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Application of liquid chromatography/mass spectrometry and nuclear magnetic resonance to the identification of degradates of a novel insulin sensitizer in aqueous solutions

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Abstract

Degradation of a novel insulin sensitizer in aqueous solutions was studied using high pressure liquid chromatography/mass spectrometry (LC/MS). The insulin sensitizer, containing a thiazolidine-2,4-dione (TZD), was a new class of antidiabetic agent for the treatment of type II diabetes. Chemical stability of the insulin sensitizer was evaluated by stressing its aqueous solutions at 40 °C for 24 h. Oxygen was removed from one of the solutions by bubbling pure nitrogen through to identify non-oxidative pathways. LC/MS analyses of the stressed solutions revealed that hydrolysis and oxidation are the primary degradation pathways for the studied compound. A α-thiol acetic acid, acyl amide, and two dimeric diastereomers were the main degradates of the insulin sensitizer. The α-thiol acetic acid served as an intermediate-like species, and oxidized to two dimeric degradates upon exposing to air. All of them were identified as ring-opening products of the TZD. The entities of the acyl amide and dimeric degradates were respectively verified by a synthetic standard or NMR following isolation of a diastereomeric degradate. Characterization using MS in both positive and negative ion scans were discussed for an isolated diastereomeric degradate. Mechanisms of fragmentation and formation for those degradates are presented based on the MS result.

Keywords: Insulin sensitizer; Thiazolidine-2,4-dione; Degradation; Dimerization

1. Introduction

The thiazolidinediones (TZDs) are an important chemical class in drug development for the treatment of diabetes [1–5]. These TZDs are new insulin sensitizers and exert antidiabetic effect via a mechanism involving activation of the peroxisome proliferator activated receptor (PPAR). They can reduce insulin resistance and improve the related syndrome called “Syndrome X”, the metabolic syndrome that includes dyslipidaemia, hypertension and impaired fibrinolysis [4,5]. These important discoveries have led to the extensive scientific exploration on TZD compounds as potential blockbuster drugs for type II diabetes as well as associated cardiovascular disease. Three TZD drugs so far reached to the market — pioglitazone, rosiglitazone and troglitazone, the third was later withdrawn from market because of its significant hepatotoxicity. These drugs were reported to effectively ameliorate glucose and lipid metabolic disturbances for type II diabetic patients. In their clinical studies, investigation of their metabolic mechanism were studied in several animal species and humans, wherein liquid chromatography/mass spectrometry (LC/MS) have been widely applied to the metabolite [6–9] and degradate identification [10]. These studies provided valuable information for understanding the metabolic pathway of the TZDs.

Recently, a novel insulin sensitizer, compound I (Fig. 1, MW 477), was reported by Merck & Co., Inc. [11,12]. This TZD compound acts as a PPAR gamma agonist to target type II diabetes. In this paper, the chemical stability of the insulin

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sensitizer was determined in situ to understand the degradation mechanism. The stability of compound I was evaluated in aqueous medium under thermo-stressed conditions. LC/MS and LC/MS/MS analyses of the stressed samples indicated that hydrolysis of the TZD ring followed by oxidation was the primary degradation pathway for the insulin sensitizer. This study of in situ degradation mechanism of TZDs will be valuable for the exploration of the chemistry for TZDs and their pharmaceutical development.

2. Experimental

2.1. Chemical and reagents

The studied insulin sensitizer (compound I) — 5-{3-[3-(4-phenoxy-2-propyl-phenoxy)-propoxy]-phenyl}-thiazolidine-2,4-dione (sodium salt, racemic), and the synthetic standard of degradate III (Fig. 1) were obtained from Merck Research Laboratories (Rahway, NJ, USA). HPLC-grade acetonitrile, methanol, and water were purchased from EM Science (Gibbstown, NJ, USA). Trifluoroacetic acid (>99%), formic acid (96%) were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA).

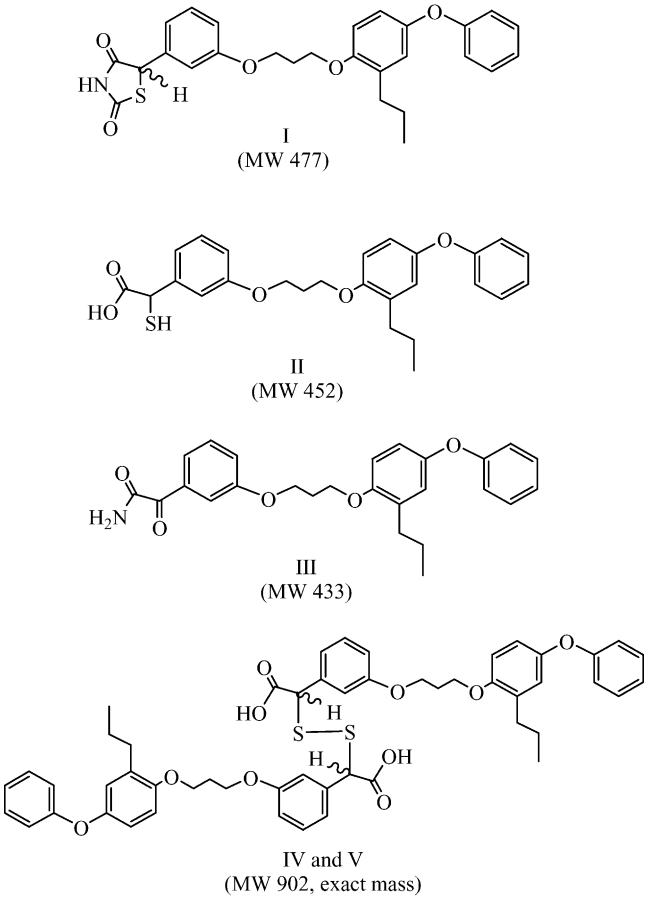


Fig. 1. Compound I and its major thermal degradates.

Deuterated acetonitrile-d_3 (99.96% D) and deuterium oxide (100% D) were purchased from Isotec Co. (Miamisburg, OH, USA). Ultra-pure nitrogen gas was supplied in-line in-house at Merck & Co., Inc.

2.2. Sample preparation

A stock solution of the studied insulin sensitizer at 1.0 mg/mL was prepared by dissolving the solid bulk drug in methanol. The stock solution was further diluted to 4 ng/μL with 50:50 acetonitrile–aqueous 0.1% formic acid for infusion experiments to optimize the analytical conditions of mass spectrometers.

Two stability-test samples of the studied insulin sensitizer were generated by dissolving the solid drug in a small amount of acetonitrile followed by diluting to 2.0 mg/mL with water. Oxygen was removed from one of the resulting solutions by bubbling pure nitrogen through to become a degassed solution. These two aqueous samples were stressed at 40 °C for 24 h to evaluate the solution stability of the insulin sensitizer. The stressed solutions were mixed with an equal volume of acetonitrile to a concentration of 1.0 mg/mL for LC/MS and LC/UV analyses.

