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Red-shifting the optical response of firefly oxyluciferin with group 15/16 substitutions

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Time-dependent density functional theory has been used to investigate the effects of group 15/16 element substitution on the optical response of firefly oxyluciferin. A range of analogues containing symmetrical substitutions at the N and S atom positions of the naturally-occurring oxyluciferin have been found to have red-shifted electronic excitation energies with the heaviest derivative investigated (As/Se) displaying a shift of -0.69 eV. Fluorescence emission wavelengths for all P- and As-containing derivatives in DMSO are estimated to lie in the 710-930 nm region making them interesting for bio-imaging applications.

1 Introduction

The chemical reaction responsible for producing the bioluminescent light emission of fireflies is well known and has received a great deal of attention from the research community both experimentally and though the application of theoretical methods.[1–8] Originally driven mostly by the desire to understand this strikingly beautiful natural phenomenon, most recent research in this area has focussed on technological applications in a wide variety of fields that include biomedical imaging of tissue structures and cellular processes and detection of the presence of undesirable microorganisms both in the environment and in factory settings within the food industry.[9]

The firefly bioluminescence reaction involves the oxidation of a substrate molecule, luciferin **1**, to form oxyluciferin **2** (Fig. 1). This reaction is mediated by the enzyme luciferase which in the presence of Mg^{2+} and ATP produces electronically excited oxyluciferin which then decays to its ground state, emitting a photon in the process.[10] The light produced by fireflies is yellow-green in colour ($\approx$ 560 nm) however it has been found that the emission wavelength is sensitive to external factors such as pH or the presence of metal dications such as Cu^{2+} and protein denaturing agents like urea.[11–16] Similarly, amino acid mutations in the luciferase enzyme itself have been used to effect alterations in the colour of light emitted by this reaction.[17–20]

Whilst some of the colour-tuning studies have been directed at understanding purely the microenvironmental factors that govern emission colour in the native biological setting, technological application has again been an important driving factor in this work. In particular, the use of the firefly bioluminescence reaction in biomedical imaging[1,17,21–25] suffers from the drawback that the natural yellow-green light is poorly transmitted through biological tissues and this limits the tissue depth at which such bio-imaging can be usefully employed. For optimal tissue penetration of the emitted light it would be preferable to shift the emission colour to the red end of the spectrum where light is much more weakly absorbed by mammalian tissues.

A number of the luciferase mutants so far produced have in fact achieved this red-shift to a greater or lesser degree and this continues to be a highly promising approach to the production of an optimised reaction system for use in bio-imaging. In addition to this there has been a significant effort to alter the

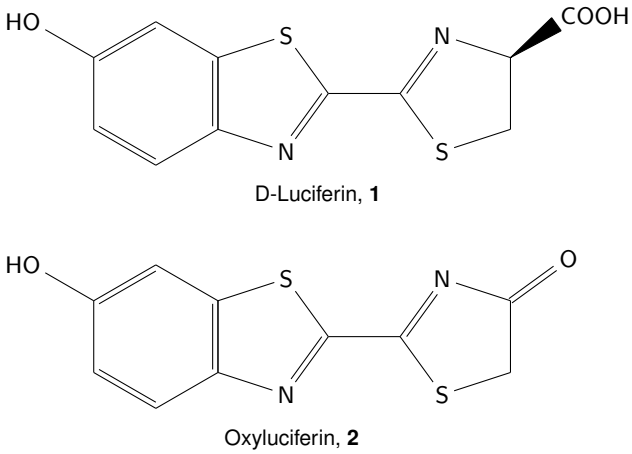

Fig. 1 Structures of natural substrate (D-luciferin **1**) and product (oxyluciferin **2**) of the firefly bioluminescence reaction.

