Title: Photoinduced dissociation mass spectroscopy of firefly oxyluciferin anions

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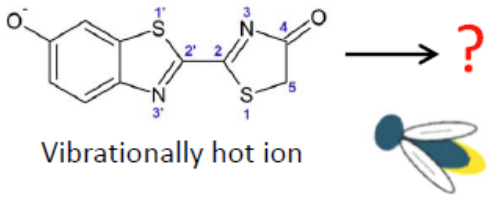
Graphical abstract



Highlights

- Fragment ion masses of photoexcited oxyluciferin anions were identified.
- Isotope-labelling reveals that the thiazolyl is most susceptible to fragmentation.
- Dominant fragment ion is deprotonated 2-cyano-6-hydroxybenzothiazole.
- In the firefly the dominant fragment ion, if formed, is recycled to D-luciferin.

Photoinduced dissociation mass spectroscopy of firefly oxyluciferin anions

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Abstract

The oxyluciferin molecule in its anionic form is responsible for light emission from fireflies and some railroad worms and click beetles. Here we have studied the breakdown of the ions after photoexcitation by 550-nm light, and identified the atom composition of eight fragment ions based on mass spectrometric experiments on isotope-labelled compounds. A sector instrument with an electrospray ion source and a pulsed laser system was used for the experiments. After

photoexcitation the time for dissociation was up to about fifteen microseconds, which is much shorter than the 100-μs time constant for dissociation after one-photon absorption. The laser power was therefore kept high to allow the oxyluciferin anions to absorb two photons to produce enough fragment ions on the instrumental relevant time scale. The reaction energies leading to these ions were obtained from density functional theory calculations. The dominant fragment ion was deprotonated 2-cyano-6-hydroxybenzothiazole. Interestingly this behavior mirrors that of oxyluciferin both *in vivo* in insects, where the same nitrile is an intermediate in the postulated regeneration of D-luciferin from oxyluciferin or *in vitro* in near-neutral aqueous buffer. Dissociation of the oxyluciferin anion into this fragment ion was calculated to require 1.86 eV, which is less than the energy of one photon (2.25 eV). Experiments done on 5,5-dimethyloxyluciferin revealed a similar fragmentation pattern.

Keywords

Oxyluciferin; photoinduced dissociation; isotope labelling; DFT calculations; bioluminescence; scrambling

Introduction

In fireflies and other light-emitting insects, the D-luciferin substrate is converted biochemically to the oxyluciferin anion (Fig. 1) in its electronically excited state by the luciferase enzyme [1-5]. The energy-rich ATP (adenosine triphosphate) is involved in the oxidative decarboxylation of luciferin together with molecular oxygen and divalent metal ions. Electronically excited oxyluciferin anions emit light (bioluminescence) in the yellow-green, orange, and red dependent on the insect species

[1-6] and with a high quantum yield of 41 ± 7.4 % [4,6]. After light emission the oxyluciferin anion is enzymatically broken down to thioglycolic acid and 2-cyano-6-hydroxybenzothiazole, and the latter in the presence of cysteine is recycled to D-luciferin thereby allowing for a new light flash [7-9].

Still, a significant fraction of ions undergo internal conversion to the electronic ground state instead of light emission, resulting in vibrationally excited ions. These ions dissipate their heat in interactions with the protein environment (typical time scale is tens of picoseconds) though it cannot be excluded that a few would have time to dissociate prior to cooling. An environment can provide an efficient way of protection by energy dissipation [10]. The dissociation time of the isolated photoexcited ions *in vacuo* is about 100 μs based on previous storage-ring experiments [11], which results in a dissociation yield of 10^{-6}-10^{-8} assuming a time constant for vibrational cooling between 1 ps and 100 ps. Each flash from a firefly contains about 10^{13} photons [12], and the same order of magnitude of hot ions is therefore produced (59 % yield versus 41 %). Hence for each flash, a rough estimate of the number of ions that dissociate is $10^5 – 10^7$. Considering that the flashing rate can be about ten per minute [13], this number will quickly multiply. For the atom economy of the firefly (and maybe also its safety) these fragments should as far as it is possible be recycled to D-luciferin.

To shed light on the fragment ions that potentially could be produced from hot oxyluciferin anions we have in this work measured photoinduced dissociation (PID) mass spectra. Isotope-labelled compounds were subjects for study (anions shown in Fig. 2) to get detailed information on the actual atoms in the fragment ions and to identify possible scrambling reactions. A wavelength of 550 nm was used as we have earlier found that this is the maximum of the absorption band [11]. For each of the identified fragments, we calculated the reaction energy associated with the dissociation channel to see if the reaction would be possible or not for

vibrationally excited (hot) oxyluciferin ions within the luciferase enzyme. The lowest-energy structure of oxyluciferin is the keto form [11] but the enol form (Fig. 1) may also be present in the ion beam. We therefore also did experiments for 5,5-dimethyloxyluciferin that is locked in the keto form (Fig. 2).

