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Tautomerism of uracil and related compounds: A mass spectrometry study

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Abstract

It has been demonstrated that uracil has a preponderant tautomeric form, but it is also known that different tautomers coexist in this equilibrium. In this work, mass spectrometry is used as a helpful tool to analyse the equilibria, using derivative compounds to forbid the presence of some tautomers and ion trap mass spectrometry to follow relevant fragmentation pathways. Theoretical calculations were performed to confirm tautomers abundance by energy minimization in gas phase. Analysis of mass spectra of uracil, three methyl-substituted uracils, 2-thiouracil and three benzouracils suggest that uracil exists mainly as three tautomers in gas phase: one major structure that corresponds to the classical structure of uracil (pyrimidine-2,4(1H,3H)-dione) bearing two carbonyls and two NH moieties, and two minor enolic forms (4-hydroxypyrimidin-2(1H)-one and 2-hydroxypyrimidin-4(1H)-one). Such tautomeric distribution is supported by theoretical calculations, which show that they are the three most stable tautomers.

Keywords

Uracil, tautomerism, mass spectrometry, theoretical calculations, thiouracil

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Introduction

Since many years ago, important efforts have been carried out to have a complete knowledge about DNA and RNA structure and their replication processes.[1,2] Approaching this purpose, not only was it found that uracil is one of the pyrimidine nucleobases of RNA (in DNA it is replaced by thymine, a 5-methyl derivative), but it has also been stated a highly probable connection between spontaneous point mutations developed during RNA replication and the occurrence of the rare enol tautomers of uracil.[3–7] Then, there has been great interest in predicting the stabilities of the various tautomeric forms of purine and pyrimidine bases due basically to two facts: first, the biological importance of mispairing by the rare tautomeric forms of these bases as it was previously mentioned, and second, the development of unnatural bases that could extend the genetic alphabet.

In addition, structural studies of uracil and all its tautomeric forms have been developed by different levels of theoretical approximations[8–11] as ab initio methods or semi-empirical correlations (with different basis sets), arriving in all cases to the conclusion that the dioxo form is, by far, the most stable one.[12] The dioxo structure is completely in accordance with Watson–Crick model of RNA, in which uracil in uridine must take its dioxo tautomeric form in order to be totally complementary with the normal amino tautomeric structure of adenosine. However, fluorescence excitation studies showed the coexistence of the most stable form and keto–enol tautomers, giving strong evidence about the possibility of having this kind of tautomerism involved and taking part at the moment of replication.[13]

In this work, electron ionization mass spectrometry is used as a powerful tool to study all the tautomeric equilibria,[14] not only on the current availability and ease of use of mass spectrometers but also on the coupling of these instruments to highly efficient chromatographic systems (GC or HPLC). Additionally,

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high sensitivity (it allows small sample handling) is another well-known feature. Unique peaks are used to show the existence and the relative abundance of all the forms previously presented.[15] Derivative compounds, in which one or more of the tautomers are blocked, were used to affirm that one specific peak comes from a particular tautomer and all fragmentation pathways are shown and confirmed with ion-trap mass spectra.

As an evidence to support obtained results about relative abundance, theoretical structure optimizations were carried out using Gaussian Program System, B3LYP level and 6-31G(d,p) plus 6-311++G(d,p) as bases. Several studies on the tautomeric equilibrium of diverse families of compounds have been carried out in our laboratory and, so far, mass spectrometry and semi-empirical calculations have proved to constitute powerful tools for prediction of fast equilibrium occurrence.[16] This methodology approach has been used in this work.

Experimental

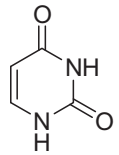
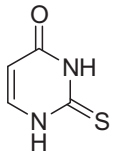
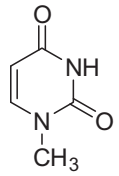
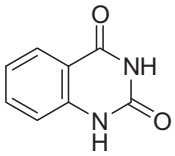
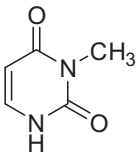
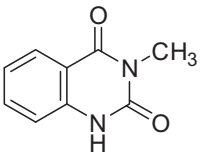
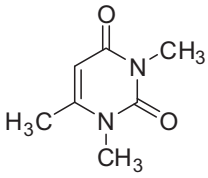
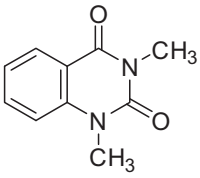
Synthesis of substituted uracils

Uracil (**1**) was commercially available (Sigma Aldrich). 1-Methyluracil (**2**), 3-methyuracil (**3**), 1,3,6-trimethyluracil (**4**), 2-thiouracil (**5**), benzouracil (**6**), 3-methyl-benzouracil (**7**) and 1,3-dimethyl-benzouracil (**8**) were synthesized according to methods from literature or their modifications.[17–20] The compounds under study were identified by ^{1}H NMR and ^{13}C NMR (Table 1).

Gas chromatography–mass spectrometry–single quadrupole

Determinations were performed by injection of methanol solutions (1 µl, 0.1%) in an HP 6890 Chromatograph coupled to an Agilent/HP 5972A mass spectrometer. The analytical column was a HP5-MS (30 m × 0.25 mm × 5 µm) using Helium as

Table 1. ^{1}HNMR and ^{13}CNMR chemical shifts (ppm) of compounds **1–8** in $CDCl_3$ at 25°C.