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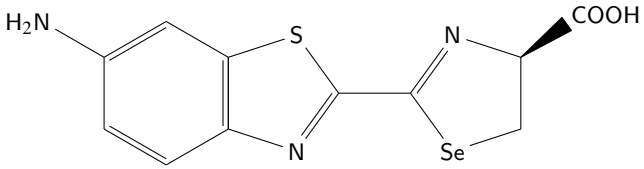


Aminoseleno-D-luciferin, **3**

Fig. 2 Synthetic luciferin analogue employed by Conley et al (2012).[28]

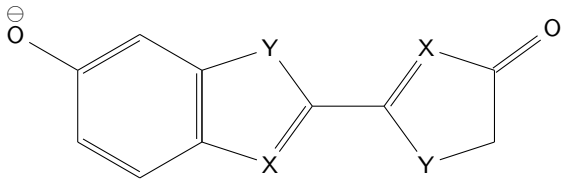
structure of the substrate molecule in such a way that the oxyluciferin produced by the bioluminescence reaction will emit light of different wavelengths to the natural system even when the native luciferase enzyme is used.[3]

Many of the luciferin modifications investigated to date have focussed on the addition of side groups or the alteration of the 6-hydroxy moiety (either its movement to a different position or replacement with e.g. an amine group).[26,27] A similar approach was taken by Conley et al (2012) in which they substituted the 6-hydroxy group with a 6-amino moiety, however, the luciferin was then further altered by the replacement of the sulfur atom in the carboxythiazoline ring with a selenium atom.[28] The luciferase reaction using this aminoseleno-D-luciferin **3** (Fig. 2) was found to produce red light whereas the amino-substitution alone produced orange light indicating that the selenium substitution had an important red-shifting effect.

In the present work theoretical calculations using time-dependent density functional theory (TDDFT) have been used to investigate the effects on the absorption spectrum of the emitter oxyluciferin of making group 15 and 16 substitutions in both the benzothiazole and thiazolinyl rings. It has been found that there is a systematic red-shift in the first excitation energy as the atomic weight of the substituted atoms increases from N (O) to As (Se). Thus a similar effect on the oxyluciferin emission wavelength is to be expected should this modification route be followed. Estimated emission wavelengths suggest that the heavier molecular weight analogues may display fluorescence at values approaching $\sim$ 930 nm.

2 Methods

Nine different substitution patterns were chosen for study representing the possible symmetric substitutions of N, O, P, S, As and Se (Fig. 3). Higher weight elements were not considered due to steric crowding in the oxyluciferin 5-membered rings and the distortions of the molecule likely to arise as a result. Only symmetrical substitutions were considered in order to avoid over-complication of the data in the present study. Asymmetrical substitutions are to be the subject of a future study. The keto-phenolate form of the oxyluciferin structure



	X	Y
4	N	O
5	N	S
6	N	Se
7	P	O
8	P	S
9	P	Se
10	As	O
11	As	S
12	As	Se

Fig. 3 Oxyluciferin keto-phenolate analogues incorporating group 15 and 16 substitutions studied in the present work.

was chosen since there is a growing consensus in the literature that this is the key emitter species involved in bioluminescent light-production.[3,29]

All calculations were performed using GAMESS-US (1st October 2010 (R1) release).[30] Ground state (GS) geometries were optimised at the DFT-CAM-B3LYP level using the 6-31+G(d) basis set.[31–37] The CAM-B3LYP functional has been found to provide a better description of bond-length alternations in conjugated systems than the standard B3LYP functional which can be important in the description of the optical absorption of these systems.[38] In order to simulate a microenvironment of medium polarity the polarisable continuum model (PCM) was used.[39,40] The chosen solvent was DMSO both because this is known from experimental studies to dissolve the oxyluciferin anion.[41]

The GS absorption spectra were calculated with time dependent density functional theory (TDDFT)[42] at the GS geometries using the TD-CAM-B3LYP method with the aug-cc-pVTZ basis set[43,44] and including PCM DMSO solvation. The GS DFT integration grids were increased above their default values of 96 for the radial grid and 302 for the Lebedev angular grid to 120 and 1202 points, respectively. Similarly, the integration grid used in the excited state TDDFT calculations was increased from the defaults of 48 (radial) and 110 (angular) to 96 and 302, respectively. These values are expected to render the integration grids negligible as a source of error in the calculations.

