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Application of LC-NMR and LC-MS to the identification of degradation products of a protease inhibitor in dosage formulations

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Abstract

LC-NMR and LC-MS were applied to the characterization of six degradation products of a protease inhibitor, N-hydroxy-1,3-di-[4-ethoxybenzenesulphonyl]-5,5-dimethyl-[1,3]cyclohexyldiazine-2-carboxamide, in a dosage formulation. A reversed-phase HPLC method was developed for the separation of the parent compound and its six degradation products. LC-MS was then utilized to obtain the molecular weight and fragmentation information using an electrospray ionization (ESI) interface in the positive ion mode. LC-NMR was employed to acquire detailed structural information using a selective solvent suppression pulse sequence in the stop–flow mode. This work demonstrated the usefulness of this integrated approach for the rapid and unambiguous identification of drug compounds and their degradation products in dosage formulations.

Keywords: LC-MS; LC-NMR; Degradation; Formulation; Protease inhibitor

1. Introduction

In drug discovery, there are many complex structure elucidation problems that require the use of more than one analytical technique for their solutions. Traditionally, LC-MS has been the method of choice for the characterization of complex mixtures. However, the structural assignments by LC-MS alone often remain tentative, especially when structural, conformational, and optical isomers need to be identified. In many cases, separate NMR experiments are required to obtain detailed structural information on each component. In doing so, each component has to be separated and isolated from an HPLC run for NMR analysis. With the advent of LC-NMR, this additional fraction collection step is eliminated and the structural characterization work is thus accelerated. Because of recent major advances, LC-NMR has been increasingly utilized to characterize in vitro and in vivo metabolites [1–3], bulk drug impurities [4], and drug components from combinatorial library synthesis [5,6]. In ad-

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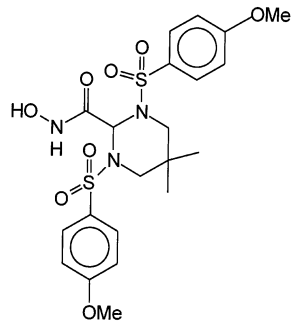


Fig. 1. Chemical structure of PGE4410186.

dition, the combined use of LC-NMR and LC-MS has received much attention lately to facilitate the characterization of in vitro metabolites [7], in vivo metabolites in rat and human urine [8,9], and combinatorial chemistry mixtures [10].

The identification of degradation products in various dosage formulations plays an important role in the drug discovery process. Information on the structures of degradation products can help synthetic chemists modify their novel compounds to prepare drug candidates with improved stability. It can also aid in development of alternative formulations for increased drug stability. When the modification of a particular dosage formulation or the structure of a test compound is not warranted, the identification of degradation products is critical to understanding the potential toxicities or side effects associated with degradation. It is not uncommon to find that many potent drug candidates undergo rapid degradation in certain dosage formulations. Therefore, a rapid and unambiguous determination of drug degradation

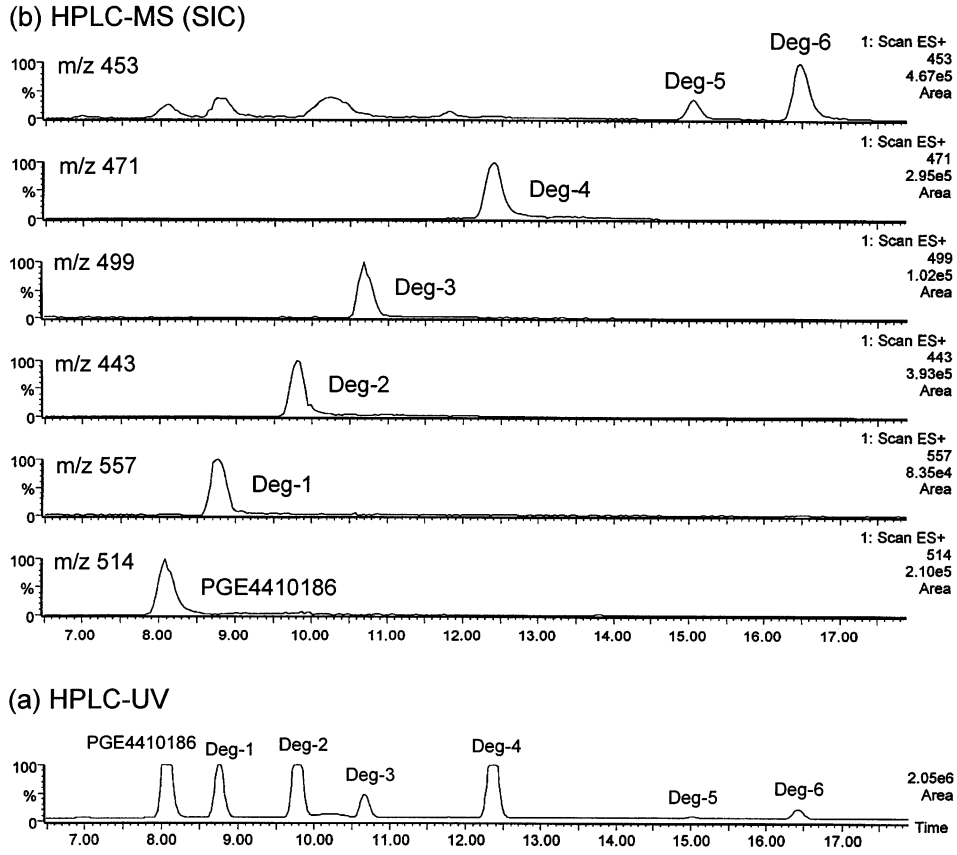


Fig. 2. Low-cone-voltage (30 V) single ion (MH^+) chromatograms (b) and HPLC-UV chromatogram (a) of the degradation sample of PGE4410186 in a dosage formulation from an LC-UV-MS experiment.

products is required to accelerate the drug discovery process. Current on-line detection and characterization of degradation products have been mainly accomplished by LC-MS and LC-MS-MS [11,12].

In this work, LC-NMR and LC-MS were employed to elucidate the structures of six degradation products of a matrix metalloprotease (MMP) inhibitor (see Fig. 1), N-hydroxy-1,3-di-[4-methoxybenzenesulphonyl]-5,5-dimethyl-[1,3] cyclohexyldiazine-2-carboxamide (PGE4410186), in a dosage formulation. This compound belongs to a class of hydroxamic acid-based compounds that has been explored for potential drug candidates in the treatment of cancers, arthritic disorders, and other connective tissue-related diseases, through the inhibition of various MMPs [13]. PGE4410186 degrades quickly in a dosage formulation designed for an animal model study. Based on LC-MS data, structures were tentatively proposed for each major degradant. LC-NMR data confirmed three degradation products and identified the structures of the other three degradants. This example demonstrated the usefulness of these two complementary techniques for rapid identification of degradation products in dosage formulations.

2. Experimental

2.1. Material

PGE4410186 was obtained from Procter & Gamble Pharmaceuticals (Mason, OH, USA). HPLC-grade acetonitrile, formic acid, monobasic sodium phosphate, and dibasic sodium phosphate were purchased from J.T. Baker (Phillipsburg, NJ, USA). NMR-grade acetonitrile and poly(ethylene glycol) were obtained from Aldrich (Milwaukee, WI, USA). Deuterated water (D_2O) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

2.2. Sample preparation

The dosing solution of the compound was made at a concentration of 10 mg ml^{-1} by dissolving the compound in a dosage formulation consisting of 10% ethanol, 65% PEG-400, and 25% aqueous phosphate buffer at pH 7.4. The solution was incubated at 50°C for 3 h and analyzed directly in the LC-NMR experiment. For LC-MS, the resulting solution was diluted to 0.5 mg ml^{-1} prior to analysis.

