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Titan's Primordial Soup: Formation of Amino Acids via Low-Temperature Hydrolysis of Tholins

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Abstract

Titan organic haze analogues, or "tholins," produce biomolecules when hydrolyzed at low temperature over long timescales. By using a combination of high-resolution mass spectroscopy and tandem mass spectrometry fragmentation techniques, four amino acids were identified in a tholin sample that had been hydrolyzed in a 13 wt % ammonia-water solution at 253 ± 1 K and 293 ± 1 K for 1 year. These four species have been assigned as the amino acids asparagine, aspartic acid, glutamine, and glutamic acid. This represents the first detection of biologically relevant molecules created under conditions thought to be similar to those found in impact melt pools and cryolavas on Titan, which are at a stage of chemical evolution not unlike the "primordial soup" of the early Earth. Future missions to Titan should therefore carry instrumentation capable of, but certainly not limited to, detecting amino acids and other prebiotic molecules on Titan's surface. Key Words: Titan—Prebiotic chemistry. Astrobiology 10, 337–347.

1. Introduction

Titan, Saturn's largest moon, is a world rich in organic molecules. Reactions occurring in Titan's dense nitrogen-methane atmosphere produce a wide range of nitrogen-bearing organic molecules (Hanel *et al.*, 1981; Kunde *et al.*, 1981; Maguire *et al.*, 1981; Waite *et al.*, 2005; Vuitton *et al.*, 2007), which subsequently rain down onto the satellite's surface. Laboratory experiments that simulate the reactions occurring in Titan's atmosphere produce many of the same gas-phase organic molecules observed by the Voyager and Cassini spacecraft (Sagan *et al.*, 1992; Cabane and Chassefière, 1995), along with organic precipitates known as tholins. Tholins have the general chemical formula $C_xH_yN_z$ and are spectrally similar to Titan's haze (Khare *et al.*, 1984). The abundance and wide variety of organic molecules available on Titan make it an interesting natural laboratory for studying prebiotic chemistry.

Water—thought to be another important ingredient for life—is likewise abundant on Titan. Theoretical models of Titan's formation and evolution (*e.g.*, Tobie *et al.*, 2005, 2006) predict that its interior consists of an ice I or methane clathrate layer several tens of kilometers thick that overlies a liquid water layer several hundred kilometers thick. Titan's ocean likely contains an antifreeze agent such as ammonia, or possibly methanol or ammonium sulfate (Fortes *et al.*, 2007), dissolved in it. Observations by Lorenz *et al.* (2008) support this model of Titan's interior. They found that Titan's rotation period is different from its orbital period, which is consistent with the seasonal exchange of angular momentum between Titan's surface and its atmosphere, but only if Titan's crust is decoupled from its core by an internal ocean.

Frozen water should therefore be abundant at Titan's surface. However, for molecules to interact and form biologically relevant species, liquid water must be present and in contact with the organics at Titan's surface. Its surface temperature of 94 K dictates that Titan is, on average, too cold for liquid water to persist at its surface, but melting caused by impacts or cryovolcanism may lead to its episodic availability. Impact melt pools on Titan would likely remain liquid for $\sim 10^2$ to 10^4 years before freezing (O'Brien *et al.*, 2005).

When these two ingredients—complex atmospheric organics and liquid water—are combined, they have been shown to produce biomolecules. Khare *et al.* (1986) first demonstrated that amino acids, as well as urea, could be formed through high-temperature acid hydrolysis of Titan tholins (in 6 *N* HCl over 20 h at 373 K). More recently, Raulin *et al.* (2007) showed that this process also applies in solutions of neutral pH (over 2 days at 343 K). However, given the high temperatures and short timescales used in both works, neither can be said to be a realistic analogue to the transient liquid water environments found on Titan. Pure water melts

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would quickly cool to the freezing point of 273 K, and if ammonia is present on Titan (as it is predicted to be), the temperature of impact melts and cryolavas could be as low as the eutectic temperature of 176 K. Since new reaction pathways can be accessed given sufficient energy, it remains unclear whether the chemistry observed by Khare *et al*. (1986) or Raulin *et al*. (2007) would occur in any significant way at the much lower temperatures encountered on Titan.

In this work, we sought to determine what biomolecules might be formed under conditions more analogous to those found in transient liquid water environments on Titan. Given the large uncertainty in the amount of ammonia one might find in these environments, we studied solutions with an ammonia concentration of 13 wt %, a composition roughly halfway between pure water (0 wt % ammonia) and pure ammonia-dihydrate (33 wt % ammonia), which is the expected composition of a peritectic melt. We hydrolyzed Titan tholins in a 13 wt % ammonia-water solution at 253 and 293 K for timescales on the order of a year. The initial stages of this reaction are reported in Neish *et al*. (2009). The tholins reacted rapidly with the ammonia-water solution, producing complex oxygen-bearing organic molecules with reaction half-lives between 0.3 and 14 days at 253 K. In addition to its antifreeze properties, ammonia was observed to participate in chemical reactions with tholins in a manner that was independent of the tholins' reactions with water. Thus, the presence or absence of ammonia on Titan will be important for understanding its chemistry.

We then used a new and novel technique to detect biomolecules within our sample. Using ultrahigh-resolution mass spectrometry (Fourier transform ion cyclotron resonance, FT-ICR), we searched for species that had the same exact mass as the 5 nucleobases and the 17 non-sulfur-containing biological amino acids. To distinguish between the many structures possible in organic molecules of the size and complexity of our tholin sample, we then selected several species for fragmentation. These fragmentation studies are necessary to prove structural identity of species, since a species with a well-defined chemical composition (which can be determined by FT-ICR) can exist in the form of many different structural isomers. By comparing the fragments of the tholin species to fragments from pure biomolecular samples, we determined whether the species were consistent with biomolecular structures.

2. Experimental

To prepare the tholins, a mixture of 2% CH_4 and 98% N_2 was exposed to an electrical discharge under slow flow (4.5 atm L h^{-1} at a pressure of 880 Pa) at a temperature of 195 K in a glass reaction vessel. Details relating to the isolation and preparation of the tholin material used in this work are given in Neish *et al*. (2009). Tholin products produced in this manner have been systematically analyzed in past work by mass spectrometry (Sarker *et al*., 2003; Somogyi *et al*., 2005). A wide variety of ions with the general formula $C_xH_yN_z$ are observed, reflecting the complexity of the products. Tandem mass spectral data and H/D exchange products in the solution phase support the presence of amino and nitrile functionalities in these highly unsaturated compounds. Previous hydrolysis experiments have also been implemented to elucidate the product functionalities in these tholins. Neish *et al*. (2008, 2009) compared growth rates of oxygenated species with decay rates of non-oxygenated species, whose masses corresponded to potential parent species, and found evidence of aliphatic amine loss. This is consistent with imine hydrolysis and amine substitution. Nonetheless, given the complexity of molecules of this size, there are insufficient data to make conclusive structural assignments for our tholin sample. The current fragmentation study is thus particularly important for understanding the structure of these complex molecules.