2.3. Liquid chromatography for degradate profiling and isolation

The HPLC system consisted of two LC-10AD pumps, a system SCL-10A controller (Shimadzu Scientific Instruments, Columbia, MD, USA), an on-line degassing system (Alltech Associates, Inc., Deerfield, IL, USA), a HP 1100 photo diode array UV detector (Agilent Technologies, Inc., Wilmington, DE, USA), and a PE 200 series autosampler (Perkin-Elmer Instruments, Norwalk, CT, USA). A linear gradient program using aqueous 0.01% trifluoroacetic acid (TFA) solution (A) and 0.01% TFA in acetonitrile (B) (t = 0–1 min, 48% B; t = 10 min, 60% B; t = 20 min, 65% B; t = 33 min, 78% B; t = 55 min, 85% B; t = 56 min, 48% B) was employed on a Zorbax Rx C8 column (4.6 mm × 250 mm, 5 μm) (SUPELCO, Bellefonte, PA, USA). The LC flow rate was 1.0 mL/min on the column, splitting at 15:85 ratio for LC/MS and LC/UV analyses. The LC column was maintained at ambient temperature to avoid any on-line degradation. The diode-array detector was monitored at both 210 and 280 nm. Test samples were kept at 15 °C during the entire analysis in the autosampler. A mixture of 50:50 acetonitrile–water was used as the needle washing solvent for autosampler.

Isolation of one dimeric degradate (RT = 41.7 min, Fig. 3; RT stands for retention time) was performed on the same HPLC system with a semi-preparative HPLC column — Zorbax Rx C8 column (9.4 mm × 250 mm, 5 μm) (Agilent Technologies, Inc., Wilmington, DE, USA) at ambient temperature. A linear gradient program of (t = 0–1 min, 78% B; t = 20–25 min, 85% B; t = 26 min, 78% B.) was used to perform the degradate isolation with the same mobile

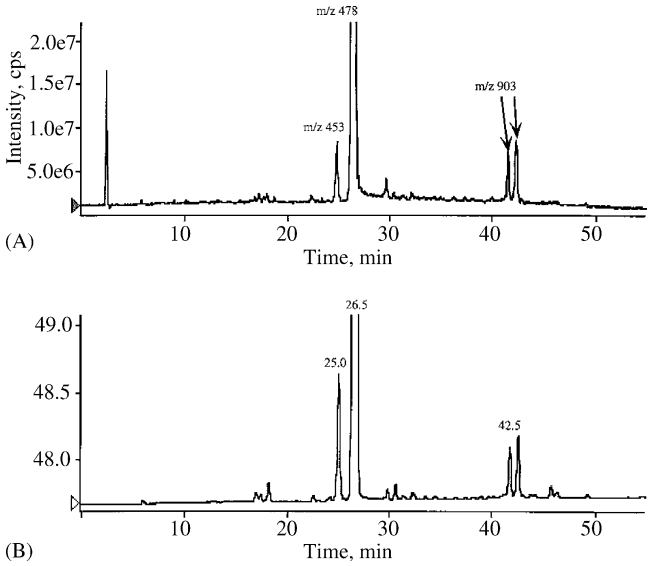


Fig. 2. LC/MS (A) and LC/UV (B: peaks labeled with RT time; λ = 280 nm) chromatograms of stressed degassed solution of compound I (40 °C/24 h).

phase solvents. The LC flow rate was 2.0 mL/min on the column. The dimeric degradate was collected in a 100 mL round flask chilled with an ice-water mixture during isolation process and immediately stored at −70 °C afterward. The collected dimeric degradate solution was dried on a Rotavapor (BÜCHI, Switzerland) at 40 °C. The purity of the isolated degradate was checked under the same LC/MS and LC/UV conditions as those for degradate identification (see the first paragraph of Sections 2.2 and 2.3) before NMR analysis.

2.4. Mass spectrometry

Applied Biosystems API 150 EX single quadrupole and API 300 triple quadrupole mass spectrometers (Foster City, CA, USA) coupled with a TurboIonSpray™ (TIS) ionization source were used for sample analysis. Both mass spectrometers were interfaced with the HPLC system at the TIS source with a ratio of 15/85 for MS and UV detectors. The ionization source was heated at 450 °C. The mass spectrometers were operated in the positive ion mode and mass spectrometer parameters were optimized using a diluted solution of the studied insulin sensitizer. High purity nitrogen was used as both heating gas in the source region and collision gas with a collision gas thickness (CGT) of 2.49×10^{15} molecule/cm^2. Hydrocarbon-free air generated by a Zero Air Generator (Whatman, Inc., Haverhill, MA, USA) was used as the nebulizer gas. A Harvard Model-22 syringe pump (Harvard Apparatus, South Natick, MA, USA) was used to directly introduce sample solutions into the mass spectrometer at 100 μL/min for instrumental optimization as well as characterization of the isolated dimeric degradate. A polypropylene glycol (PPG) calibration solution provided by Applied Biosystems was used for the mass axis calibration of the mass spectrometer. Applied Biosystems MacQuan/MultiView version 1.3 and Analyst™ software version 1.1 were used for the data acquisition and data processing.

Characterization of the isolated dimeric degradate of the insulin sensitizer was conducted under the established MS conditions in both positive and negative ion modes. Product ion scan as well as quasi-MS/MS/MS experiments were applied to the acquisition of fragment information for the isolated degradate.

2.5. NMR instrumentation

The isolated dimeric degradation product of the novel insulin sensitizer and the parent compound I were dissolved in 75/25 deuterated acetonitrile:deuterium oxide. All spectra were acquired on a Varian Unity Inova 600 MHz spectrometer (Varian, Palo Alto, CA, USA) equipped with a 5 mm ^{1}H{^{13}C/^{15}N} PFG triple resonance probe. ^{1}H-1D, 2D-DQF-COSY, and ^{1}H–^{13}C correlation spectra of HSQC and HMBC were obtained at 25 °C to characterize the structure of the isolated degradation product.

3. Results and discussion

Identification of degradates for compound I was conducted using LC/MS and LC/MS/MS with simultaneously detection by an UV detector. Figs. 2 and 3 show the degradate profiles of compound I (RT = 26.5 min) in degassed and undegassed solutions, respectively. LC/MS chromatograms are on the top panel and LC/UV chromatograms are on the bottom panel in both figures. In the degassed solution, a profile including a major degradate with molecular weight (MW) of 452 Da (RT = 25.0 min) and two major degradates (RT = 41.7 min,

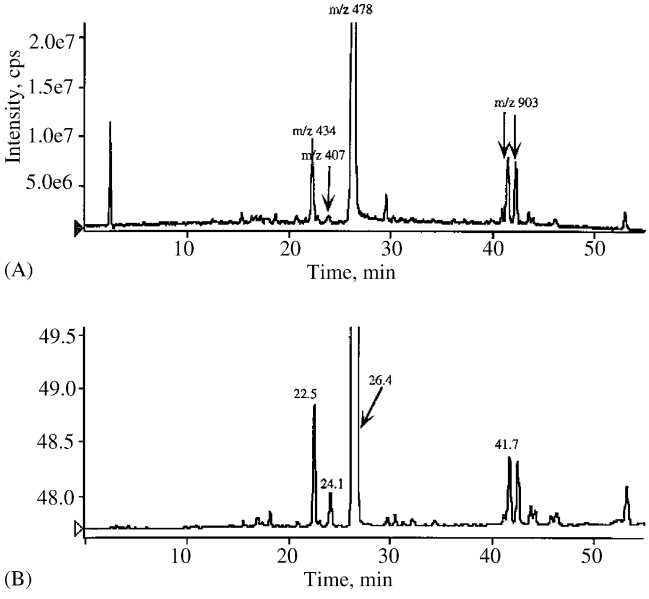


Fig. 3. LC/MS (A) and LC/UV (B: peaks labeled with RT time; λ = 280 nm) chromatograms of stressed undegassed solution of compound I (40 °C/24 h).

