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Abstract

In order to assist with the development of more selective and sensitive methods for thyroid hormone analysis the [M-H]– anions of the iodothyronines; T4, T3, rT3, (3,5)-T2 and the non-iodinated thyronine (T0) have been generated by negative ion electrospray mass spectrometry. Tandem mass spectra of these ions were recorded on a triple quadrupole mass spectrometer and show a strong analogy with the fragmentation pathways of the parent compound, tyrosine. All iodothyronines also show significant abundances of the iodide anion in their tandem mass spectra, which represents an attractive target for MRM analysis, given that iodothyronines are the only iodine bearing endogenous molecules. Characteristic fragments are observed at m/z 359.7 and 604.5 for rT3 but are absent in the spectrum of T3 thus differentiating the two positional isomers. The striking difference in the fragmentation patterns of these regioisomeric species is attributed to the increased acidity of the phenol moiety in rT3 compared with T3.

Keywords

thyroid hormone, iodothyronines, tyrosine, mass spectrometry, negative ion, electrospray ionization, collision induced dissociation, CMMB

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Tandem mass spectrometry of deprotonated iodothyronines

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Abstract

In order to assist with the development of more selective and sensitive methods for thyroid hormone analysis the $[M-H]^-$ anions of the iodothyronines; T4, T3, rT3, (3,5)-T2 and the non-iodinated thyronine (T0) have been generated by negative ion electrospray mass spectrometry. Tandem mass spectra of these ions were recorded on a triple quadrupole mass spectrometer and show a strong analogy with the fragmentation pathways of the parent compound, tyrosine. All iodothyronines also show significant abundances of the iodide anion in their tandem mass spectra, which represents an attractive target for MRM analysis, given that iodothyronines are the only iodine bearing endogenous molecules. Characteristic fragments are observed at *m/z* 359.7 and 604.5 for rT3 but are absent in the spectrum of T3 thus differentiating the two positional isomers. The striking difference in the fragmentation patterns of these regioisomeric species is attributed to the increased acidity of the phenol moiety in rT3 compared with T3.

Keywords thyroid hormone, iodothyronines, tyrosine, mass spectrometry, negative ion, electrospray ionization, collision induced dissociation

Introduction

Thyroid hormones are iodothyronines derived from the amino acid tyrosine and are the only endogenous molecules to contain iodine. They are secreted by the thyroid gland, which, in humans is a butterfly-shaped gland located just below the larynx in the neck.[1] These compounds are involved in the regulation of various pivotal biological processes including growth and development, carbohydrate metabolism, oxygen consumption, protein synthesis and foetal development.[2-4] It has also been proposed that these molecules may act as antioxidants.[3,5]

Thyroxine (T4 or 3,5,3′,5′-tetraiodothyronine) was first crystallised as early as 1914 and its structure was correctly determined by 1926.[6] T4 is a hydroxyphenyl ether of tyrosine containing four iodine atoms located in the 3- and 5-positions of each benzene ring (Figure 1a). Other homologues have also been subsequently characterized including 3,5,3′-triiodothyronine (T3), 3,3′,5′-triiodothyronine (rT3) and 3,5-diiodothyronine (T2) for which structures are shown, along with the parent thyronine (T0), in Figure 1. As the structure of these molecules suggests, thyroid hormones are highly lipophilic at physiological pH with T4 having a phosphatidyl choline/aqueous partition coefficient of between 12,000-23,500.[7,8] As a consequence, more than 99% of thyroid hormones (T4 and T3) circulating in the plasma are bound to plasma proteins such as thyroxine-binding globulin.[9]

[FIGURE 1]

Measurement of thyroid hormone levels in blood are essential to be able to assess thyroid function, that is, to monitor hyper- or hypo-thyroidism. For example, measurement of high levels of plasma T3 is critical for diagnosing hyperthyroidism. Reliable measurement is also important for adjusting antithyroid drug dose.[10] The detection and differentiation of thyroid hormone homologues is also important for understanding the biochemical relationships and varying functions between

these species *in vivo*. For example, T4 is known to be secreted directly from the thyroid gland, while most T3, and all rT3, T2 and T0 are produced by peripheral deiodination of T4.[3]

There are numerous problems associated with the analysis of thyroid hormones which can cause disagreement among values obtained by different analytical methods.[11] This has lead to a recent interest in the use of liquid chromatography-mass spectrometry (LC-MS),[10-15] which has several advantages over existing radio-immunoassay (RIA) and gas chromatography-mass spectrometry (GC-MS) methods.[16] LC-MS analyses of thyroid hormones generally utilize single ion monitoring (SIM) whereby the $[M+H]^+$ or $[M-H]^-$ ions are detected by electrospray ionisation mass spectrometry (ESI-MS) in chromatographic effluent. Negative ion analysis has some advantages given the acidity of the thyroid hormones and the potential to observe unique iodide fragment anions by tandem mass spectrometry. For example, Soukhova *et al*. utilized single reaction monitoring (SRM) techniques to monitoring the $[T4-H]^-$ (*m/z* 777) $\rightarrow I^-$ (*m/z* 127) fragmentation channel for T4 quantitation using a triple-quadrupole mass spectrometer.[10] Such methods can enhance the sensitivity and selectivity of thyroid hormone detection and quantitation, particularly in complex matrices. Although several simple fragmentations have been utilized in SRM experiments, to-date there has been no comprehensive study investigating the collision induced dissociation of this class of molecule. The ability to measure and differentiate between the various iodothyronines in the same sample is an important investigative tool for understanding the various deiodinative pathways involved in the metabolism of thyroid hormones.

As a part of a larger study to investigate the role of thyroid hormones as potential membrane anti-oxidants, we present here a detailed study of the fragmentation pathways of thyronines upon collision induced dissociation and propose mechanisms to account for the product ions observed.

Experimental

Mass Spectrometry

Thyronines, tyrosine, tyrosine methyl ester and 4-hydroxycinnamic acid were obtained from Sigma-Aldrich (Castle Hill, Australia) and were used without further purification. Standard solutions of 10 μM were prepared in methanol:chloroform (approximately 2:1 by volume) with the pH adjusted to 9-10 using aqueous ammonia. Deuterium exchange was performed using d_1-methanol (99.8 atm%). Mass spectra were obtained using a QuattroMicro triple quadrupole mass spectrometer (Waters, Manchester, UK). Spectra were obtained by infusion of the standard solution (10 μL/min), typical settings were cone voltage 60 V, capillary voltage 3 kV, source temperature 80 ºC. ESI-MS spectra were obtained by scanning Q1 while operating Q3 in R_f-only mode. Resolution for ESI-MS and ESI-MS/MS experiments was typically 0.7 Th across the entire mass range. ESI-MS/MS spectra were obtained by mass-selecting the parent ion using Q1 and scanning for product ions using Q3. Argon was used as the collision gas at a pressure of 4×10^{-3} Torr. ESI-MS and ESI-MS/MS spectral data presented in this paper result from the average of at least 50 scans. The data was baseline subtracted (40% background subtract with a first order polynomial) and smoothed (two mean smooths using a peak width of 0.7 Th) using the MassLynx software (Waters, Manchester UK). The high resolution ESI-MS/MS spectrum of deprotonated tyrosine was obtained using a Q-ToF2 quadrupole-time of flight instrument (Waters, Manchester, UK).

Results and Discussion

The negative ion ESI-MS/MS spectra of the $[M-H]^-$ anions formed from the thyronines T4, T3, rT3, T2 and T0 were recorded using a triple quadrupole mass spectrometer and the spectra are presented in Figure 2. In order to rationalise the fragments, the corresponding spectrum of the $[M-H]^-$ anions formed from the ESI of tyrosine was also recorded under the same instrumental conditions and is presented in Figure 3.