Throughout this work, we refer to the organic compounds produced in our laboratory as "tholins." It is important to note, however, that this term has been used to describe organic compounds that were formed at a variety of temperatures and pressures and produced with different energy sources (*e.g.*, UV photolysis or electrical discharge). In particular, tholins formed at pressures lower than 160 Pa and produced via radio frequency discharge appear to have spectral signatures more representative of Titan's haze than those formed at higher pressures (Imanaka *et al*., 2004). However, to make the amount of tholins required for the hydrolysis study, it was necessary to use the AC discharge technique at the higher pressure.

From the tholin sample produced in this work, we prepared two different samples, one for each temperature of interest (253 and 293 K). We dissolved $\sim$50 mg of the tholins in 2 ml of acetonitrile and placed 100 μl of this solution in a mixture containing 1 ml of a $\sim$14 *N* aqueous solution of NH_4OH ($\sim$26 wt % ammonia, EMD Chemicals Inc.) and 1 ml of sonicated double deionized water. The ammonia-water solution was thermally equilibrated in a constant temperature bath prior to the introduction of the tholin and maintained a temperature stability of roughly $\pm$1 K over the course of the experiment. Immediately after the solutions were made, the vials were sealed from air with airtight septa. Note that a small amount of air was present in the headspace of the vials before they were sealed, and a small amount of dissolved oxygen may therefore have been present in the sample. However, this oxygen has been shown to be unreactive compared to the much more concentrated water source. In a previous study (Neish *et al*., 2009), reactions were observed in labeled $H_2^{18}O$, and only those products containing ^{18}O (not ^{16}O, as would be found in dissolved oxygen) were observed to grow over time. This confirmed water as the source of the oxygen in the products.

After 1 year, the products of the hydrolysis reactions were analyzed via FT-ICR ultrahigh-resolution mass spectrometry. A 25 μl aliquot of the complete mixture in solution was quenched in 200 μl of CH_3OH:CH_3CN (1:1) and injected directly into an electrospray ionization source of a Bruker Daltonics (Billerica, MA) Apex-ultra 9.4 T FT-ICR instrument with normal electrospray ionization conditions. After the chemical formulas for the components were determined by accurate mass measurements, select samples were chosen for tandem mass spectrometric fragmentation via low-energy quadrupole collision-induced dissociation (QCID) and infrared multiphoton dissociation (IRMPD). QCID fragments were formed by colliding select ionic species with N_2 molecules with a laboratory collision energy in the range of 8–12 eV. The QCID fragments were then transferred to the ICR cell where they were analyzed with ultrahigh-resolution mass spectrometry. IRMPD fragments were generated by

irradiating select precursor ions with a CO_2 infrared laser for 0.7–1.0 seconds with a laser power of 50–60% in the ICR cell. Nucleobase and amino acid standards were also analyzed and fragmented under the same mass spectral conditions for comparison. Fragmentation spectra depend on the ion activation technique used, so it is desirable to apply two different and independent techniques.

Note that, for the first time in tholin sample analyses by FT-ICR, all data were collected in the negative ion mode (in which the species are deprotonated, $[M\text{-}H]^-$). In all previous studies (Sarker *et al.*, 2003; Somogyi *et al.*, 2005; Neish *et al.*, 2008, 2009), the data were collected in the positive ion mode (in which the species are protonated, $[M+H]^+$). It is important to study products in both the positive and the negative mode of the FT-ICR instrument, since different species are observed in the different modes. In the negative mode, more unsaturated species are observed; in the positive mode, more saturated species are observed. This study allows us to investigate products hitherto unseen.

3. Results

Each sample analyzed contained several thousand compounds with detectable intensities at masses in the range 110–400 Da. For each sample, we generated an accurate mass/intensity list by calibrating the mass spectrum, using the exact mass of several ions whose masses were unambiguously determined in previous work (Somogyi *et al.*, 2005; Neish *et al.*, 2008). We imported these lists into a program that compared the masses to a list of known compounds to identify the chemical formulas of the compounds. This is possible due to the high mass resolution of the FT-MS, which regularly gives mass over charge (m/z) measurements to better than ± 0.0004 Da accuracy. We searched the data for molecules with the same exact mass as the 5 nucleobases and the 17 non-sulfur-containing biological amino acids. The results are given in Table 1, with the qualitative strength of the signal of each mass peak given for the two temperatures studied. Note that we could not detect species with masses less than $m/z \sim 110$, since the ion optics–determined sensitivity of the FT-ICR instrument used in this study is insufficient in that range.

Molecular formulas are a necessary, but not sufficient, criteria for determining the presence of amino acids in a sample. Given the large sample diversity, tholins are comprised of many different structures. Even species with a well-defined chemical composition can have different structural isomers. Therefore, we used tandem mass spectrometry fragmentation to help distinguish between those isomeric structures. We fragmented those tholin species that had the same exact mass as a biomolecule and compared the results to fragmentation data taken from pure standard samples of the same biomolecule under identical experimental conditions. [Biomolecule standards were acquired from Sigma-Aldrich, St. Louis, MO, and dissolved in CH_3OH:CH_3CN (1:1) prior to analysis.] All fragmentation studies were conducted in negative ion mode by using both IRMPD and low-energy (eV) QCID fragmentation modes available for our FT-ICR instrument.