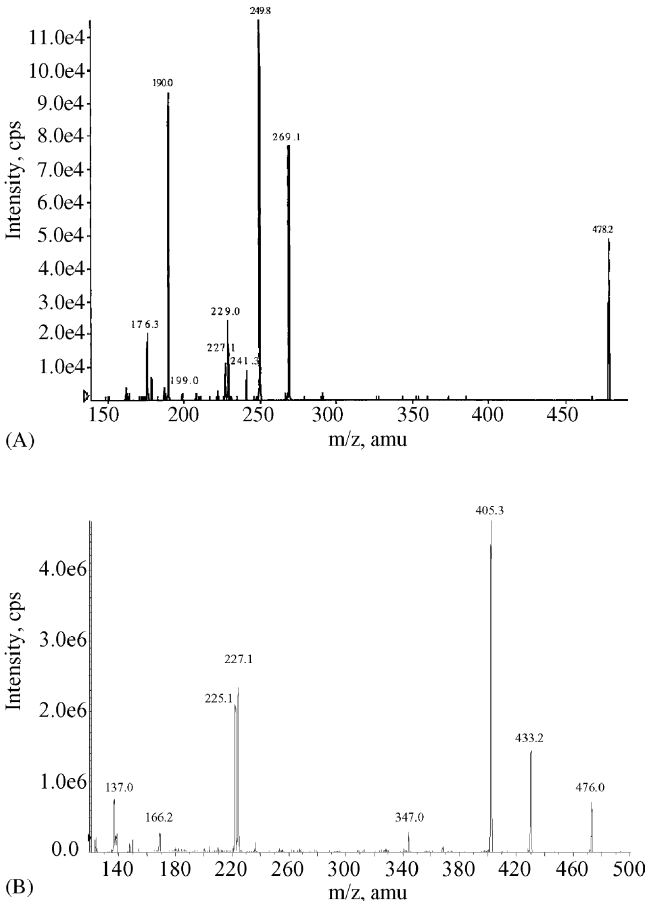


Fig. 4. (A) Product ion scan spectrum of protonated molecule at *m/z* 478 of compound I (collision energy (CE): 16 eV) (top panel). (B) Product ion scan spectrum of deprotonated molecule at *m/z* 476 of compound I (CE: 38 eV) (bottom panel).

42.5 min) with the same MW of 902 Da (exact mass − same in the rest of text) were obtained. While in the undegassed solution, the same two major degradates (MW 902 Da) were detected along with a major degradate with MW of 433 Da (RT = 22.5 min). It was interesting to point out that, upon exposing the degassed solution to the air for 4 h, the degradate (452 Da) became undetectable. As a result, the level of the two late-eluted degradates (MW 902 Da) were increased. This experimental result suggested that the degradate (MW 452 Da) was unstable and could be the precursor for the two late-eluted species (MW 902 Da). Since all of these species were degradation products of compound I, in order to identify these degradates by LC/MS, it is important to firstly understand the gas-phase chemistry of their parent molecule.

3.1. Fragmentation mechanism of compound I

A study on the fragmentation of compound I was performed by MS/MS scan of the protonated molecule at *m/z* 478. A product ion scan spectrum of compound I is shown in Fig. 4A, in which ions at *m/z* 269, 250, 229, 227, 190, and 176 were observed. A mechanism for the formation of these ions is illustrated in Scheme 1. It was indicated that fragmentation could easily occur at the central ether linkages of compound I, yielding intense ions at *m/z* 269, 250, 229 and 190. The cleavage of the bond of 4-phenoxy-2-propyl-phenoxyl could form a counter-ion intermediate as shown in Scheme 1, leading to the formation of the ions at *m/z* 250 and *m/z* 229. The ion at *m/z* 250 was further fragmented to an ion at *m/z* 190 by a neutral loss of S=C=O from the TZD ring. The ion at *m/z* 269 was generated by the loss of 3-TZD-phenoxyl group. The ion at *m/z* 176 is likely formed by the loss of a phenolate radical from the ion at *m/z* 269, leading to a radical cation. A quasi-MS/MS/MS experiment of the ion at *m/z* 269 generated a fragment at *m/z* 241 with a loss of an ethylene, a phenoxyl quinone oxinium ion at *m/z* 227, 199 [13], and a minor ion at *m/z* 176.