Materials and Methods

Experiments were done with a sector instrument equipped with an electrospray ion source [14, 15]. Oxyluciferin was dissolved in methanol and electrosprayed to produce the anions. After a heated capillary and tube-lens skimmer region, the ions were stored in an RF-only octopole trap. The trap was emptied every 25 ms, and all ions were accelerated to 50-keV energies. Those of interest according to their mass-to-charge ratio (*m/z*) were selected by an electromagnet. In a field free region, the ions were photoexcited by a nanosecond laser pulse. The laser is a Nd:YAG laser where the third harmonic is led into an optical parametric oscillator to generate visible light at 550 nm. The repetition rate of the laser is 20 Hz so only every second ion bunch was irradiated to subtract a background signal due to collision-induced dissociation from residual gas in the beam line (laser on – laser off). After photoexcitation, the time for dissociation was a few microseconds. Daughter ions were selected by a hemispherical electrostatic analyzer and counted by a channeltron detector. The detection efficiency is high as the ions have high velocities.

The synthesis of both 5,5-dimethyloxyluciferin and the isotope-labelled oxyluciferins was performed following established synthetic pathways towards firefly luciferin [16], but using appropriately ^{13}C- and ^{15}N-labelled precursors. Eventually, the labelled oxyluciferins were obtained according to Goto's method by condensation of 2-cyano-6-hydroxybenzothiazole with ethyl mercaptoacetate [17, 18], taking advantage of recent improvements for this capricious key step [19].

The samples displayed a chemical purity of >98%, and the labelling in the respective positions was close to 99% according to NMR. Full experimental details will be reported elsewhere.

Theoretical calculations were done with the Gaussian03 program package [20]. Geometries were first optimized at the B3LYP/6-31+G(d) level of theory and vibrational frequencies calculated to verify that the structures are local minima and not transition states. Single-point energies were calculated at the higher B3LYP/6-311++G(2d,p) level of theory, and these were corrected for zero-point kinetic energies. All structures, energies and zero-point corrections can be found as supporting information.

Results and Discussion

A PID mass spectrum of oxyluciferin (*m/z* 249) is shown in Fig. 3. Eleven fragment ions are evident with *m/z* 221, 206, 175, 150, 149, 143, 82, 73, 58, 42, and 26. Dissociation is the result of absorption of two photons according to power-dependence measurements (see supporting information and ref. [11]). The most dominant fragment ion is *m/z* 175 followed by *m/z* 206, 42, and 221. We discuss the eight most abundant fragment ions separately in the following, leaving out *m/z* 143, 82 and 73, beginning with the highest *m/z* and ending with the lowest.

m/z 221 + mass 28

Narrow-scan spectra are shown for the ions labelled A to H in Fig. 4; see Fig. 2 for labelling. The neutral fragment is less than 32 and therefore only one peak is seen in the H spectrum. The fragment ion mass is one higher in spectra B, C, D, E and G and unchanged in F, which implies that the ion contains 3'-N, 3-N, 2'-C, 2-C, and 5-C but not 4-C. Based on this we assign the neutral

mass to be ^{4}CO, that is, loss of carbon monoxide from the five-membered thiazolyl ring and not from the phenolate. A possible ion structure is given in Fig. 5.

m/z 206 + mass 43

Fig. 6 shows the narrow-scan spectra in the relevant mass region. Spectrum H contains one peak which not surprisingly implies that both sulphurs belong to the fragment ion. Spectra B, D, E, and G reveal that the ion contains 3'-N, 2'-C, 2-C, and 5-C but not 3-N and 4-C. Based on this we assign the neutral fragment to H^{3}N^{4}CO, and a possible fragment ion structure is shown in Fig. 5.

m/z 175 + mass 74

Now spectrum H is a clear double peak (Fig. 7), and the neutral fragment therefore contains one sulphur atom. The fragment ion is composed of 3-N, 3'-N, 2-C, and 2'-C but not 4-C and 5-C. Hence the neutral fragment is $^{5}CH_2{}^{4}C(O)(S)$, and the structure of the anionic fragment is given in Fig. 5.

m/z 149 (150) + mass 100 (99)