To increase the likelihood of obtaining observable fragments, we chose to fragment only those tholin species that had reasonably strong intensities in the mass spectrum. This limited us to the tholin species with the same exact mass as thymine, asparagine, aspartic acid, glutamine, glutamic acid, and histidine. The structures of these six molecules are given in Fig. 1. We can assert with reasonably high confidence that the observed asparagine, aspartic acid, glutamine, and glutamic acid molecules were not contaminants, as the masses

Table 1. Signal Intensity of Deprotonated Species Detected in the Hydrolyzed Tholin Sample with the Same Exact Mass as a Biomolecule

		Signal intensity in tholin sample	
Biomolecule	*Exact mass (Da)*	*253 K*	*293 K*
Glycine ($C_2H_4NO_2^-$)	74.0248	Too small to detect	Too small to detect
Alanine ($C_3H_6NO_2^-$)	88.0404	Too small to detect	Too small to detect
Serine ($C_3H_6NO_3^-$)	104.0353	Too small to detect	Too small to detect
Cytosine ($C_4H_4N_3O^-$)	110.0360	Weak	Weak
Uracil ($C_4H_3N_2O_2^-$)	111.0200	Weak	Weak
Proline ($C_5H_8NO_2^-$)	114.0561	Not detected	Very weak
Threonine ($C_4H_8NO_3^-$)	118.0510	Not detected	Very weak
Thymine ($C_5H_5N_2O_2^-$)	125.0357	Weak	Strong
Isoleucine ($C_6H_{12}NO_2^-$)	130.0874	Very weak	Very weak
Asparagine ($C_4H_7N_2O_3^-$)	131.0462	Strong	Weak
Aspartic acid ($C_4H_6NO_4^-$)	132.0302	Weak	Strong
Adenine ($C_5H_4N_5^-$)	134.0472	Weak	Weak
Glutamine ($C_5H_9N_2O_3^-$)	145.0619	Strong	Strong
Lysine ($C_6H_{13}N_2O_2^-$)	145.0983	Very weak	Very weak
Glutamic acid ($C_5H_8NO_4^-$)	146.0459	Weak	Strong
Guanine ($C_5H_4N_5O^-$)	150.0421	Very weak	Very weak
Histidine ($C_6H_8N_3O_2^-$)	154.0622	Strong	Weak
Phenylalanine ($C_9H_{10}NO_2^-$)	164.0717	Very weak	Weak
Arginine ($C_6H_{13}N_4O_2^-$)	173.1044	Very weak	Very weak
Tryosine ($C_9H_{10}NO_3^-$)	180.0666	Very weak	Weak
Tryptophan ($C_{11}H_{11}N_2O_2^-$)	203.0826	Very weak	Very weak

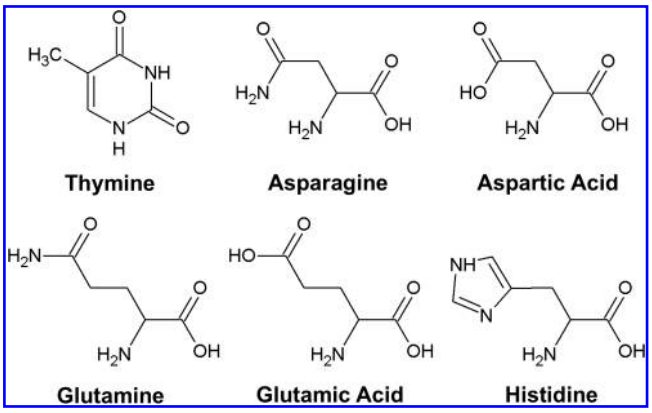

FIG. 1. Structures of the six biomolecules that have the same exact mass as species in the hydrolyzed tholin sample with relatively strong intensities in the negative ion mode.

corresponding to those molecules were not present in a nonhydrolyzed reference tholin that had been kept in CH_3OH:CH_3CN (1:1) at $-80°C$ for a year. Small peaks consistent with the exact mass of deprotonated thymine and histidine are observed in the reference tholin but not in a fresher sample analyzed in the positive ion mode.

After the species had been fragmented, we calculated the difference between each observed parent molecule and each observed fragment. We searched this list for neutral losses that corresponded to one of 43 different formulas within ± 0.001 Da. Those formulas consisted of H_2O, $2H_2O$, H_4O, H_2O_2, NH_3, $2NH_3$, HCN, NH_2CN, CO, CO_2, CH_3OH, C_2H_5OH, CH_3NO, C_2H_5NO, CH_3NH_2, $C_2H_5NH_2$, HCOOH, CH_3COOH, COOHOH, $COOHNH_2$, $CH_2(OH)_2$, CH_2O, C_2H_4O, CH_2, NH, CH_3, HCCH, CH_4, C_2H_4, H_2CNH, C_2H_6, CH_6N, CH_2CN, C_3H_4, CH_3CN, C_3H_6, C_2H_5N, CH_4N_2, C_3H_8, C_3H_3N, C_3H_4N, C_2H_5CN, and $C_2H_4N_2$. Example fragmentation spectrum for thymine, asparagine, aspartic acid, and histidine, along with their tholin mass-equivalent counterparts, are given in Figs. 2–5. Parent species and notable fragments are labeled. The complete list of all fragments for the six biomolecules considered in this work are given in Table 2.

4. Discussion

Up to this point, all studies of Titan tholin hydrolysis have focused on the products of chemistry that occur over hours, days, or weeks, at temperature and pH conditions likely not encountered on Titan's surface. Our present investigation represents the first study to observe the products of chemistry that occur on timescales approaching those for which liquid water may be expected to persist on Titan, at temperatures similar to those that would be encountered in impact melts and cryolavas. We therefore have a unique opportunity to examine the long-term products of tholin hydrolysis.

In our hydrolyzed tholin samples, we found species that had molecular formulas consistent with the 5 nucelobases and the 17 non-sulfur-containing biological amino acids. Of the molecules massive enough to be observed in our sample, most biomolecule species were present, though with differing ion intensities. Structure could only be inferred for those species with intensities strong enough to allow fragmentation into their constituent parts. These species include those with the same exact mass as thymine, asparagine, aspartic acid, glutamine, glutamic acid, and histidine. In this section, we will consider the evidence for the presence of each of these biomolecules separately.

4.1. Thymine

We fragmented $C_5H_5N_2O_2^-$ (m/z 125.0357) in both a pure sample of thymine and a tholin sample that had been hydrolyzed at 253 K for a year, using the negative ion mode with the available IRMPD and QCID ion activation modes of the FT-ICR instrument. The observed fragments are reported in Table 2. Though a variety of fragmentation energies were tried, thymine did not show any observable fragments in either fragmentation mode under the explored fragmentation conditions (Fig. 2). The tholin sample showed a similar dearth of fragments and presented only a HCN fragment in the IRMPD mode. It is possible that two structural isomers are present in the tholin sample—one consistent with thymine and one that produces an HCN fragment—but it is impossible to confirm or deny the presence of thymine, given its lack of identifiable fragments. Unfortunately, none of the other four nucleobases had signals strong enough for fragmentation analyses, so it remains unclear whether purines or pyrimidines could be formed in a hydrolyzing tholin sample.