Compound	^{1}HNMR	^{13}CNMR	Compound	^{1}HNMR	^{13}CNMR
1 	1H (s) 11.023, 1H (s) 10.821, 1H (*J* = 7.5 Hz) (d) 7.406, 1H (*J* = 7.5 Hz) (d) 5.473	166.75 149.28 144.25 99.32	**5** 	1H (s) 11.055, 1H (s) 10.931, 1H (*J* = 10.6 Hz) (d) 7.610, 1H (*J* = 10.6 Hz) (d) 5.715	174.95 161.75 142.33 104.62
2 	1H (s) 10.845, 1H (*J* = 10.3 Hz) (d) 7.590, 1H (*J* = 10.3 Hz) (d) 6.085, 3H (s) 3.420	165.90 154.05 147.45 101.37 36.23	**6** 	1H (s) 11.060, 1H (s) 10.785 4H (m) 7.663–7.351	165.49 148.34 142.18 133.47 128.74 123.07 117.23 117.12
3 	1H (s) 11.051, 1H (*J* = 10.7 Hz) (d) 7.548, 1H (*J* = 10.7 Hz) (d) 5.895, 3H (s) 3.271	164.32 156.78 142.48 102.45 28.53	**7** 	1H (s) 11.078, 4H (m) 7.662–7.324, 3H (s) 3.312	163.80 152.39 138.38 133.47 126.75 126.68 116.32 114.68 26.87
4 	1H (s) 5.906, 3H (s) 3.385, 3H (s) 3.312, 3H (s) 2.510	164.94 152.93 146.48 100.35 40.15 28.98 21.12	**8** 	4H (m) 7.625–7.358, 3H (s) 3.455, 3H (s) 3.324	166.14 152.22 142.05 132.98 128.04 123.81 116.21 116.01 32.05 27.87

carrier gas (0.6 ml/min). The temperatures set points were: 200°C at the injector, 300°C at the interface, 185°C at the ion source and the oven ramp was 40°C (5 min), 20°C/min, 290°C. The electron energy was 70 eV and the pressure in the mass spectrometer was low enough ($<10^{-5}$ torr) as to preclude ion-molecule reactions (no autoprotonation observed). No tautomer separation could be achieved within the experimental conditions used in this work. Total ion chromatograms for compounds **1–8** are presented as Supplementary Material.

Gas chromatography–mass spectrometry–ion trap

These determinations were performed by injection of methanol solutions (1 µl) in a Thermo Quest Trace 2000 coupled to Finnigan Polaris ion trap detector (unit mass resolution) under the same conditions already mentioned for the single quadrupole GC/MS system. This instrumentation was utilized to confirm proposed fragmentation pathways by CID (collision induced dissociation) using helium as collision gas, a CID voltage of 5–7 eV and an excitation energy of 0.35–0.45 eV (values were optimized for each ion transition). These experiments were done by selecting a precursor ion from the full-scan spectrum and carrying out the corresponding MS/MS product ion scan.

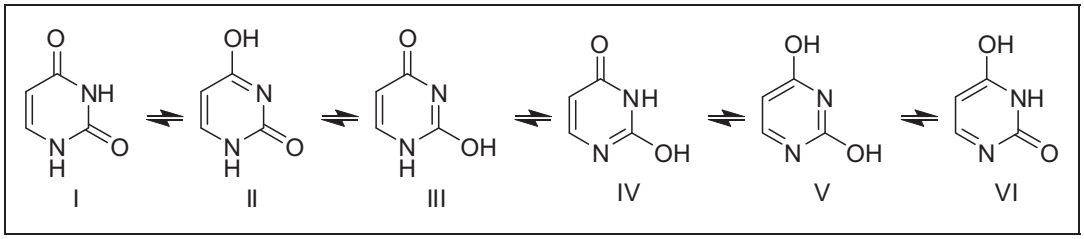
Theoretical calculations

There are several computational procedures for treating tautomeric equilibria, being density functional theory (DFT) methods[21] preponderant over the last few decades. Given the enormous number of available functionals, the prediction of the tautomerism by quantum chemistry depends strongly on the DFT functional and basis set used.[22] All tautomers of compounds under study were subjected to geometry optimizations using the DFT. In order to achieve this, B3LYP hybrid exchange-correlation functional[23] together with the 6-31G(d,p) basis set as implemented in the Gaussian 03 package[24] was used. Numerous conformations were computed to ensure that the lowest energy conformation was obtained for each molecular system. All geometrical parameters were optimized without constraints. Structures of minimum energy drawn from these curves were re-optimized using the 6-311++G(d,p) basis set.

Results and discussion

Uracil (**1**) can exist, at least at first, as six possible tautomeric forms (named **I–VI** from now on), which are shown in Scheme 1.

Figure 1 shows the mass spectrum of uracil, in which the spectra of all tautomers in equilibrium (that arrive to the ion source) are overlapped.



Scheme 1. Tautomeric forms of uracil.

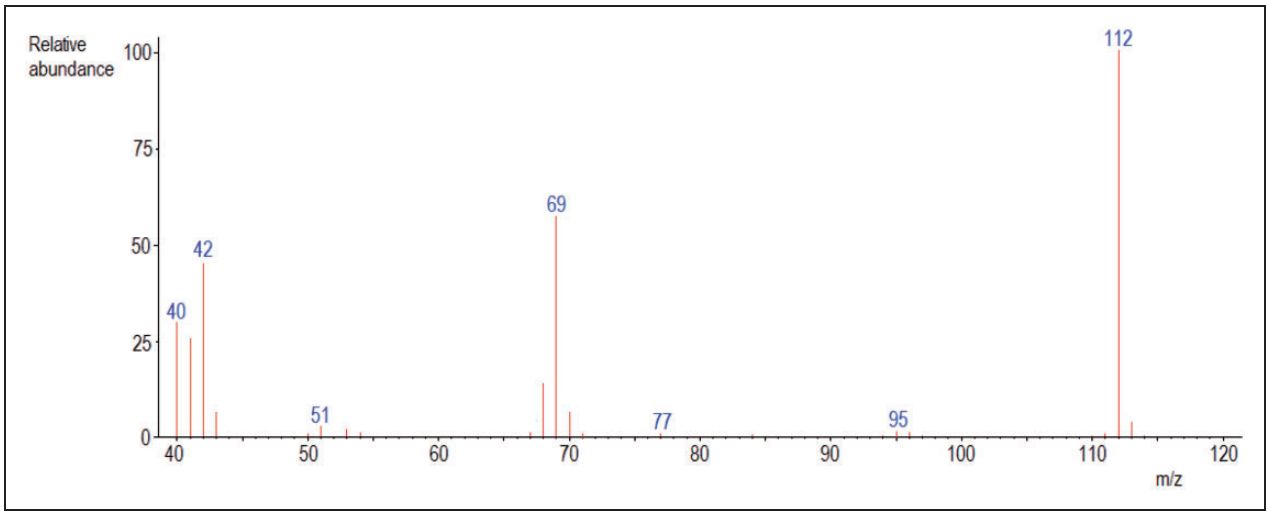


Figure 1. Mass spectrum of uracil (**1**).