The spectra in this region are shown in Fig. 8. The signals for the H ions were too weak and are not included. Overall these spectra are of lower quality as the count rate is much lower. They do, however, indicate that there are two different fragment ions with *m/z* 149 and *m/z* 150 that are formed after breaking of the central bond between the two ring systems as the ionic fragments contain 2'-C and 3'-N and the neutral one 2-C, 3-N, 4-C, and 5-C. The difference between the two

ionic fragments is whether bond breakage is associated with hydrogen transfer from the five-membered ring to the other or not (see structures in Fig. 5).

m/z 58 + mass 191

This fragment ion clearly contains one sulphur atom as spectrum H is a double peak (Fig. 9), and the ion must therefore be SCN^-. Considering the structure of oxyluciferin, there are two obvious candidates for SCN^-, either $^{1'}S^{2'}C^{3'}N$ or $^{1}S^{2}C^{3}N$. However, the isotope experiments unequivocally establish the fragment ion to be $^{1}S^{2}C^{3}N^-$.

m/z 42 + mass 207

The fragment ion does not contain sulphur and is most likely NCO^-. Based on the oxyluciferin structure, an obvious candidate is $^{3}N^{4}CO$, which is verified from the spectra shown in Fig. 10. This ion is simply formed after the breakage of the bond between ^{2}C and ^{3}N and the one between ^{4}C and ^{5}C.

m/z 26 + mass 223

The fragment ion must be CN^- but there is more than one possibility for this ion. Spectra B and C (Fig. 11) reveal that the ion can contain either one of the nitrogens but that there is a preference for ^{3}N based on the relative intensities. Spectrum D shows that the ion originating from the benzothiazole ring can be $^{2'}C^{3'}N^-$. Spectra E-G show that the ion originating from the thiazolyl ring

can only be $^{2}C^{3}N^{-}$. This fragment ion is the only one where our data have indicated two different origins.

PID of 5,5-dimethyloxyluciferin

The PID mass spectrum of 5,5-dimethyloxyluciferin (*m/z* 277) is shown in Fig. 12 together with that of the ion containing one S-32 and one S-34 to clearly reveal what fragments contain only one sulphur atom (double peak), *e.g.*, the prevalent *m/z* 175 and *m/z* 58 ions. The fragment ion masses are similar to those for oxyluciferin except for a new peak at *m/z* 234, which is the result of loss of mass 43 (HNCO). However, this neutral loss was also seen for oxyluciferin. A possible structure of the ion is shown in Fig. 13.

Calculated reaction energies

Based on the mass spectrometric data and the assignments, we did DFT calculations to obtain information on the reaction energy associated with each dissociation channel. These energies set lower limits for the internal energy required for the dissociation as higher-energy transition state structures may be involved. The optimized structures of the oxyluciferin parent ion and the lowest-energy products are given in Fig. 14 for each channel together with the corresponding reaction energy. In the case of 5,5-dimethyloxyluciferin we limited the calculations to the channel resulting in a new fragment ion (*m/z* 234) (Fig. 15).

Channels leading to the formation of ions with *m/z* 221, 206, 175, and 58 are all open after one-photon absorption (addition of 2.25 eV), and they could therefore be of potential relevance for the decomposition of oxyluciferin within the luciferase enzyme. However, it should be

emphasized that we have neglected the barrier for the reverse reaction. Indeed, the required energy for the formation of *m/z* 58 is the lowest, only 1.12 eV, but this fragment ion is not dominant in the spectrum, which seems to indicate that its formation is associated with a high activation barrier. All the other fragmentation channels observed in this study are likely too costly to be of relevance in the protein. Reaction along the HNCO loss channel for 5,5-dimethyloxyluciferin costs 1.04 eV (Fig. 15) in agreement with the abundant formation of *m/z*-234 ions (second most abundant fragment ion in the PID mass spectrum, see Fig. 13).

The dominant fragment at *m/z* 175 (deprotonated 2-cyano-6-hydroxybenzothiazole) corresponds to a breakdown product of oxyluciferin that has been observed both *in vitro* in near-neutral pH buffers [7] but is also believed to be a product of enzymatic decomposition of oxyluciferin *in vivo* [8]. Hence our work has shown that cold "oxyluciferin" and hot "oxyluciferin" ions break down to the same product that can be recycled to D-luciferin! There are of course some minor products that may be formed from vibrationally excited oxyluciferin, but whether these matter or not, we cannot tell from this work. However, our work suggests fragments to look for in the future.

