS^n without further treatment.

2.3. LC/MS^n analyses

A Perkin Elmer Series 200 pump equipped with a series 200 autosampler and a model 235C PDA detector (Norwalk, CT) was the LC system employed. A gradient elution with an inertsil phenyl column (4.5 × 250 mm, 5 μm particle size, MetaChem, Torrance, CA) was used along with NH_4OAc (5–10 mM, pH 5.8)/ACN mobile phases. Column temperature was set at ambient and flow rate was 1.5 ml/min. The following gradient conditions were used: a linear gradient from 10 to 25% ACN over 30 min and then a second linear gradient from 25 to 55% ACN over the next 15 min. UV spectra were recorded from

200 to 350 nm with a scan rate of 1 nm per scan and a single channel monitoring was set at 245 nm. The injection volume was 10–200 μl depending upon samples analyzed.

A Finnigan LCQ mass spectrometer equipped with an ESI interface (ThermoFinnigan, San Jose, CA) was used for LC/MS^n analyses. The ESI energy was 4.5 kV in the positive ion mode. The mean scan speed in the range of 50–1100 amu was 0.8 s, averaging three microscans that have the maximum inject time of 50 ms/microscan. The relative collision energies for LC/MS^n analyses ranged from 12 to 60% depending upon compounds and fragment ions analyzed. The collision energy was calibrated by ThermoFinnigan using the vendor-defined procedure. Selected ions for LC/MS^n were then resonantly excited in the presence of helium (about 10^{-5} Torr) to produce collision induced dissociations. Sample analyses were performed by sequential UV and MS. The LC eluent from the diode array detector was split by using a micro-splitter valve (Upchurch Scientific, Oak Harbor, WA) at a ratio of about 1/10 (MS to waste).

3. Results and discussion

3.1. Degradate identification

Stability indicating LC/UV methods (SIM) using phosphate or other non-volatile buffers are often developed, validated and applied in order to study drug product stability profiles during product development. For this project, the SIM was originally developed utilizing a phosphate buffer mobile phase. The mobile phase for the LC/MS^n method was modified to replace the phosphate buffer with the volatile 5–10 mM ammonium acetate buffers. Gradient conditions in the LC/MS^n method were then optimized to obtain compatible chromatographic profiles between LC/MS^n and LC/UV SIM methods. Initial LC/MS experiments were conducted in a full scan MS mode on the Compound **A** sample in order to obtain the formula (FW) or molecular weights (MW) of all the possible degradates and impurities. Fig. 1 shows a typical LC/UV trace of a representative Compound **A** sample used to assess the product safety by using the LC/MS^n methods. The number of degradates chosen for detailed identification was based upon their UV and MS abundance. Among eight degradates selected in this work, seven were chosen based on UV abun dances over a threshold of 0.1% of the parent drug and one degradate (Degradate **2**) was also characterized due to its strong MS response. The degradates studied are numbered according to their elution order in the chromatogram. The mass-to-charge (m/z) values of molecular (or quasi-molecular) ions are also labeled in Fig. 1. Degradate **2** with m/z 170 is an UV silent species that has very little response at the detection wavelength of 245 nm. The peak observed between Degradates **2** and **3** is associated with sucrose that is present in sample solutions (Fig. 1). The peak present at 14.74 min is a known impurity, an isomer of Compound **A**. Although degradation profile remained unchanged between the original thawed −70 °C product (4% degradation, by UV area%) and the LC/MS^n samples, total amount of degradation increased to approximately 20% (UV area%) in LC/MS^n samples that had been stored for 6 days at 5 °C. Particularly, Degradates **4** and **6** grew significantly with storage at 5 °C from the original −70 °C product.