Previous works carried out on a wide variety of carbonyl and thiocarbonyl compounds[25–31] support the following two assumptions and they all lead to the conclusion that the observed spectrum is the superposition of all tautomers:

1. Once the tautomers are turned into radical cations in the ion source, they do not interconvert (i.e. the molecular ion does not appear to suffer tautomerization).
2. The tautomers are not separated in the chromatographic system (even when such separation has been exceptionally seen for some compound families).[32]

In order to have a clue on the tautomers that are really taking place in the equilibrium (at least in appreciable concentration), the mass spectra of methyl-substituted uracils were analysed. Figures 2 to 4 show the mass spectra of 1-methyluracil (**2**), 3-methyluracil (**3**) and 1,3,6-trimethyluracil (**4**).

It is important to notice at this step that **2** can exist as tautomers **I**, **II** and **III**; **3** can exist as tautomers **I**, **IV** and **VI**; and **4** can only exist as tautomer **I**.

MS spectra of compounds **1** and **3** show a peak at m/z 68, which can be rationalized from the fragmentation pathway shown in Scheme 2. Analogously, MS spectra of compounds **2** and **4** show a peak at m/z 82, that can be explained following the same fragmentation pathway. These ions, with proposed formula $RN{=}C{=}CHCO^+$, can be assigned to tautomer **I** (the only possible common tautomeric structure for all four compounds).

The peak at m/z 42 (which appear in the four spectra) is not specific given that it can correspond either to NCO^+ or to $C_2H_2O^+$ and it will be not included in the analysis.[33] Peaks at m/z 69 and 83 (whose abundance is relatively high in the spectra) can be obtained from several of the tautomeric forms proposed in Scheme 1 and so they are not useful for discrimination of tautomers.

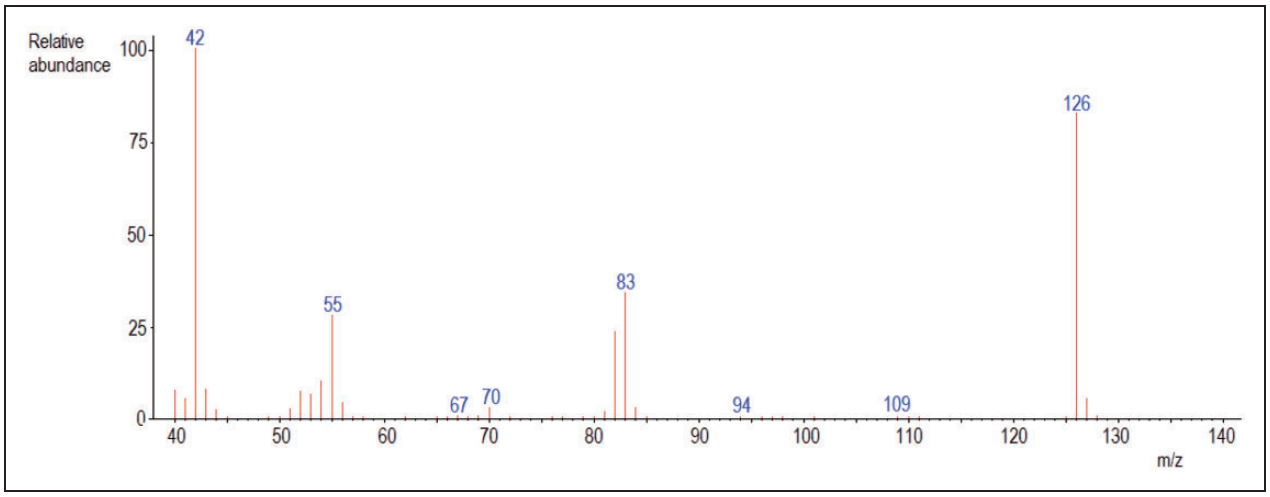


Figure 2. Mass spectrum of 1-methyluracil (**2**).

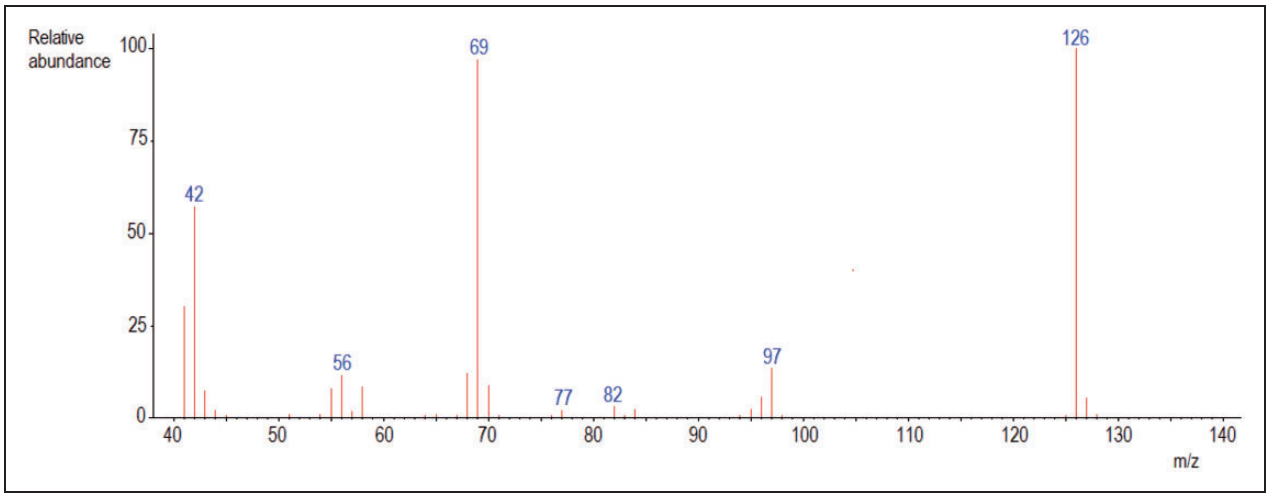


Figure 3. Mass spectrum of 3-methyluracil (**3**).