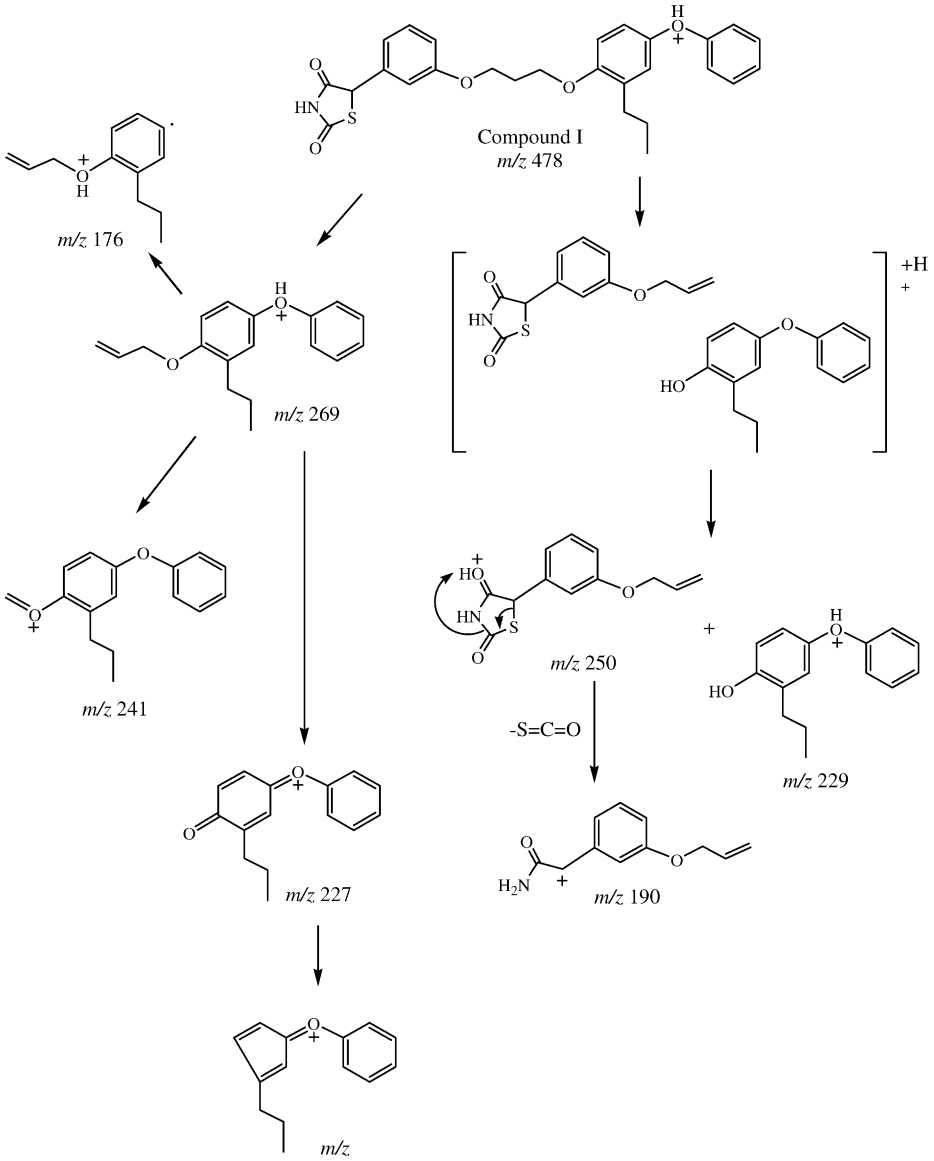
MS/MS scan of the deprotonated molecule at *m/z* 476 of compound I generated major fragments at *m/z* 433, 405, 227, 225, and 137 (Fig. 4B). Similarly as the fragmentation of the protonated molecule, sequential neutral losses of HN=C=O and CO molecules led to the formation of the fragments at *m/z* 433 and 405 (Scheme 2). The latter ion yielded fragments at *m/z* 227 and 137 corresponding to phenoxyl-phenolate and 3-thiolketene-phenolate anions.

The understanding of the fragmentation mechanism of compound I was a crucial step to localize the degradation sites and laid the foundation for the structural elucidation of the degradation products.

3.2. Degradate identification for compound I by LC/MS

3.2.1. Degassed solution of compound I

The product ion scan of the protonated molecule at *m/z* 453 (RT 25.0 min in LC/UV chromatogram) produced ions at *m/z* 407, 373, 279, 269, 267, 227, 199, 191, 176 and 147 (Fig. 5). By comparing with the fragmentation of the parent molecule, the observation of fragments at *m/z* 269, 227 and 199 suggested that (4-phenoxy-2-propyl-phenoxy)propoxyl moiety was intact and degradation occurred at the phenyl-TZD part. Since oxygen was removed from this test solution, hydrolysis was most likely the primary degradation pathway for compound I rather than oxidation. Based on understanding of the chemistry for compound I, a structure with a thiol-acetic acid moiety is assigned for the degradate with MW of 452 Da (Scheme 3). The structure was well-fitted in a fragmentation mechanism based upon acquired MS data. The presence of the thiol-acetic acid was especially evidenced by the observation of ions at *m/z* 407, 373, and 191. As illustrated in Scheme 3, a neutral loss of 46 Da (HCOOH) from the protonated molecule at *m/z* 453 resulted in the formation of the ion at *m/z* 407. This ion fragmented to the ion at *m/z* 373 as a consequence of another neutral loss of 34 Da — a H_2S molecule. The presence of the carboxylic acid was further supported by the ion at *m/z* 191, one dalton greater than the ion at *m/z* 190 of compound I, differentiating by –COOH from $–CONH_2$. Although this degradate was not isolated and characterized by NMR, the proposed structure was strongly supported by the MS result.



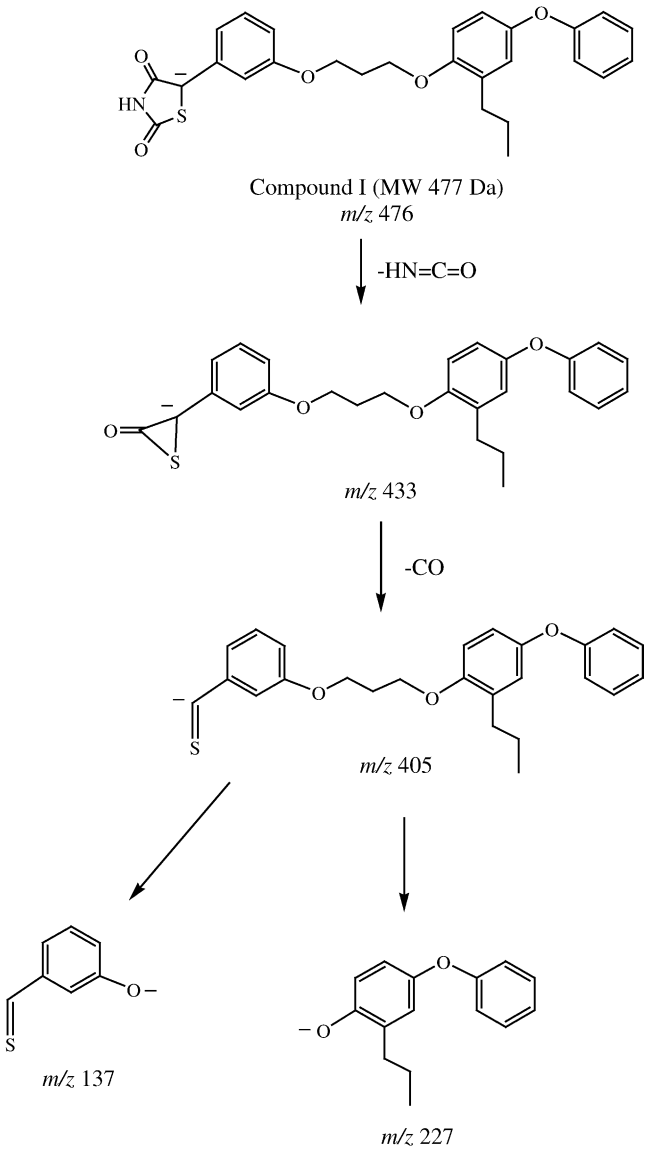
Scheme 1. Proposed fragmentation mechanism for compound I in the positive ion scan mode.

It should be pointed out that disulfide formation could be anticipated for the thiol group upon oxidation, resulting the generation of secondary degradates. Actually, this phenomena indeed happened to the degradate (MW 452 Da) and will be addressed later along with discussion.

