Gas-phase fragmentation of protonated benzodiazepines

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Protonated 1,4-benzodiazepines dissociate in the gas phase by the common pathway of CO elimination and by unique pathways dictated by the substituents; the latter typically differentiate one benzodiazepine from another. Protonated 3-dihydro-5-phenyl-1,4-benzodiazepin-2-one, the base diazepam devoid of substituents, dissociates by eliminating CO, HNCO, benzene, and benzonitrile. Mechanisms of these reactions are proposed with ionic products being resonance stabilized. The abundant [MH−CO]+ ion dissociates to secondary products via elimination of benzene, benzonitrile, the NH2 radical, and ammonia, yielding again ionic products that are stabilized by resonance. Copyright © 2007 John Wiley & Sons, Ltd.

Benzodiazepines were discovered serendipitously in 1957 in the Roche laboratories.1 The benzodiazepine class of drugs consists of approximately 50 commercially available compounds with anxiolytic, muscle-relaxant and sedative effects.2,3 In clinical practice these drugs are widely prescribed for the treatment of insomnia and anxiety disorders. Benzodiazepines are central nervous system (CNS) depressants4,5 and are characterized by a strong and specific interaction with local γ-aminobutyric acid chloride channel receptor (GABA_A) sites.6–10 Various benzodiazepine derivatives were originally marketed as an improvement over the then available barbiturates with regard to overdose effects, drug interactions, dependence development, and illicit abuse. Over-prescription in the 1970s and 1980s revealed varying biological activity that each of these will form,2,3,12,16 benzodiazepines, together with the multiple metabolites of benzodiazepines by analyzing the CID of a skeletal end, we first examine the intrinsic dissociation reactions when a new benzodiazepine is encountered. Towards this end, we first examine the intrinsic dissociation reactions of benzodiazepines by analyzing the CID of a skeletal benzodiazepine, 3-dihydro-5-phenyl-1,4-benzodiazepin-2-one (see Scheme 1 for the structure), that is devoid of any functional group which typically differentiates the various benzodiazepine products. For simplicity, this benzodiazepine will be referred hereafter as the base diazepam. Characteristic fragmentations of benzodiazepine products will then be introduced and discussed with respect to features that will aid identification of these products by recognizing the functional groups or substituents that they carry.
EXPERIMENTAL

Materials
Nitrazepam, norfludiazepam, lorazepam (see Scheme 1 for structures and molecular weights) were obtained from Cambridge Isotope Laboratories (Quebec, Canada) as solutions at a certified concentration of 1 mg/mL in methanol. All other benzodiazepines, including base diazepam, nordiazepam, clonazepam, diazepam, oxazepam, temazepam, and flunitrazepam (Scheme 1), at 10 ng/μL in water/methanol, were a gift from MDS SCIEX (Concord, ON, Canada). Sample solutions for MS analysis were at a concentration of 4 mM in 50:50 water/methanol containing 1% acetic acid. Methanol and acetic acid were from Sigma (St. Louis, MO, USA); water was purified using a Milli-Q system (Millipore, Billerica, MA, USA). All diazepam materials were used as received and stored at 4°C except for analysis.

Mass spectrometry
Experiments were conducted on a pre-commercial version of the QTRAP linear ion-trap mass spectrometer (Applied Biosystem/MDS SCIEX). Sample solutions were infused into the pneumatically assisted ESI source at a flow rate of 2–3 μL/min using a syringe pump. The typical ESI voltage was between 5 and 5.5 kV. Dry air was used as the nebulizer gas. The orifice and lens voltages were optimized for maximum precursor ion signal intensity and between 100 and 200 scans were summed to produce a mass spectrum. In a typical MS/MS experiment, the precursor ion was mass-selected using the first quadrupole (Q1), allowed to collide with nitrogen at a pressure of approximately 7.5 mTorr (CAD gas setting of 4) in the second quadrupole (q2), and mass-analyzed using the third quadrupole (Q3). For chlorine-containing benzodiazepines, unless otherwise stated, the reported results are based on analyses of the $^{35}$Cl-containing ions, although the $^{35}$Cl- and the $^{37}$Cl- containing ions were both examined to verify ion assignments. For CID, the laboratory collision energy ($E_{lab}$) typically varied between 5 and 50 eV (100 eV for the base diazepam). The ion lineage of specific product ions was further investigated using the enhanced product ion (EPI) scan mode, which traps and accumulates product ions in Q3 for better sensitivity.