2.3. LC-MS

HPLC experiments were carried out on a Hewlett Packard 1100 HPLC with an autosampler and a variable-wavelength UV detector. A 20 min linear gradient elution from mobile phase A (35:65:0.1, HPLC-grade acetonitrile:water:formic acid, v/v/v) to mobile phase B (80:20:0.1, HPLC-grade acetonitrile:water:formic acid, v/v/v) was performed on a Waters Symmetry C-18 column (3.9×150mm) with a flow rate of 1.0 ml min^{-1} and UV detection at 240 nm. The injection volume was 5 μL. Mass spectrometry experiments were run on a Micromass Platform II mass spectrometer interfaced with HPLC. Mass spectra were obtained using an electrospray ionization (ESI) interface, in the positive ion mode, at a source temperature of 120°C. Two mass ranges, m/z 300–700 (for 30 V cone voltage) and m/z 100–660 (for 80 V cone voltage) were alternately scanned in 1 s. The post-column split ratio between the UV detector and ESI-MS detector is 9:1.

2.4. LC-NMR

The HPLC conditions for the LC-NMR experiment were identical to those of the LC-MS experiment, except that NMR-grade acetonitrile was employed and D_2O was substituted for H_2O. The injection volume was 25 ml. The outlet of the UV detector was connected to a Bruker LC-NMR probehead via a polyethylether ketone (PEEK) capillary. NMR spectra were generated using a Bruker Avance DRX600 NMR spectrometer equipped with a 3 mm inverse dual $^1H/^{13}C$ probehead (60 μl active cell volume) with an actively shielded Z-gradient coil. Free induction decays

Table 1
Structures of degradation products of PGE4410186 in a dosing formulation as determined by LC–MS and LC–NMR

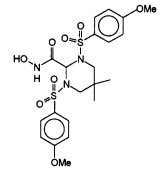
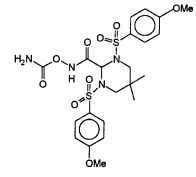
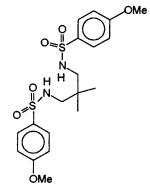
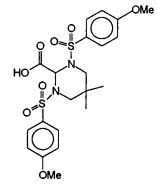
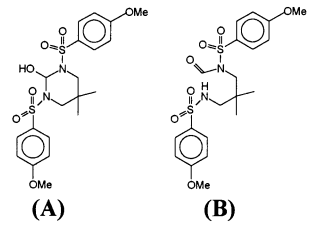
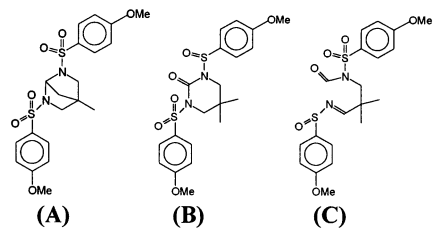
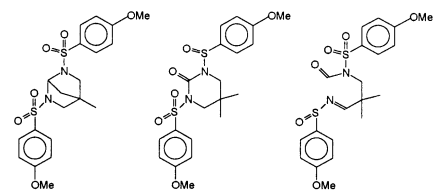
Component	t_R (min)	M.W. (LC–MS)	Proposed Structure (LC-MS)	Structure confirmation (LC-NMR)
PGE4410186	8.07	513 514 (MH^+) 531 (MNH_4^+) 536 (MNa^+)		confirmed
Degradant-1	8.78	556 557 (MH^+) 474 (MNH_4^+) 479 (MNa^+)		confirmed
Degradant-2	9.80	442 443 (MH^+) 460 (MNH_4^+) 465 (MNa^+)		confirmed
Degradant-3	10.69	498 499 (MH^+) 516 (MNH_4^+) 521 (MNa^+)		confirmed
Degradant-4	12.38	470 471 (MH^+) 488 (MNH_4^+) 493 (MNa^+)	 **(A)** **(B)**	LC-NMR is consistant with structure B.
Degradant-5	15.03	452 453 (MH^+) 475 (MNa^+)	 **(A)** **(B)** **(C)**	LC-NMR is consistant with structure B.

Table 1 (Continued)

Component	t_R (min)	M.W. (LC–MS)	Proposed Structure (LC-MS)	Structure confirmation (LC-NMR)
Degradant-6	16.42	452 453 (MH^+) 475 (MNa^+)	 **(A)** **(B)** **(C)**	LC-NMR is consistant with structure C.

(FIDs) were collected into 32K data points with a spectral width of 15 015.02 Hz. Solvent (CH_3CN and HDO) resonances were suppressed with a WET pulse sequence that uses a train of four selective shaped RF pulses with each followed by a dephasing field gradient pulse [14,15]. Carbon decoupling was employed during the four selective RF pulses sequence. The composite 90° read pulse was utilized in all experiments. All FIDs were processed using a convolution baseline correction method for further suppression of the largest solvent signal from acetonitrile at 2 ppm [16]. The LC-NMR experiments were conducted in the stop–flow mode, where LC flow was stopped while the NMR was acquiring data on an LC peak. The number of transients used for signal averaging was varied from 24 to 640 depending on the amount of each component in the LC-NMR flow cell. All chemical shifts were referenced to that of acetonitrile at 2 ppm.

3. Results and discussion

3.1. HPLC-UV-(ESI)MS

The separation of parent compound and degradation products is particularly important for LC-NMR experiments because NMR data need to be acquired on a single component, sequentially isolated within the LC-NMR flow cell. Thus, an HPLC separation was developed to baseline-resolve components of the PEG4410186 degradation sample. The resulting UV chromatogram, along with low-cone-voltage (30 V) MS profiles obtained in parallel, is shown in Fig. 2. These data revealed peaks corresponding to parent compound plus six significant degradants. For illustration, single ion chromatographic profiles represent each of six distinct MH^+ values observed (seven related compounds, including a pair of isomers). Definitive molecular weight assignments were made for each component, based on molecular adduct ions observed in corresponding 30 V ESI mass spectra, as listed in Table 1.

High-cone-voltage (80 V) ESI mass spectra were also obtained for each compound during this same HPLC run. Spectra, along with partial interpretation, are shown in Fig. 3a–g. These data led to tentative structure assignments, as displayed in Table 1. Note that when MS fragmentation data were less definitive, more than one possible structure was proposed. For example, molecular weight and fragmentation data on Degradant-4 were consistent with inclusion of an alcohol moiety, but its tautomeric form could also be rationalized. Also, as Degradant-5 and Degradant-6 were of the same molecular weight and yielded identical fragmentation patterns as shown in Fig. 3f and g, it was likely that they were structural isomers. In both cases, three possible structures were rationalized. It was apparent that additional characterization information would be required to properly assign structures.