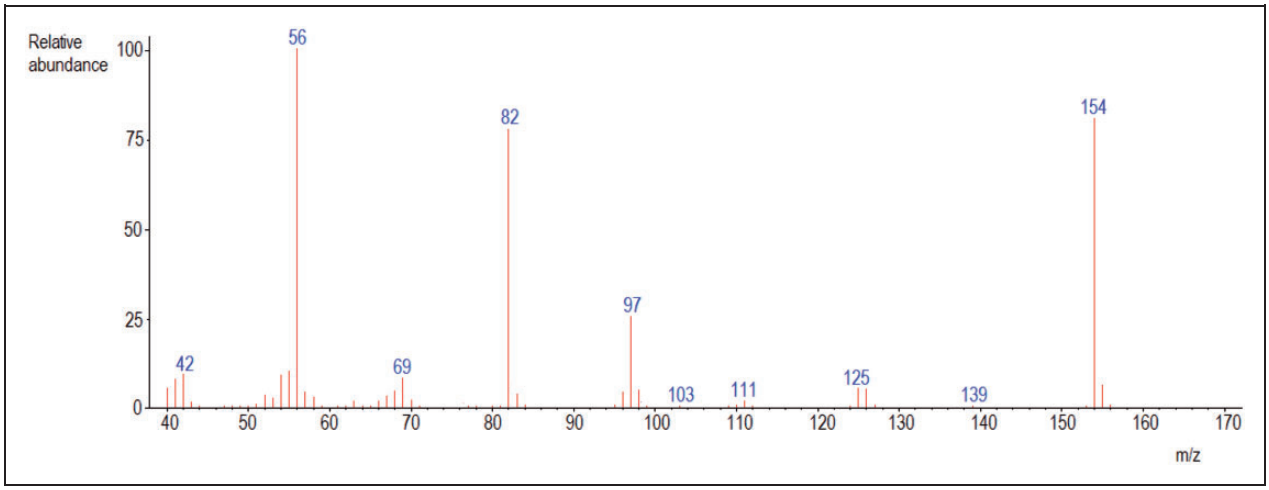
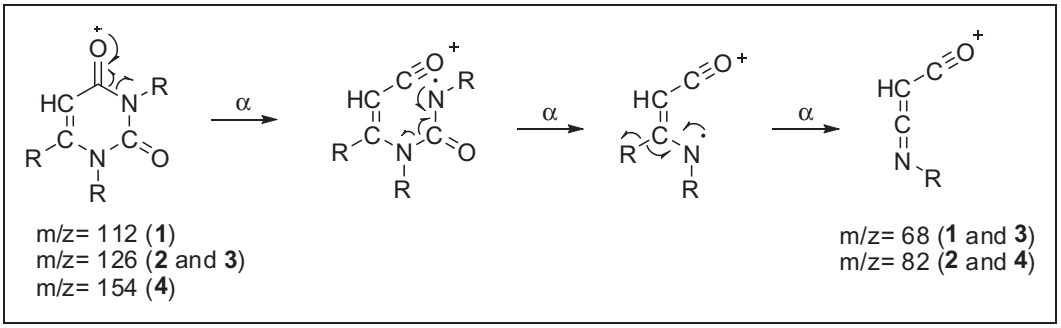


Figure 4. Mass spectrum of 1,3,6-trimethyluracil (**4**).



Scheme 2. Proposed fragmentation pathway for N,N-disubstituted uracils.

Mass spectrum of uracil (Figure 1) shows a small but detectable peak at m/z 95 $(M-17)^+$ which can be assigned to the loss of OH. This peak can correspond to any of the enolic tautomers **II**, **III**, **IV**, **V** or **VI**. Thus, it confirms the presence of other tautomers besides **I**, without specifying which of them. In fact, **4** (which can exist only as **I** tautomer) lacks completely of peak $(M-17)^+$.

Mass spectrum of **2** (which can exist as **I**, **II** and **III**) shows an $(M-17)^+$ peak at m/z 109, and this suggest that **II** and/or **III** are present in the equilibrium of uracil. Besides, mass spectrum of **3** (which can only exist as **I**, **IV** and **VI**) does not show a detectable $(M-17)^+$ peak. This fact precludes the presence of tautomers **IV** and **VI**.