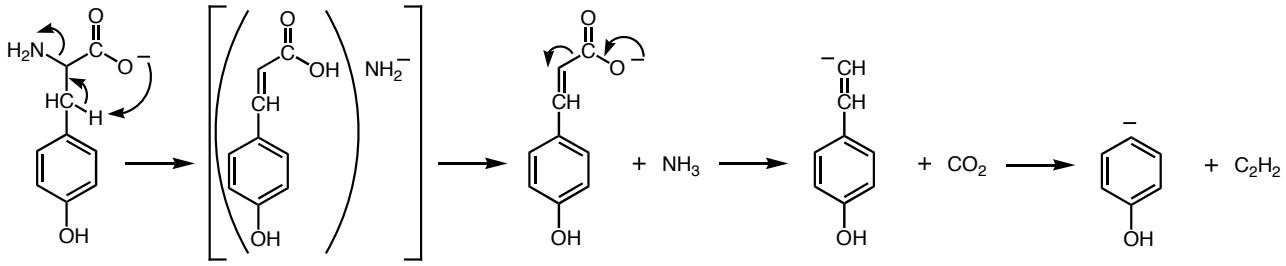
[FIGURE 2 and 3]

Tyrosine and Phenylalanine

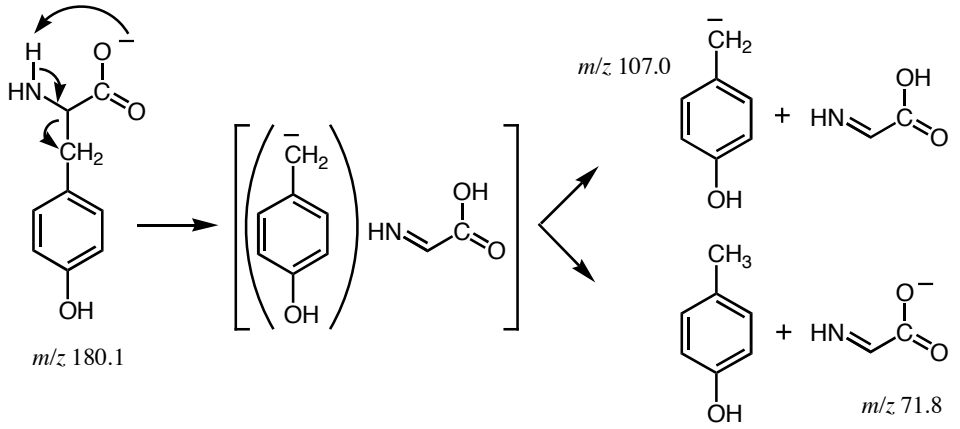
The major fragment ions observed from $[Tyr - H]^-$ are *m/z* 163.0 and *m/z* 119.0 which are consistent with the observations of Bowie and co-workers,[17,18] who have carried out extensive studies of the fragmentation of deprotonated amino acids and small peptides.[19,20] The fragment ion at *m/z* 163.0 corresponds to a loss of a nominal mass of 17 Da from the precursor anion and can be attributed to the loss of molecular ammonia but the low resolution data does not exclude the possibility of hydroxyl radical loss. The high resolution ESI-MS/MS spectrum of $[Tyr - H]^-$ was obtained using a quadrupole-time of flight instrument and the 17 Da loss was measured to be precisely 17.0231 Da: confirming fragmentation via the loss of ammonia (theoretical mass 17.0265 Da) rather than hydroxyl radical (theoretical mass 17.0027 Da). The mechanism for NH_3 loss, as previously proposed by Bowie and co-workers,[18] involves the abstraction of an acidic hydrogen from the benzylic position by the carboxylate anion to facilitate the elimination of NH_2^- and formation of an ion-molecule complex, as shown in Scheme 1. The strongly basic amide anion can then abstract the proton from the carboxylic acid moiety to form the stable carboxylate anion at *m/z* 163.0 with concomitant loss of ammonia. Decarboxylation of the nascent ion in a secondary fragmentation process yields a deprotonated styrene anion at *m/z* 119.0 that undergoes loss of acetylene to give *m/z* 92.9. This mechanism is supported by the ESI-MS/MS spectrum of source formed *m/z* 163.0 (Table 1) which also shows the loss of 44 Da and subsequent loss of 26 Da and is similar to the ESI-MS/MS spectrum of the $[M-H]^-$ anion formed from authentic 4-hydroxycinnamic acid (*p*-coumaric acid) run in a separate experiment under identical conditions (Table 1).[21] The mechanism given in Scheme 1 is further supported by the CID spectrum of $[Phe - H]^-$, listed in Table 1, which also shows major fragmentation via consecutive losses of NH_3 and CO_2. The strong similarities between the CID spectra of $[Tyr - H]^-$ and $[Phe - H]^-$ suggest that most of the

fragmentation observed in the CID of $[Tyr - H]^-$ is driven by the carboxylate rather than the phenolate anion. Both deprotonated tyrosine and phenylalanine also show a significant abundance of a fragment ion at *m/z* 71.8 (Figure 3 and Table 1, respectively) that can be rationalized in terms of an elimination mechanism driven by abstraction of a proton from the amine by the carboxylate anion, as outlined for $[Tyr - H]^-$ in Scheme 2. This mechanism leads to the formation of didehydroglycine and a benzylic anion, which may then effect deprotonation of the carboxylic acid. This proposal is supported by the observation of the intermediate benzylic anion at *m/z* 106.9 and the analogous *m/z* 90.9 fragment ion in the $[Phe - H]^-$ spectrum (Table 1). Loss of 46 Da is also observed from both $[Tyr - H]^-$ and $[Phe - H]^-$, although in each case this is a minor fragment. This pathway represents the loss of formic acid or concomitant loss of carbon monoxide and water. Whilst it is not possible to categorically differentiate between these two possibilities, both pathways are likely to proceed by initial abstraction of a benzylic proton by the carboxylate anion followed by elimination of the carboxylic acid moiety, as outlined for $[Tyr - H]^-$ in Scheme 3.

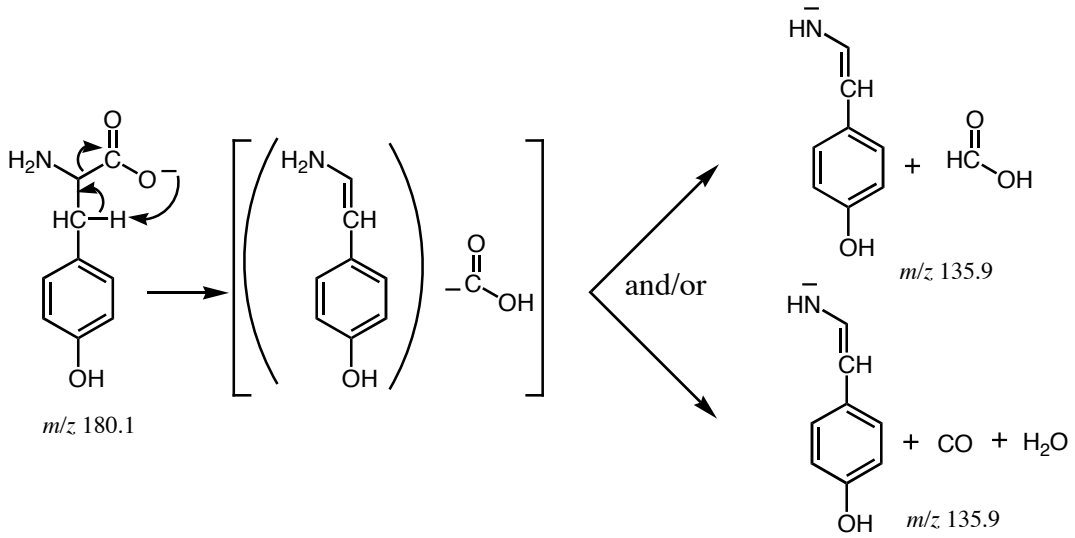
Scheme 1.