The TD-CAM-B3LYP approach, which was designed to provide a more accurate description of long-range interactions such as those important in charge-transfer type excitations, was previously found to produce absorption energies almost identical to those obtained with the much more computation-

Table 1 TD-CAM-B3LYP/aug-cc-pVTZ/DMSO first excitation energies and oscillator strengths (f) for X,Y analogues shown in Fig. 3. Estimated fluorescence emission values given in parentheses.

	X	Y	eV	nm	f
4	N	O	2.84 (2.26)	437 (549)	0.975
5	N	S	2.60 (2.02)	477 (614)	0.943
6	N	Se	2.54 (1.96)	488 (633)	0.948
7	P	O	2.33 (1.75)	532 (709)	0.764
8	P	S	2.15 (1.57)	577 (790)	0.755
9	P	Se	2.13 (1.55)	582 (800)	0.782
10	As	O	2.13 (1.55)	582 (800)	0.670
11	As	S	1.95 (1.37)	636 (905)	0.670
12	As	Se	1.91 (1.33)	649 (932)	0.700

Table 2 CAM-B3LYP/aug-cc-pVTZ/DMSO frontier orbital energies / eV.

	X	Y	HOMO	LUMO	Δ
4	N	O	-6.31	-1.41	4.90
5	N	S	-6.29	-1.65	4.64
6	N	Se	-6.27	-1.69	4.58
7	P	O	-6.24	-1.78	4.46
8	P	S	-6.27	-2.02	4.25
9	P	Se	-6.28	-2.07	4.21
10	As	O	-6.14	-1.92	4.22
11	As	S	-6.14	-2.15	3.99
12	As	Se	-6.15	-2.22	3.93

ally demanding equation of motion coupled cluster singles and doubles (EOM-CCSD) method for the oxyluciferin anion in the gas phase.[6] Furthermore, the aug-cc-pVTZ basis set used here is of similar quality to the large Def2-TZVPPD basis set used by Støchkel et al and can be expected to provide well-converged results.

3 Results and discussion

3.1 Optical response

The calculated excitation energies are presented in Table 1. The GS absorption value for the natural oxyluciferin **3** was found to be 2.60 eV (477 nm) which is in good agreement with the experimental absorption peak value of 476 nm obtained for the oxyluciferin anion by Naumov et al (2009) in DMSO solvent with one molar equivalent of base.[41] The TD-CAM-B3LYP method was previously found to perform well for native oxyluciferin and related systems.[6,27] In addition to the general properties of the CAM-B3LYP functional and the large size and flexibility of the aug-cc-pVTZ basis set this provides good evidence of the suitability of the methodology employed in the current work.

In the same paper, Naumov et al found the fluorescence emission maximum of oxyluciferin in DMSO to occur at 617 nm representing a shift of –0.58 eV.[41] Using this as an estimate of the possible fluorescence shift that could be expected in the proposed oxyluciferin analogues, potential emission values have been added in parentheses to Table 1.

It is highly likely that differences in the vibrational behaviour of the various analogues would in reality alter this fluorescence emission by changing the exact nature of the vibrational relaxation involved in the Stokes process and this must be borne in mind when evaluating these estimates. Furthermore, direct comparison with the firefly bioluminescence maximum is not entirely appropriate due to the fact that fluorescence emission is not exactly the same process as that involved in bioluminescence and also that the DMSO solvent is likely not an ideal model of the luciferase active site microenvironment. However, the values presented in the current work are believed to provide a reasonable indication of the linear optical response properties of the compounds under study.

Bearing these caveats in mind, the results presented in Table 1 indicate that the first excitation energy can be red-shifted significantly relative to the natural N/S containing oxyluciferin by substitution with heavier group 15 and 16 elements in the 5-membered rings of the molecule. The exception to this being the N/O analogue **4** which has its absorption energy blue-shifted relative to that of the natural species.