Two major degradates with the same MW of 902 Da were detected at RT of 41.7 min and 42.5 min (LC/UV chromatogram). The observation of the same product ion profile from in-source CID — ions at *m/z* 925 (Na^+ adduct), 903, 885, 857, 839, 811, and 629 (Fig. 6) suggested that they were isomers and easy to fragment. Their MW (902 Da) were greater than its parent molecule by a 425 Da, suggesting possible degradate–degradate or degradate–compound I adducts. The molecular weights — 902 Da were just twofold of the MW of [452-H] Da implied possible dimerization products of the degradate (MW 452 Da). In fact, the changes of their relative concentrations in the degassed solution after exposure to air, as discussed earlier in Section 3, had provided the experimental support for the statement. Based on the above information and understanding of the chemistry of thiols [14,15], diastereomeric structures with a disulfide linkage were proposed for these two degradates (MW 902 Da, IV and V in Fig. 1). A further structural investigation for these two diastereomeric degradates will be discussed in Section 3.3.

3.2.2. Undegassed solution of compound I

An ion profile from MS/MS scan of the protonated degradate (MW 433) molecule included ions at *m/z* 406, 389, 371, 269, 267, 241, 227, 199, 176, 161, and 135 (Fig. 7).



Scheme 2. Proposed fragmentation mechanism for compound I in the negative ion scan mode.

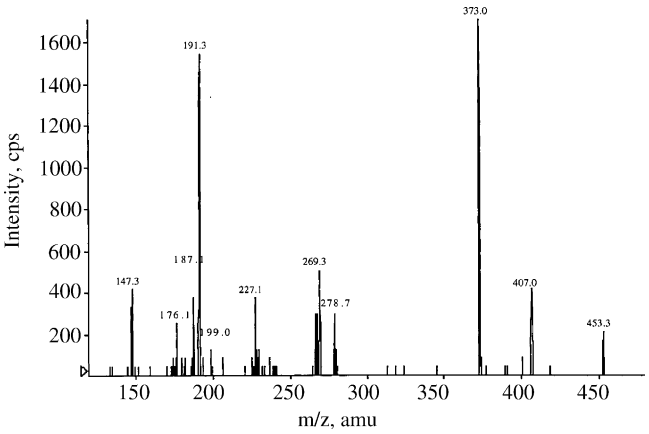


Fig. 5. MS/MS spectrum of the degradate with MW of 452 Da (CE: 18 eV).

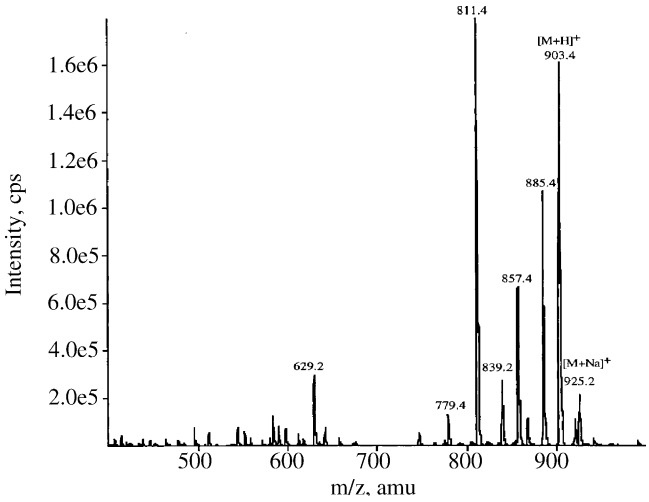


Fig. 6. A representative MS spectrum of the protonated molecules at *m/z* 903 for the two diastereomeric degradates (MW 903 Da) (decluster potential: 10 eV).

Similarly as discussed previously for the degradate (MW 452 Da), the formation of ions at *m/z* 269, 227, 199, and 176 suggested no structural change of compound I occurred at 4-phenoxy-2-propyl-phenoxyl moiety rather than the TZD ring. The degradate was believed to be generated via opening of the TZD ring followed by oxidation. A structure with an acyl amide moiety (III in Fig. 1) was assigned for this degradate (MW 433 Da). The proposed structure was verified with a synthetic standard by both RT and MS spectra.

Similar to the degassed solution of compound I, two late-eluted major degradates with MW of 902 Da were detected. The identical ion profiles as reported previously along with the same RT clearly indicated that they were the same diastereomeric species from the degassed solution.

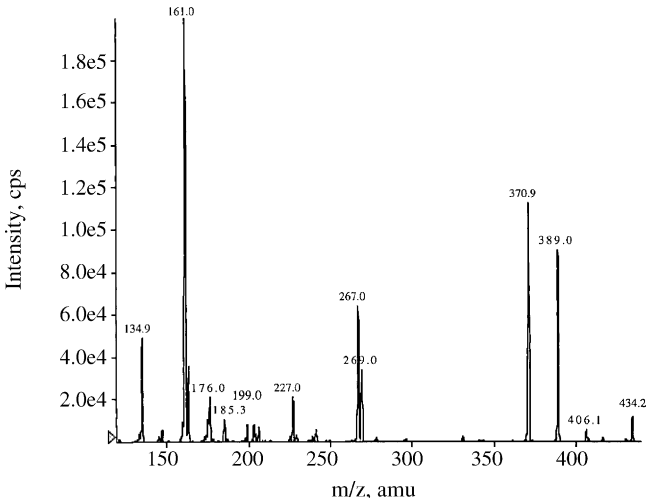
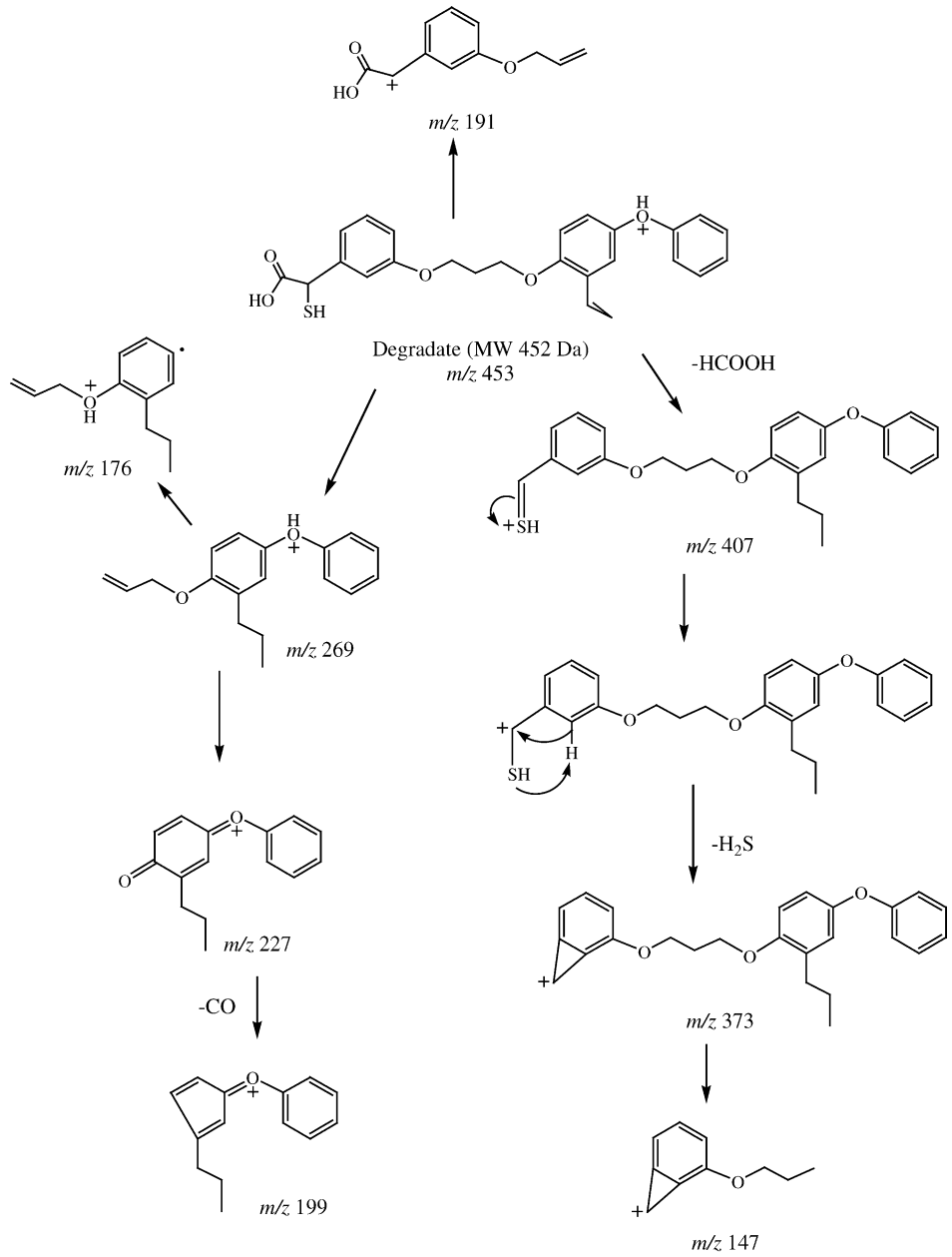