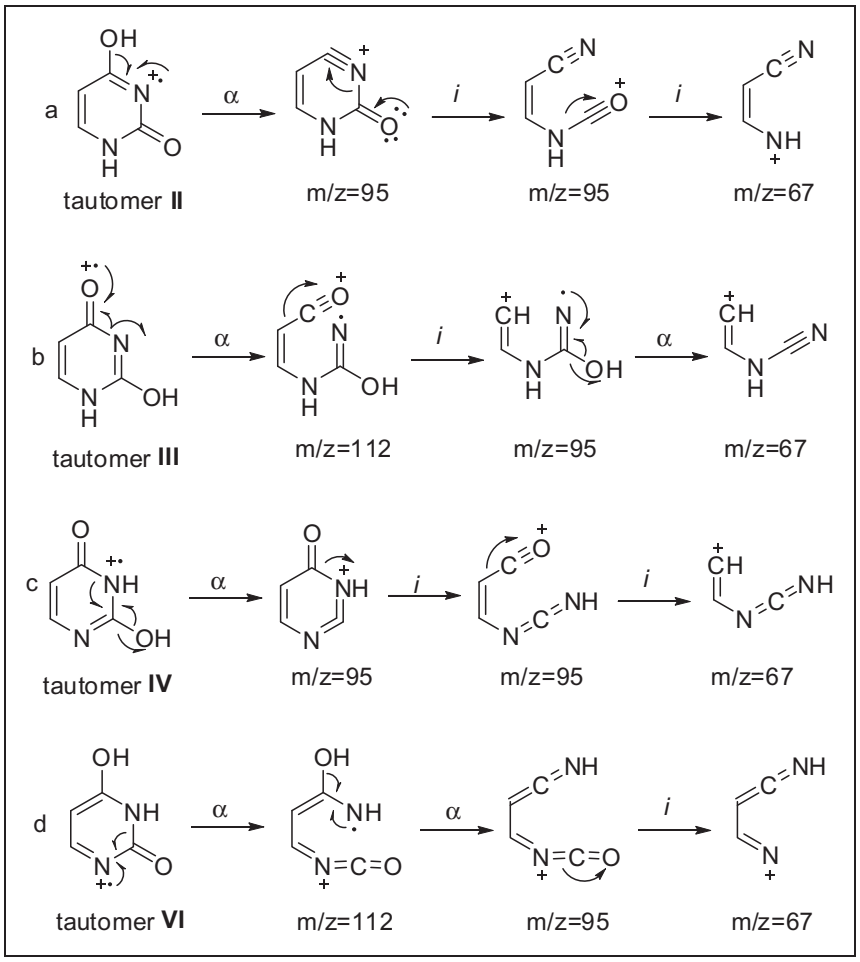
Additionally, peak at m/z 67 in Figure 1 can only be rationalized from tautomers **II**, **III**, **IV** and **VI** (but not from **I**), as shown in Scheme 3. The corresponding peak in the spectrum of **3** should be m/z 81 and it is not present in its mass spectrum; while the corresponding peak for **2** should be m/z 81 for tautomers **II** and **III**. This peak appears in the spectrum of **2** and this fact suggests again that tautomers **II** and/or **III** are present.

The corresponding fragmentation pathway for tautomer **IV** in **3** (Scheme 3c) should give ions at m/z 126-109-109-81, while the fragmentation pathway for tautomer **VI** (Scheme 3d) should be 126-126-109-81. Neither m/z 109 nor m/z 81 ions are seen in Figure 3, and this fact supports the absence of tautomers **IV** and **VI**.

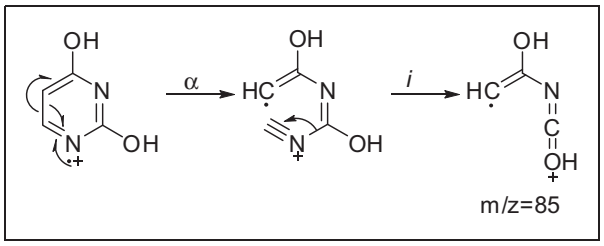
The corresponding fragmentation pathways for tautomers **II** and **III** in **2** (Scheme 3a and b) would be m/z 126-109-109-81 and 126-126-109-81, respectively. All these ions are present in the spectrum of **2**.

If tautomer **V** was present, then Scheme 4 shows a plausible fragmentation pathway for it, which would give a peak at m/z 85 and this peak can only be rationalized from this specific tautomer. Such peak is not detectable in the mass spectrum of uracil, and this fact suggests that tautomer **V** is negligible in the equilibrium of uracil.

In order to confirm the previous assumption about tautomer **V**, mass spectrum of 2-thiouracil (**5**) was measured and it is shown in Figure 5. If tautomer **V** was present in this compound (which would be more plausible than for uracil according to previous studies[16,25]), then a fragmentation pathway analogous to Scheme 4 should take place and give a peak at m/z 101. Such peak is not detectable in the spectrum of **5** and then it supports the assumption that tautomer **V** is not present.



Scheme 3. Proposed fragmentation pathways to explain fragments at m/z = 95 and 67 from tautomer **I**, **II**, **IV** and **VI**.



Scheme 4. Proposed fragmentation pathway to show an exclusive fragment from tautomer **V**.

Benzouracil (**6**), 3-methyl-benzouracil (**7**) and 1,3-dimethyl-benzouracil (**8**) mass spectra have also been recorded in order to confirm the previous assumptions (Figures 6 to 8). **6** can exist as **I–VI** tautomers, but only **I**, **IV** and **VI** are possible for **7**. **8** can only exist as tautomer **I**.

Some conclusions that support the previous assumptions can be drawn from these three spectra:

- The peak at m/z 92 in **6** and **7** spectra may correspond to $PhNH_2^+$, which corresponds to tautomer **I** present in each species (according to fragmentation pathway shown in Scheme 5a and b). Their analogue in compound **8** is the ion at m/z 104, corresponding to $PhNHCH_3^+$.
- The ion at m/z 117 (analogous to m/z 67 in uracil) is present in **6**, but that one at m/z 131 (which would correspond to tautomers **IV** and **VI** according to Scheme 3c and d) do not appear in the spectrum of **7**. This fact supports the absence of tautomers **IV** and **VI** in the system.
- The peak at m/z 117 in **6** corresponds to tautomers **II** and **III**, following a fragmentation pathway analogous to that shown in Scheme 3a and b.
- Compound **6** shows an $(M\text{-}17)^+$ peak at m/z 145, but **7** and **8** lack of $(M\text{-}17)^+$ ion, which should appear at m/z 159 and 173, respectively. Then, **7** shows no **IV** nor **VI** tautomers.
- Compound **6**, the only one capable of existing as tautomer **V**, lacks of peaks at m/z 111 and 85. These two peaks should appear (according to Scheme 6) if tautomer V was present. This fact suggests that tautomer V does not exist in equilibrium (or, if it does, it is not detectable).