3.2. HPLC-UV-NMR

In order to provide complementary structural information on the six PGE4410186 degradants, LC-NMR experiments were conducted under the identical chromatographic conditions as those in the LC-MS experiments. A UV signal was obtained during the LC-NMR run to correlate the LC peaks with the NMR spectra. Shown in Fig. 4a–g are representative ^{1}H NMR spectra of the

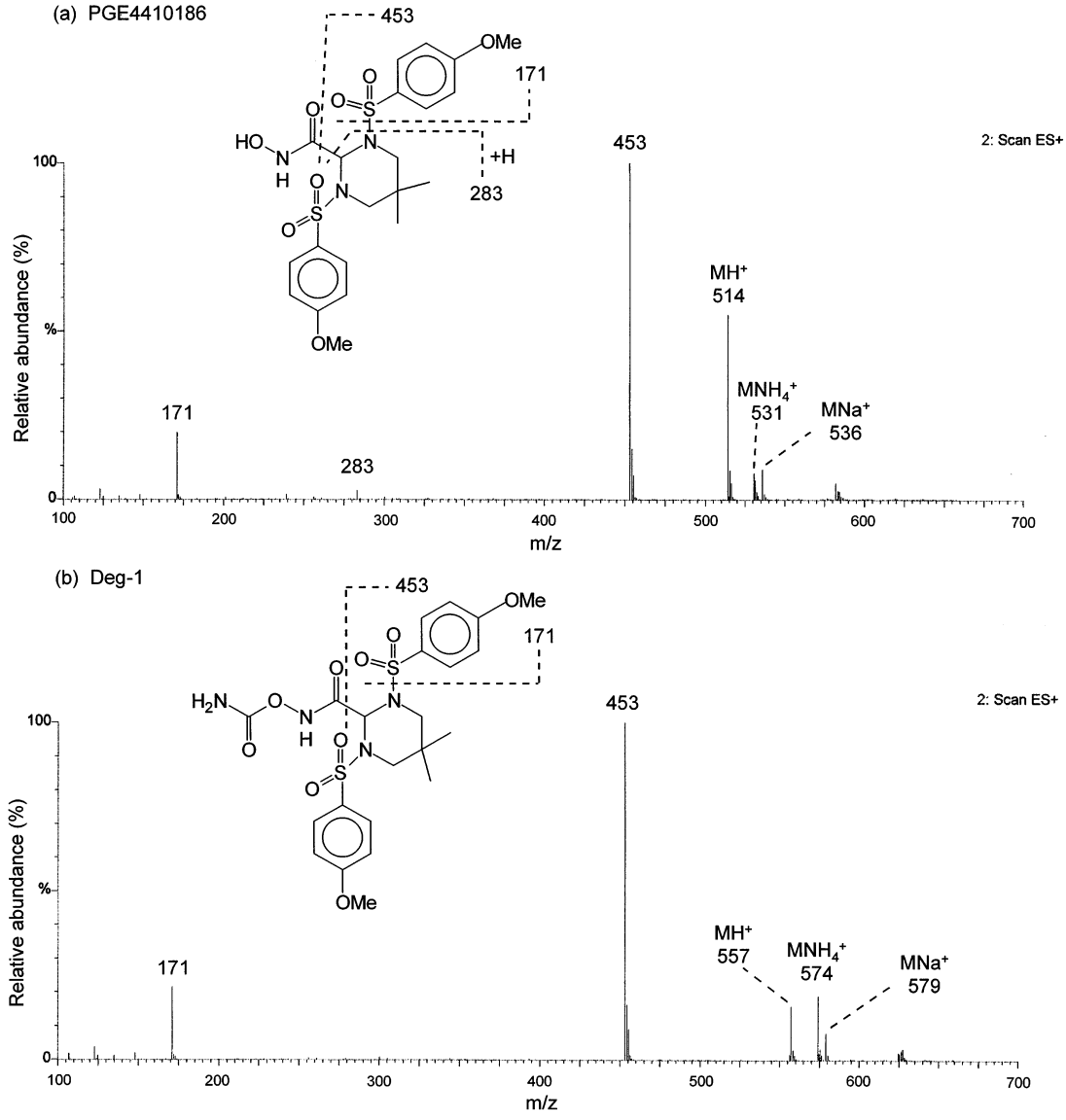


Fig. 3. Positive ion, high-cone-voltage (80 V) ESI mass spectra obtained from an LC-MS experiment on the degradation sample of PGE4410186 in a dosage formulation. Spectra (a)–(g) represent parent compound and Degradants 1–6, respectively.

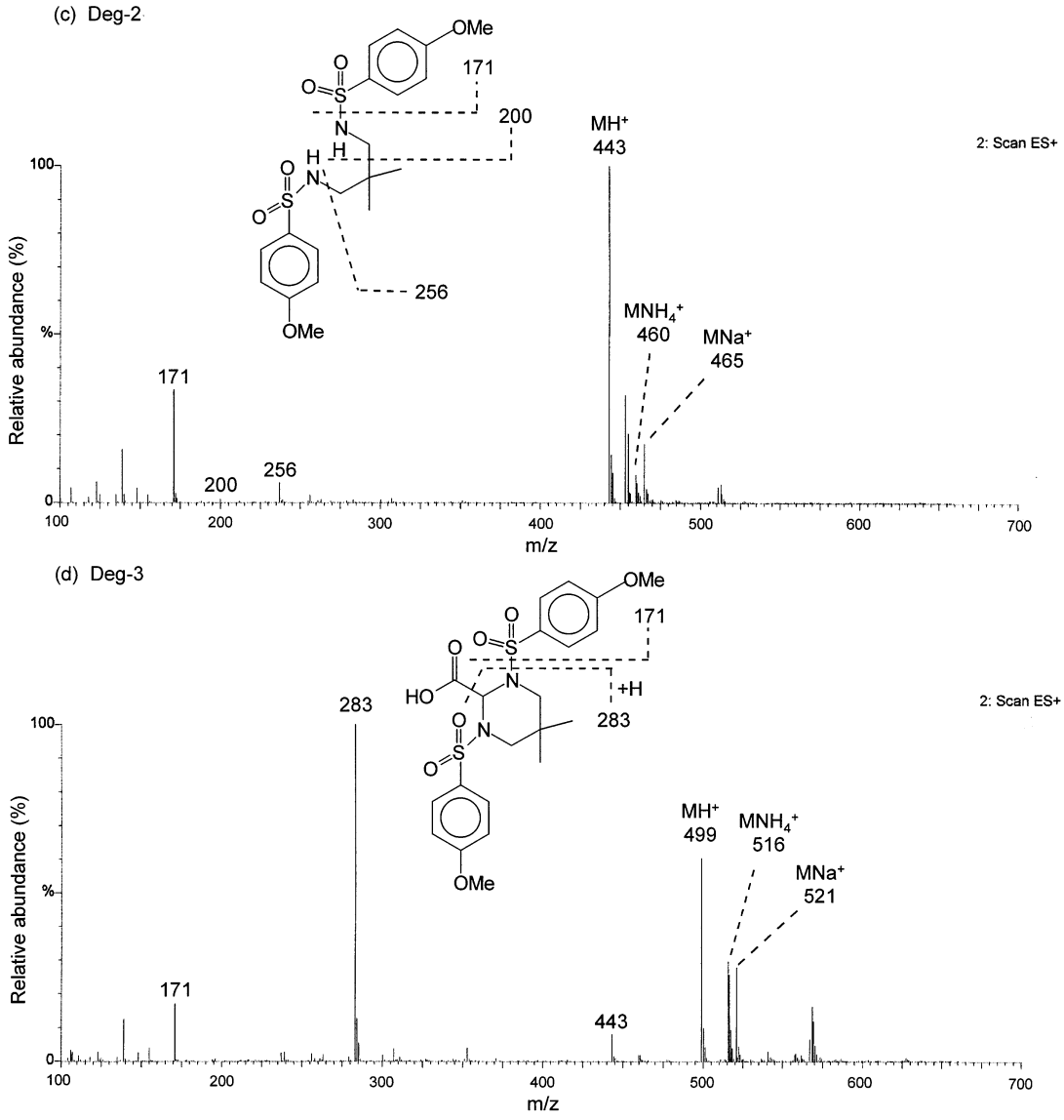


Fig. 3. (*Continued*)