Conclusions

We have identified the relevant dissociation channels of photoexcited oxyluciferin anions and the minimum energies required. Our work has clearly established that the five-membered thiazolyl ring is most susceptible to fragmentations leaving the benzothiazole ring undamaged except in the case of CN^- formation that occurs with a low probability. Interestingly, there is no indication of scrambling of carbon atoms, which allows for a clear assignment of the fragment ions. The dominant fragment ion (deprotonated 2-cyano-6-hydroxybenzothiazole) is the same as the one

formed in fireflies after the enzymatic break down of oxyluciferin, which implies that the recovery of D-luciferin is more or less independent on whether electronically excited oxyluciferin emits light or not.

Acknowledgements

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Figures

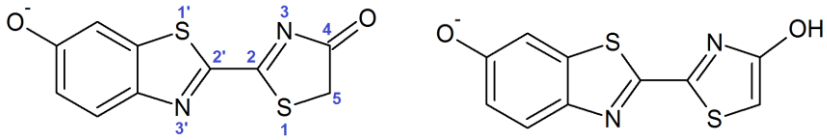


Figure 1. Two tautomeric structures of the oxyluciferin anion. Left: keto form with atom numbering. Right: enol form. Atoms that are isotope labelled in this work are numbered 2, 2', 3, 3', 4, and 5, either as C-13 or N-15. Experiments were either done with both sulphurs (atoms 1 and 1') being S-32 or one being S-32 and the other S-34, randomly.

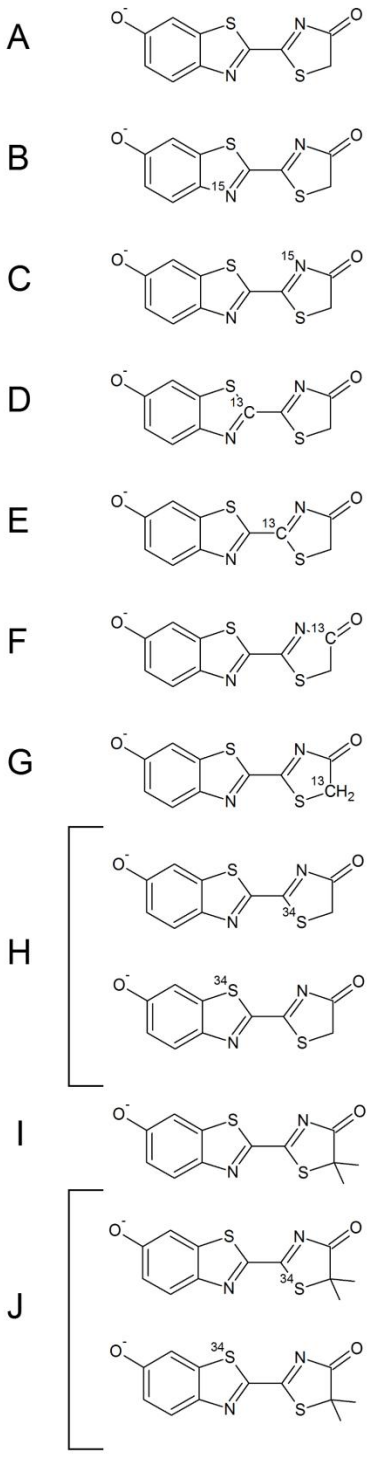


Fig. 2. Oxyluciferin and 5,5-dimethyl oxyluciferin anions subject for study.