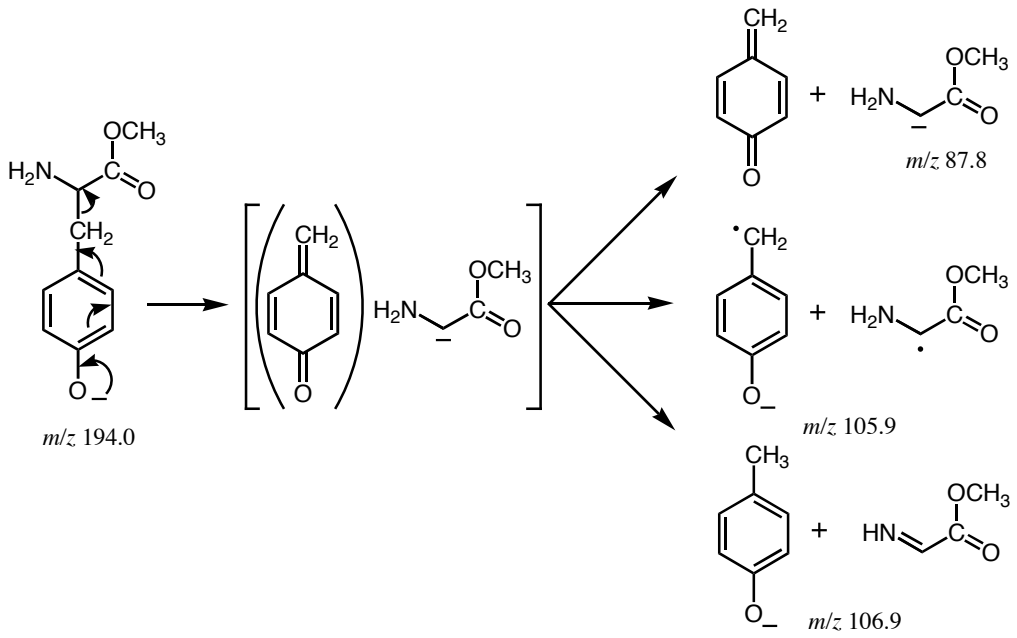
Scheme 2.



Scheme 3



Scheme 4.



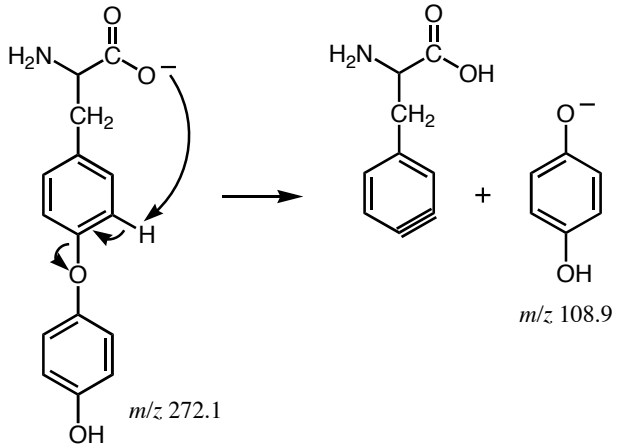
The contribution of the phenolate anion to the observed fragmentation of $[Tyr - H]^-$ was investigated by measuring the ESI-MS/MS spectrum of tyrosine methyl ester (Figure 3b). The major fragments in the CID spectrum of $[Tyr(OCH_3) - H]^-$ are observed at *m/z* 87.8, 105.9 and 106.9 which may all be rationalized in terms of charge driven dissociation of the tyrosine side chain to form the $[Gly(OCH_3) - H]^-$ and $CH_2C_6H_4O$, as outlined in Scheme 4. Direct dissociation of the ion-dipole complex yields $[Gly(OCH_3) - H]^-$ at *m/z* 87.8 while donation of an electron or a hydride anion from the anion to $CH_2C_6H_4O$, within the complex, gives rise to the observed fragments at *m/z* 105.9 ($CH_2C_6H_4O^-$) and 106.9 ($CH_3C_6H_4O^-$), respectively. These data suggest that fragmentation of the tyrosine phenolate anion may account for the observation of *m/z* 73.8, 106.0 and 106.9 in the CID spectrum of $[Tyr - H]^-$ (Figure 3a) as suggested by Bowie and co-workers.[22] Given that these fragments are extremely minor in the tyrosine spectrum, accounting for *ca*. 5% of total ion current, it can be safely assumed that carboxylate anions form that major component of the $[Tyr - H]^-$ ion beam in the negative ion electrospray ionisation of tyrosine. This is perhaps not surprising, given solution and gas phase acidities of the two portions of the molecule both strongly favour formation of the carboxylate anion. In solution the pK_a values for the amino acid and phenol side-chain moieties are 2.2 and 10.8,[23] respectively while in the gas phase the acidity of tyrosine, $\Delta_{acid}H_{298}[Tyr\text{-}H] = 1407 \pm 2.9$ kJ mol^{-1}, is closer to that of glycine, $\Delta_{acid}H_{298}[NH_2CH_2CO_2\text{-}H] = 1429 \pm 9$ kJ mol^{-1}, than to phenol, $\Delta_{acid}H_{298}[PhO\text{-}H] = 1466.1 \pm 2.5$ kJ mol^{-1}.[24-26]

T0

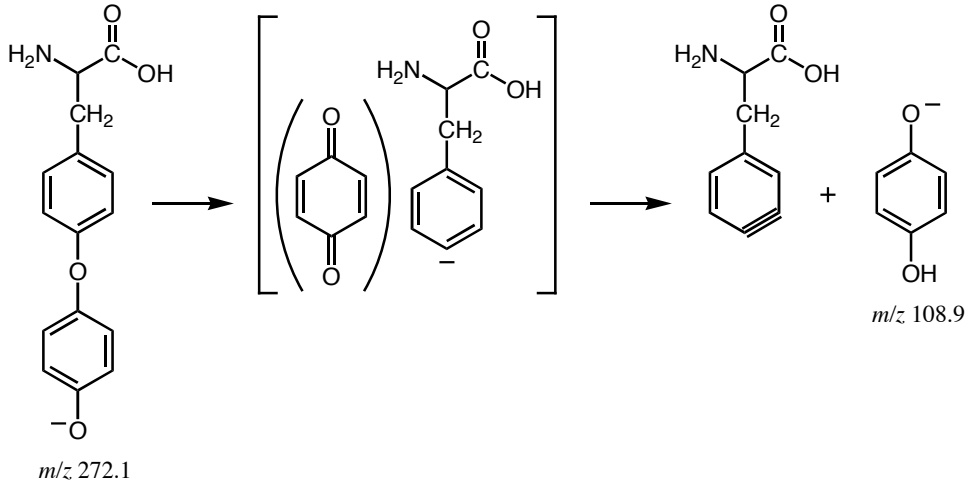
The ESI-MS/MS of the $[T0\text{-}H]^-$ anions is given in Figure 2(e) and shows major fragment ions at *m/z* 255.0 and *m/z* 211.0, which corresponds to the loss of NH_3 and subsequent loss of CO_2 from the precursor ion at *m/z* 272.1. These observations are directly analogous to the fragmentation observed

for $[Tyr\text{-}H]^-$ and $[Phe\text{-}H]^-$ discussed above and can therefore be rationalized according to the mechanism outlined in Scheme 1. Similarly, the observation of an intense peak at *m/z* 71.8 in the CID spectrum of $[T0\text{-}H]^-$ is consistent with $[Tyr\text{-}H]^-$ and $[Phe\text{-}H]^-$ and can be rationalized by the fragmentation pathway in Scheme 2. This proposal is further supported by the observation of the minor fragment at *m/z* 199.0 corresponding to the loss of $HNCHCO_2H$ (loss of 73 Da, *cf.* Scheme 2). The *m/z* 71.8 fragment ion and the corresponding loss of a 73 Da neutral are observed for all the thyronines in this study (Figure 2a-e), which is consistent with the common tyrosine structural motif of these molecules. The abundant ion at *m/z* 108.9 in the $[T0\text{-}H]^-$ spectrum corresponds in mass to deprotonated 1,4-dihydroxybenzene and is a significant fragmentation, as it is the only major pathway resulting in fission of the diphenyl ether linkage. Formation of this fragment through a direct elimination mechanism (Scheme 5a) seems unlikely on steric grounds. The possibility of homolytic or heterolytic (Scheme 5b) cleavage driven from the phenoxide anion cannot be rigorously excluded, however model studies on tyrosine (discussed above) suggest that deprotonation from the amino acid moiety out-competes deprotonation of the phenol and thus formation of a significant population of the phenoxide anion from T0 also seems unlikely. A more plausible rationale for the observed fragment ion at *m/z* 108.9 is the elimination of the phenoxide fragment by the highly basic amide anion within an anion neutral complex (Scheme 5c). The mobility of anions within an ion-neutral complex has previously been demonstrated.[27-29]