The greatest effect is observed with the heaviest group 15/16 elements As and Se. Independently, arsenic effects a 0.65 eV reduction in the first excitation energy of **11** relative to the natural oxyluciferin molecule. Selenium alone shifts the absorption energy of **6** by only -0.06 eV relative to the natural species indicating that the greatest effect arises from the substitution of the group 15 element. When both As and Se are combined in analogue **12** the shift is -0.69 eV, approximately the sum of the two individual shifts and suggesting that the mechanisms by which the elements from the different groups contribute to this shift may be relatively independent although additive. Interestingly, the red-shift observed by Conley et al when utilising the aminoseleno-D-luciferin substrate **3** in the bioluminescence reaction was found to be mediated mainly by the substitution of the 6-hydroxy group by 6-amino with the substitution of the thiazolinyl sulfur by selenium only producing a further shift of approximately -0.05 eV, similar to that observed in the present work.[28]

3.2 Frontier electronic states

Alterations in the first excitation energy can be expected to be reflected in changes in the frontier electronic states (those likely to be most heavily involved in the lowest energy excitations). The energies of the HOMO and LUMO states are

given in Table 2 along with the energy difference between these states, Δ. From these data it can be seen that there is a decrease in the value of Δ on going down the table that follows the trend seen for the excitation energies in Table 1.

The origins of this decrease come from alterations in the energies of both HOMO and LUMO caused by the group 15/16 substitutions. As was seen with the excitation energies in Table 1 the frontier state energies are most strongly perturbed by the group 15 substitution with smaller effects being seen on alteration of the group 16 atoms.

Spatial distributions for the HOMO and LUMO states of the natural oxyluciferin keto-phenolate **5** and the selenarsole derivative **12** are shown in Fig. 4. The analogue **12** was chosen because it displayed the greatest red-shift in the TDDFT calculations and might be expected to also display any alterations to the charge density distributions of the frontier states caused by the group 15/16 substitutions.

In both the HOMO and LUMO it is possible to see obvious changes in **12** when compared to the natural compound. The HOMO of oxyluciferin **5** is mainly localised on the phenoxybenzothiazole moiety with some density evident on the thiazolinyl nitrogen and a smaller amount visible on the keto oxygen whilst the LUMO is more delocalised but has a large portion of its density located on the thiazolinyl ring.

In contrast, the HOMO of analogue **12** is clearly delocalised onto the arsenic of the selenoarsolinyl ring and keto oxygen. In addition, the LUMO of **12** is more localised in the As-C-C-As region of the molecule with no density evident on either of the Se atoms unlike natural oxyluciferinate anion **5** where both sulfurs play a significant role in the LUMO distribution. Changes in the electronic structure in this bridging region of the molecule have previously been suggested as being highly important in modulating the oxyluciferin optical response.[45]

From these spatial features of the frontier states it appears that the degree of charge-transfer nature in a HOMO-LUMO transition should be less in the case of **12** than in oxyluciferin **5** and therefore the energy of the transition would be expected to be higher, contrary to the TDDFT results obtained in the present work.[46]

Fortunately, it is possible to better characterise the nature of an electronic transition calculated with TDDFT by using the Λ diagnostic of Tozer and co-workers (2008).[31] Λ provides a measure of the degree of spatial overlap between all occupied and unoccupied electronic states participating in a given electronic excitation and takes a value in the range $0 \leq \Lambda \leq 1$. Values near 0 correspond to highly delocalised excitations (e.g. Rydberg excitations) whilst values approaching 1 indicate very localised character. In the original paper an upper bound of $\Lambda \approx 0.55$ was found for intermolecular charge–transfer type excitations using the TD-CAM-B3LYP method.[31]

The Λ values corresponding to the TD-CAM-B3LYP/aug-cc-pVTZ excitations calculated in the present work are presented in Table 3. The value obtained for **5** was 0.667 which suggests that in the natural oxyluciferin the first excitation already displays a distinctly local character. With the exception of the N/O species **4** all other analogues were found to have Λ values greater than that of the natural compound and this value

