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Full Length Article

Dissociative protonation and long-range proton migration: The chemistry of singly- and doubly-protonated tigecycline

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ABSTRACT

Tigecycline is an antibacterial drug in the tetracycline class. In electrospray mass spectrometry both singly-protonated (MH^+) and doubly-protonated molecules (MH_2^{2+}) are formed in abundance. The major fragmentation reactions of the molecular ions in both charge states take place around the amido group at the C2-position on the A-ring and the t-butylamino group of the side chain at the C9-position on the D-ring. Deuterium labeling reveals that losses of ammonia from C2 and isobutene from C9 from both MH^+ and MH_2^{2+} take place in a consecutive fashion, and elimination of the constitutive t-butylamine as a distinct neutral species does *not* occur as suggested intuitively. In addition, it is found that the elimination of isobutene from MH_2^{2+} is more than 10-fold as much as that from MH^+ relative to the ammonia loss. This suggests that in the "double engine" molecular ion (MH_2^{2+}), the first protonation occurs on the *N*,*N*-dimethylamino substituent on the A-ring, responsible for the loss of ammonia from the adjacent C2-amido group, and the second protonation occurs on the t-butylamino moiety on the D-ring to initiate the removal of isobutene. Since these two reactions are also observed with the "single engine" molecular ion (MH^+), it is clear that upon the first neutral loss (either ammonia or isobutene), a proton is required to travel across the tetracycline core to trigger the second neutral loss. Multistage mass spectrometry confirms that the structure of the final product ion is the same regardless of the order in which the two neutral species are eliminated, supporting the requirement for a long-range proton migration.

1. Introduction

Mass spectrometry is recognized as an essential technology in drug discovery and development for a variety of indispensable roles in both qualitative and quantitative analyses. In terms of qualitative structural analysis [1,2], whether it is early in the discovery stage or late in the clinical development phases, mass spectrometry has become the primary tool for the identification of drug metabolites [3,4] for scientists who strive either to improve the overall compound quality [5], including metabolic stability [6], or to understand the cause of a specific toxicity that involves reactive metabolites [7,8]. The superior sensitivity that requires only a small amount of samples and the vigorous versatility in dealing with complex matrices are the major attributes to enable mass spectrometry to reveal fingerprint information for structural determination. However, in strong contrast to the relative adroitness in obtaining affluent mass spectral data, the interpretation of these data to suggest sensible structures still remains clumsy even in the age of highly advanced artificial intelligence.

In mass spectrometry with the contemporary electrospray ionization (ESI) or the classical chemical ionization (CI), the molecules of interest are ionized primarily by protonation, and the resulting protonated molecules (MH^+) are brought to produce molecular structural fingerprint through fragmentation. The difficulty in mass spectral interpretation arises obviously from the combined complexities in both protonation and fragmentation. Protonation on its own is an active and broad area for extensive research using either experimental techniques (including mass spectrometry) or theoretical calculations. Firstly, we are curious about the exact position in a molecule that the ionizing proton is attached to. A simple protonation reaction, shown as $M + H^+ \rightarrow MH^+$, is an exothermic process [9]. Therefore, from the thermodynamic perspective, the proton affinity (PA), which is an intrinsic molecular property, should generally direct where the proton goes. Given the fact that a diatomic molecule such as carbon monoxide [10] may be "ambidextrous" in protonation, a multifunctional drug molecule containing various heterocyclic rings [11], such as atorvastatin [12], may accept a proton at many basic sites to form a highly heterogeneous MH^+ population. Structural features such as neighboring groups [13–15]

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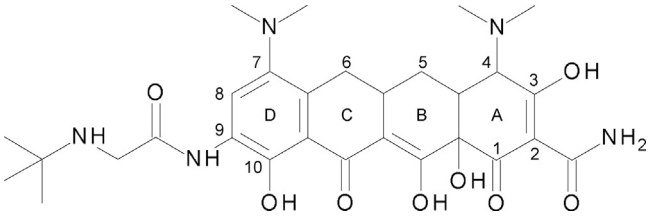

Scheme 1. Chemical structure of tigecycline and position labels.

may alter the route of protonation through facilitating intramolecular hydrogen bond to stabilize the MH^+ ion. In addition, the kinetics of protonation in atmospheric pressure ionization may be found to overcome the thermodynamic factors in a few cases [16,17], similar to what was proposed to explain competitive fragmentation pathways observed in chemical ionization [18,19], although it should be emphasized that a non-favorable site of protonation [16] is *not* necessarily a site of protonation under kinetic control. Furthermore, an extrinsic factor, which is the solvent effect, has also been reported to alter protonation site. In continued efforts to further understand the mechanism of ion production in ESI, it was found that the same molecules showed different fragmentation patterns when different solvents were used, leading to a hypothesis that the site of protonation is solvent dependent [20–22], which could trace several decades back to similar investigations into the role of solvents used in CI as the ionization reagents [23,24].