Fig. 7. MS/MS spectrum of the degradate with MW of 433 Da (CE: 30 eV).

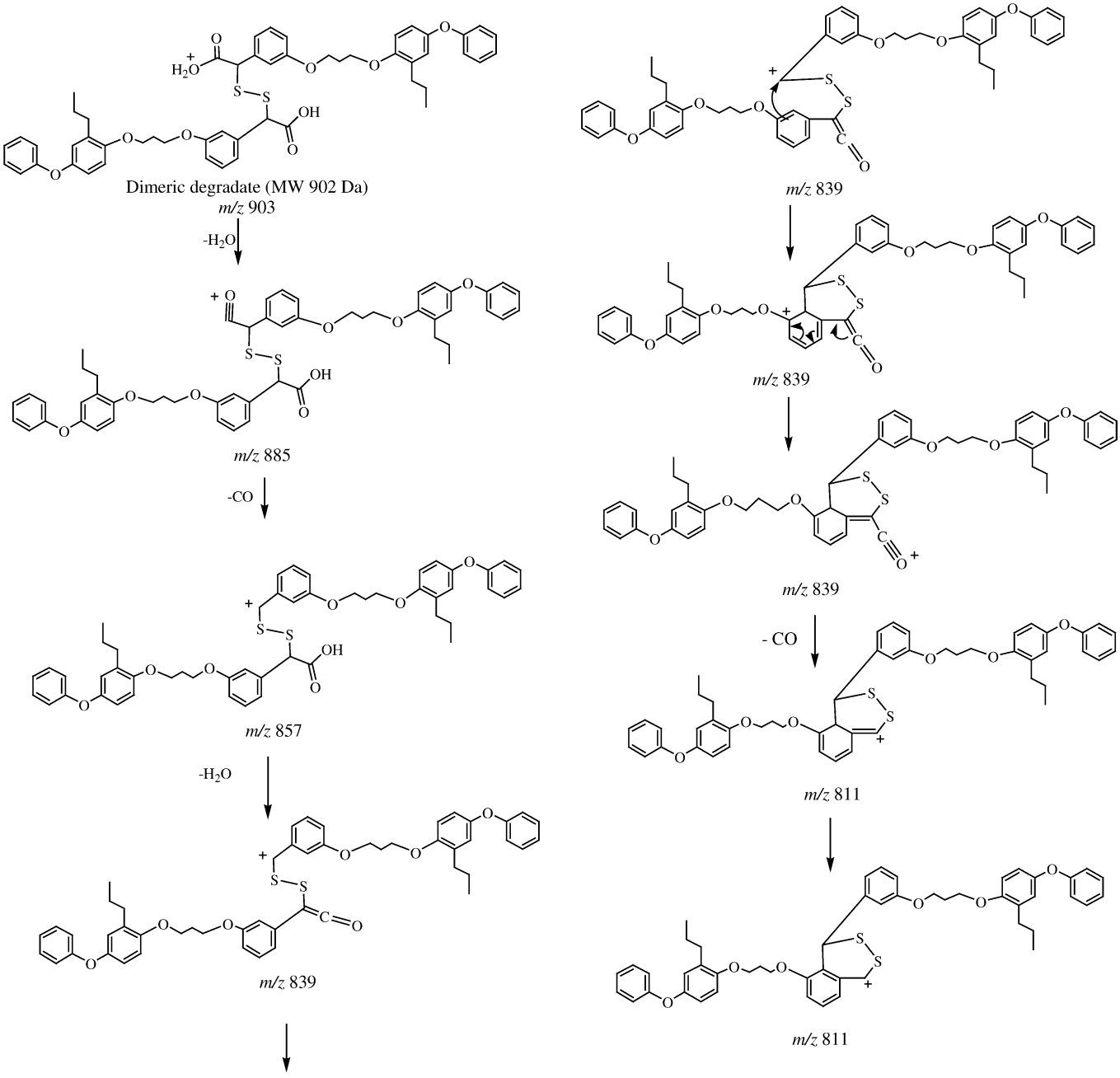


Scheme 3. Proposed fragmentation mechanism for the degradate with MW of 452 Da in the positive ion scan mode.

3.3. Mass spectrometric study of the diastereomeric degradates

To further confirm the structures of the two dimeric degradates, one of the diastereomers was isolated by semi-preparative HPLC and characterized by MS in both positive and negative ion modes. The isolated degradate was re-injected into the LC/MS system under the same instrumental conditions. An identical MS spectrum (Fig. 6) and retention time verified that no chemical change occurred to the isolated degradate during the isolation process. By infusing solutions of the isolated degradate into MS, the product scan results of the protonated (*m*/*z* 903) and deprotonated (*m*/*z* 901) molecules were acquired (data not shown; major fragments of the ion at *m*/*z* 903 were the same as shown in Fig. 6). A fragmentation mechanism of the ion at *m*/*z* 903 is proposed in Scheme 4. Through successive two neutral losses of H_2O and CO, the protonated molecule at *m*/*z* 903 yielded ions at *m*/*z* 885 and 857, the latter underwent another neutral loss of H_2O to generate an ion at *m*/*z* 839. Following a cyclization involving the adjacent phenyl ring [16], the ion at *m*/*z* 839 was rearranged to an acylium ion intermediate and further fragmented to the base ion at *m*/*z* 811 after a loss of CO molecule.