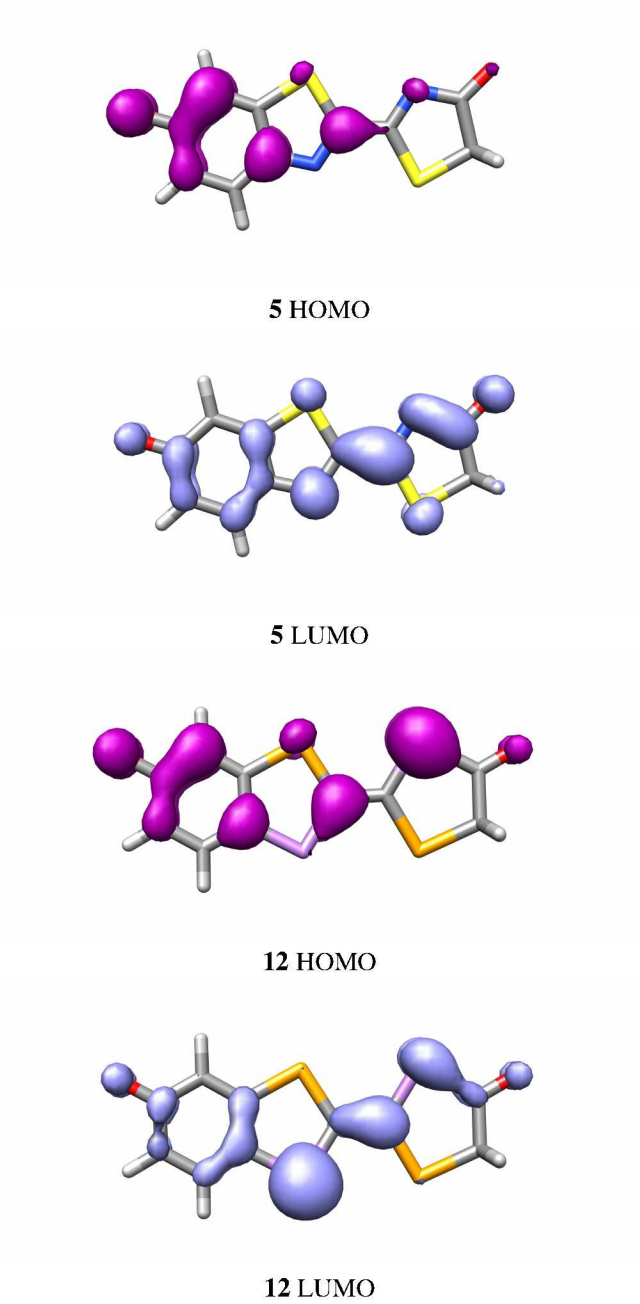


Fig. 4 Frontier electronic state isosurfaces (cut-off = 0.05 a.u.) for natural oxyluciferinate keto-phenolate anion **5** and As/Se analogue **12**.

Table 3 TD-CAM-B3LYP/aug-cc-pVTZ/DMSO Λ diagnostic in arbitrary units.

	X	Y	Λ
4	N	O	0.641
5	N	S	0.669
6	N	Se	0.686
7	P	O	0.671
8	P	S	0.694
9	P	Se	0.704
10	As	O	0.712
11	As	S	0.705
12	As	Se	0.718

Table 4 (TD-)CAM-B3LYP/aug-cc-pVTZ/DMSO electric dipole moments μ in Debye units.

	X	Y	μ^{GS}	μ^{ES}	$\Delta\mu$ / %
4	N	O	12.63	6.34	49.8
5	N	S	12.51	5.43	56.6
6	N	Se	13.19	5.65	57.2
7	P	O	12.44	6.68	46.3
8	P	S	12.13	6.01	50.4
9	P	Se	12.73	6.76	46.9
10	As	O	14.15	7.92	44.0
11	As	S	13.62	7.63	44.0
12	As	Se	13.45	8.21	39.0

exceeded 7 in the heaviest analogues **8 – 12**. The substitution of the N atoms with P and As was found to produce a larger shift towards local character than the group 16 elements which again served more to fine–tune the final Λ value.