All these observations support the previous assumptions: keto tautomer **I** is present in the system, and

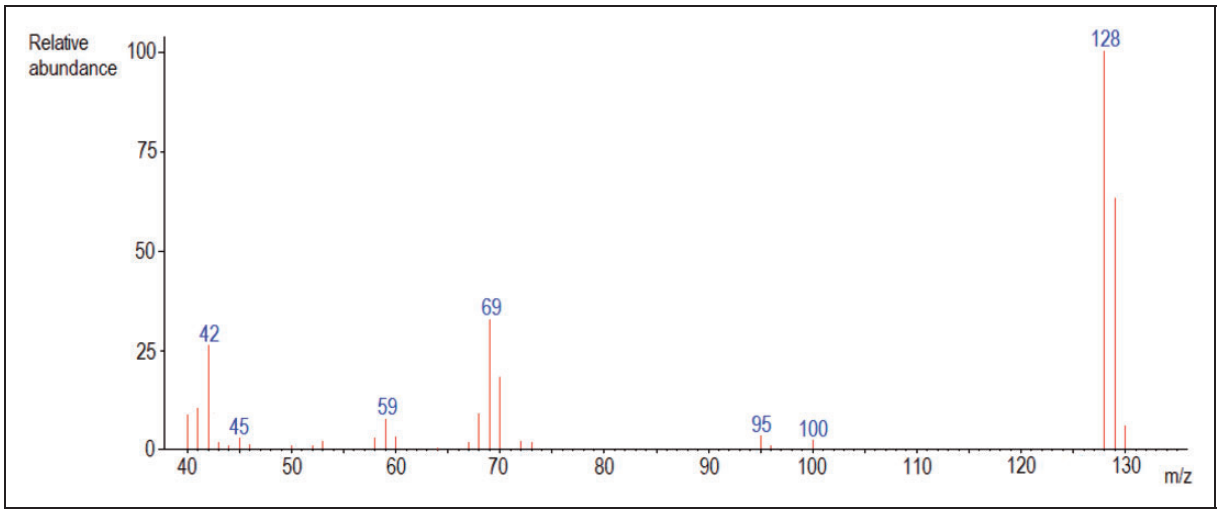


Figure 5. Mass spectrum of 2-thiouracil (**5**).

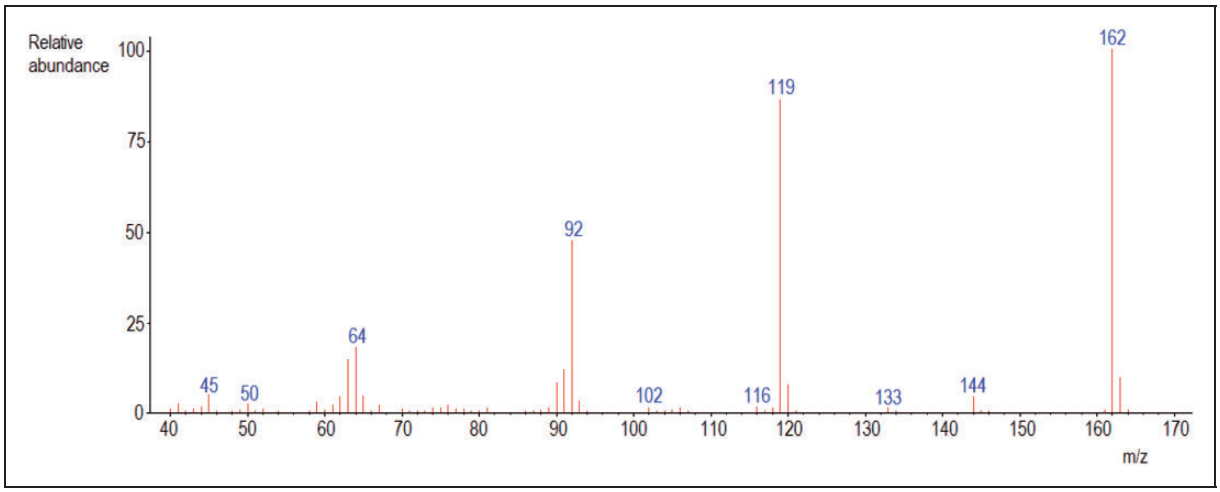


Figure 6. Mass spectrum of benzouracil (**6**).

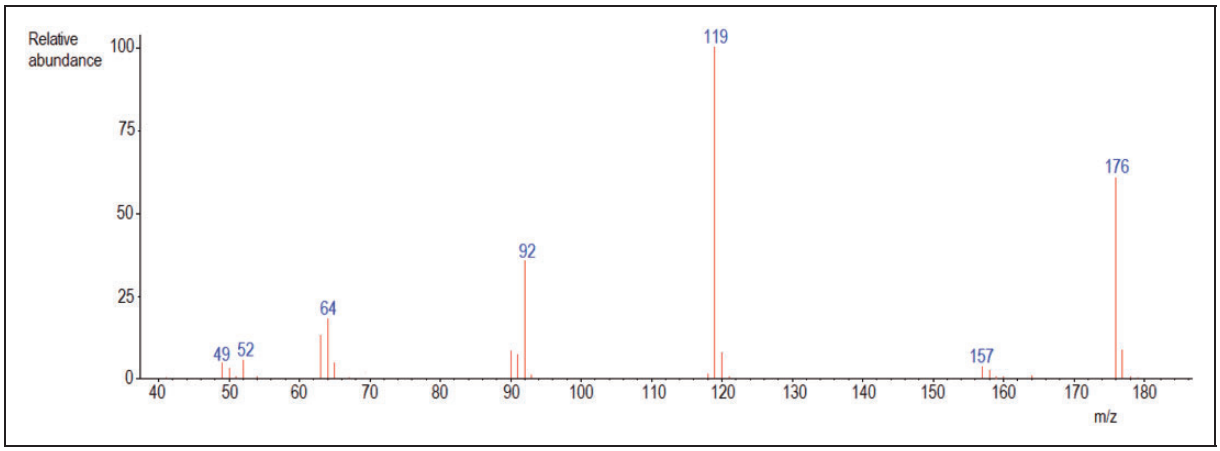


Figure 7. Mass spectrum of 3-methyl-benzouracil (**7**).