MS/MS scan of the deprotonated molecule at *m*/*z* 901 yielded two predominant fragments at *m*/*z* 451 and 407. The



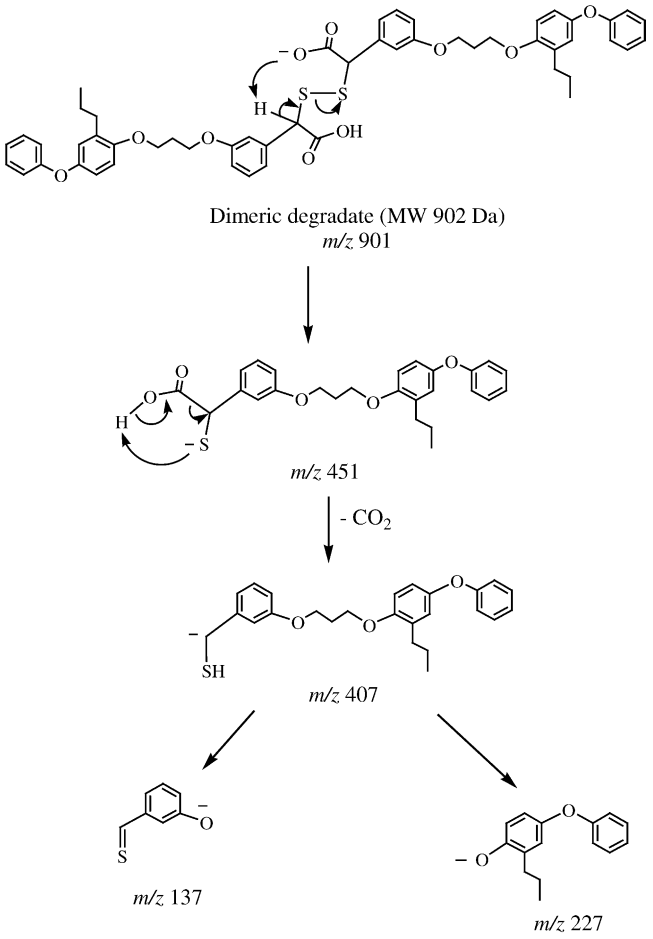
Scheme 4. Fragmentation mechanism of the dimeric degradate (902 Da) in the positive ion scan mode.

former ion was formed, as shown in Scheme 5, by cleaving at the disulfide bond of the deprotonated molecule. Followed by a neutral loss of a CO_2, the ion at *m*/*z* 451 further fragmented to the latter ion at *m*/*z* 407. Quasi-MS/MS/MS scan of the ion at *m*/*z* 407 produced fragments of 4-phenoxylphenolate anion at *m*/*z* 227 and *p*-thioketone phenolate anion at *m*/*z* 137, both ions were also the major fragments of the deprotonated molecule at *m*/*z* 476 of compound I (Scheme 3).

In brief, the characterization by MS provides strong evidence that the two diastereomeric degradates had a disulfide moiety while retaining the same non-TZD structural feature as their parent drug molecule. Dimerization following the TZD ring-opening of compound I was believed their formation pathway.

3.4. Characterization of the isolated dimeric degradate using NMR

The dimeric degradate (MW 902 Da; RT = 41.7 min), isolated from the stressed aqueous solution of compound I using preparative HPLC, was further studied by ^{1}H and ^{13}C NMR analyses. The NMR spectra of compound I were also acquired as reference. For the dimeric degradate, the peak integrals from 1D proton spectrum (Fig. 8A)



Scheme 5. Fragmentation mechanism of the dimeric degradate (902 Da) in the negative ion scan mode.

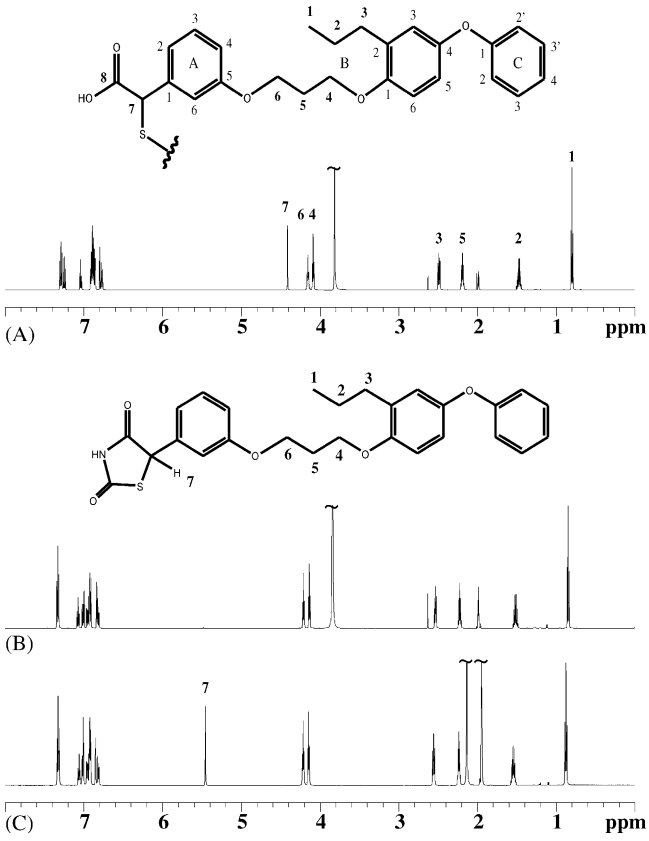
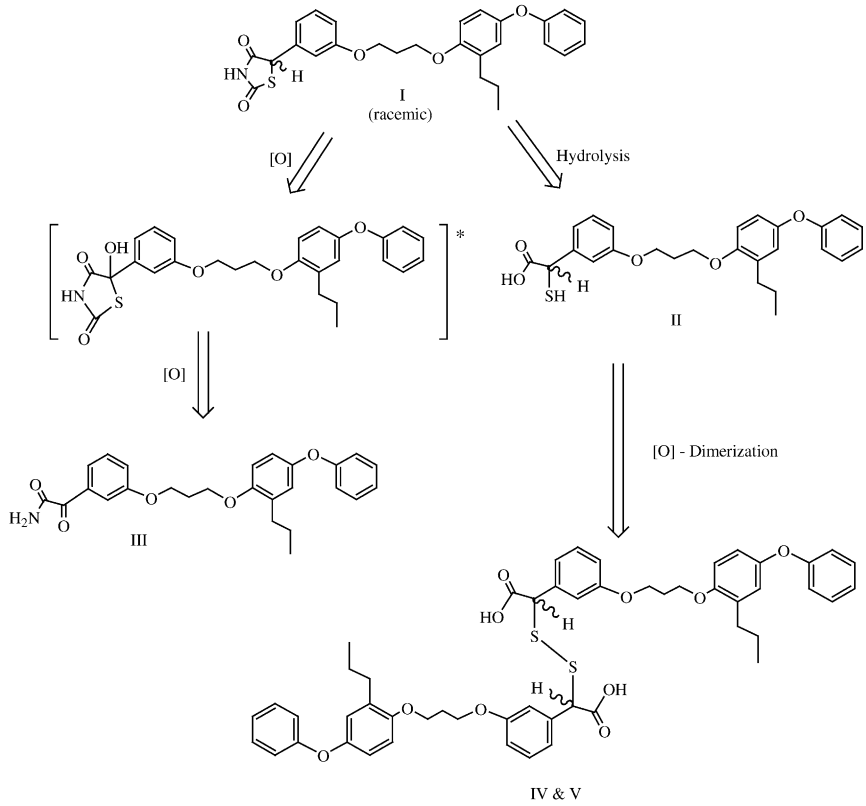