These results suggest that in fact there is perhaps little charge–transfer character in the first electronic excitation of the keto–phenolate form of the oxyluciferin anion **5** and that what charge–transfer character there may be in this excitation is in fact reduced on going to the heavier group 15/16 analogues. Thus, it seems more likely that instead of drastically altering the fundamental nature of the excitation itself, these substitutions are more likely to be inducing the observed color shift simply due to alterations in the potential experienced by the electronic states involved in this transition. Such a change in the intramolecular potential acting on the frontier electrons is consistent with the results obtained here and also fits with the conclusions of previous studies that have shown that an intermolecular or artificially applied external potential can produce large color shifts in the oxyluciferin system.[6,16,45,47,48]

3.3 Electric dipole moment

An important feature of the interaction of the oxyluciferin emitter species with the active site of the luciferase enzyme has been shown to be the way in which the molecule is polarised by the active site local electric field.[49] This is important not only in determining the emission wavelength by altering the energy levels and/or orbital overlap involved in the electronic transition but is also likely important in the stages of the reaction that forms the emitter species. Unfavourable electrostatic interactions with the environment could be expected to adversely effect the course of the reaction itself by altering details of the potential energy surface corresponding to the reaction process.

A useful metric that provides some indication of how similarly or differently the oxyluciferin analogues studied here might interact with the luciferase active site is the electric dipole moment μ. Table 4 contains values of μ calculated at the ground state geometry and reported in Debye units (D). An important aspect is how μ might be expected to change between the ground (GS) and excited state (ES) (as both feature in the reaction process) and for this reason both μ^{GS} and μ^{ES} are provided.

The values obtained here are larger than those previously calculated in the gas phase for uncharged oxyluciferin **2** (μ^{GS} = 7.13 and μ^{ES} = 4.36).[26] However, given the fact that the current work deals with the anionic species plus the inclusion of DMSO solvation and differences in the (TD)DFT methods used this is not unexpected.

The data in Table 4 indicate a general increase in the values of both μ^{GS} and μ^{ES} on going from N to As. The change in dipole moment between the GS and ES $\Delta\mu$ drops somewhat for the As-containing species relative to the lighter group 15 analogues but still indicates lower polarity in the electronically excited state. This reduction in $\Delta\mu$ is in line with smaller charge-transfer character in the excitation as would be expected with the changes in the HOMO/LUMO density distributions seen above. The values of $\Delta\mu$ obtained here are consistent with the value of 38.8 % obtained in the gas phase.[26]

The changes in dipole moment relative to the natural species **5** are negligible for the N- and P-containing compounds (with the exception of the N/Se analogue **6**) and suggest that all of these might interact with the luciferase active site in similar ways. The greater increase shown by the As-containing analogues might be a cause for concern in relation to their suitability for involvement in the bioluminescence reaction but as a number of luciferin derivatives with much more prominent substitutions have been shown to be suitable substrates for the luciferase reaction this concern may prove unwarranted.

3.4 Geometry

The optimised geometries of all oxyluciferin analogues studied were found to be approximately planar and the differences caused by the incorporation of the various substitutions were mainly localised within the rings containing the substitu-

tions. As the first excitation in oxyluciferin is mostly HOMO-LUMO in nature and the majority of the charge density associated with population of these orbitals is located separately on the benzothiazole (HOMO) and thiazolinyl (LUMO) components of the molecule, two important geometrical parameters are, first, the overall length of the oxyluciferin analogue and second, the degree of rotation of the 5-membered rings relative to one another. In Table 5 both the distance between the 6-hydroxyl oxygen and the keto oxygen at the opposite end of the molecule and the X-C-C-X dihedral angle are given. A reduction in the angle formed by the two terminal oxygens and the group 15 atom in the benzothiazole moiety was evident in the optimised structures (see Fig. 4) and so this angle is also included in Table 5.