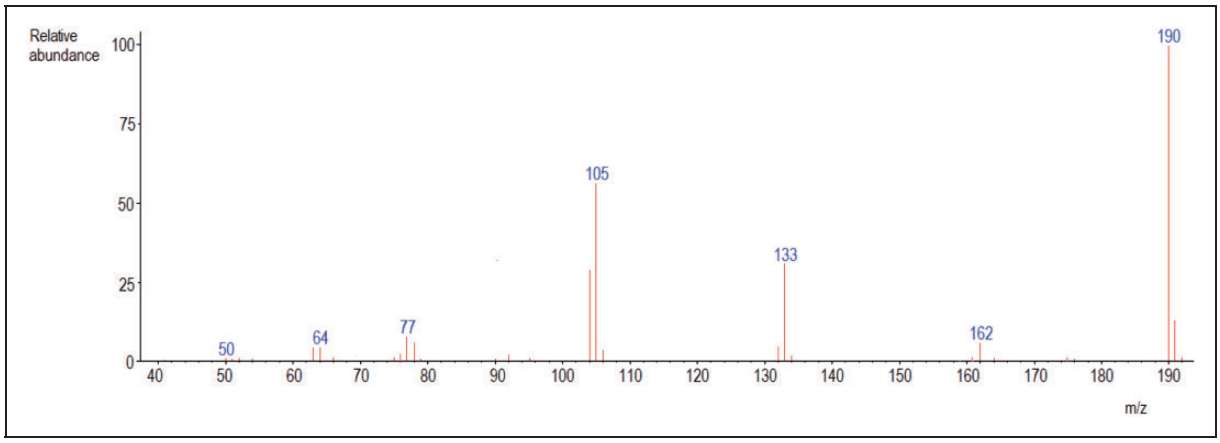
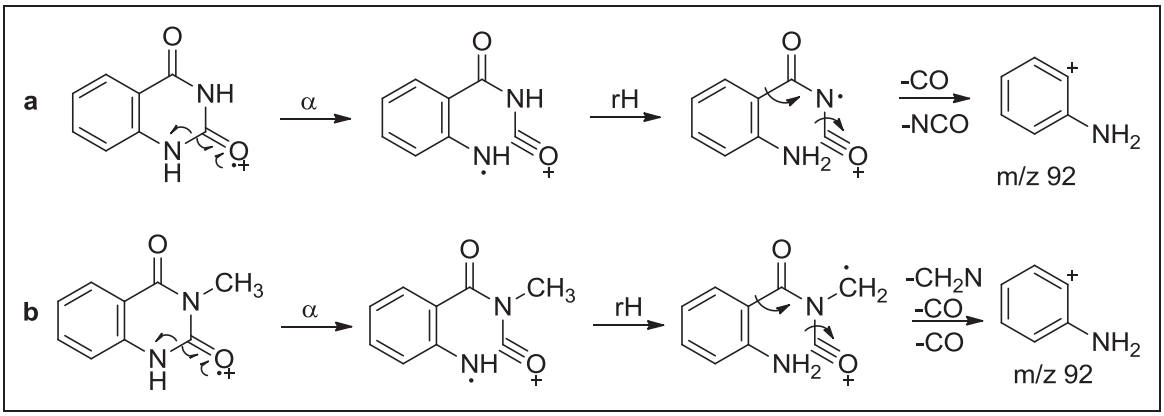
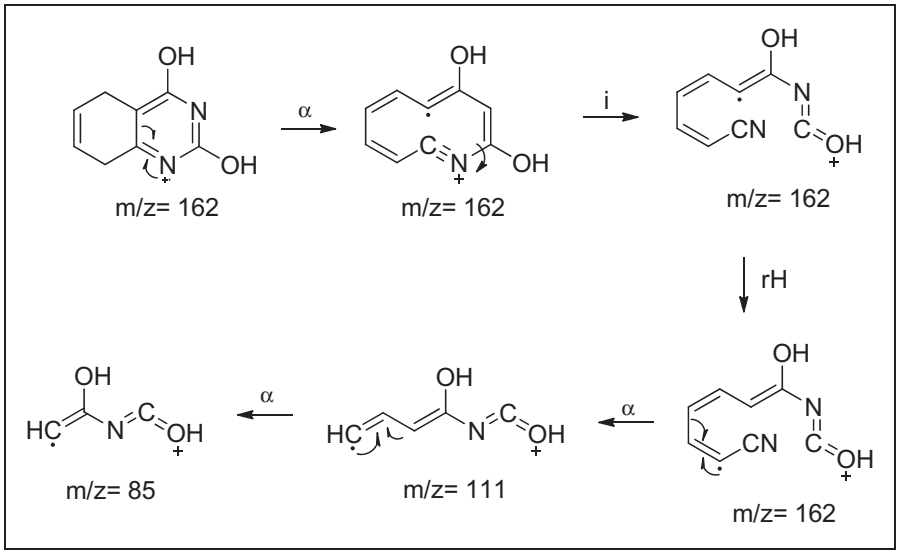


Figure 8. Mass spectrum of 1,3-dimethyl-benzouracil (**8**).



Scheme 5. Proposed fragmentation pathway to explain the fragment at m/z 92 from benzouracil.



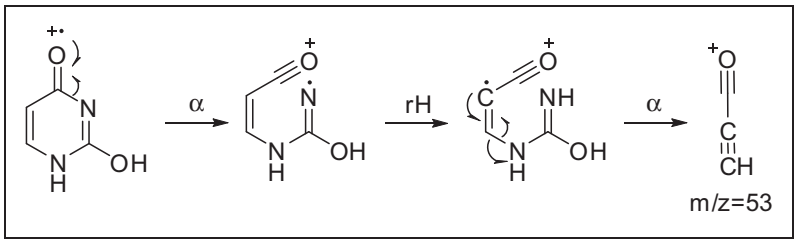
Scheme 6. Proposed fragmentation pathway to show the absence of tautomer **V** in compound **6**.

enolic tautomers **II** and **III** are also detectable, but not tautomers **IV**, **V** or **VI**.