4.2. Asparagine

We next compared a pure sample of asparagine to a tholin sample that had been hydrolyzed at 253 K for a year by fragmenting the $C_4H_7N_2O_3^-$ species (m/z 131.0462), using the IRMPD and QCID fragmentation modes of the FT-ICR instrument. The observed fragments are reported in Table 2. For the two overlapping data sets, the fragments agree remarkably well. In the negative QCID mode, both asparagine and the tholin species show an intense NH_3 loss and a slightly less intense H_2O loss (Fig. 3c, 3d). These are both expected fragments for amino acids, which have terminal amine and carboxyl groups. Asparagine showed smaller $2H_2O$ and H_4O losses not observed in the tholin sample, but it is possible that the intensity of these peaks is too small to be detected within the signal-to-noise range of the tholin sample. The $2H_2O$ loss from asparagine is $\sim$10 times less intense, and the H_4O loss is $\sim$40 times less intense than the larger NH_3 loss. In the negative ion IRMPD mode, both asparagine and the tholin species show a large water loss and a smaller $2H_2O$ loss (Fig. 3a, 3b). No NH_3 loss is observed amongst the tholin fragments, though, again, it is possible that the intensity is too small to be detected. The NH_3 loss from asparagine is $\sim$40 times less intense than the observed H_2O loss. These data do not confirm that the species we see in the tholin sample is asparagine, but it is consistent with that hypothesis.

4.3. Aspartic acid

We then fragmented $C_4H_6NO_4^-$ (m/z 132.0302) in both a pure sample of aspartic acid and a tholin sample that had been hydrolyzed at 293 K for a year, using the negative ion IRMPD and QCID modes of the FT-ICR instrument. The observed fragments are reported in Table 2. The tholin species $C_4H_6NO_4^-$ shows remarkable similarities to the

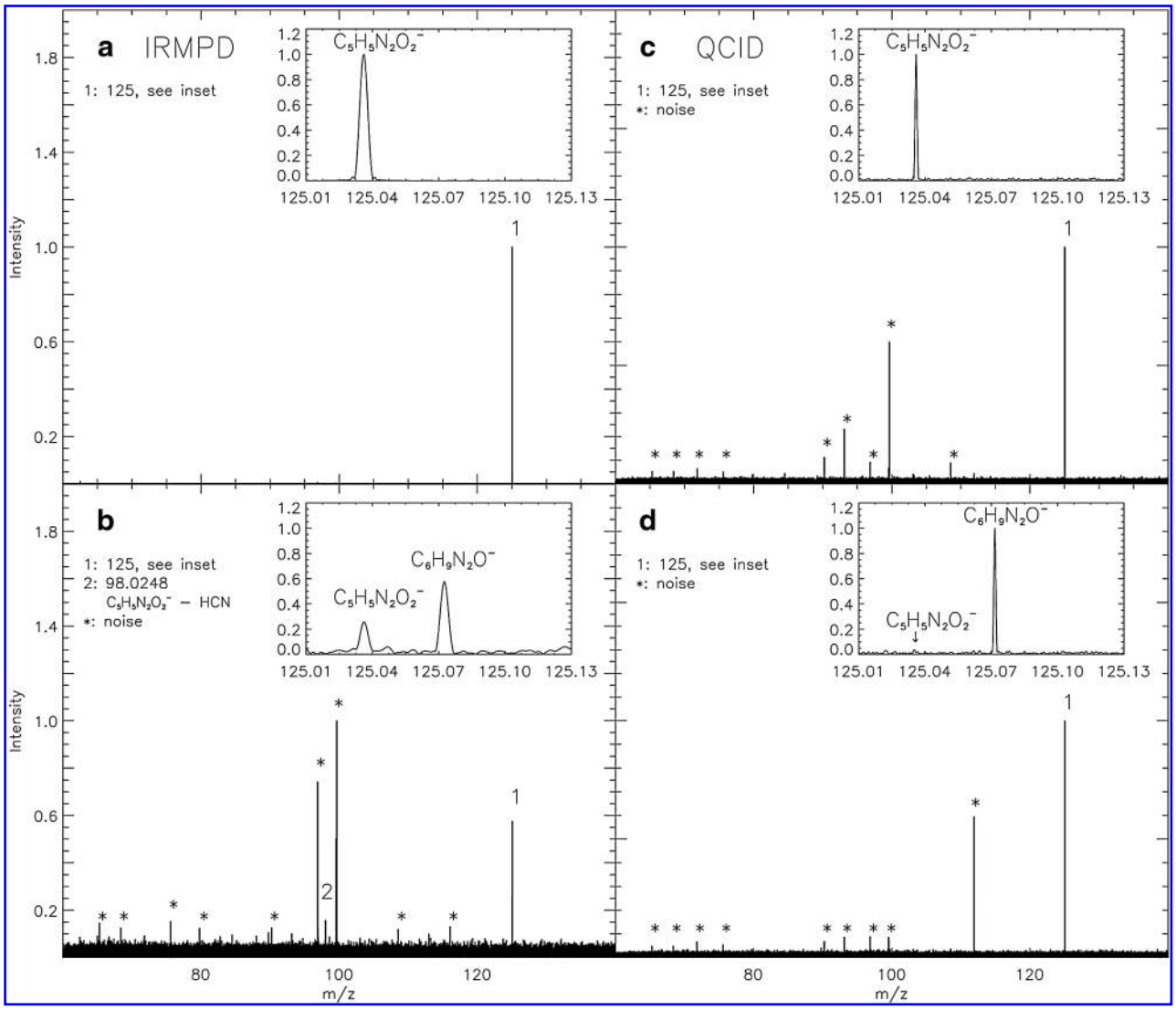

FIG. 2. Fragmentation mass spectrum of the nucleobase thymine at nominal mass m/z 125 via (**a**) IRMPD and (**c**) QCID in the negative ion mode. Fragmentation mass spectrum of a tholin sample hydrolyzed at 253 K for 1 year at nominal mass m/z 125 via (**b**) IRMPD and (**d**) QCID in the negative ion mode. The parent peaks are labeled by the number one, with a high-resolution expansion given in the inset at top right. Notable fragments are labeled by numbers, and instrumental noise peaks are marked with asterisks. The masses of the fragments are given at top left.

fragmentation spectrum of aspartic acid. In the negative ion QCID mode, both samples show an intense NH_3 loss and a much less intense H_2O loss (Fig. 4a, 4b). There are also many similarities between the fragmentation spectra in the negative ion IRMPD mode (Fig. 4c, 4d). Both spectra show CO_2, $COOHNH_2$, H_2O, NH_3, and H_2O_2 loss. CO_2, H_2O, and NH_3 are all expected fragments for an amino acid, representing carboxylic acid, alcohol, and amine functional groups. Given the structure of aspartic acid, $COOHNH_2$ and H_2O_2 may seem like odd fragments to be lost. It is most likely that these fragments represent the successive loss of two molecules. In the former case, we could be observing the loss of CO_2 and NH_3; and, in the latter case, 2OH losses. Again, the data do not confirm that the species we see in the tholin sample is aspartic acid, but the similarities between the fragmentation spectra of the species found in the tholin and that of the standard provide strong support for the presence of aspartic acid in the hydrolyzed tholin sample.