Little deviation from planarity was observed in the X-C-C-X dihedral angle for any of the N- or P- containing oxyluciferin analogues. Less than 4° out-of-plane twist was found for the series of derivatives **4**–**9**. A larger deviation from planarity was observed for the As/S and As/Se species **11** and **12**. Here the dihedral angle dropped to $\sim 170°$, possibly due to the steric hindrance caused by the close proximity of these relatively bulky atoms.

The length of the molecule as measured by the distance between the two terminal oxygens was found to increase in a manner that depended on both the group 15 and group 16 substitutions with heavier elements leading to longer O-O distances. In the case of the As/Se species **12** the length of the molecule was found to increase by ~ 1 Å relative to natural oxyluciferin.

Overall, the O-X-O angle was reduced by up to $\sim 15°$ on going from N to As. The magnitude of this reduction also depended on the group 16 element which countered the group 15 effect on the angle to some extent.

As with the alterations in dipole moment discussed above, whether or not the observed changes in the oxyluciferin geometry could have an adverse impact on the interaction with the luciferase active site is difficult to assess and really the only way to answer this would be to synthesise the corresponding luciferin species and test them experimentally as luciferase substrates.

4 Conclusion

In the present work the effects on the optical response of the oxyluciferinate keto-phenolate anion **5** of substitution of the N and S atoms with group 15/16 elements from the first three rows of the periodic table were investigated. Time-dependent density functional theory was used in order to evaluate the possibility of using this as a method by which the bioluminescence emission colour of the firefly luciferase reaction might be modulated to provide red-shifted emission wavelengths more useful in bio-imaging applications.

Table 5 CAM-B3LYP/6-31+G(d)/DMSO selected geometrical parameters. Bond length in Å and angles in degrees.

	X	Y	O-O	O-X-O	X-C-C-X
4	N	O	9.943	135.07	176.28
5	N	S	10.433	135.55	179.22
6	N	Se	10.559	138.86	179.76
7	P	O	10.357	114.54	178.64
8	P	S	11.071	125.24	179.90
9	P	Se	11.257	128.34	179.72
10	As	O	10.437	111.97	178.03
11	As	S	11.212	122.85	169.05
12	As	Se	11.420	126.09	172.07

A range of symmetrically-substituted oxyluciferin analogues were investigated and it was found that the first excitation energy could be reduced by ~ 0.7 eV on going from the natural N,S-containing compound **5** to the As,Se-containing derivative **12**. Incorporating the experimental data obtained by Naumov et al it was estimated that this could be expected to shift the fluorescence in DMSO solution from 614 nm (**5**) to 932 nm (**12**).

Analysis of the frontier electronic state energies and spatial distributions indicated that the substitutions lead to a reduction in HOMO-LUMO energy gap with a concomitant change in spatial distribution suggesting some reduction in charge-transfer character in the associated electronic transitions. Increases in the value of the Λ diagnostic for the first excitations of the heavier analogues support this conclusion. HOMO-LUMO overlap at the position of the thiazolyl N atoms was previously proposed as playing a key role in determining the features of the first electronic excitation in oxyluciferin and this is supported by the present results.[45] Thus, the most important effect of altering the substitution pattern is most likely in changing the potential influencing these frontier states. This is in agreement with previous theoretical results that showed that an external applied potential could be used to produce a similar effect by longitudinal polarisation of the charge density in oxyluciferin.[48]

Whilst some geometrical changes were observed in the analogues compared with the natural compound the planarity of all species was essentially maintained. The overall length increased by ~ 1 Å in the heaviest As- and Se-containing derivatives, possibly being the most significant geometrical effect observed, however it is felt that these changes are unlikely to adversely influence the interaction of these compounds with the luciferase enzyme active site. Similarly, alterations in the ground- and excited-state electric dipole moments showed that the polarity of the heavy analogues would be greater than that of natural oxyluciferinate anion. Once again, it seems unlikely that this would to any great extent adversely effect the ability

of the corresponding luciferin molecules to act as suitable luciferase substrates.