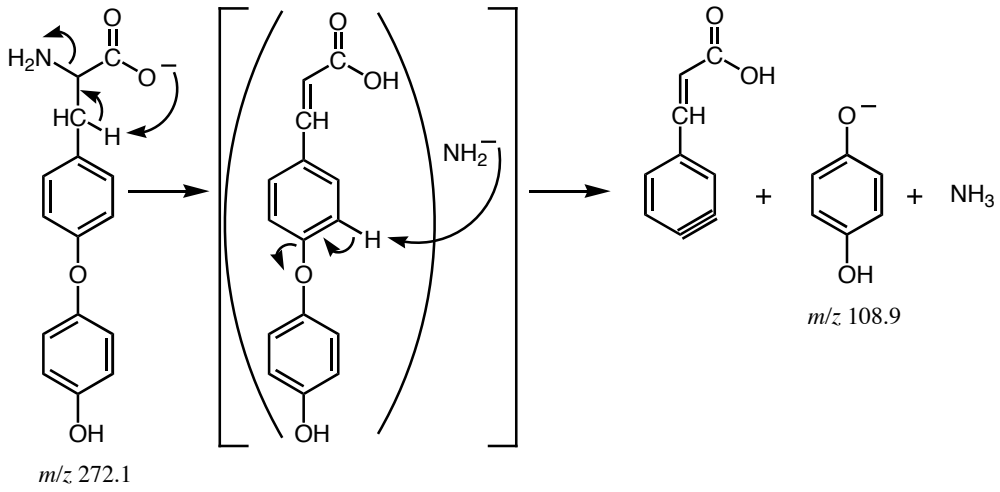
Scheme 5a



Scheme 5b



Scheme 5c

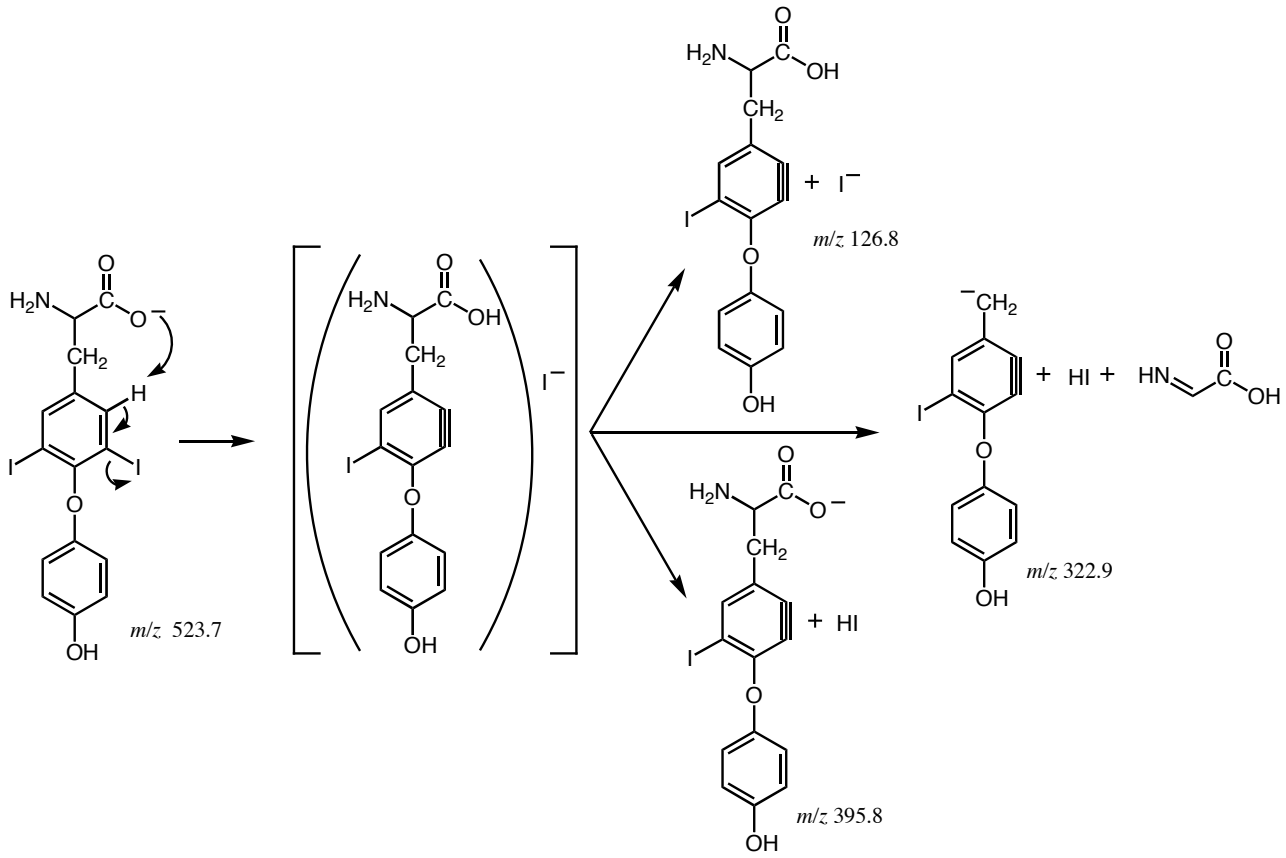


T2

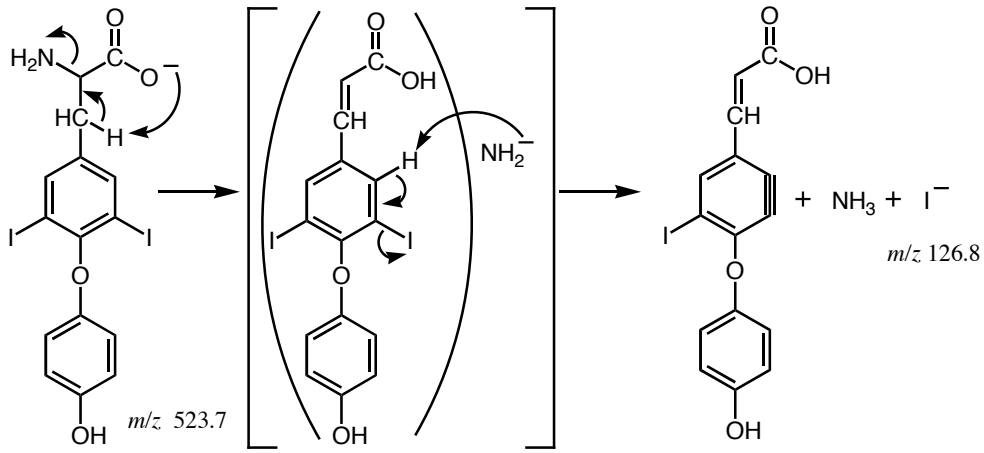
CID of the $[T2 - H]^-$ molecular anion, *m/z* 523.7, yields fragment ions at *m/z* 506.7, 71.9 and 126.8 (Figure 1d). The observation of these anions can be simply rationalized as loss of NH_3 (*cf.* Scheme 1), formation of $HNCHCO_2^-$ (*cf.* Scheme 2), and formation of the iodide anion, respectively. The observation of the iodide anion is hardly surprising as the high electron affinity of the halogen atom is a strong driving force for anion dissociation (*EA*[I] = 3.059038 eV).[30] It may be formed by a direct elimination driven by the carboxylate anion (Scheme 6a) or, alternatively, the amide anion may again play a role within the ion-neutral complex (Scheme 6b). The minor fragment ion observed at *m/z* 395.8 corresponds to the loss of hydrogen iodide and supports the mechanism proposed in Scheme 6a, where the nascent iodide anion may abstract a proton. Such a proton transfer is estimated to be endothermic by *ca.* 92 kJ mol^{-1} ($\Delta_{acid}H_{298}$[Tyr-H] = 1407 ± 2.9 kJ mol^{-1} kJ mol^{-1} compared with $\Delta_{acid}H_{298}$[HI] = 1315.3 ± 0.3 kJ mol^{-1} kJ mol^{-1})[25,30] which is consistent with the relative abundance of the *m/z* 395.8 and *m/z* 126.8 fragment ions. The minor fragment observed at *m/z* 322.9 may also be rationalised via the ion-molecule complex shown in Scheme 6a. In this case the iodide anion can bring about elimination of $HNCHCO_2H$ and formation of a stabilized benzylic anion. It is significant to note that the ring fission pathway observed for T0 (Scheme 5c) is effectively switched off for T2. This is not surprising given that the mechanism proposed for this pathway (Scheme 5c) relies on proton abstraction from the *meta* position, both of which are blocked by iodine in the structure of T2. Homolytic cleavage of the carbon-iodine bond is also observed in the fragmentation of T2 with the resulting ions observed at *m/z* 396.9 (loss of I) and *m/z* 379.9 (loss of NH_3 and I). Such fragmentation is unusual in even electron anions but it is reasonable in this case because of the low energy of the carbon-iodine bond (DH_{298}[Ar-I] = 280.8 ± 5.9 kJ mol^{-1}).[31,32] It is significant to note that for deprotonated T2 the loss of CO_2 from the $[T2\text{-}H\text{-}NH_3]^-$ anion is not observed in contrast to both $[Tyr\text{-}H]^-$ and $[T0\text{-}H]^-$. This is most probably because further activation of the nascent carboxylate anion results in elimination of I^-, as outlined

in Scheme 7, in preference to the decarboxylation observed for the non-iodonated compounds. This proposal is supported by the CID spectrum of the source formed carboxylate anion, which shows I^- as the only major fragment (Table 1).