4.4. Glutamine

We compared a pure sample of glutamine to a tholin sample that had been hydrolyzed at 253 K for a year by fragmenting the $C_5H_9N_2O_3^-$ species (m/z 145.0619) with the negative ion IRMPD and QCID modes of the FT-ICR instrument. The observed fragments are reported in Table 2. There are some similarities between the fragmentation spectra but also some differences. In the QCID mode, both the pure glutamine sample and the tholin sample show H_2O, NH_3, $2H_2O$, and H_4O loss. However, the glutamine sample also shows small CO, HCOOH, CH_5NO, and $CH_2(OH)_2$ losses, while the tholin sample shows a fairly strong CH_3NH_2 loss. In the IRMPD mode, both the pure glutamine sample and the tholin sample show H_2O and $2H_2O$ loss. However, the glutamine sample also shows NH_3, HCOOH, CH_3NO losses, and the tholin sample shows a fairly strong CH_5NO loss. This pattern could be explained by two

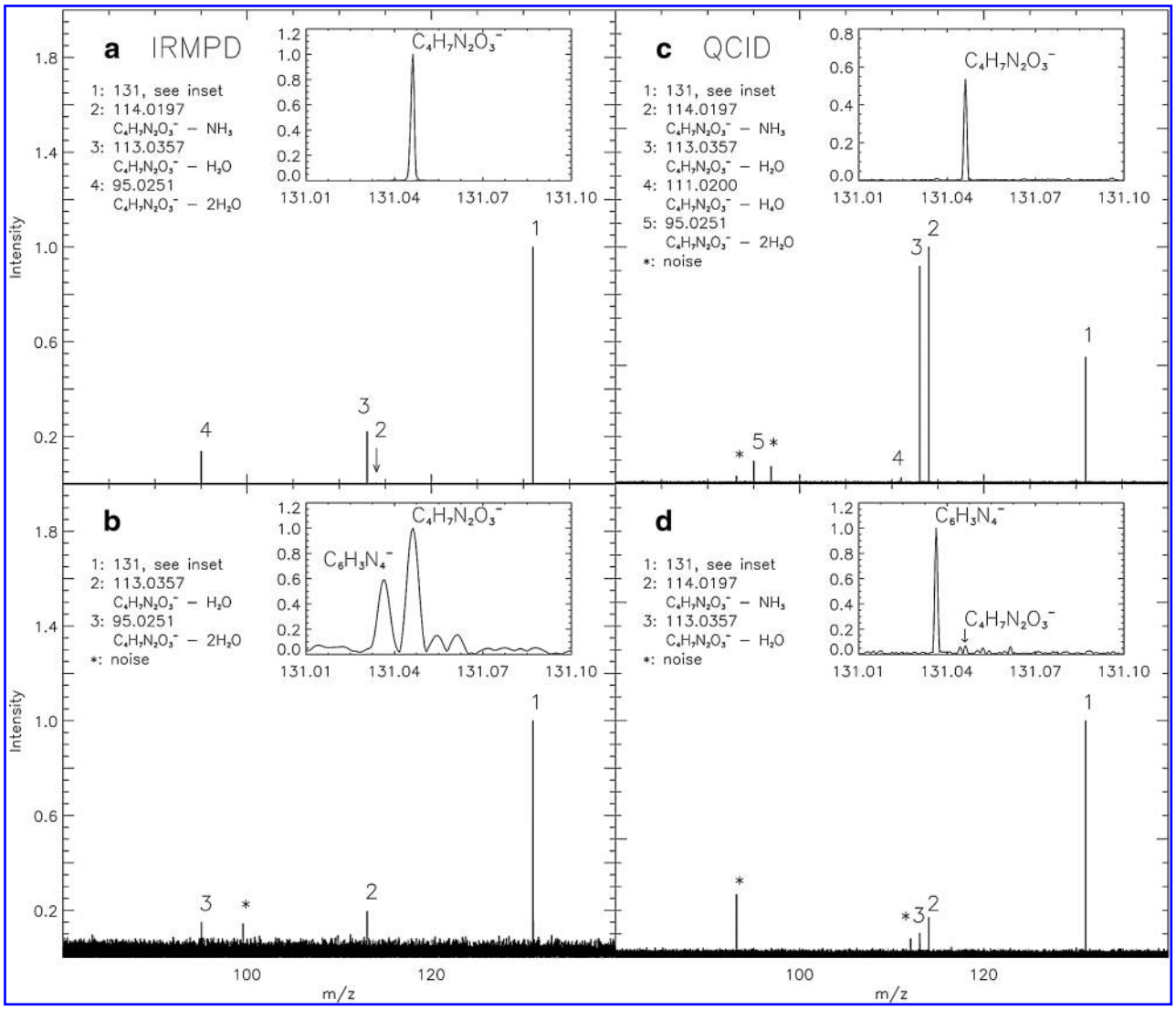

FIG. 3. Fragmentation mass spectrum of the amino acid asparagine at nominal mass m/z 131 via (**a**) IRMPD and (**c**) QCID in the negative ion mode. Fragmentation mass spectrum of a tholin sample hydrolyzed at 253 K for 1 year at nominal mass m/z 131 via (**b**) IRMPD and (**d**) QCID in the negative ion mode. The parent peaks are labeled by the number one, with a high-resolution expansion given in the inset at top right. Notable fragments are labeled by numbers, and instrumental noise peaks are marked with asterisks. The masses of the fragments are given at top left.

structural isomers being present in the tholin sample: one consistent with glutamine and another that produces the CH_3NH_2 and CH_5NO losses. In general, the data show reasonable consistencies between glutamine and the tholin species, but enough differences remain that it is unclear whether glutamine is exclusively present in the sample.