In addition to the intrinsic and extrinsic factors described above that influence the site of protonation, a stunning feature that dramatically complicates the chemistry of protonated molecules is the ability of the proton to move across the entire molecules, which has been coined as the mobile proton model [25,26]. Although this model was born in the era when mass spectrometry was extensively applied to the sequencing of peptides and proteins [27], the mobility of proton was first observed and unequivocally characterized with rigid aromatic molecules [28,29]. In the well-designed terphenyl derivatives [30], for example, the proton specifically placed on a carbonyl group on one end of the molecules was found to walk [31] across three phenyl rings to a methoxylmethyl group on the other end, leading to the loss of a neutral methanol. For a given multifunctional molecule, there are various MH^+ ions. On the potential energy surface, the one that has the proton attached to the most favorable position resides in the global minimal energy well whereas other ions in local minimal energy wells. The observed proton mobility indicates that when conversion of the MH^+ ions from one tautomeric form to another takes place *prior* to fragmentation, the ions are adequately activated to overcome the central energy barrier between the two forms. It should be recognized that some MH^+ ions exist as non-dissociating species, and activation by way of collisions promotes proton migration to trigger fragmentation. The amide is a well-documented example, which is protonated primarily at the carbonyl oxygen [32–34] leading to a non-dissociating MH^+ ion. When this ion is activated to break the $C(O\mathbf{H}^+)–NR_2$ bond, the eliminated amine ($\mathbf{H}NR_2$) carries the external proton that has migrated from O to the N atom. To describe such positions in a molecule as the nitrogen in the amide that leads to immediate simple bond cleavage upon protonation, we have introduced the term of "dissociative protonation site" [35–38], which is a dynamic starting point to rationalize fragmentation reactions in mass spectrometry.

Recently we came across tigecycline (see Scheme 1 for its structure and positional labels), which is a new antibacterial drug molecule in the tetracycline class [39]. In a study to evaluate its efficacy, an analytical method based on ESI mass spectrometry was required. During method development, the compound was infused in a typical acidic aqueous solution. What we found is that the MH^+ ion (*m/z* 586) is entirely buried in the chemical noise whereas the doubly-charged ion at *m/z* 293.5 is outstanding. Since it is not often for a small molecule to capture two protons, we decided to take a close look at the chemistry of both the MH^+ and MH_2^{2+} ions of tigecycline by various tandem mass spectrometry methods combined with deuterium labeling. Major fragmentation reactions of the MH^+ ion are (1) the loss of ammonia (NH_3) from the simple amido group (H_2NCO-) at the C2-position on the A-ring, and (2) the loss of isobutene (i-C_4H_8) from the *N*-t-butyl group on the side chain at the C9-position on the D-ring. Behind these simple reactions is the loss of a neutral [C_4, H_{11}, N] species, which is found *not* to be the constitutive t-butylamine ($C_4H_9NH_2$) of the C9 side chain but a combination of distinctive ammonia from C2 and isobutene from C9, in both orders. This third reaction requires long-range proton migration either from the A-ring to the D-ring or vice versa. We report here the detailed characterization of these reactions as well as some minor ones that are also counter-intuitive.

Table 1
Model molecules and their proton affinities [9] resembling functionalities of tigecycline.

Model	PA (kcal/mol)	Site of protonation	Tigecycline relevance
Benzamide	212.2	O	C2-amido (O)
Trimethylamine	226.8	N	C4-dimethylamino (N)
N,*N*-Dimethylaniline	224.9	N	C7-dimethylamino (N)
t-Butylamine	223.3	N	C9-*t*-butylamino (N)
Glycine	211.9	N	C9-glycyl (O)[a]
Acetamide	206.4	O	C9-glycyl (O)

[a] Glycine is preferably protonated at the amino group, so PA at this oxygen could be even lower (similar to acetamide).

2. Experimental

Tigecycline and other reagents in this study were purchased from Sigma-Aldrich (St Louis, MO) and used as received without further purification. The compound were introduced to the ion source as a solution at a concentration of approximately 100 ng/mL in methanol/water (50/50, v/v) with 0.1% formic acid. The solution was delivered through an infusion pump at a rate of 10–50 μL/min. For experiments on the deuterated ions, the solvent was changed to CH_3OD/D_2O (50/50, v/v). Since tigecycline contains eight exchangeable labile protons, to ensure they are completely exchanged with deuterium, blank CH_3OD/D_2O was infused to the ion source at the same rate for 10–15 min to remove water residue on the surface. The complete exchange is indicated by a dominant signal at *m/z* 595 for the $[M\text{-}d_8 + D]^+$ ion, which should be ~100x higher than the adjacent ion at *m/z* 594 and other lighter isotopomers.

The low resolution CID mass spectra were obtained from an LTQ linear ion trap mass spectrometer, and high resolution spectra from an Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA), both of which were equipped with an electrospray ion source and operated with the Xcalibur software package. The source temperature was kept at 200 °C, and the capillary voltage (10 V) and the tube lens voltage (80–120 V) were optimized for sensitivity. To activate the selected ions, helium was used as the collision gas, and the "normalized collision energy" was varied in a range of 10–30%. For the purpose of comparison, a Sciex API 4000 triple quadrupole mass spectrometer (Sciex, Toronto, Ontario, Canada) with an orthogonal turbo V spray ion source was also used. The ion source was operated at a temperature of 300 °C and an electrospray voltage of 5000 V, and nitrogen served as the collision gas. Unless otherwise indicated, only data obtained from the ion trap are discussed.