parent compound and its six degradants obtained from this experiment. It is evident that high quality and high resolution NMR spectra were obtained with an estimated 5–50 μg on-column for each component, depending on the actual amount of each component in the LC-NMR flow cell. The NMR resonance peaks were assigned as indicated in the spectra. The solvent resonances (acetonitrile at 2 ppm and HDO at 4.2 ppm) were well suppressed as shown in the NMR spectra in Fig. 4. The third solvent resonance peak at 8.2 ppm was from

formic acid used as a mobile phase modifier. It was not necessary to suppress this signal due to its relatively weak intensity, compared to the strong resonance peak from acetonitrile. The resonance peaks at 3.6 ppm came from an impurity in the mobile phase. This resonance appeared more pronounced in some NMR spectra because more scans were used to acquire the spectra of the corresponding components in those cases.

The ^{1}H NMR spectrum of the parent compound is shown in Fig. 4a. The resonances were assigned as shown in the figure. The two methyl groups were conformationally non-equivalent, giving rise to two resonances at δ 0.32 and δ 0.78 (denoted as peaks 1 and 1′). The two methylene groups were similarly non-equivalent and showed two doublets at δ 3.02 and δ 3.26 (peaks 2 and 2′). The geminal coupling of about 13 Hz of the methylene protons

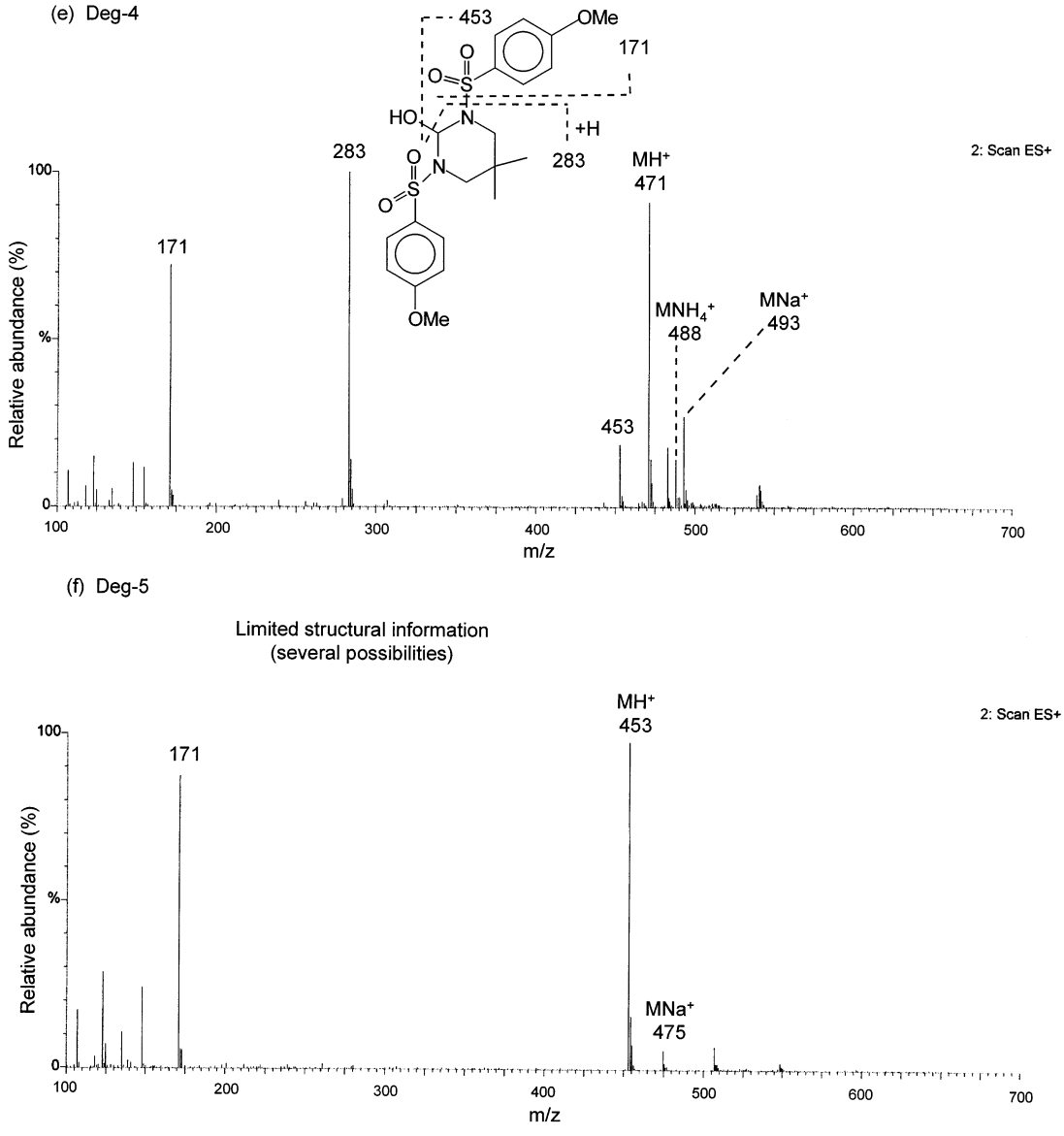
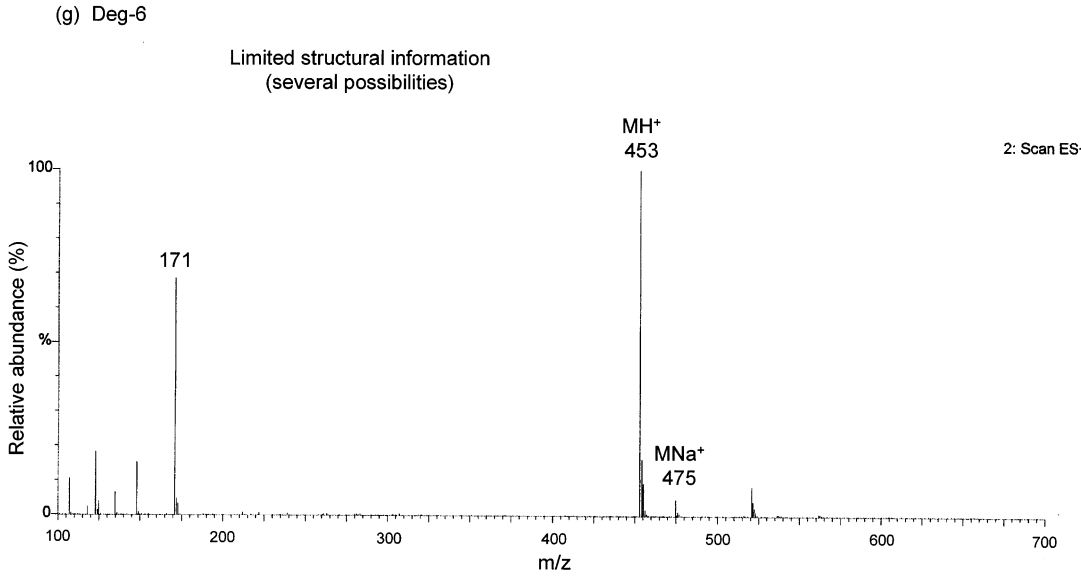