4.5. Glutamic acid

We then fragmented $C_5H_8NO_4^-$ (m/z 146.0459) in both a pure sample of glutamic acid and a tholin sample that had been hydrolyzed at 293 K for a year, using the negative ion IRMPD and QCID modes of the FT-ICR instrument. The observed fragments are reported in Table 2. The fragments agree very well between the two data sets. In the QCID mode, both samples show a strong H_2O loss, with smaller CO_2 and NH_3 losses. In the IRMPD mode, both samples show strong H_2O loss, with somewhat weaker CO_2 and $COOHNH_2$ losses. There are a few differences between the two spectra, but they are unlikely to invalidate the tentative identification of glutamic acid in the tholin sample. The COOHOH loss associated with the pure sample is likely due to the successive loss of CO_2 and H_2O (both observed in the tholin spectrum), and the $2H_2O$ loss is almost certainly too small to be observed in the noisier tholin sample (it is $\sim$5000 times less intense than the much stronger water loss). The small losses of CH_4O_2 and NH_3 in the tholin sample may be due to a second, less intense structural isomer. Despite these small differences in the fragmentation spectra, the observed fragments are generally consistent with the presence of glutamic acid in the tholin sample.

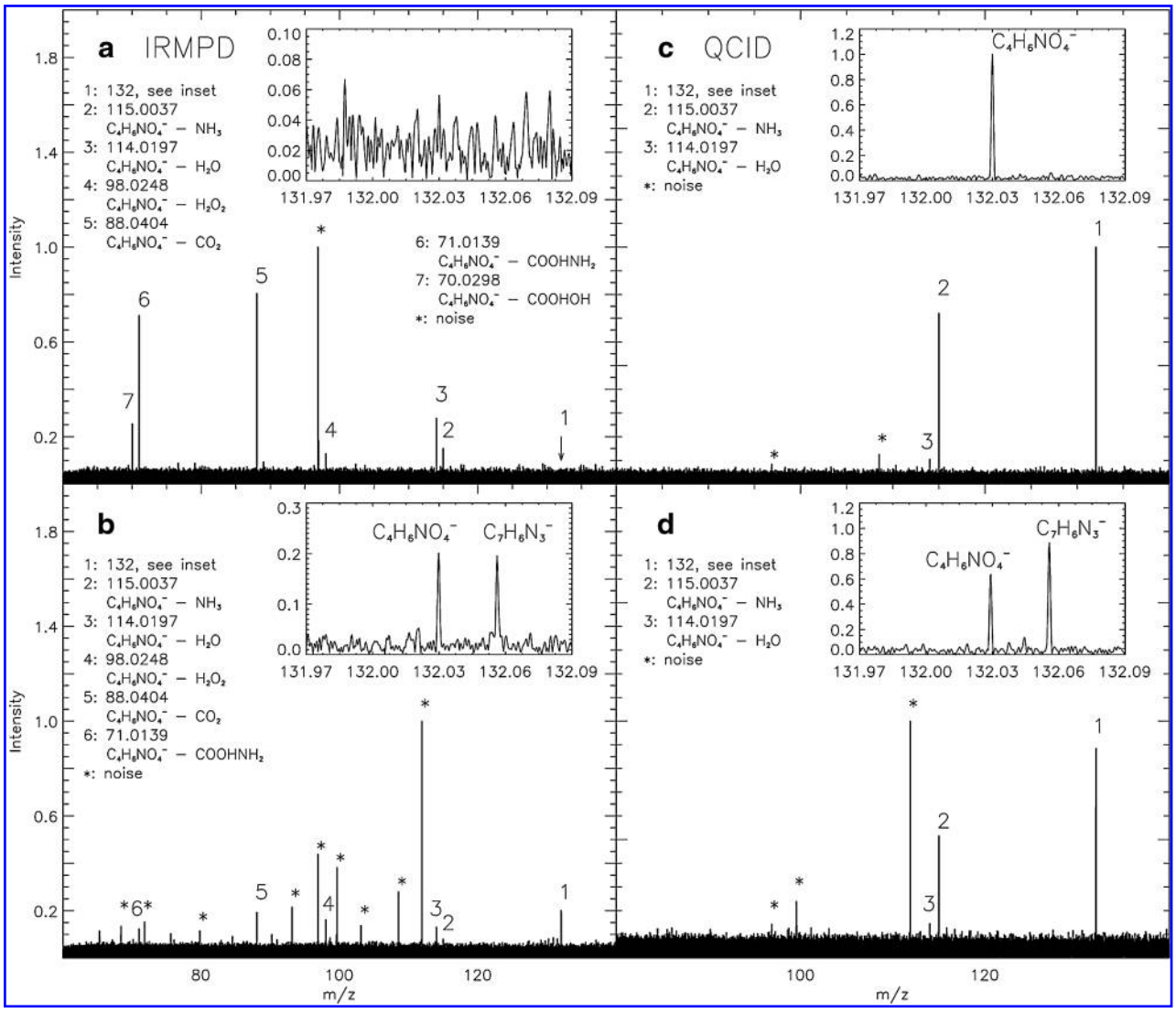

FIG. 4. Fragmentation mass spectrum of the amino acid aspartic acid at nominal mass m/z 132 via (**a**) IRMPD and (**c**) QCID in the negative ion mode. Fragmentation mass spectrum of a tholin sample hydrolyzed at 293 K for 1 year at nominal mass m/z 132 via (**b**) IRMPD and (**d**) QCID in the negative ion mode. The parent peaks are labeled by the number one, with a high-resolution expansion given in the inset at top right. Notable fragments are labeled by numbers, and instrumental noise peaks are marked with asterisks. The masses of the fragments are given at top left.

4.6. Histidine

Lastly, we fragmented $C_6H_8N_3O_2^-$ (m/z 154.0622) in both a pure sample of histidine and a tholin sample that had been hydrolyzed at 253 K for a year, using the negative ion IRMPD and QCID modes of the FT-ICR instrument. The observed fragments are reported in Table 2. The fragments from the tholin sample and the histidine sample do not agree very well. The ratio of the intensities between the NH_3 fragment and the H_2O fragment is similar in both QCID spectra, but two cyanides—C_2H_5N and HCN—are also present in the tholin spectrum (Fig. 4b). These are not observed in the fragmentation spectrum of the histidine standard, which shows more oxygen-rich fragments, such as CO_2 and HCOOH, expected fragments for a carboxylic acid (Fig. 5a). A similar pattern is observed in the IRMPD mode (Fig. 5c, 5d). Most of the larger, oxygen-rich fragments are missing from the tholin spectrum, though a cyanide loss, $C_2H_4N_2$, is observed. It therefore seems likely that the predominant structural isomer of $C_6H_8N_3O_2^-$ in the tholin sample is not histidine. This "negative" example shows the importance of fragmentation studies: the agreement between exact masses of tholin components and biomolecules is not enough to unambiguously determine structural isomerism.