Fig. 8. 1D proton NMR spectra of (A) the isolated dimeric degradate (MW 903 Da, RT = 41.8 min) in 75% CD_3CN and 25% D_2O, (B) compound I in 75% CD_3CN and 25% D_2O, and (C) compound I in CD_3CN.

indicated that the ratio of $CH:CH_2:CH_3$:aromatic protons is 1:10:3:12, the same ratio as that of compound I by comparison. The COSY spectrum established the fragments of ($CH_3CH_2CH_2$), ($CH_2CH_2CH_2$), and the connections of aromatic protons in different rings. The one-bond C–H correlations were obtained from the HSQC data. The HMBC spectrum provided two-bond H–C–C and three-bond H–C–C–C correlations to put together the structural information obtained from the COSY and HSQC spectra. Key correlations in the HMBC experiment were two bond H–C–C correlation from H7 to C8 and aromatic CA1, and three bond H–C–C–C correlation from H7 to aromatic CA2 and CA6. Results from these data are summarized in Table 1 for the assignment of proton and carbon chemical shifts of the isolated dimeric degradate. NMR analysis together with the MS result indicated the dimer formed via a disulfide linkage for the two diastereomeric degradates (MW 902 Da). It should be noted that H7 in the parent compound has a different chemical environment. When compound I was dissolved in 75% CD_3CN and 25% D_2O, no appreciable H7 peak was observed in the 1D spectrum (Fig. 8B). This is because H7 underwent the keto-enol tautomerism, and in the enol form, the hydroxyl proton exchanged with deuterium of D_2O. However, when compound I was dissolved in CD_3CN, the H7 peak appeared at 5.456 ppm (Fig. 8C). This tautomerism phenomenon did not occur at the H7 proton of the dimeric degradate.

3.5. Proposed degradation mechanism of compound I and formation of the dimeric degradates

MS and NMR results clearly pointed out that degradation of compound I occurred at the TZD ring in aqueous medium. It is rationalized in Scheme 6 that, in the absence of oxidants such as oxygen in the degassed solution, hydrolysis was the primary degradation process for compound I. As a result, scission of the TZD ring led to the formation of the degradate (MW 452 Da) [17]. This degradate acted as an intermediate-like species and dimerized to the diastereomeric degradates (MW 902 Da). This proposed mechanism was supported by the undetected degradate (MW 452 Da) in the stressed degassed solution after exposing to air for 4 h. It should be noted that the complete removal of oxygen in the aqueous solution would be very difficult. The residual oxygen could result in the formation to the dimeric degradates (MW 902 Da) since thiol can be easily oxidized to a disulfide [14,18].



Scheme 6. Proposed degradation mechanism for compound I in aqueous medium. **Note*: This intermediate species was not identified.

Table 1
Proton and carbon chemical shift for the isolated dimeric degradate with MW of 902 Da

Assignment		Carbon, δ_C (ppm)	Proton, δ_H (ppm)
1	CH_3	15.89	0.799 (t, J = 7.4 Hz)
2	CH_2	25.42	1.473 (m)
3	CH_2	34.49	2.490 (t, J = 7.5 Hz)
4	CH_2	67.61	4.087 (t, J = 6.1 Hz)
5	CH_2	31.60	2.190 (m)
6	CH_2	67.37	4.154 (m)
7	CH	60.73	4.414 (s)
8	C	174.15	
A1	C	139.72	
A2	CH	123.49	6.910 (obscured)
A3	CH	132.69	7.249 (t, J = 7.8 Hz)
A4	CH	117.24	6.899 (obscured)
A5	C	161.67	
A6	CH	117.83	6.855 (s)
B1	C	155.79	
B2	C	135.62	
B3	CH	123.99	6.799 (s)
B4	C	152.40	
B5	CH	120.25	6.771 (d, J = 8.5 Hz)
B6	CH	115.44	6.874 (obscured)
C1	C	161.10	
C2	CH, H′	120.06	6.885 (obscured)
C3	CH, H′	132.45	7.292 (m)
C4	CH	125.25	7.042 (m)

The structure with hydrocarbon assignment was shown in Fig. 8. δ_C referenced to DSS-d_6 at 0.00 ppm; δ_H referenced to DSS-D_6 at 0.00 pp.

Because compound I was started as a racemic mixture, two enantiomeric degradation products, *R*/*S* 2-thio-2-{3-[3-(4-phenoxy-2-propylphenoxy)propoxy]phenyl}acetic acid, were expected to be generated via the TZD ring-opening process, assuming the same reactivity of the stereoisomers. As a result, theoretically, three dimeric degradates should be observed (*RR*, *SS*, and *RS* — identical to *SR* because of a center of symmetry). However as shown in Figs. 2 and 3, only two peaks were detected. This experimental result could be due to possible coelution (one peak for *RR*/*SS*; another for *RS*) on a non-chiral HPLC column employed in this study, which was also evidenced by a single HPLC peak of the studied racemic insulin sensitizer. Nevertheless, the observation of the degradate (MW 452) and identification of the dimeric degradates by MS and NMR provided strong supports for the degradation mechanism of the TZD ring-opening followed by dimerization.

In the presence of oxidants such as oxygen in the undegassed solution, hydrolysis was competed by an oxidation process, resulting in the formation of degradation products from both pathways. The oxidation could initiate at the benzyl position of the TZD ring and generated the corresponding acyl amide (MW 433 Da). This degradate was also detected as a major product in the solid formulation of compound I stressed under heated conditions (data not included). Such species was reported as a major

product under oxidation conditions with hydrogen peroxide [19].

4. Conclusions

Assessment of drug stability in aqueous media is an important step in the drug development process. The result can help formulation scientists select processes that are appropriate to the drug candidates. Hydrolysis and oxidation are two mostly common causes of degradation for drugs in aqueous media. This was demonstrated by the tested compound I under thermo-stressed conditions. The tested insulin sensitizer in aqueous solutions underwent degradation and formed TZD-ring opening products as the result of both hydrolysis and oxidation. Therefore, careful steps such as application of antioxidants in formulations or desiccants in storage should be taken in drug development of such compounds in liquid vehicles as well as solid dosage forms.

Our experimental results also demonstrated that a combination of LC/MS and NMR is a powerful technique in identification and characterization of drug degradates, study of degradation mechanism, and predication of degradation pathways.

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