Fig. 3. (*Continued*)



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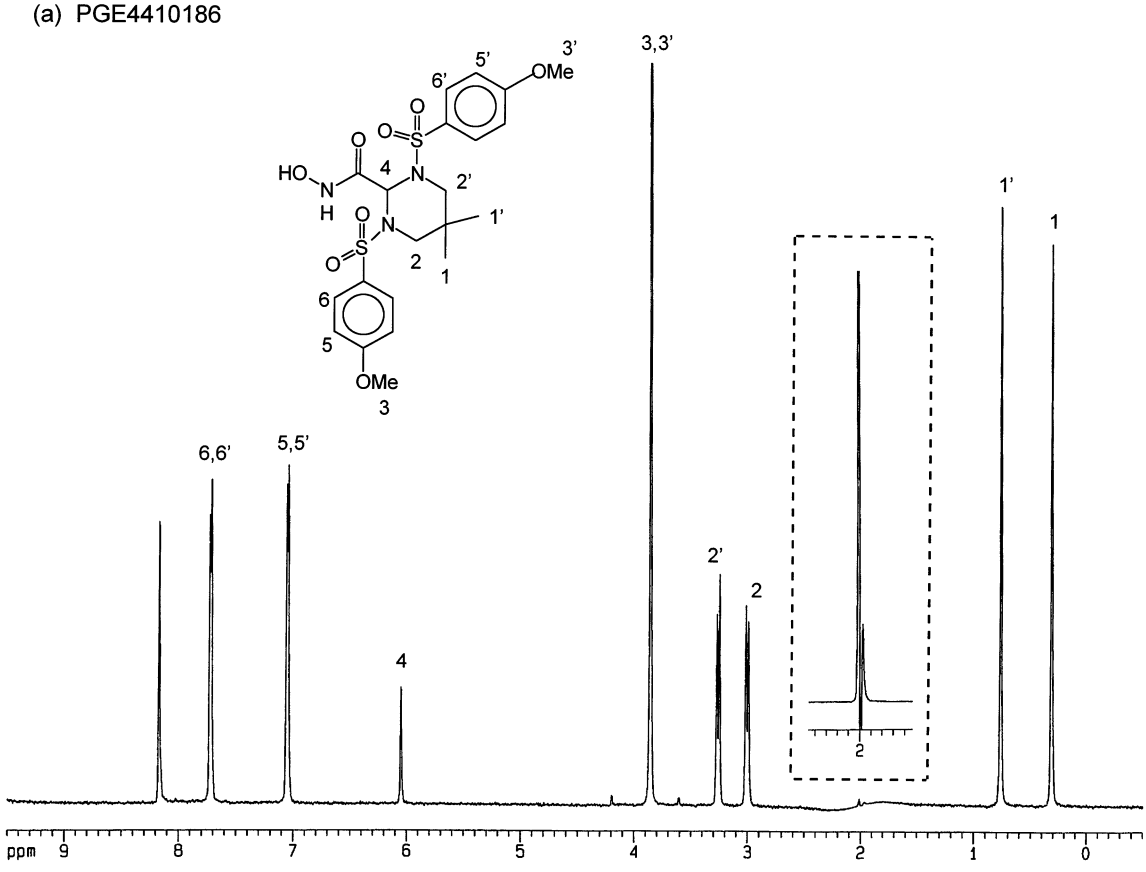

Fig. 4. 600-MHz ^{1}H NMR spectra obtained during a stop-flow LC-NMR experiment on the degradation sample of PGE4410186 in a dosage formulation. Spectra (a)–(g) correspond to parent compound and Degradants 1–6, respectively. In (a), the inset above 2 ppm shows the WET pulse sequence-suppressed acetonitrile resonance peak prior to a convolution baseline correction.

was clearly resolved and demonstrated the high quality of spectral resolution obtained in our experiments. The methine proton was found at δ 6.05 (peak 4). The two methoxy groups were equivalent resulting in a single resonance peak at δ 3.87 (peak 3, 3′). Because of the symmetry of the molecule, only two pairs of doublets, characteristic of vicinal couplings, were observed for the aromatic resonance peaks at δ 7.04 and δ 7.72 (peaks 5, 5′ andy 6, 6′). None of the NH and OH protons in the molecules were observed because these labile protons exchanged with deuterium from D_2O in the mobile phase. The NMR spectrum is consistent with the structure of the parent compound.

The NMR spectrum of Degradant-1 was similar to that of the parent compound, as shown in Fig. 4b, and was consistent with its proposed structure from the LC-MS experiment. The exchangeable protons of the carbamate functionality on the hydroxamic acid moiety were not observable. The NMR spectrum of Degradant-2 shown in Fig. 4c was different from those of the parent compound and Degradant-1 in several respects—the resonance peak of the methine proton was absent, two peaks for the methyl groups merged into a singlet at δ 0.78, and the two doublets for the methylene groups appeared as another singlet at δ 2.58. This is consistent with the MS-proposed open-ring structure of Degradant-2. The ring

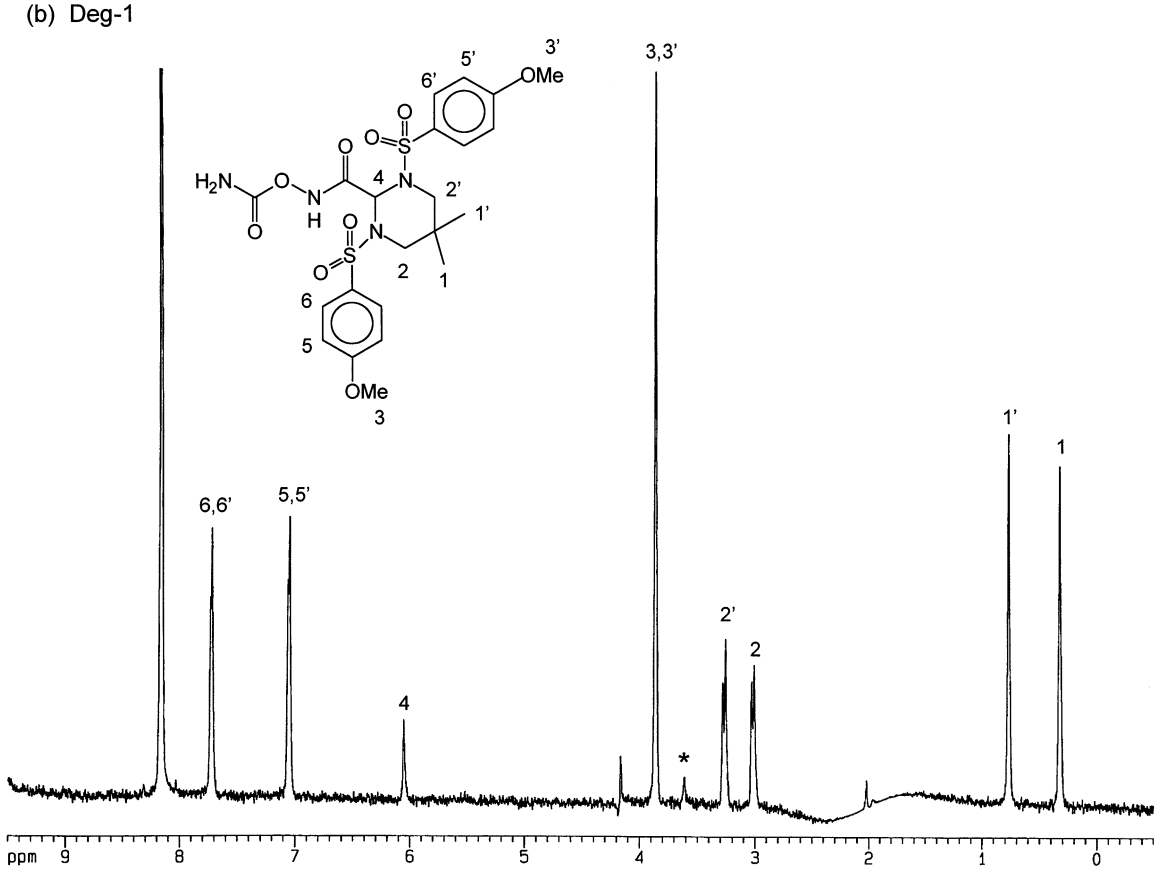

Fig. 4. (*Continued*)