The tandem mass spectrum of the parent compound is briefly analyzed (M^+ is denoted as the quasi-molecular ion and MH^+ is the protonated molecular ion) in order to help the discussion of degradate identification. As is shown in Fig. 2a, the major fragments of Compound **A** are at m/z 580 [M^+–CO_2], 552 [580 – CO], 538 [M^+ –$CH_3CH(OH)CH{=}C{=}O$], 494 [538 – CO_2], 401, and 244. Fig. 2b shows the proposed fragmentation pathway of Compound **A**. Decarboxylation (an ion at m/z 580) is the most prominent fragmentation pathway for Compound **A**. The second most characteristic ion in the tandem mass spectrum of Compound **A** is an ion at m/z 401, attributed to a naphthosultamyl moiety, which is a unique feature of this compound. The neutral loss of 86 amu is a characteristic fragmentation of the β-lactam moiety of Compound **A**. The fragment at m/z 602 ([2M–CO_2]$^{2+}$) was found to be a doubly charged ion, the charge state readily was

determined by recording the MS/MS spectrum of the parent ion (m/z 624) in a wide scan window (± 2 amu). The ion at m/z 602 is thought to be formed from decarboxylation of a non-covalent dimer of the parent ion as an artifact of the LCQ ion trap mass spectrometer, since this ion was not observed when utilizing a triple quadruple instrument (ThermoFinnian TSQ7000 mass spectrometer) to analyze these same sample solutions. The detailed description of structural analyses of observed Compound **A** degradates is presented below in the chromatographically observed elution sequence.

3.1.1. Degradate *1*

The tandem mass spectrum and proposed structure of Degradate **1** is shown in Fig. 3a. Degradate **1** has a molecular ion at m/z 242 and elutes in the solvent front. Characteristic fragments of Degradate **1** are at m/z 224 [MH^+–H_2O], 198 [MH^+–CO_2], 180 [224 – CO_2], and 152 [180 – CO]. The UV spectrum of Degradate **1** indicates the absence of the naphthosultamyl group. Both MS/MS and UV data suggest that Degradate **1** is the ring-opened β-lactam portion of Compound **A**. The proposed structure of Degradate **1** is consistent with results published previously [11].

3.1.2. Degradate *2*

The formula weight of Degradate **2** was found to be 170 (Fig. 3b). The UV-silent nature of Degradate **2** is due to a non-UV functional group, the DABCO moiety of Compound **A**. The most prominent fragments of Degradate **2** were detected at m/z 125 [M^+–$HCONH_2$] and 112 [M^+–CH_2CONH_2].

3.1.3. Degradate *3*

Degradate **3** has the same UV spectrum as Compound **A** and a MW of 641, indicating water addition to Compound **A** (FW 624). Major fragments of Degradate **3** were detected at m/z 620,