Computational methods
Geometry optimizations and energy calculations were performed with Gaussian 03 [35] using the non-local hybrid
three parameter B3LYP density functional theory (DFT) approach \cite{36,37} with the 6-31+G(d) split-valence basis sets. All stationary points were characterized by harmonic frequencies as local minima (all real frequencies) and saddle points (one imaginary frequency). Connections between transition states and corresponding minima were verified using the intrinsic reaction coordinate method. \cite{38,39}

**RESULTS AND DISCUSSION**

A benzodiazepine has three heteroatoms on the seven-membered ring and each potentially can accommodate the ‘ionizing’ proton to give a protonated benzodiazepine (MH$^+\$). These are the imine nitrogen at N4, the amide oxygen attached to C2, and the amide nitrogen at N1 (Scheme 1),

![Figure 1. Product ion spectrum of protonated base diazepam, m/z 237, at E$_{lab}$ = 30 eV.](image)

![Figure 2. (a) Energy-resolved CID results of MH$^+$, m/z 237 and (b) details of secondary products of the [MH–CO]$^+$ ion, m/z 209.](image)
in decreasing order of basicity. DFT calculations show that protonation on the imine nitrogen produces ion I that is 23.1 and 36.9 kcal/mol lower in enthalpy ($\Delta H^\circ$) than the ions produced by protonation on the amide oxygen, II, and on the amide nitrogen, III, respectively (Scheme 2).

Figure 1 shows the CID spectrum (product ion scan) of protonated base diazepam at a laboratory collision energy ($E_{lab}$) of 30 eV. Energy-resolved CID results are shown in Fig. 2 for the MH$^+$ ($m/z$ 237) as well as details of secondary products from a prominent product ion at $m/z$ 209, formed by elimination of CO. The loss of CO is a feature shared by all benzodiazepines.\textsuperscript{11,17,28–34} Figure 3 gives the energy profiles for the loss of CO from ion I directly and indirectly via ion III. The former has a higher enthalpy barrier ($\Delta H^\circ = 73.3$ kcal/mol) than the latter (56.3 kcal/mol). Protonation on the amide nitrogen weakens the amide bond (a fact well known in the CID of protonated peptides).\textsuperscript{40} It is, therefore, not surprising that the reaction pathway involving ion III has a lower energy barrier. The barrier against conversion of ion I into ion III, involving a 1,4-proton shift, is 54.2 kcal/mol – slightly lower than the barrier against the loss of CO from III.

In addition to the loss of CO, protonated base diazepam can also lose neutrals of 43, 78, and 103 Da to give relatively minor product ions at $m/z$ 194, 159, and 134, respectively (Figs. 1 and 2(a)). Plausible mechanisms are shown in Scheme 3. In pathway B, ring contraction leads to the loss of HNCO, 43 Da. The resulting ion at $m/z$ 194 delocalizes its positive charge onto the original N4 (IV), the benzylic C5, and both benzene rings. In pathway C, a 1,3-proton shift from N4 to C1' on the phenyl ring induces elimination of the phenyl ring as benzene, 78 Da, giving a benzodiazepine ion at

Figure 3. Energy profile for the elimination of CO from protonated base diazepam at B3LYP/6-31+G(d).

Scheme 2.
the energy-resolved results shown in Fig. 2(b), and ignoring product ions with abundances <5%, it is apparent that the following secondary product ions, m/z 131, 106, 193, and 192, are formed directly from the [MH−CO]⁺ ion at m/z 209, and with relatively low barriers. Mechanisms that can lead to these ions are shown in Scheme 4. In pathway E, a 1,5-proton shift in P from N1 to C1' or a 1,3-proton shift in P' from N4 to C1' induces elimination of the phenyl ring as benzene and gives the protonated 2-methaniminobenzonitrile ion (VII) at m/z 131. Ring contraction in pathway F can occur from either P or P' forming either protonated benzoazetine (ion VIII) at m/z 106, or protonated benzonitrile at m/z 104. Ion VIII is 16.9 kcal/mol higher in enthalpy (ΔH°) than protonated N-methylenebenzenamine, Ph−NH⁺=CH₂; however, conversion from the former into the latter is a high-energy process, requiring a 1,3-proton shift and cleavage of a C–C bond. The calculated proton affinities (PAs) of 1,2-dihydro-4-phenylquinazoline (that protonates to give VII) and benzonitrile are 218.2 and 194.4 kcal/mol at B3LYP/6-31+G(d); the latter is in good agreement with the reference PA of 194.9 kcal/mol for benzonitrile.41 The observation of protonated benzonitrile being formed at higher threshold energies suggests that the dominant pathway is via ion P'. At low collision energies, the lifetime of the initially formed PhCNH⁺/benzoazetine complex will be sufficiently long to permit efficient proton transfer and protonated benzoazetine will be the major product; at higher collision energies, the lifetime of the complex will be shorter and direct dissociation yields protonated benzonitrile. Pathways G and H result in losses of the NH₂ radical and NH₃ leading to the product ions at m/z 193 and 192, respectively. The initial step is identical and involves cleavage of the N1–C2 bond in P, giving IX, an ion in which there is extensive charge delocalization. Elimination of NH₃ (pathway G) yields a benzyne ion X at m/z 192 that is similarly resonance stabilized. Alternatively, elimination of NH₂ (pathway H) results in a distonic phenyl radical XI at m/z 193 that is also resonance stabilized.