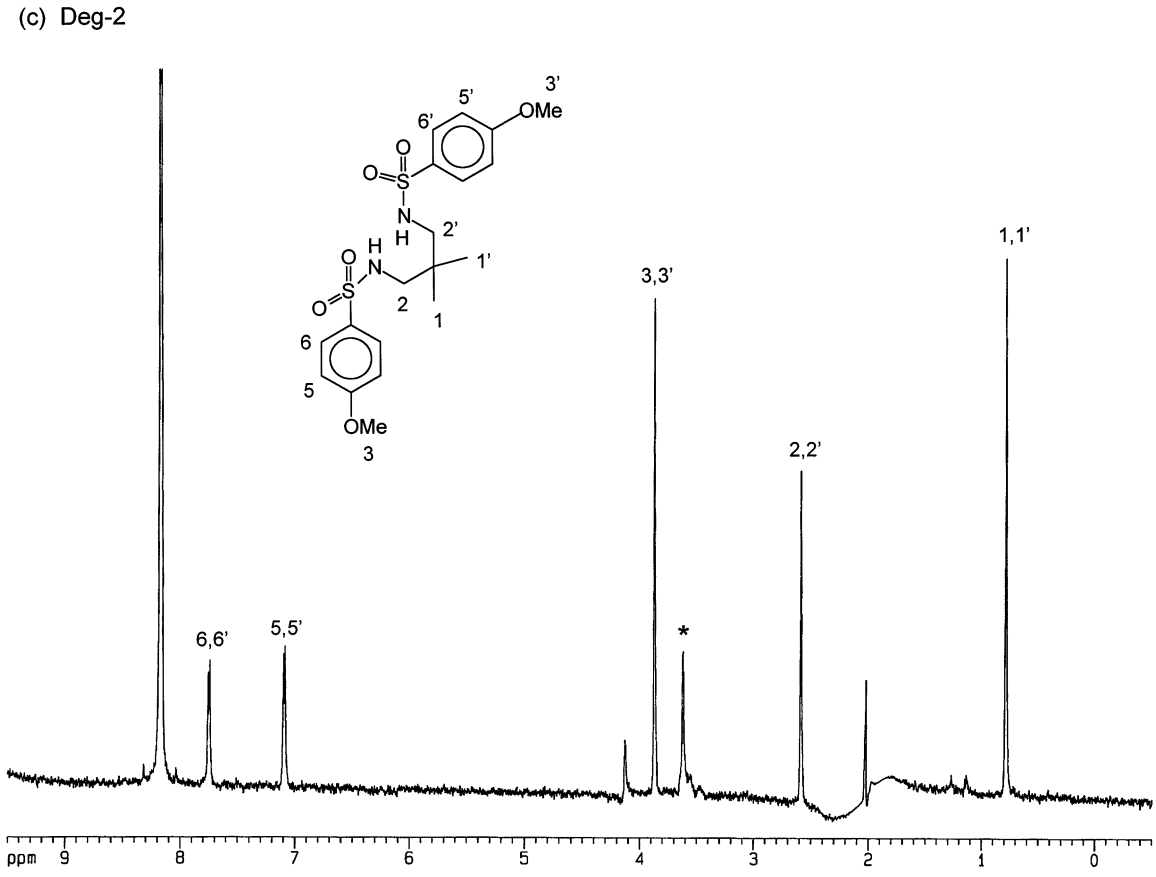

Fig. 4. (*Continued*)

opening occurred at position 4, leading to the loss of the methine proton. The conformational flexibility of the open-ring structure also made the methyl groups and the methylene groups equivalent leading to the respective singlets. The NMR spectrum of Degradant-2 matched that of a synthetic reference standard, confirming its structural assignment.

For Degradant-3, the NMR spectrum (Fig. 4d) verified the structure proposed by LC-MS as the carboxylic acid form of the parent compound. The -COOH group at position 4 caused a downfield shift to the methine proton which appeared at δ 6.30. Because of its closed-ring structure, the two methyl groups and the two methylene groups were non-equivalent (as in the parent compound), resulting in two sets of resonances.

The molecular weight and fragmentation pattern suggested an alcohol form for Degradant-4, as shown in Table 1. The NMR spectrum shown in Fig. 4e, however, was not consistent with an alcohol form. The resonance peak at δ 9.02 is characteristic of the aldehyde proton. Consistent with an open-ring structure, the methine proton resonance peak around 6 ppm was missing and the protons from two methyl groups gave only one resonance peak at δ 0.88. This structural information pointed to an open-ring aldehyde, instead of the alcohol tautomeric form. In the aldehyde form, the aromatic protons became doublets of doublets, indicating that the protons at positions 5 and 6, as well as positions 5′ and 6′, were no longer equivalent due to the addition of the aldehyde group at position 7. Because of the

loss of their symmetry, the two methylene groups showed two resonance peaks, while the methoxy groups appeared as doublets. The alcohol, as postulated by LC-MS, and the aldehyde, as postulated by LC-NMR, are tautomeric forms with the same molecular weight. It is most likely that the aldehyde, which is the stable form in solution, had undergone a gas phase conversion to the alcohol during ESI and subsequent collision with surrounding gas molecules in the LC-MS experiment.

Although LC-MS showed the same molecular weight and fragmentation patterns for Degradant-5 and Degradant-6, as illustrated in Fig. 3f and g and Table 1, their NMR spectra were different in several aspects, indicating that their structures were different. The molecular weight of Degradant-5 or Degradant-6 differed from that of Degradant-4 by 18, the molecular weight of H_2O. As shown in Table 1, three possible structures (A, B or C) were proposed to account for the loss of an H_2O molecule from Degradant-4. The NMR spectrum of Degradant-5 was not reconcilable with structure A which contained three methyl protons, six methylene protons and one methine proton, whereas the NMR spectrum of Degradant-5 (Fig. 4f) indicated six methyl protons, four methylene protons and no methine proton (around δ 6.0). Structure C was eliminated because of the absence of the aldehyde proton