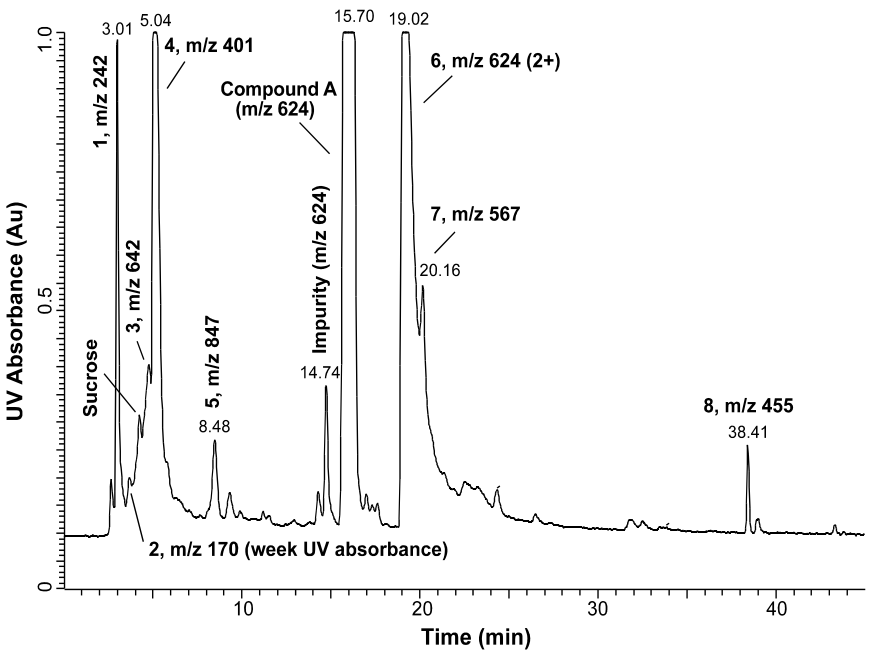


Fig. 1. HPLC/UV chromatogram of an approximately 20% degradated Compound **A** sample in an aqueous matrix. Chromatographic conditions: column: inertsil phenyl, 4.6 × 250 mm; column temperature: ambient; mobile phases: A: 5 mM NH_4OAc in water (pH 5.8) and B: acetonitrile; gradient: 30 min from 10%B to 25%B and 15 min from 25%B to 55%B; flow rate: 1.5 ml/min; injection volume: 200 μl; detection wavelength: 245 nm.

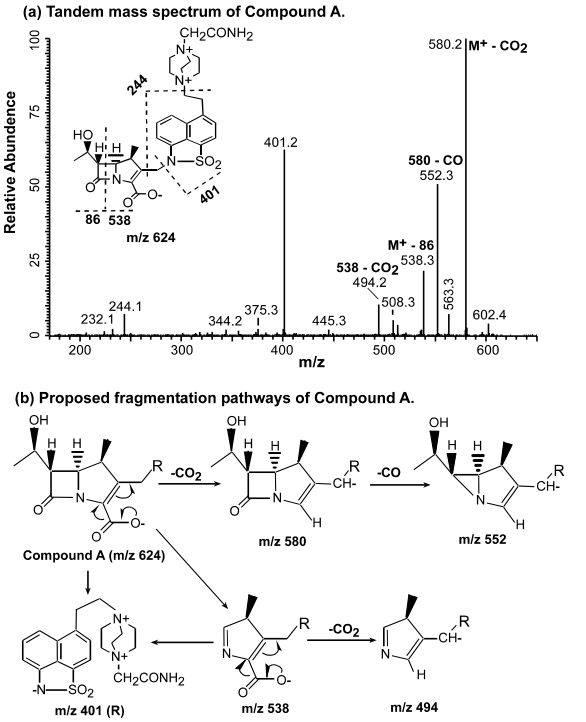


Fig. 2. Proposed fragmentation and tandem mass spectrum of Compound **A**. Chromatographic conditions are the same as in Fig. 1. Mass spectrometer: interface: Finnigan ESI source; electrospray voltage: 4.5 kV; heated capillary temperature: 200 °C; sheath and auxiliary gas: 75 and 35 units, respectively; relative collision energy: 25%.

598 [MH^+–CO_2], 570 [598 – CO], 429, 385 [429 – CO_2] and 303. Fragmentation patterns of Degradate **3** are slightly different from the parent compound, which may be due to chemical environment differences around the β-lactam moiety. The facile loss of two molecules of CO_2 (ions at m/z 429 and 385) from Degradate **3** in the MS/MS spectrum suggests that Degradate **3** has two carboxylic groups. The species at m/z 620 that is a doubly charged ion may be formed from the decarboxylation of the in-source dimer at m/z 642 (doubly charged), which may be similar to the ion at m/z 602 in the tandem mass spectrum of the parent drug (m/z 624). The proposed structure of Degradate **3** and its tandem mass spectrum are shown in Fig. 4a.

3.1.4. Degradate **4**

The counterpart of Degradate **1**, Degradate **4**, is a naphthosultamyl moiety of Compound **A**. The UV spectrum of Degradate **4** was found to be similar to that of Compound **A**. The characteristic fragments of Degradate **4** are detected at m/z 356 [M^+–$HCONH_2$], 343, 232 and 170 (Fig. 4b) and are the same as the MS/MS spectrum of its authentic standard. Subsequently, this structural assignment was confirmed with authentic standard spiked into the chromatographic method exhibiting co-elution. Both of these degradates may come from the β-lactam ring-opened degradate, which is Degradate **3** (an unstable intermediate, see above for its structural analysis).