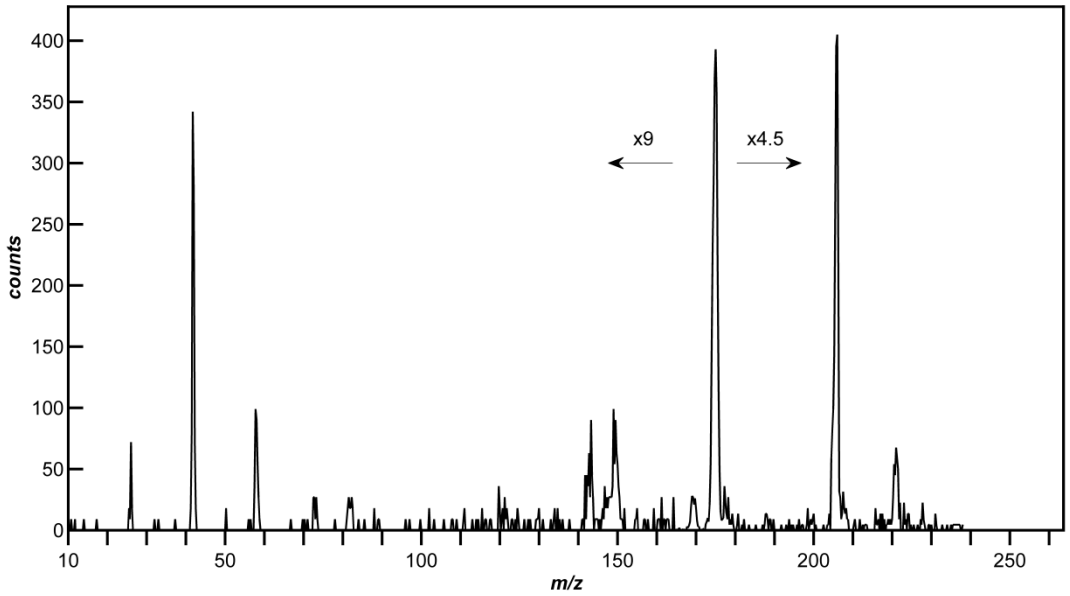


Figure 3. PID mass spectrum of oxyluciferin anions (*m/z* 249). The spectrum ends before the parent ion to avoid saturation of the detector. The peak at *m/z* 170 is an artefact from *m/z*-175 ions being deflected in the analyzer into the channeltron detector.

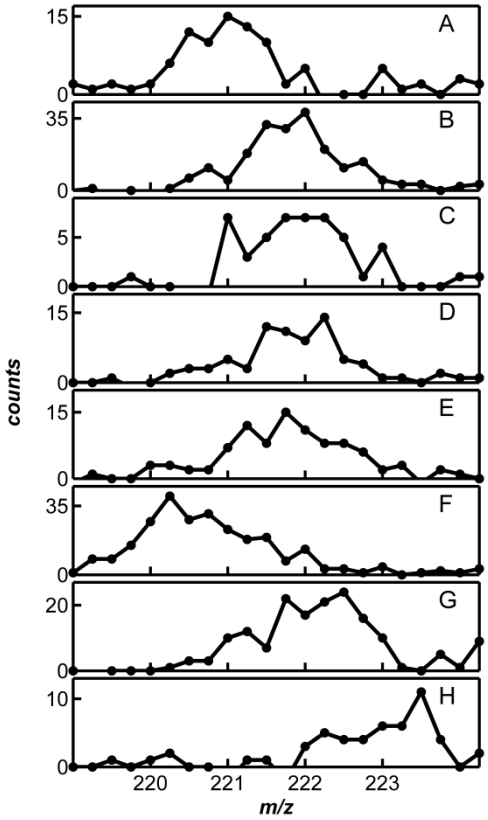


Figure 4. Narrow-scan mass spectra. The A-H labelling refers to the parent ion, see Fig. 2. The mass of the daughter ion with all carbons being C-12, both nitrogens N-14, and both sulphurs S-32 is 221 (see Fig. 5).

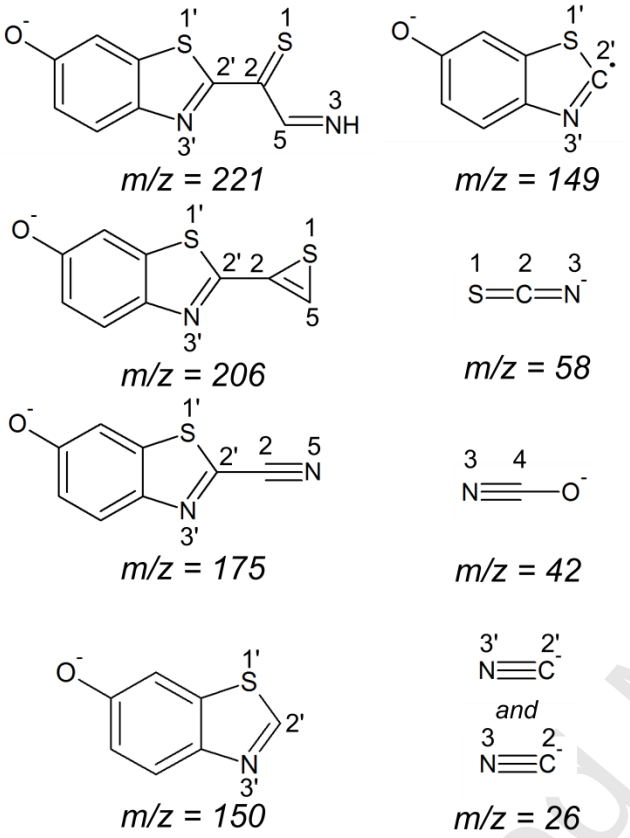


Fig. 5. Summary of assigned fragment ions.