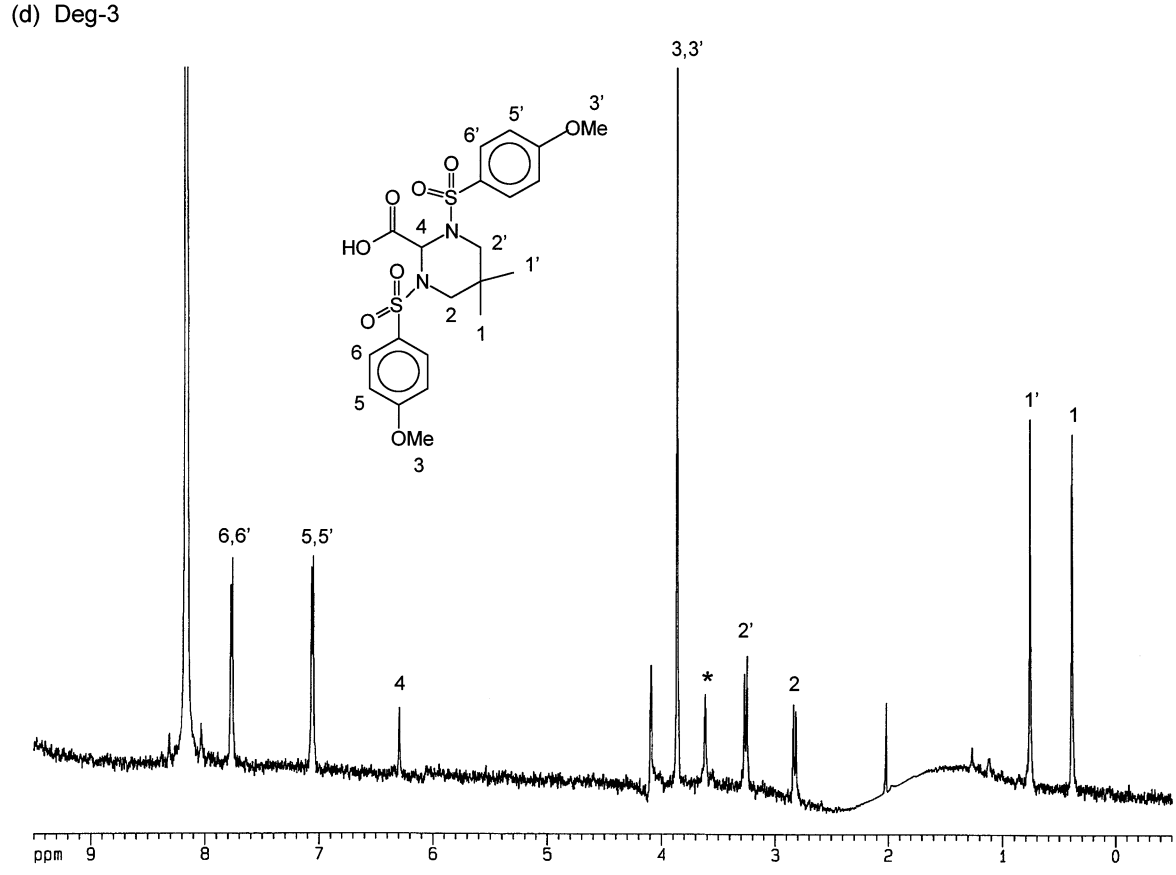

Fig. 4. (*Continued*)

(e) Deg-4

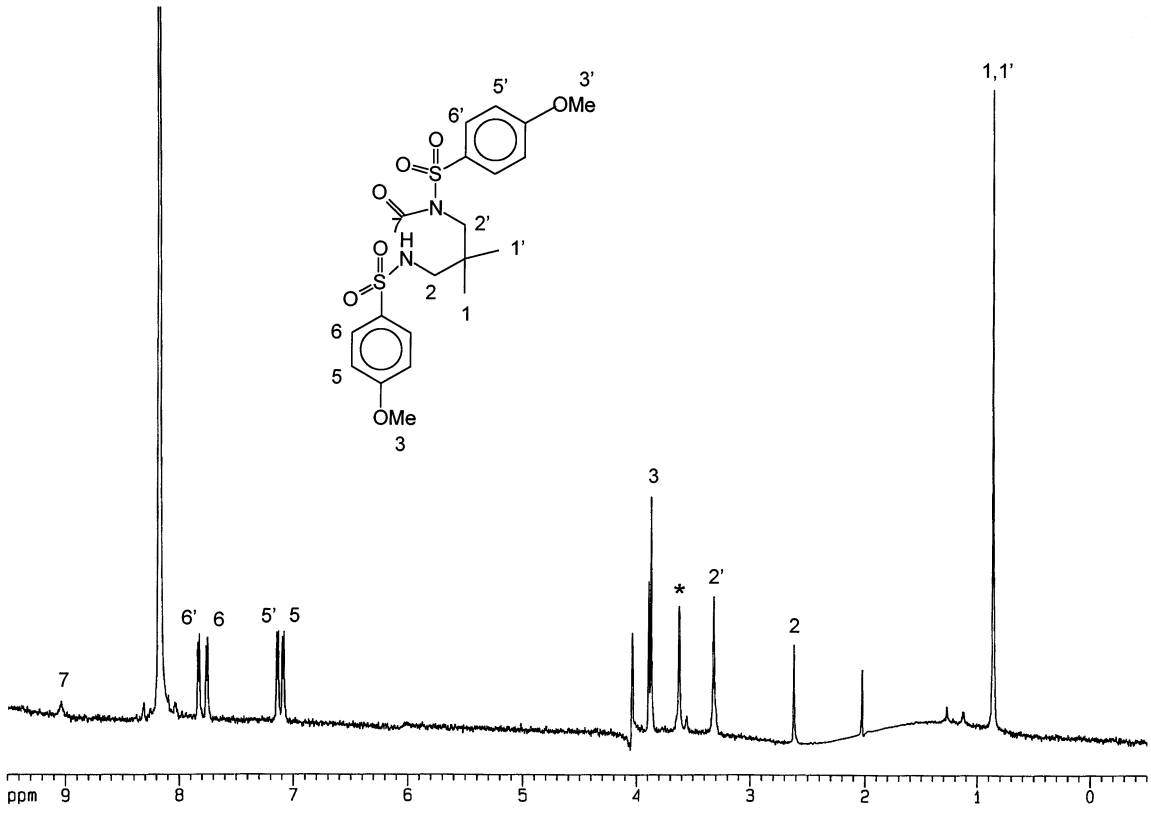

Fig. 4. (*Continued*)

signal at about δ 9.0. The NMR spectrum of Degradant-5 supported structure B. The conformationally equivalent methyl groups and the methylene groups in structure B appeared as a single resonance peak at δ 0.79 and δ 2.59, respectively, as shown in Fig. 4f. The absence of a methine proton signal at about δ 6.0 and an aldehyde proton signal at about δ 9.0 were consistent with the cyclic urea structure. The effect of an S=O group versus that of an O=S=O group on the chemical shifts of the surrounding protons may be minimal such that the overall symmetry of the aromatic and the methoxy groups are still maintained.

The NMR spectrum of Degradant-6 in Fig. 4g showed a resonance at δ 9.01, characteristic of an aldehyde proton. The presence of the aldehyde group made the two aromatic and the two methoxy groups asymmetric, giving rise to two sets of peaks, as observed for Degradant-4. The relative ratio of signal intensities for the methylene (δ 3.30) to the methyl (δ 0.87) protons was reduced, indicating the loss of one methylene proton. In addition, the lack of the resonance peak of a methine proton at about δ 6.0 and the appearance of the resonance peak of a conjugated imine proton at δ 9.40 supported the open-ring aldehyde structure C for Degradant-6, as shown in Fig. 4g and Table 1.