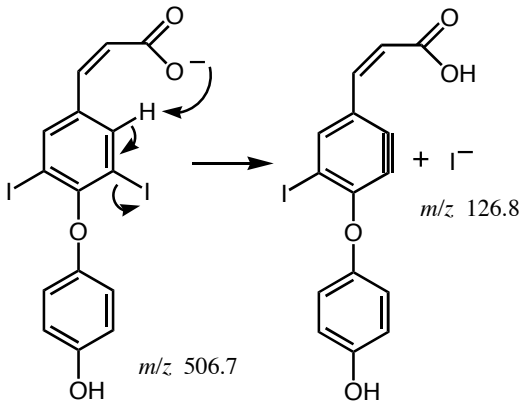
Scheme 6a



Scheme 6b



Scheme 7.

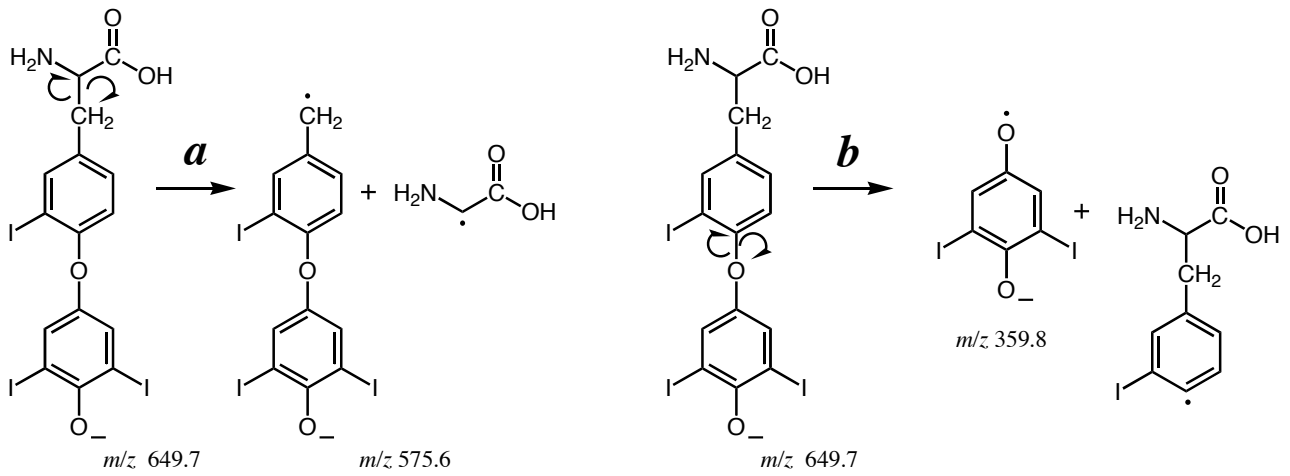


T3 and rT3

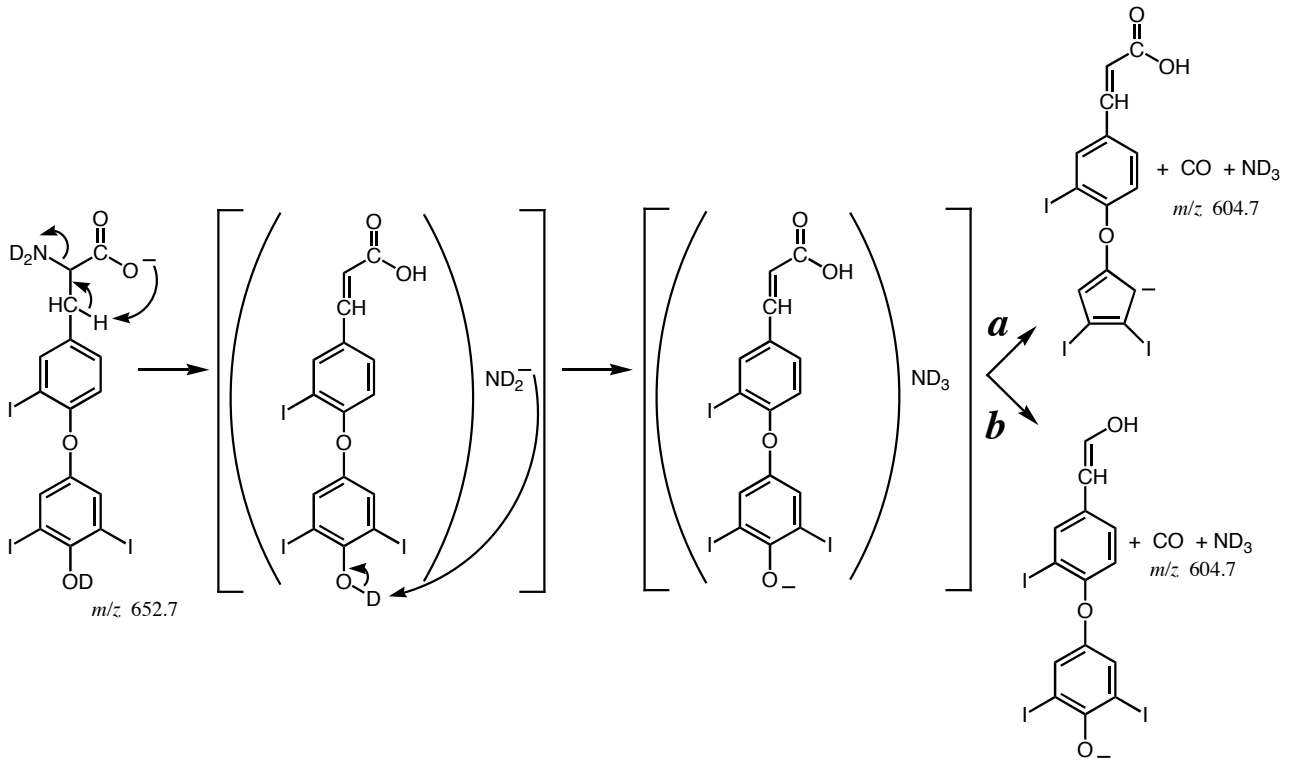
The ESI-MS/MS spectrum of $[T3\text{-}H]^-$ is given in Figure 2(b) and demonstrates fragmentation directly analogous to the $[T2\text{-}H]^-$ homologue described above. The major fragments correspond to loss of NH_3 at *m/z* 632.6, formation of the iodide anion at *m/z* 126.8 and formation of the $HNCHCO_2^-$ anion at *m/z* 71.8. Minor fragments are also observed at *m/z* 448.8 and 505.7, corresponding to losses of HI + $HNCHCO_2H$ (*cf.* Scheme 6a), and NH_3 + I, respectively. The strong similarities in the fragmentation of T3 and T2 suggest that addition of the iodine to the outer benzene ring does not significantly alter the fragmentation energetics of the molecule. Interestingly, rT3 shows significantly different fragmentation to its structural isomer (Figure 2c). The most obvious difference is that the abundance of the iodine anion (*m/z* 126.8) is significantly lower, relative to other fragments, than is observed for either T3 or T2. This observation is consistent with the mechanism proposed in Scheme 6a where the carboxylate abstracts a proton from the *ortho* position to effect vicinal elimination of iodide from the top ring. The characteristic amino acid cleavages are also observed for rT3 at *m/z* 632.6 ($-NH_3$) and *m/z* 71.8 ($HNCHCO_2^-$) but at significantly lower abundances than observed for T2 and T3. Significantly, rT3 also shows a fragment ion at *m/z* 588.6, in contrast to T3 and T2, but consistent with the consecutive NH_3 and CO_2 losses observed for tyrosine and T0. This supports the previous hypothesis that