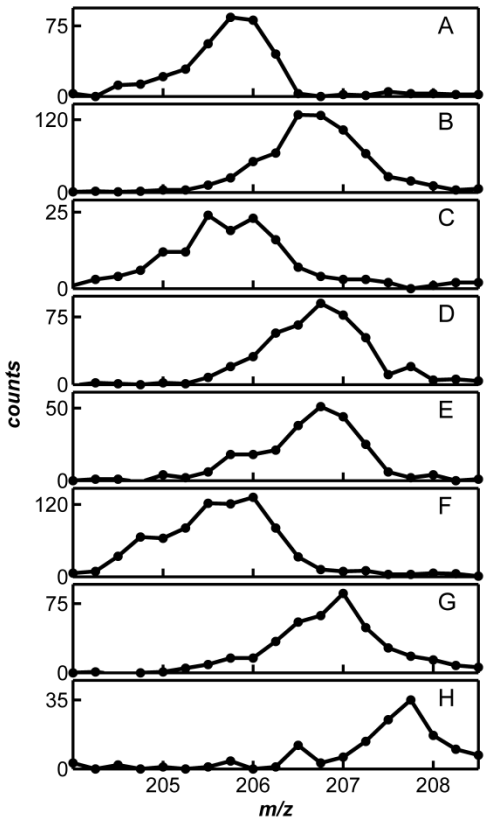


Figure 6. Narrow-scan spectra. The A-H labelling refers to the parent ion, see Fig. 2. The mass of the daughter ion with all carbons being C-12, nitrogen N-14, and both sulphurs S-32 is 206 (see Fig. 5).

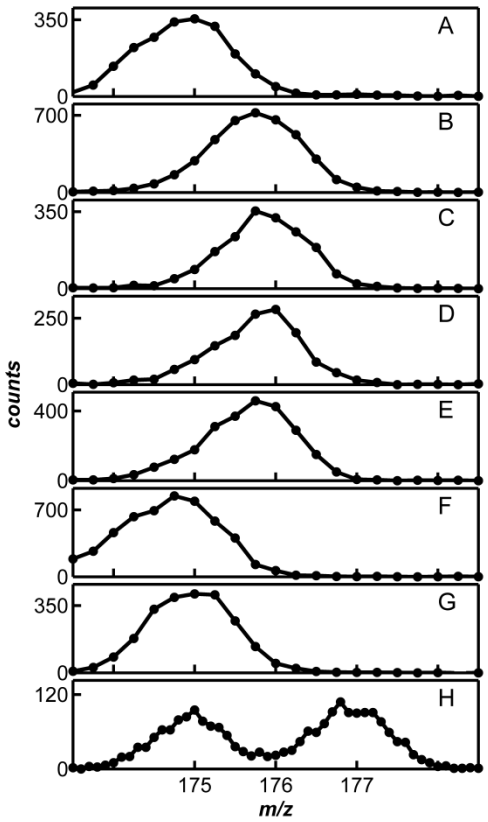


Figure 7. Narrow-scan mass spectra. The A-H labelling refers to the parent ion, see Fig. 2. The mass of the daughter ion with all carbons being C-12, both nitrogens N-14, and sulphur S-32 is 175 (see Fig. 5).

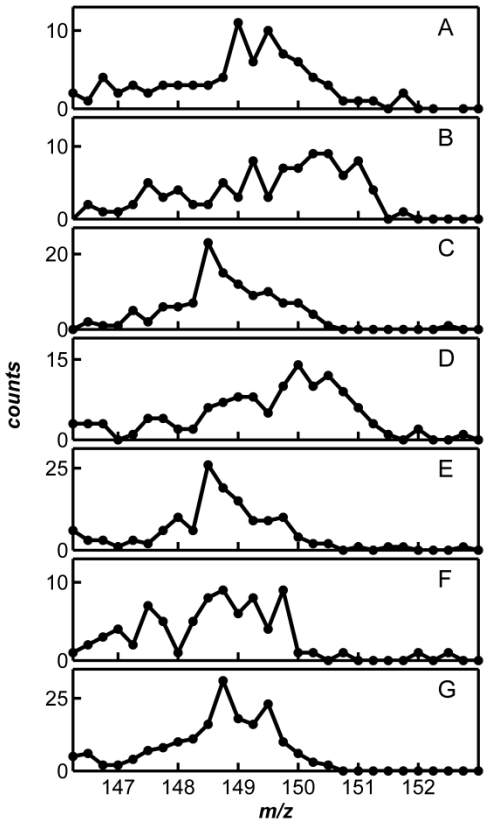


Figure 8. Narrow-scan mass spectra. The A-H labelling refers to the parent ion, see Fig. 2. The masses of the two relevant daughter ions with all carbons being C-12, nitrogen N-14, and sulphur S-32 are 149 and 150 (see Fig. 5).