Overall, the group 15 substitutions were found to play the largest role in altering the optical response with much smaller contributions coming from the group 16 elements. This is in agreement with previous experimental results and provides good evidence that this may indeed be a profitable approach to the development of bioluminescent photo-labels possessing specific emission properties.[28]

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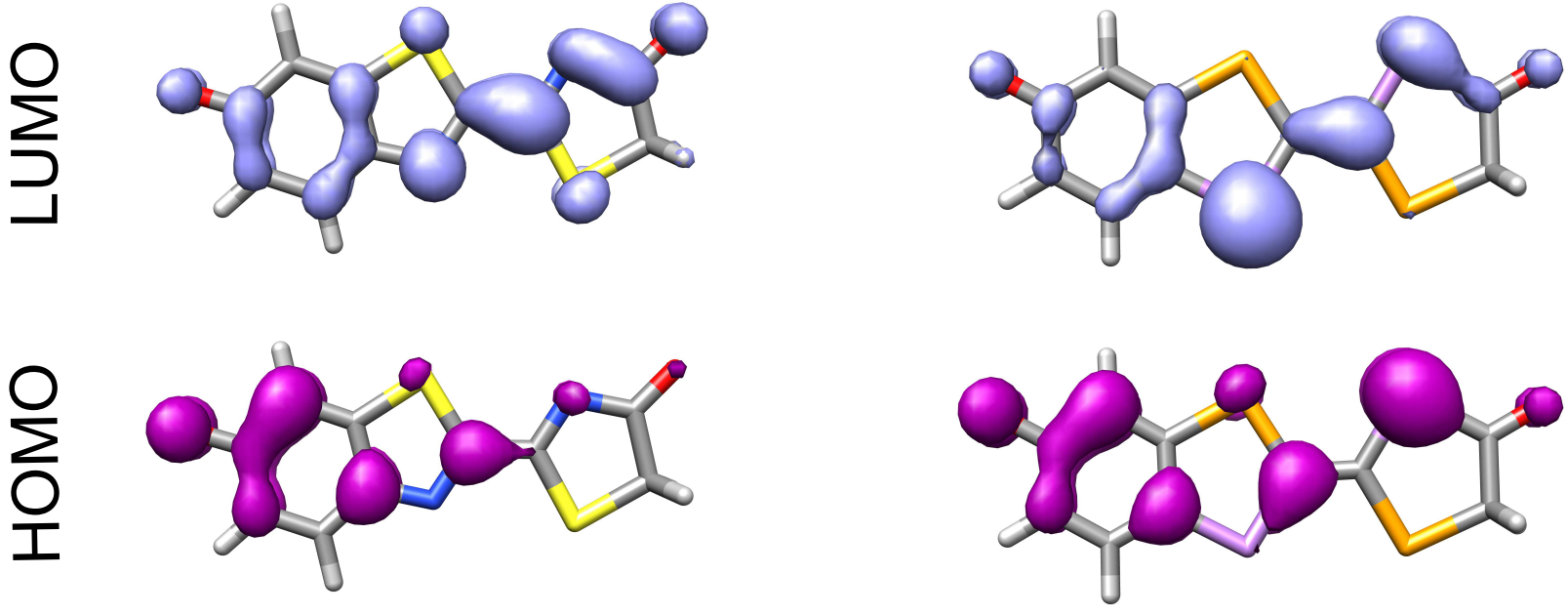
$\Delta E^{S0\rightarrow S1}$ (N/S→As/Se) = -0.69 eV



A range of firefly oxyluciferin analogues symmetrically substituted with group 15 and 16 elements have been found to have red-shifted first excitation energies with the heaviest derivative investigated (As/Se) displaying a shift of −0.69 eV.