4.7. Summary

By comparing fragmentation spectra from hydrolyzed tholin samples and biomolecules with the same exact mass, we found good evidence for asparagine, aspartic acid, and glutamic acid in our tholin sample, reasonable evidence for glutamine, and little to no evidence for histidine. We were unable to determine whether thymine was present in our sample, since the pure standard did not fragment under the

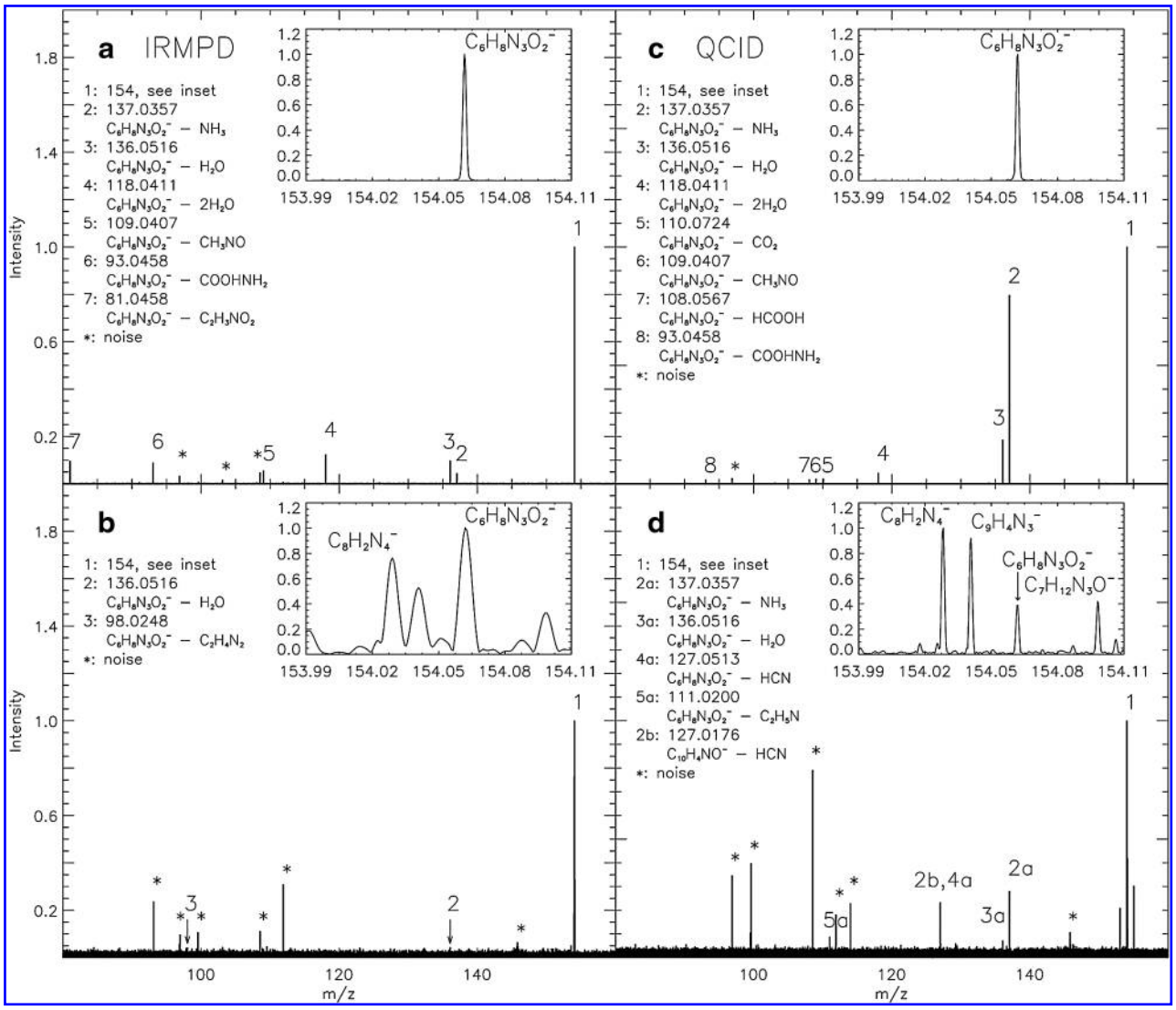

FIG. 5. Fragmentation mass spectrum of the amino acid histidine at nominal mass m/z 154 via (**a**) IRMPD and (**c**) QCID in the negative ion mode. Fragmentation mass spectrum of a tholin sample hydrolyzed at 253 K for 1 year at nominal mass m/z 154 via (**b**) IRMPD and (**d**) QCID in the negative ion mode. The parent peaks are labeled by the number one, with a high-resolution expansion given in the inset at top right. Notable fragments are labeled by numbers, and instrumental noise peaks are marked with asterisks. The masses of the fragments are given at top left.

explored conditions to produce an identifiable fragmentation spectrum. However, the behavior of the thymine standard was essentially that seen for our hydrolysis component at that mass. As mentioned earlier in the paper, it is extremely unlikely that the observed asparagine, aspartic acid, glutamine, and glutamic acid molecules were contaminants, as the masses corresponding to those molecules were not present in a nonhydrolyzed reference tholin.

It is interesting to note that, of the four biomolecules identified in this work, all have homologous structures. Glutamine is the CH_2 homologue of asparagine, while glutamic acid is the CH_2 homologue of aspartic acid. Asparagine has the same structure as aspartic acid, with the side chain carboxylic group replaced by an amide group. These similarities suggest that the particular structure these molecules possess—a hydrocarbon chain terminated by a carbonyl group—is favored in hydrolyzing tholin samples. It is also interesting to note that those molecules containing carboxylic acid functional groups (aspartic acid, glutamic acid) appear to form more readily at higher temperatures than those molecules containing amide functional groups (asparagine, glutamine). We expect that two different reactions form the two different types of molecules. These reactions almost certainly have different activation energies, which accounts for the temperature dependence. Further kinetic studies will be required to determine the activation energies of these reactions in a more quantitative manner.