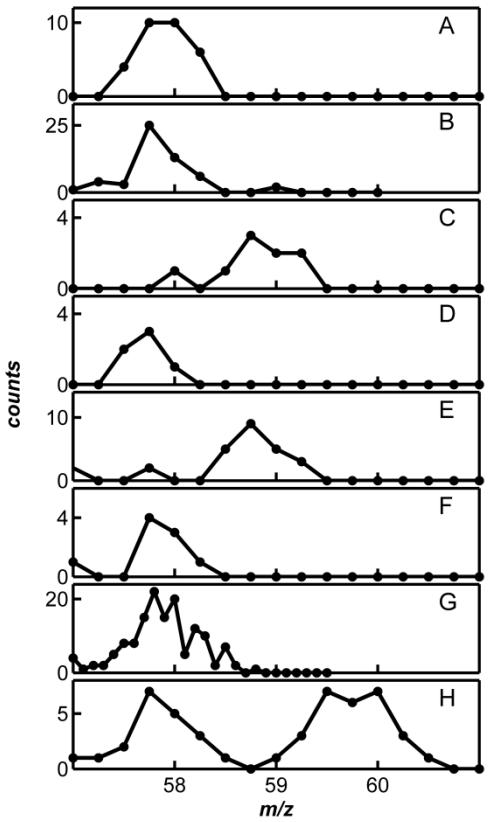


Figure 9. Narrow-scan mass spectra. The A-H labelling refers to the parent ion, see Fig. 2. The mass of the daughter ion with carbon being C-12, nitrogen N-14, and sulphur S-32 is 58 (see Fig. 5).

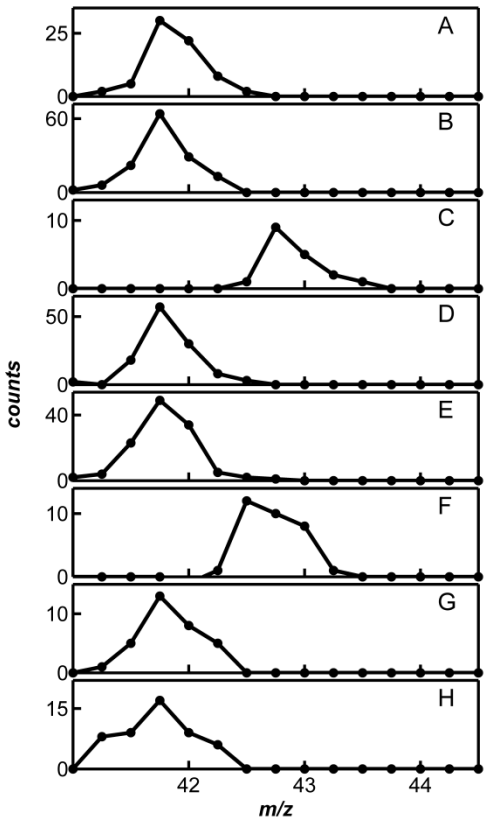


Figure 10. Narrow-scan mass spectra. The A-H labelling refers to the parent ion, see Fig. 2. The mass of the daughter ion with carbon being C-12 and nitrogen N-14 is 42 (see Fig. 5).

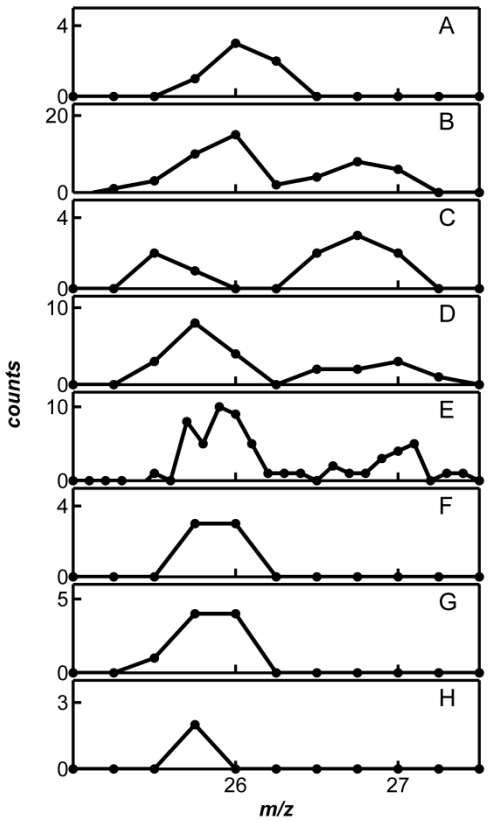


Figure 11. Narrow-scan mass spectra. The A-H labelling refers to the parent ion, see Fig. 2. The mass of the daughter ion with carbon being C-12 and nitrogen N-14 is 26 (see Fig. 5).