decarboxylation is suppressed in T3 and T2 due to preferential elimination of iodine as shown in Scheme 7. In rT3 however, with only one iodine substituent on the top ring, the iodine elimination and decarboxylation processes are competitive since both products are observed in the CID spectrum. CID of $[rT3\text{-}H]^-$ also gives rise to a number of fragments not observed for the other homologues that are indicative of the increased acidity of the phenol moiety in rT3. For example, the observation of abundant fragments at *m/z* 575.6 and *m/z* 359.8 suggests a significant proportion of the phenoxide anion is being generated during ionization, with the resulting ion favouring homolytic decomposition *via* the processes outlined in Schemes 8a and 8b. One of the major fragments in the CID spectrum of $[rT3\text{-}H]^-$ is observed at *m/z* 604.6 and corresponds to a surprising loss of 45 Da. Such a loss is not observed for any of the other thyronine homologues discussed thus far. Deuterium exchange of rT3 was carried out to assist with the characterization of this product channel. The analogous fragment in the $[D_4\text{-}rT3\text{-}D]^-$ spectrum (Table 1) is also observed at *m/z* 604.6 corresponding to a loss of 48 Da, thus identifying the fragmentation as the concomitant loss of ND_3 and CO. This result further attests to the mobility of the amide anion within the ion-neutral complex prior to dissociation, indicating that it is capable of migration from the amino acid portion of the molecule to affect deprotonation at the phenol moiety. It strongly suggests that the increased acidity of the phenol moiety in rT3 due to the presence of the two iodines on the outer ring is a critical factor in altering the fragmentation pattern of this species relative to the T3 isomer. The loss of CO has previously been observed in the high energy CID studies of phenoxide anions[33] and is favoured due to the formation of the aromatic cyclopentadienyl anion. Such a precedent might suggest that in this instance that decarbonylation of the bottom ring arises from the exothermic abstraction of the phenol proton as outlined in Scheme 9a, however, the possibility that CO is lost from the amino acid moiety, as indicated in Scheme 9b, cannot be excluded.

Scheme 8.



Scheme 9.



The fragment at *m/z* 478.7 in the CID spectrum of rT3 corresponds to an unusual loss of 171 Da, which suggests a concomitant loss of atomic iodine and carbon dioxide. Such a fragmentation is necessarily a radical process. This proposal is supported by the CID spectrum of source formed *m/z* 478.7 (Table 1), which also fragments *via* radical processes, namely, the loss of atomic hydrogen to give *m/z* 477.7 and loss of the NH_2 radical to form *m/z* 462.6. These ions are also observed in the CID spectrum of $[rT3\text{-}H]^-$ (Figure 2c), suggesting that they are secondary products from the primary fragment at *m/z* 478.7. The relatively high abundance of ions at *m/z* 478.7, 477.7 and 462.8

also points to a significant proportion of phenolate anions in the $[rT3\text{-}H]^-$ anion population compared with deprotonated T3.

T4

Examination of the CID spectrum of T4 (Figure 2a) reveals fragmentation types observed in both T3 and rT3 consistent with two iodines substituted on each of the top and bottom benzene rings. For example, the ions at *m/z* 758.6 ($-NH_3$), *m/z* 126.9 (I^-) and *m/z* 71.9 ($HNCHCO_2^-$) are observed as major fragments and are comparable to T3, whereas *m/z* 730.5 ($-NH_3$ and CO), *m/z* 702.6 ($-NH_2CHCO_2H$) and *m/z* 359.7 ($OC_6H_2I_2O^-$) are also observed and are comparable to rT3. In contrast to rT3, there is no loss of CO_2 following loss of NH_3 (expected ion at *m/z* 714.6) further supporting the proposal that decarboxylation does not compete with iodide elimination when two iodines are present on the top ring. However, the losses of 171 Da (*m/z* 604.7) and 187 Da (*m/z* 588.7) are due to radical processes, directly analogous to the pathways observed for rT3 (*cf.* Scheme 8).

Conclusions

The thyronines examined in this study were readily ionisable by negative ion ESI, forming abundant $[M\text{-}H]^-$ anions. Fragmentation suggests that they are mostly carboxylate anions, although rT3 and T4 show limited fragmentation driven by the phenoxide anion. CID of the mass-selected precursor ions show two sets of fragment ions common to all thyronines, namely, (i) loss of NH_3 and formation of the $HNCHCO_2^-$ anion at *m/z* 71.8 by analogy with the decomposition of $[Tyr\text{-}H]^-$ and $[Phe\text{-}H]^-$ anions, and (ii) formation of I^- for all iodothyronines. The latter observation suggests that precursor ion mass spectrometry is the most appropriate method for thyroid hormone analysis in complex matrices[10] especially given that iodothyronines are the only endogenous compounds

known to contain iodine. Comparison of the CID spectra of T3 and rT3 reveal several distinct fragments. Of particular importance are the ions observed at *m/z* 604.6 and *m/z* 359.8 from $[rT3\text{-}H]^-$ which do not appear at all in the corresponding $[T3\text{-}H]^-$ spectrum. Such a dramatic difference in the CID mass spectra of positional isomers is unusual. More commonly positional isomers produce the same fragment ions but in different ion abundances. In this case the differences strongly suggest the participation of the phenoxide anion in the fragmentation of rT3 compared with T3, which is consistent with the additional anion stabilization afforded by the two iodines substituted on the bottom ring in the former. Thus, tandem mass spectrometry provides excellent method to distinguish these isomers in native samples with the transitions, 649.6 → 604.6 and 649.6 → 359.8, particularly amenable to SRM experiments in an analytical context. Finally, the dramatic differences in unimolecular gas phase chemistry of these T3 and rT3 may well be indicative of significantly different chemistries for these molecules *in vivo*.

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21. It should be noted that slight differences are observed between the source CID-MS^3 spectrum of $[Tyr - H - NH_3]^-$ and the MS/MS spectrum of the $[M-H]^-$ anion formed from 4-hydroxycinnamic acid. The most significant of these are low abundance fragment ions observed at *m/z* 148 and *m/z* 133 in the $[Tyr - H - NH_3]^-$ spectrum. This may suggest a minor competitive pathway for the loss of NH_3 from $[Tyr - H]^-$. For example, a competitve *syn* elimination of NH_3 may result in formation of the *cis* alkene which may fragment differently to the predominantly *trans* 4-hydroxycinnamic acid. More likely however, is that the spectral differences are the result of isobaric interferences in the ion source and would be absent in a true MS^3 experiment.

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Table 1. The tandem mass spectra of additional precursor ions examined in this study (*i.e.*, those not given as figures). All ESI-MS/MS spectra reported were recorded on a triple quadrupole mass spectrometer.