It is worth mentioning that this work represents the first identification of asparagine, glutamine, and glutamic acid in a hydrolyzed tholin sample. These species were not observed in the earlier work of Khare *et al*. (1986) or Raulin *et al*. (2007). These authors observed mostly glycine, alanine, and aspartic

Table 2. Neutral Losses from the Six Species Considered in This Work, Listed in Order of Intensity for the Two Different Fragmentation Techniques

	QCID		*IRMPD*	
Species	*Standard*	*Tholin*	*Standard*	*Tholin*
Thymine ($C_5H_5N_2O_2^-$)	—	—	—	HCN
Asparagine ($C_4H_7N_2O_3^-$)	NH_3, H_2O, $2H_2O$, H_4O	NH_3, H_2O	H_2O, $2H_2O$, NH_3	H_2O, $2H_2O$
Aspartic acid ($C_4H_6NO_4^-$)	NH_3, H_2O	NH_3, H_2O	CO_2, $COOHNH_2$, H_2O, $COOHOH$, NH_3, H_2O_2	CO_2, H_2O_2, H_2O, $COOHNH_2$, NH_3
Glutamine ($C_5H_9N_2O_3^-$)	H_2O, NH_3, $2H_2O$, H_4O, CO_2, $HCOOH$, CH_5NO, $CH_2(OH)_2$	H_2O, CH_3NH_2, $2H_2O$, H_4O, NH_3	H_2O, NH_3, $2H_2O$, $HCOOH$, CH_3NO	H_2O, CH_5NO, $2H_2O$
Glutamic acid ($C_5H_8NO_4^-$)	H_2O, CO_2, NH_3	H_2O, CO_2, NH_3	H_2O, $COOHOH$, CO_2, $COOHNH_2$, $2H_2O$	H_2O, CO_2, $COOHNH_2$, CH_4O_2, NH_3
Histidine ($C_6H_8N_3O_2^-$)	NH_3, H_2O, $2H_2O$, CO_2, CH_3NO, $HCOOH$, $COOHNH_2$, H_4O	NH_3, C_2H_5N, H_2O, HCN	$2H_2O$, $C_2H_3NO_2$, H_2O, $COOHNH_2$, CH_3NO, NH_3, $HCOOH$, C_5NO_2	H_2O, $C_2H_4N_2$

Neutral losses are presented for both the pure biomolecule standards and species of the same exact mass observed in the hydrolyzed tholin sample.

acid in their samples. Glycine and alanine are too small to be detected with our instrumentation, and aspartic acid was confirmed in the tholin sample hydrolyzed at 293 K. A species consistent with aspartic acid is also present in the 253 K sample, but the signal is too weak to confirm the structure via fragmentation.

We wish to comment briefly on the yields of our products. Though the yield of any individual oxygenated species in our sample represents a relatively small portion of the sample mass (since it represents only one of hundreds of molecular formulas observed), the total amount of oxygen incorporation into our sample is quite high. Using Equation 5 from Neish *et al*. (2009), we were able to calculate the mass percent oxygen incorporated into our hydrolyzed tholin sample. Over the course of a year, the tholins incorporated 6% oxygen by mass at 253 K and 12% oxygen by mass at 293 K. This is a substantial increase from the ~2–3% oxygen yield observed after 51 hours (293 K) and 526 hours (253 K) (Neish *et al*., 2009).

It remains to be seen how much more oxygen could be incorporated into Titan's organic inventory over the longer timescales necessary for impact melts to freeze (10^2 to 10^4 years) and at the much lower temperatures encountered in cryovolcanic partial melts. Laboratory work suggests the activation energies of these reactions may be 60 ± 10 kJ/mol (Neish *et al*., 2008). If the reactions follow simple Arrhenius behavior (in which reaction rates drop off exponentially with temperature), any reaction that requires ~1 year to occur at 253 K would take 2×10^5 years at 176 K. Note that this calculation ignores any possible changes in rate due to the increasing NH_3 concentration of the solution as it freezes, and it assumes that the reactions observed to occur near 273 K have the same activation energy as reactions that may occur at 176 K, which may or may not be valid.

It is also unclear how the higher viscosity of ammonia-water solutions at low temperature may affect these reactions. At temperatures of ~200 K, the viscosity of an ammonia-water slurry could be as much as 10^3 to 10^4 K (Kargel *et al*., 1991), which is comparable to that of terrestrial basalts (10^1 to 10^4 Pa). The higher viscosity may impede the reactions. On the other hand, the concentrations of reactants at the microscopic scale may be enhanced at the freezing interface, and dramatic acceleration of reaction rates may occur. Takenaka *et al*. (1996) found that the oxidation of nitrite can be accelerated by a factor of 100,000 by freezing. In any event, given the rapid reaction timescale observed in the laboratory and the long timescales over which liquid water may be available on Titan, it seems likely that Titan's regolith—especially those areas affected by impact cratering or volcanism—contains a substantial fraction of oxygen-containing organic molecules.

5. Conclusions

In this study, we analyzed the long-term hydrolysis products of Titan tholins at low temperature (253 and 293 K) in 13 wt % ammonia water. With high-resolution mass spectrometry, we identified molecules with the same exact mass as most of the 5 nucleobases and 17 non-sulfur-containing amino acids. Using two independent fragmentation techniques (QCID and IRMPD), we were able to confirm the presence of four biological amino acids. These four species have the same molecular formula and remarkably similar fragmentation spectra to the amino acids asparagine, aspartic acid, glutamine, and glutamic acid. Aspartic acid has been detected in previous studies of tholin hydrolysis, but this represents the first detection of asparagine, glutamine, and glutamic acid in a hydrolyzed tholin sample. It also represents the first detection of biologically relevant molecules created under conditions similar to those found in impact melt pools and cryolavas on Titan. Though the formation of biomolecules on Titan is likely an ongoing process occurring even in the present age, in terms of chemical evolution, these transient liquid water environments may

represent the closest Solar System analogue to the primordial soup of life. Any future missions to Titan (for example, Coustenis *et al*., 2009) should therefore carry instrumentation capable of, but certainly not limited to, detecting amino acids and other prebiotically interesting molecules.

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Author Disclosure Statement

No competing financial interests exist.

Abbreviations

FT-ICR, Fourier transform ion cyclotron resonance; IRMPD, infrared multiphoton dissociation; m/z, mass over charge; QCID, quadrupole collision-induced dissociation.

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