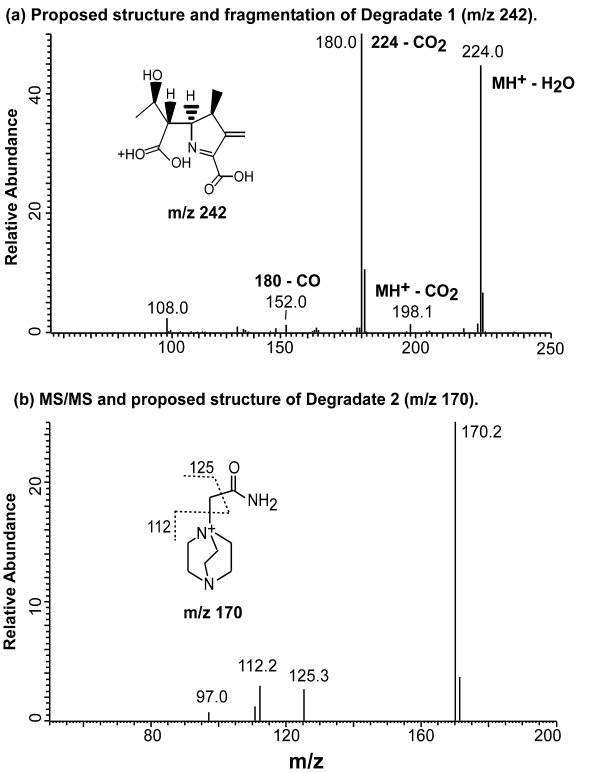


Fig. 3. Proposed structures and tandem mass spectra of Degradates **1** and **2**. Chromatographic conditions are the same as in Fig. 1. Mass spectrometer: interface: Finnigan ESI source; electrospray voltage: 4.5 kV; heated capillary temperature: 200 °C; sheath and auxiliary gas: 75 and 35 units, respectively; relative collision energies for Degradates **1** and **2**: 25 and 12%, respectively.

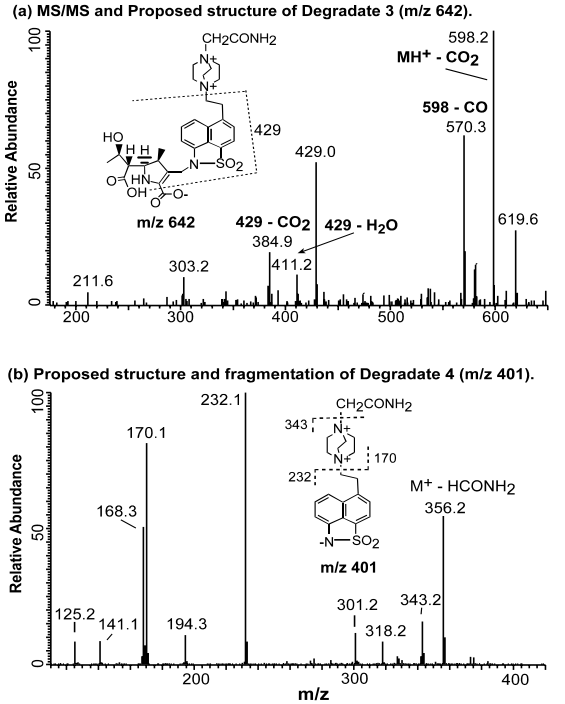


Fig. 4. Proposed structures and tandem mass spectra of Degradates **3** and **4**. Chromatographic conditions are the same as in Fig. 1. Mass spectrometer: interface: Finnigan ESI source; electrospray voltage: 4.5 kV; heated capillary temperature: 200 °C; sheath and auxiliary gas: 75 and 35 units, respectively; relative collision energies for Degradates **3** and **4**: 25 and 20%, respectively.

*3.1.5. Degradate **5***

Tandem mass spectrum and proposed structure of Degradate **5** with its UV spectrum similar to Compound **A** are shown in Fig. 5a. Major fragments of Degradate **5**, formula weight of 848, are at m/z 803 [MH^+–CO_2], 759 [MH^+–$2CO_2$], 624, and 401. The characteristic fragment at m/z 624 clearly indicates that Degradate **5** may be formed from the combination of Compound **A** and an additional group with the mass of 224 amu, the exact weight of the β-lactam moiety of Compound **A**. The presence of the ion at m/z 401 shows that the naphthosultamyl moiety is intact as well. The existence of the ion at m/z 624 also suggests that Degradate **5** is a so-called 'diketopiperazine ring opened' species since the intact diketopiperazine group should not be easily fragmented under the soft electrospray ionization conditions used in this study.