	Product ions *m/z* (%abundance)[a]
$[Tyr - H]^-$	180.1(24.0), 163.0(29.6), 136(1.3), 134.0(0.8), 119.0(22.7), 106.9(0.7), 106.0(1.4), 92.9(5.6), 73.8(3.5), 71.8(9.5), 44.8(0.3), 41.6(0.2), 27.8(0.1), 25.9(0.3)
$[Tyr - H - NH_3]^-$	163.0(17.1), 148.0(2.2), 133.9(3.3), 119.0(61.2), 92.8(14.3)
$[M - H]^-$ 4-hydroxycinnamic acid	163.0(3.0), 148.0(>0.05), 145.0(0.1), 120.9(0.1), 119.0(93.2), 103.9(0.1), 92.9(3.1), 90.9(0.4), 89.0(>0.05), 76.9(>0.05), 64.8(>0.05)
$[Phe - H]^-$	164.0(19.5), 147.0(37.2), 119.1(0.3), 118.0(0.3), 102.9(23.5), 90.9(2.9), 76.9(0.3), 71.8(16.0)
$[Tyr(OCH_3) - H]^-$	194.1(23.5), 106.1(11.6), 105.9(36.0), 87.8(29.0)
$[D_4\text{-}T2 - D]^-$	526.6(25.0), 507.7(18.1), 400.0(0.5), 398.9(0.3), 398.0(0.3), 395.1(0.2), 381.9(0.3), 381.0(1.1), 354.0(0.1) 352.0(0.1), 338.2(0.1), 323.0(0.9), 227.2(0.1), 226.2(0.2), 222.9(0.1), 126.9(39.7), 72.9(11.3), 71.8(1.3)
$[D_4\text{-}T2 - D - ND_2H]^-$	507.8(43.5), 399.8(0.6), 380.9(5.3), 126.8(50.6)
$[rT3 - H - CO_2 - I]^-$	478.8(3.2), 478.0(5.4), 462.8(42.3), 233.8(3.4), 126.8(45.6)
$[D_4\text{-}rT3 - D]^-$	652.7(24.6), 634.6(0.6), 633.7(2.9), 605.7(2.7), 604.7(9.9), 589.6(0.4), 588.7(1.3), 577.6(0.8), 576.7(1.3), 575.6(6.4),

	524.9(0.2), 506.8(0.2), 481.9(2.4), 480.8(0.5), 479.8(0.8), 464.7(0.8), 463.8(4.6), 449.8(0.3), 448.8(0.4), 436.5(0.1), 420.9(0.1), 359.8(7.0), 343.7(0.5), 322.1(0.1), 253.8(0.1), 126.9(26.0), 72.9(1.7), 72.0(0.1)
$[D_4\text{-}T3 - D]^-$	652.7(10.2), 634.6(4.4), 633.6(12.0), 578.8(0.3), 577.6(0.9),525.9(1.0), 507.7(1.0), 506.8(3.0), 481.8(0.7), 450.7(0.4), 449.9(1.5), 448.8(3.0), 399.8(0.3), 353.1(0.4), 352.2(0.2), 253.8(0.1), 126.9(51.7), 72.9(7.7), 71.8(1.4)

[a] Ion abundance is normalized to the sum of all ion abundances *i.e.*, I_1(normalized) = I_1(%base peak)/$\Sigma_i I_i$(%base peak)

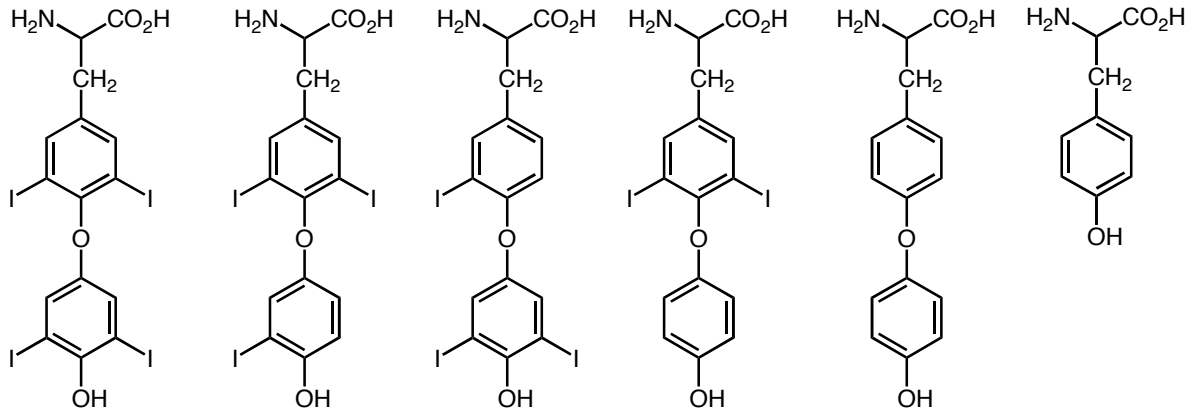
Figure Captions

Figure 1. The structures of thyroid hormones; (a) T4, (b) T3, (c) rT3, (d) T2, (e) T0, and (f) the amino acid tyrosine.

Figure 2. The ESI-MS/MS spectra obtained from 10 μM solutions of; (a) T4, (b) T3, (c) rT3, (d) T2, and (e) T0 in methanol:chloroform (2:1 v/v) at pH 9-10 using a Waters QuattroMicro.

Figure 3. The ESI-MS/MS spectra obtained from 10 μM solutions of (a) tyrosine and (b) tyrosine methyl ester in methanol:chloroform (2:1 v/v) at pH 9-10 using a Waters QuattroMicro.

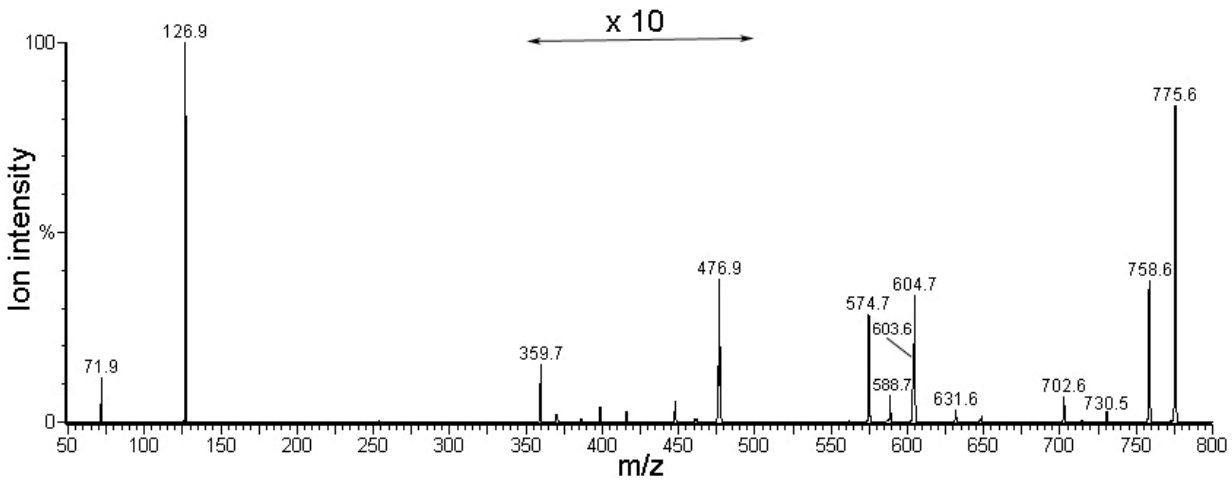
FIGURE 1



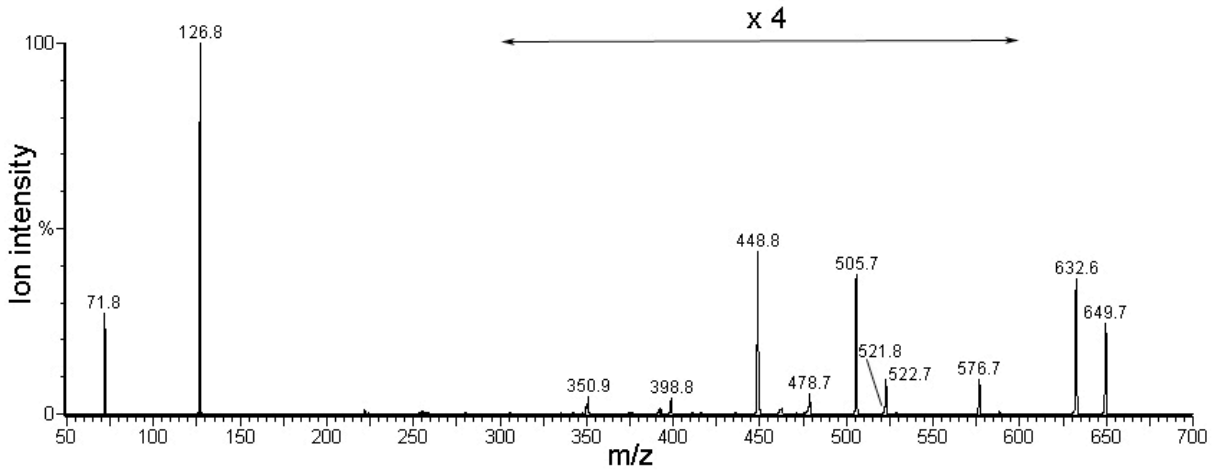
(a) T4 (b) T3 (c) r-T3 (d) T2 (e) T0 (f) Tyr