Figure 4 shows the CID results of (a) protonated nordiazepam and (b) protonated norfludiazepam, both at Elab of 30 eV. Both benzodiazepines are simple derivatives of the base diazepam: nordiazepam has a Cl substituent at the C7 position, a rather common substitution pattern among commercially available diazepam, while norfludiazepam carries, in addition to the Cl substituent, a F substituent at the C2' position, which is less common than the former (Scheme 1). As observed for protonated base diazepam, protonated nordiazepam (m/z 271) dissociates under CID conditions by losing the following neutrals: CO, HNCO, and Cl, giving the protonated Ph-CN=CH² ion at m/z 209, and protonated norfludiazepam shows the same loss of 78 Da (HNCO+Cl, Fig. 3(b)) and none at 96 Da (the mass of fluorobenzene). In addition, this interpretation is in agreement with a previous one based on H/D-exchange.31 Protonated norfludiazepam (m/z 289) dissociates analogously, via losses of CO (28 Da), Cl+HNCO (78 Da), and

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fluorobenzonitrile (121 Da). The [MH–CO]⁺ ions of both diazepam derivatives dissociate facilely to give secondary product ions, as was observed for the [MH–CO]⁺ ion of the base diazepam. For nordiazepam, the losses are Cl, benzene, and benzonitrile; for norfludiazepam, they are Cl, fluoro- benzene, and fluorobenzonitrile. The resulting ions should have structures analogous to those postulated in Scheme 4.

Substituting the N1 hydrogen of nordiazepam with a methyl group results in diazepam; the CID spectrum of protonated diazepam at E_{lab} of 30 eV is shown in Fig. 5. It
shows many similarities to the CID spectrum of protonated nordiazepam in Fig. 2(a). The two new features are the elimination of neutrals of 57 Da from protonated diazepam and of 29 Da from the [MH–CO]⁺ ion. The 57 Da neutral that is lost is interpreted as CH₃NCO; the mechanism for its elimination is postulated to be analogous to that of HNCO shown in Scheme 3. Protonated nordiazepam does not appear to lose HNCO alone, but concomitantly with Cl. The 29 Da neutral lost from the [MH–CO]⁺ ion corresponds to methyl nitrene, CH₃N (or the lower energy H₂C=NH), forming protonated isoindole.

Substituting the C7 hydrogen on nordiazepam with a nitro group results in nitrazepam; the CID spectrum of protonated nitrazepam at Elab of 30 eV is shown in Fig. 6(a). The most prominent feature is the loss of NO₂, 46 Da. This same feature is evident in the CID of protonated flunitrazepam (Fig. 6(b)), which differs from nitrazepam in having a methyl substituent on N1 and a fluoro substituent on C2', and clonazepam, a derivative of nitrazepam that contains a chloro substituent at the C₂' position.

Replacing the C3 hydrogen on nordiazepam with a hydroxyl group produces oxazepam, an additional substitution of Cl at the C₂' position results in lorazepam. Similarly, replacing the C3 hydrogen on diazepam with a hydroxyl group produces temazepam. The CID spectra of the [M+H]⁺ ions of these OH-bearing derivatives are dominated by two common neutral losses: that of water (18 Da), followed by CO (28 Da). The spectrum for protonated oxazepam is shown in Fig. 7. An additional prominent loss involves the loss of a 56 Da neutral. This loss is also
evident in protonated lorazepam, albeit much less notably. The proposed mechanism for the loss of water, shown in Scheme 5, is in agreement with H/D exchange results. Nucleophilic attack by the hydroxyl oxygen on the N4 hydrogen, followed by ring contraction and elimination of water, results in ion XIII at m/z 269. Ring contraction and cleavage of CO produces ion XIV at m/z 241 that is resonance stabilized. The 56 Da neutral loss directly from protonated oxazepam is postulated to be that of ethylenedione, C2O2, a short-lived molecule that rapidly dissociates into two molecules of CO. This hypothesis of C2O2 is consistent with the absence of the stepwise elimination of the CO molecules.

CONCLUSIONS

Substitution of chloro, hydroxyl, and nitro groups on the base diazepam has a dramatic effect on gas-phase dissociation chemistry. The losses of these groups are the most facile and dominate the CID chemistry. The loss of CO is a common, facile dissociation pathway of all benzodiazepines, in evidence even in the aforementioned substituted benzo- diazepines. Other common losses include the phenyl ring at the C5 position, as either (substituted) benzene or benzonitrile. Ion structures and fragmentation mechanisms have been postulated towards building a knowledge- and/or intuition-based benzodiazepine information bank whose contents will lead to eventual understanding of how structure drives fragmentation chemistry and determines products.

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REFERENCES