*3.1.6. Degradate **6***

A doubly-charged molecular ion of Degradate **6** was found at m/z 624, consistent with a dimerization of Compound **A** (singly charged at m/z 624). The observed UV spectrum of Degradate **6** is also very similar to Compound **A**. Major fragments in its tandem mass spectrum were observed at m/z 847 $[M - 401]^+$, 803 $[847 - CO_2]$, 624 $[M]^{2+}$, 602 $[M–CO_2]^{2+}$, and 401 (Fig. 6b). The fragment ion at m/z 847 may be formed by the loss of the intact naphthosultamyl group, indicating that dimerization is on the β-lactam moiety of Compound **A**. A small amount of Degradate **6** was chromato-

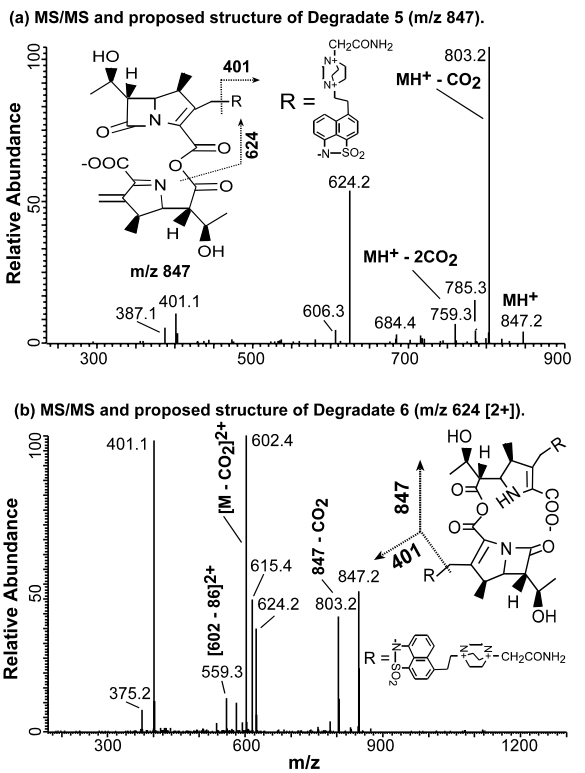


Fig. 5. Proposed structures and tandem mass spectra of Degradates **5** and **6**. Chromatographic conditions are the same as in Fig. 1. Mass spectrometer: interface: Finnigan ESI source; electrospray voltage: 4.5 kV; heated capillary temperature: 200 °C; sheath and auxiliary gas: 75 and 35 units, respectively; relative collision energies for Degradates **5** and **6**: 25 and 18%, respectively.

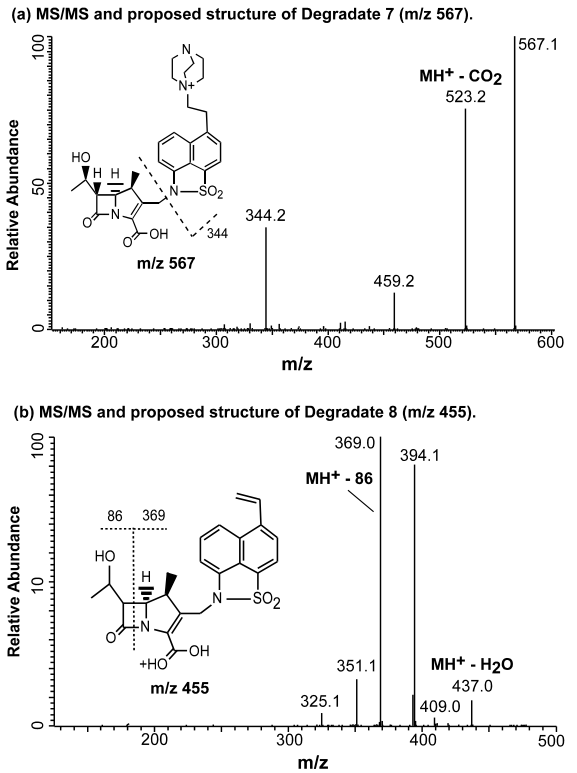
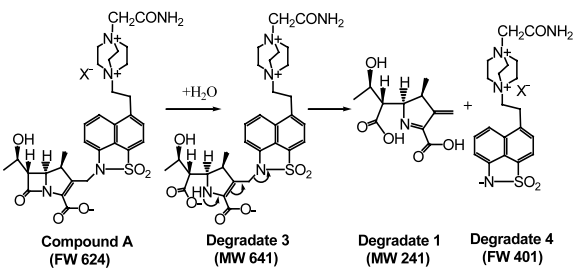