FIGURE 2

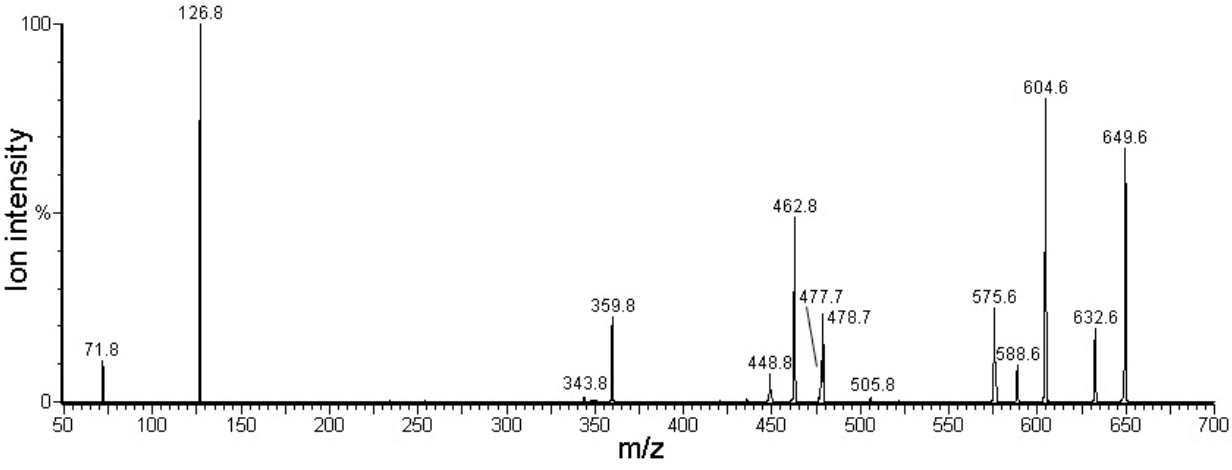
(a) T4



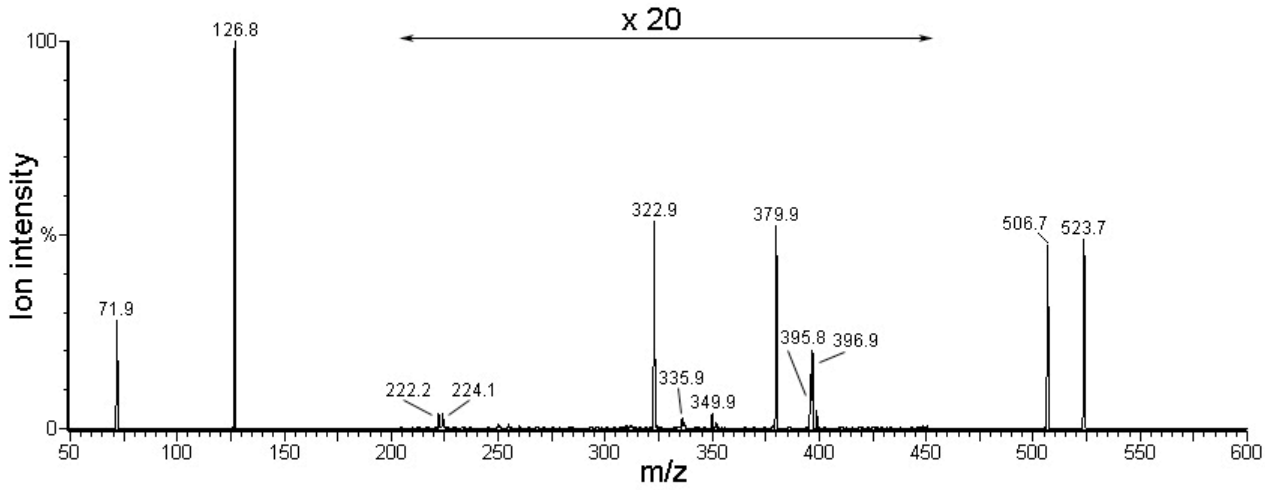
(b) T3



(c) rT3



(d) T2


x 20
126.8
322.9
379.9
506.7
523.7
71.9
395.8
396.9
335.9
222.2
224.1
349.9
Ion intensity
%
m/z

(e) T0

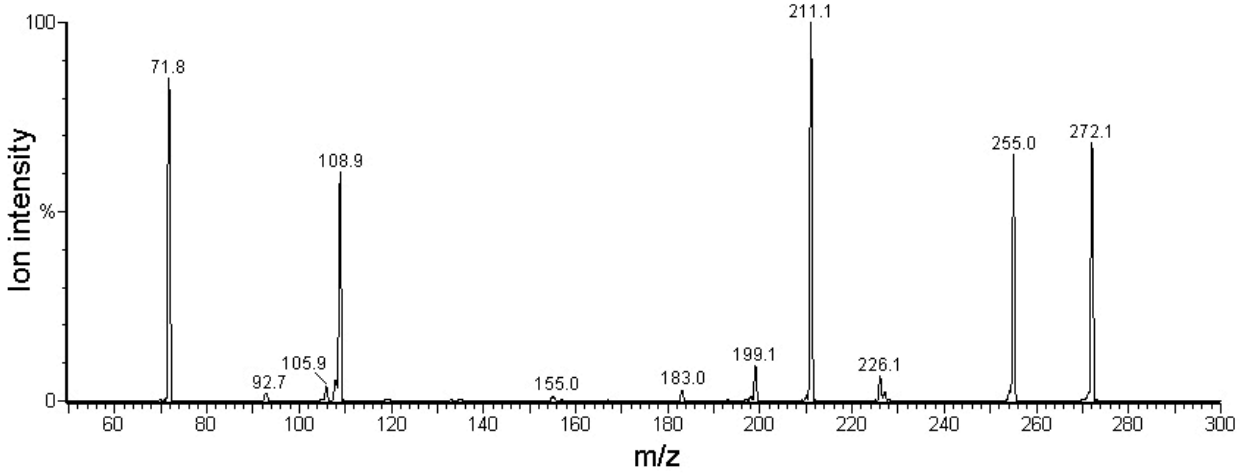
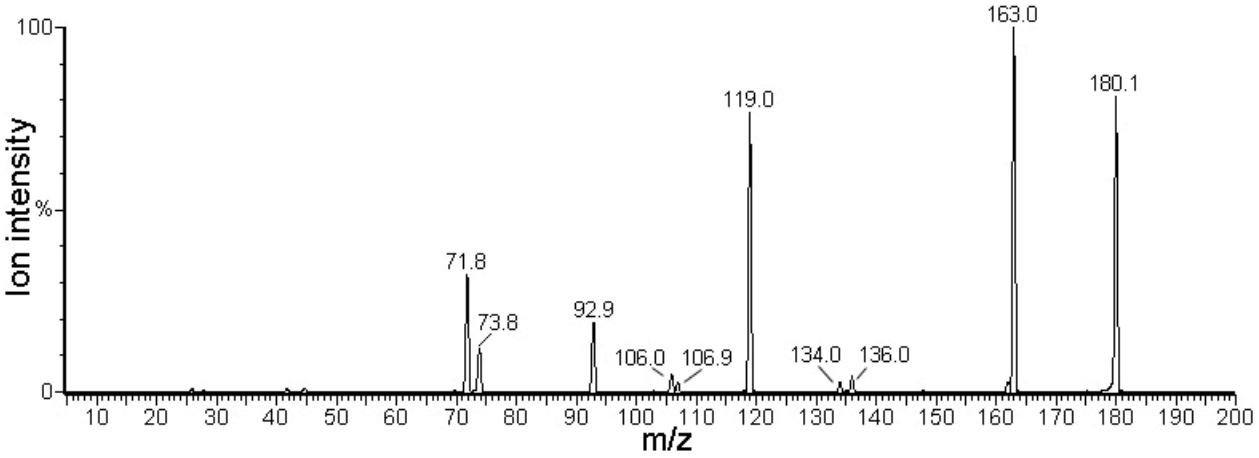

211.1
71.8
255.0
272.1
108.9
105.9
92.7
199.1
226.1
155.0
183.0
Ion intensity
%
m/z

FIGURE 3

(a) [Tyr – H]$^-$



(b) [Tyr(OCH$_3$) – H]$^-$