The structures of the parent compound and its six degradation products identified by LC-MS and LC-NMR are all listed in Table 1.

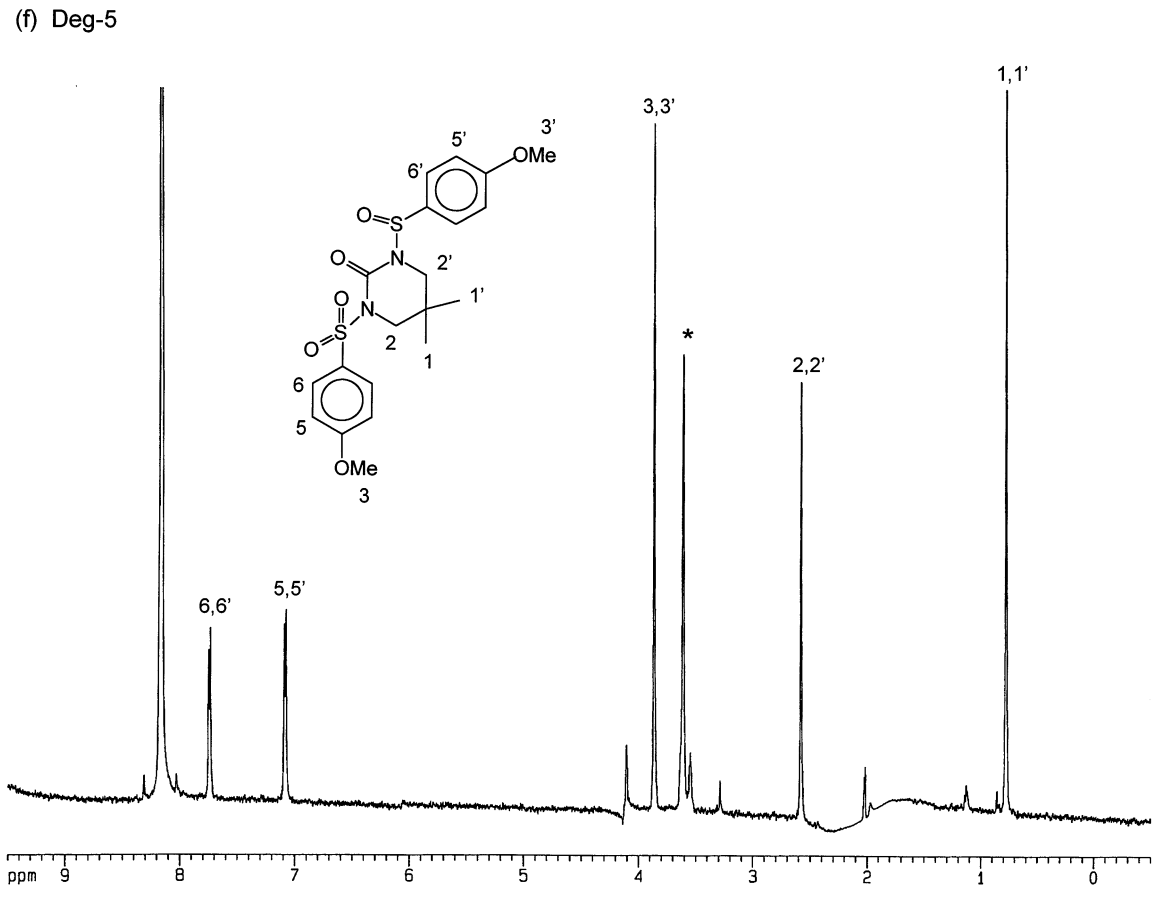

Fig. 4. (*Continued*)

4. Conclusions

LC-NMR and LC-MS have proven to be powerful complementary techniques, as utilized to characterize six degradation products of a protease inhibitor, PGE4410186, in a dosage formulation. The parent compound and its six major degradation products were identified based on the detailed structural information from LC-NMR and the molecular weight and fragmentation information from LC-MS. The combined use of LC-NMR and LC-MS provided more definitive structural assignments than either alone and, equally importantly, allowed a rapid characterization of this unknown mixture.

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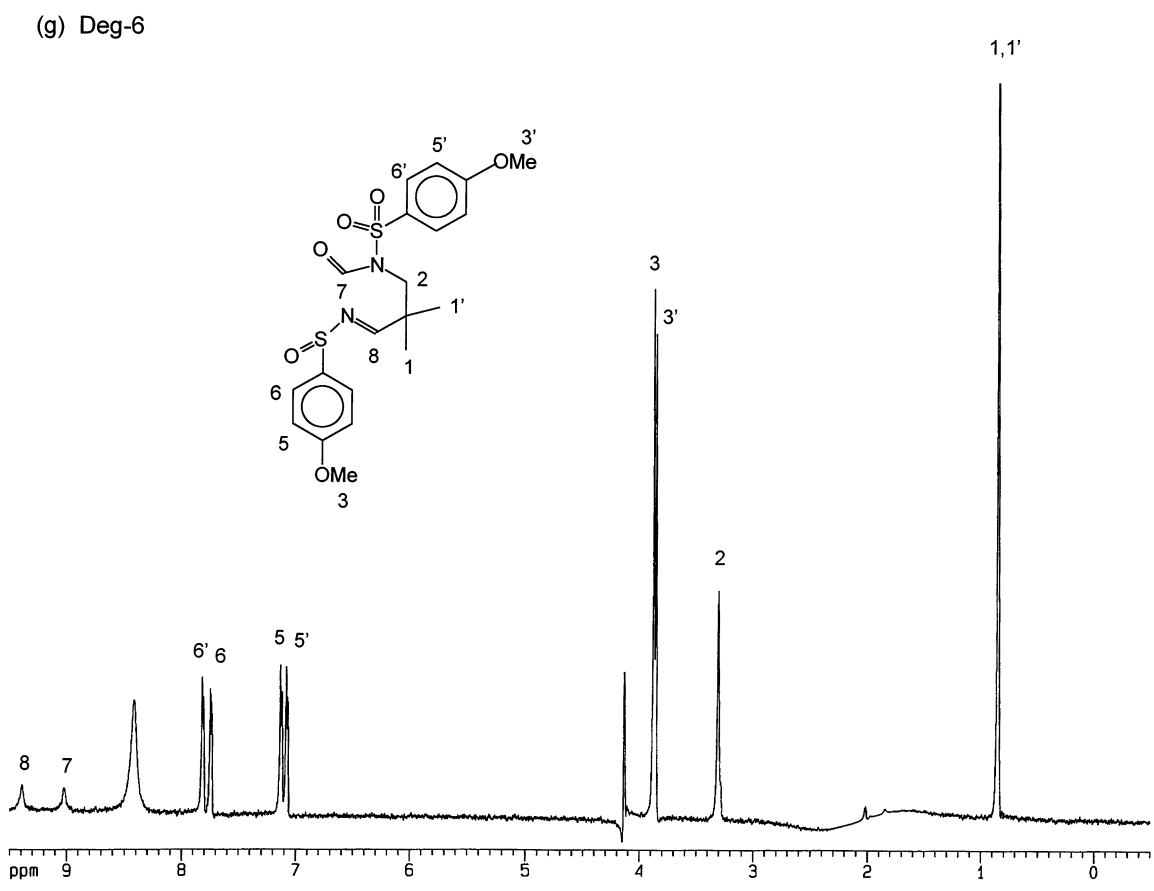

Fig. 4. (*Continued*)

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