Fig. 6. Proposed structures and tandem mass spectra of Degradates **7** and **8**. Chromatographic conditions are the same as in Fig. 1. Mass spectrometer: interface: Finnigan ESI source; electrospray voltage: 4.5 kV; heated capillary temperature: 200 °C; sheath and auxiliary gas: 75 and 35 units, respectively; relative collision energies for Degradates **7** and **8**: 25 and 20%, respectively.

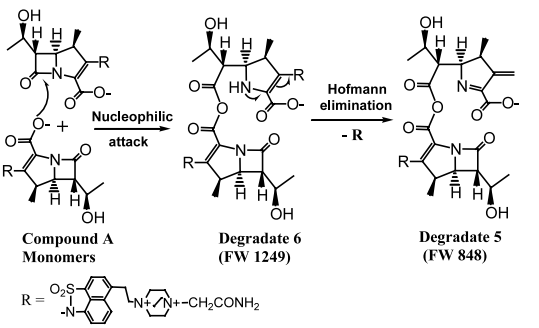


Scheme 1. Proposed formation pathways for Degradates **1**, **3** and **4**.

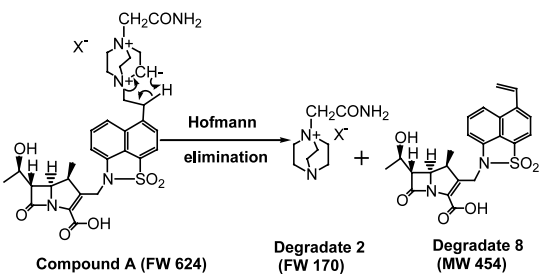
graphically isolated, then subsequently analyzed by MS^3 analysis for the fragment ion at m/z 847. The observed mass spectrum of the fragment ion at m/z 847 is identical to Degradate **5**, suggesting that Degradate **6** is also a diketopiperazine ring-opened dimer. Degradate **6** was apparently formed during 6-day storage at 5 °C since the dimer was not detected in fresh preparations from the original −70 °C samples that initially contained about 4% total degradates.

3.1.7. Degradate 7

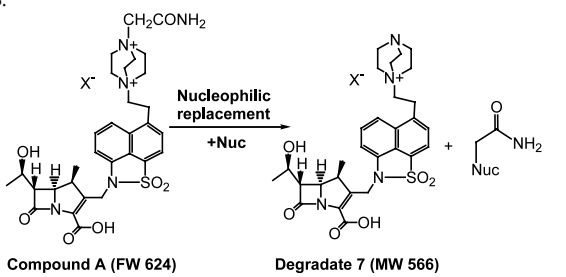
A MW of 566 was obtained for Degradate **7**, which also has a very similar UV spectrum as Compound **A**. Major fragments observed in the tandem mass spectrum were at m/z 523 [$MH^+ - CO_2$], 459 and 344 (Fig. 6a). The presence of the fragment ion at m/z 344, formed by the loss of the



Scheme 2. Proposed formation pathway of Degradates **5** and **6**.



Scheme 3. Proposed formation pathway of Degradates **2** and **8**.



Scheme 4. Proposed formation pathway of Degradate **7**.