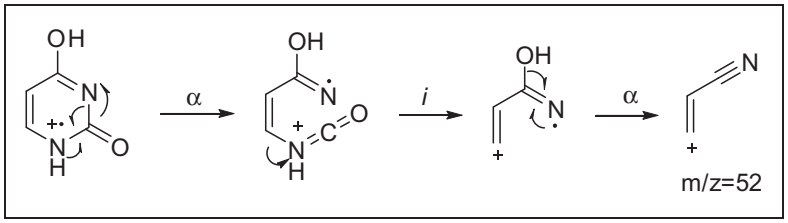
There are two peaks that are specific for tautomers **II** and **III** and confirm their presence in uracil:

The peak at m/z 53 (Figure 1) can come from tautomers **III**, **IV** or **V**, but not from **I**, **II** or **VI** (Scheme 7). Since **IV** and **V** are not present (at least in appreciable concentrations), then this peak confirms the presence of tautomer **III**.

As expected, this peak appears in the MS of **2** and **5** (in which tautomer **III** is possible), and it is absent in the MS of **3** (in which tautomer **III** is not an option).



Scheme 7. Proposed fragmentation pathway to explain the formation of the fragment at m/z 53.



Scheme 8. Proposed fragmentation pathway to confirm the presence of tautomer **II** in uracil.

As expected, this ion (or its analogues) do not appear in the spectra of **6** or **7**, since the fragmentation pathway proposed in Scheme 7 cannot take place in these compounds; however, the absence of this fragment is not an evidence neither for nor against the presence of tautomer **III** in compound **1**.

The peak at m/z 52 (Figure 1) can come from tautomers **II** or **V**, but not from **I**, **III**, **IV** or **VI**. Since **V** is not present, then this peak confirms the presence of tautomer **II** in the system. The ion may be formed from tautomer **II** following the fragmentation pathway shown in Scheme 8.

In fact, this ion appears in the spectrum of **2** (in which tautomer **II** is possible) but does not appear in the spectrum of **3** (in which **II** is not possible). The analogous peak at m/z 102 is observed in **6** but not in **7** or **8**.

These two facts highlight that, besides keto tautomer **I**, tautomers **II** and **III** are present in the equilibrium.

MS/MS experiments

The fragmentation pathways proposed in Scheme 6 (and their analogues for compound **6**) were confirmed by GC-MS-ion trap experiments: mass spectra of the $(M\text{-}17)^+$ fragments for compounds **1**, **2** and **6** have been recorded, collecting their product ions. Table 2 shows the most abundant product ions for precursor $(M\text{-}17)^+$ ions, i.e. m/z 95, 109 and 145 for **1**, **2** and **6**, respectively.

The ions at m/z 41, 67 and 69 are generated from the ion at m/z 95 in uracil (**1**), while the ions at m/z 55, 81 and 83 are generated from the ion at m/z 109 in **2** and those at m/z 91, 117 and 119 come from m/z 145 in **6**. These results confirm the presence of tautomers **II** and/or **III** in **1**, **2** and **6** (according to Scheme 3), not being specific about which of them is present and which one not.

Table 2. MS^2 Data for uracil (**1**), 1-methyluracil (**2**) and benzouracil (**6**).

Compound	Precursor ion (m/z)	Relevant product ions (m/z)
1	95	69, 67, 41
2	109	83, 81, 55
6	145	119, 117, 91

Table 3. Energy values (kJ/mol) for tautomers I-VI at B3LYP/6-311++G(d,p) theory level.

Tautomer	I	II	III	IV	V	VI
Energy (kJ/mol)	0.0	50.6	47.7	81.6	79.1	80.8

Ions at m/z 41, 69 (for **1**), m/z 55 and 83 (for **2**) and m/z 91 and 119 (for **6**) can be rationalized following classical fragmentation pathways[34] for tautomers **I**, **II** and **III**, and thus they do not give specific information.

Theoretical calculations

Theoretical calculations have been carried out in order to support the experimental data. In principle, different conformations are expected for enolic tautomers **II**, **III**, **IV**, **V** and **VI** depending on the mutual orientations of the pyrimidine ring and the OH moieties. Thus, the computed potential energy curves around the C–N–O–H dihedral angles involved in these conformations were explored (using B3LYP method and 6-31G(d,p) basis set) and the structures of minimum energy drawn from these curves were re-optimized using the 6-311++G(d,p) basis set.

Table 3 contains the energies corresponding to each tautomer from compound **1**, relative to tautomer **I** which bears the lowest energy value. It can be seen that tautomers **II** and **III** show the lowest energies among the enolic tautomers. **IV**, **V** and **VI** have energy values more than 30 kJ/mol higher than **II** and **III** and then they are not present in appreciable amount. Then, theoretical calculations clearly support the experimental observations.

Conclusions

Mass spectra of uracil and three methyl derivatives suggest that the former exists mainly as three tautomers in gas phase (at least under the experimental conditions of this work): one major structure corresponding to the classical structure of uracil (pyrimidine-2,4(1H,3H)-dione, tautomer **I**) bearing two carbonyls and two NH moieties, and two minor enolic forms (4-hydroxypyrimidin-2(1H)-one and 2-hydroxypyrimidin-4(1H)-one, tautomers **II** and **III**, respectively). The remaining three possible tautomeric forms were not detected. Analysis of mass spectra of thiouracil and benzouracil analogues confirm the predominance of such tautomers over the other possible structures. GC-MS-ion trap experiments confirmed the proposed fragmentation pathways. Theoretical calculations confirm these assumptions, since the three proposed tautomers show the lowest energy values. This job shows that mass spectrometry (together with theoretical calculations) is a very useful tool for studying tautomerism of organic compounds in the gas phase.

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