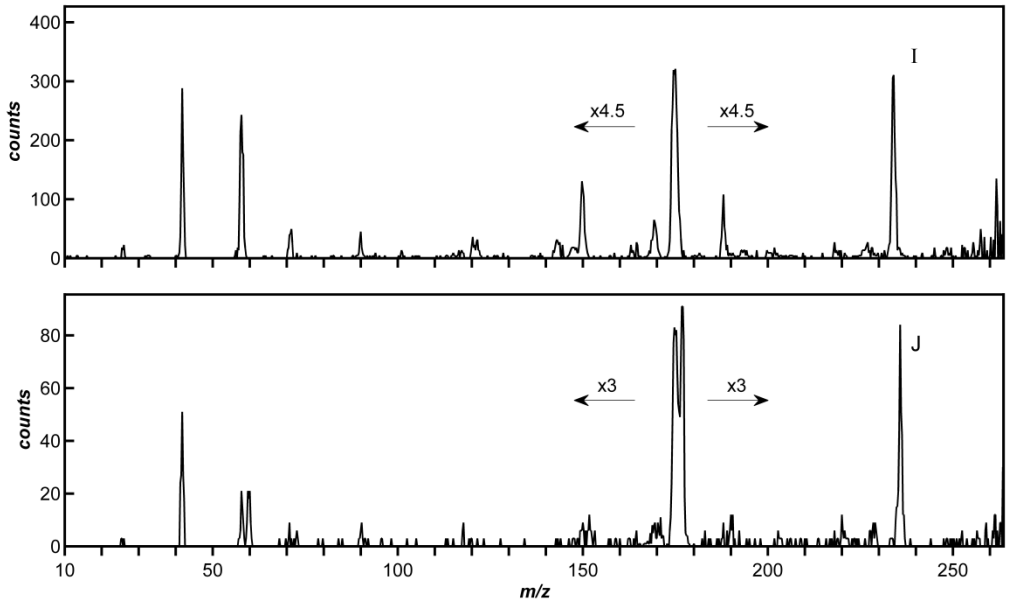
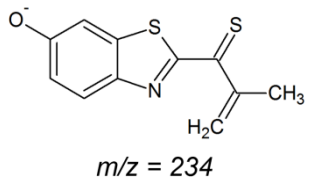
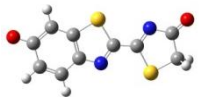


Figure 12. PID spectra of 5,5-dimethyloxyluciferin. Top spectrum: *m/z* 277 (both sulphurs are S-32). Botom spectrum: *m/z* 279 (one S-32 and one S-34).



m/z = 234

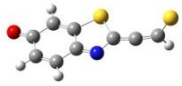
Figure 13. Suggested structure of the *m/z*-234 fragment ion formed from 5,5-dimethyloxyluciferin.



Oxyluciferin (*m/z* 249)

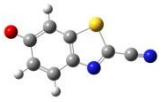
m/z 221

mass 28 1.41 eV



m/z 206

mass 43 2.08 eV



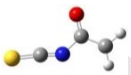
m/z 175

mass 74 1.86 eV

m/z 150

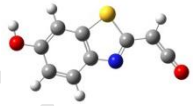
mass 99 2.44 eV

m/z 149



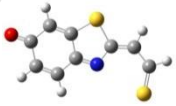
mass 100 4.60 eV

m/z 58



mass 191 1.12 eV

m/z 42

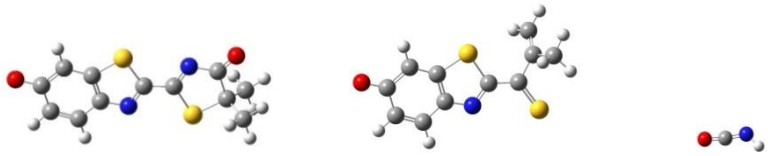


mass 207 2.62 eV

m/z 26

mass 223 2.80 eV

Figure 14. Optimized structures and reaction energies.



m/z 277 *m/z* 234 mass 43

Figure 15. Optimized structures of 5,5-dimethyloxyluciferin, the *m/z*-234 fragment ion and the mass-43 fragment. The energy required for the fragmentation is calculated to be 1.04 eV.