Table 1
Summary of degradate identification

Compound ID	Parent ion (m/z)	Chromophore[a] (UV spectrum)	Proposed reaction type	Standard synthesized
Compound **A**	624	Type 1	Not applicable	Yes
Degradate **1**	242	Type 2	Reversal of the Michael reaction	No
Degradate **2**	170	Type 3	Hofmann elimination	No
Degradate **3**	642	Type 1	Reversal of the Michael reaction	No
Degradate **4**	401	Type 1	Nucleophilic reaction (hydrolysis)	Yes
Degradate **5**	847	Type 1	Dimerization/Hofmann elimination	No
Degradate **6**	624 (2+)	Type 1	Dimerization	No
Degradate **7**	567	Type 1	Displacement reaction	No
Degradate **8**	455	Type 4	Hofmann elimination	No

[a] Chromophores are classified based upon characteristics of the UV spectrum: Type 1 is (dominated by) the absorbance of the naphthosultamyl moiety; Type 2 is (dominated by) the absorbance of the β-lactam ring; Type 3 is dominated by the absorbance of an amide bond in the DABCO group; Type 4 is similar to Type I, however, with a red shift attributed to a double bond in conjugation with the naphthosultamyl moiety.

β-lactam moiety, suggests that the β-lactam group is intact and that the site of degradation is on the naphthosultamyl side chain.

*3.1.8. Degradate **8***

The counterpart of Degradate **2** is Degradate **8** with a MW of 454. Major fragments for Degradate **8** are at m/z 437 [MH^+–H_2O], 394, 369 [MH^+–$CH_3CH(OH)CH{=}C{=}O$], 351 [369 – H_2O], and 325 [369 – CO_2] (Fig. 6b). The lost of 86 amu [$CH_3CH(OH)CH{=}C{=}O$] is the characteristic fragmentation of the β-lactam ring of Compound **A**. The structural assignment of Degradate **8** was also confirmed by a red shift in its UV spectrum compared to Compound **A**. The red shift in the UV spectrum is readily attributed to an additional double bond formed in conjugation to the 1,8-naphthosultamyl moiety.

3.2. Proposed degradation mechanisms

Understanding the degradation mechanism is an important part of degradate identification, which then provides guidance for both analytical method and formulation development. Degradates **1** and **4**, two of the most prominent degradates observed in the Compound **A** sample in the aqueous matrix (Fig. 1), are likely formed by the reversal of the Michael reaction [16] (Scheme 1) from Degradate **3**, which is clearly formed by hydrolysis of the β-lactam ring [17,18]. The relatively low level of Degradate **3** observed in the Compound **A** sample (Fig. 1) confirms that Degradate **4** is a good leaving group, the key feature in the design of Compound **A**.

The next most prominent degradate observed in the sample is Degradate **6**. Degradate **6** is thought to form through the nucleophilic attack of the carboxylic acid in one molecule of Compound **A** on the β-lactam moiety in a second Compound **A** molecule (Scheme 2). The reaction seems plausible at the high drug concentration (45 mg/ml). Degradate **6** can then subsequently undergo the reversal of the Michael reaction to yield Degradate **5** (Scheme 2) to a minor extent since the levels of Degradate **5** observed in the sample are significantly less than Degradate **6** (Fig. 1).

The formation of Degradates **2** and **8** are coupled (Scheme 3), both occurring at low levels in the current Compound **A** sample (Fig. 1), and are attributed to a Hofmann elimination reaction of Compound **A** [19]. Even though Degradate **2** is a weak chromophore at the method detection wavelength, 245 nm, Degradate **8** should still have a reasonable response at this wavelength and thus provides a more accurate reflection of the significance of this reaction scheme in the drug sample.

Finally, Degradate **7** also forms at relatively

low levels in the Compound **A** sample as well and is thought to form through a replacement reaction of Compound **A**, resultant of nucleophilic attack on the α-carbon of the acetamide group (Scheme 4).

4. Conclusions

In this work, a total of eight potential degradates of Compound **A** were characterized in the Compound **A** sample in the aqueous matrix by LC/MS^n and other techniques. Detailed structural analysis of the potential degradates and proposed degradation pathways are summarized in Table 1. The proposed structures of some degradates were also confirmed by studying synthetic and authentic standards using LC/UV and LC/MS^n. These degradates are proposed to form through one of five different pathways: reversal of the Michael reaction, Hofmann elimination, nucleophilic reaction, replacement reaction, and dimerization. Several degradates are proposed to form through a combination of these degradation pathways.

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