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Synthesis and bioluminescence of electronically modified and rotationally restricted colour-shifting infraluciferin analogues

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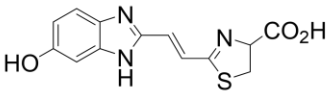

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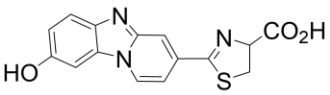

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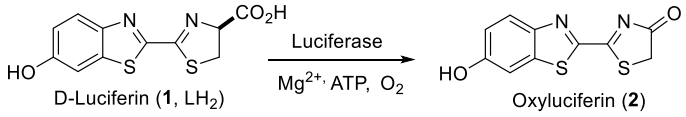
ABSTRACT

Synthetic nIR emitting luciferins can enable clearer bioluminescent imaging in blood and tissue. A limiting factor for all synthetic luciferins is their reduced light output with respect to D-luciferin. In this work we explore a design feature of whether rigidification of an exceptionally red synthetic luciferin, infraluciferin, can increase light output through a reduction in the degrees of freedom of the molecule. A rigid analogue pyridobenzimidazole infraluciferin was prepared and its bioluminescence properties compared with its non-rigid counterpart benzimidazole infraluciferin, luciferin, infraluciferin and benzimidazole luciferin. The results support the concept that synthetic rigidification of π-extended luciferins can increase bioluminescence activity while maintaining nIR bioluminescence.

1. Introduction

The Coleopteran Luciferin-Luciferase system is widely used in bioluminescence imaging (BLI) to track disease and other biological functions *in-vitro* and *in-vivo*.[1] The most commonly used substrate in this assay is the naturally occurring D-luciferin (**1**, LH_2, λ_{max} = 558 nm). In the presence of molecular oxygen, Mg^{2+} ions and ATP, LH_2 is oxidised by luciferase to form oxyluciferin (**2**), an excited-state ketone species that releases a photon of light (558 nm) as it relaxes back to its ground-state (Scheme 1).[2]



Scheme 1

However, the light emitted by D-luciferin is strongly absorbed by melanin, blood and tissue and this makes imaging in small mammals particularly difficult. To allow better image resolution the light emitted should be of higher wavelength in the near Infra-red (nIR) region (600 – 750 nm). Fortunately, the luciferase enzyme has shown considerable tolerance to different substrates and several synthetic nIR luciferins have been made and tested.[3-5]

A pioneer in this area, White prepared the amino-luciferin analogue **3** which had a red-shifted emission spectrum (λ_{max} = 593 nm) *in-vitro* compared to LH_2,[6] but was only about 10% as bright as D-luciferin. Cyclic amino modifications (**4**) by Miller and co-workers resulted in emissions greater than 600 nm.[7] Maki removed the benzothiazole core and replaced it by extended π-conjugation (**5**), which led to substantial increases in emission wavelength (λ_{max} = 675 nm).[8] This molecule as the HCl salt has been shown to be particularly effective for sensitive bioluminescence deep tissue imaging[9] and noninvasive imaging in moving animals.[10] More recently extending conjugation of the benzothiazole by substituting for a suitable napthothiazole resulted in very red shifted analogues **6a,b** (λ_{max} = 730 nm and 743) with mutant CBR2opt luciferases that proved particularly useful for highly resolved deep tissue imaging and tomography.[11] Modifications to the thiazoline portion of LH_2 have shown poorer results, with substitutions by lighter elements (**7a** and **7b**) resulting in no or slight emission and heavier elements (**7c**) resulting in red-shifted weak emission (Fig. 1).[12,13] Recently, compound **8** and the HCl salt of **5** have shown brighter emission than LH_2 at low substrate concentrations.[9,14] Luciferase mutants have also been engineered to give brighter light for some analogues[11,15] including compound **4** under sub-saturating conditions.[16] In 2014, we reported a π-extended analogue of luciferin infraluciferin (**9**, iLH_2) that exhibited nIR bioluminescence (λ_{max} = 706 nm).[17,18] Like other red-shifted synthetic luciferins, iLH_2 (**9**) was less bright than LH_2, but because of the higher proportion of light output above λ = 600 nm there is less attenuation and scatter of the signal by blood and tissue. This leads to better penetration because of a reduced signal to noise effect caused by light scattering.[17] We are still interested in improving the light output of iLH_2 and are investigating mutated luciferases and alternative iLH_2 structures for wider applicability in a variety of challenging bioluminescence imaging applications.

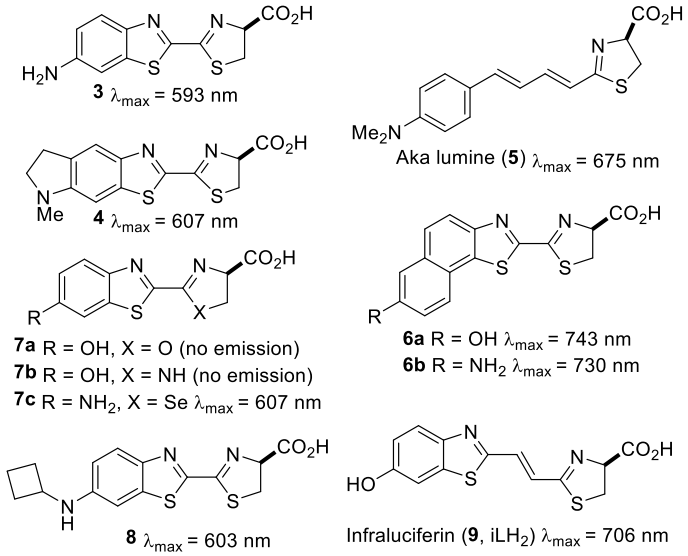


Figure 1. Bioluminescence emissions of some synthetic red shifted luciferins

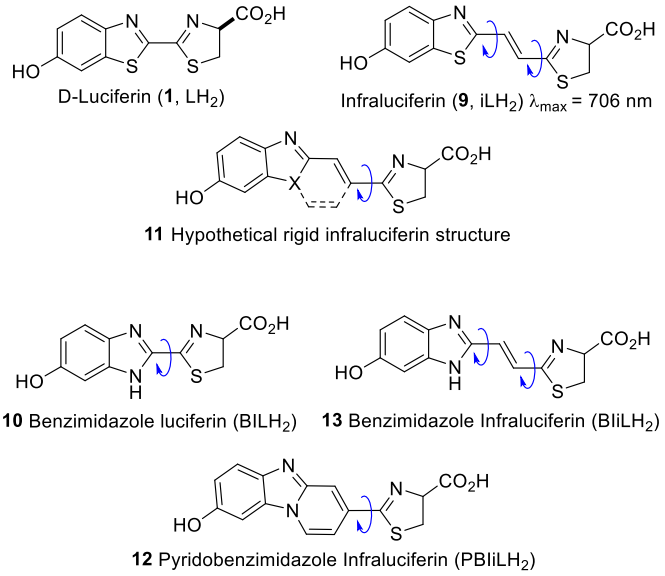


Figure 2. Design hypothesis for rigid infraluciferins

The relatively lower light output of iLH_2 (**9**) compared to LH_2 (**1**) could be due to a number of factors, such as catalytic turnover, product inhibition and radiationless decay caused by rotatable bonds. While we know that the K_m values for iLH_2 (**9**) and LH_2 (**1**) with certain Fluc enzymes is comparable, the specific activity is 100 times less and the L-enantiomer of iLH_2 (**9**) causes some enzyme inhibition,[17] the question of radiation-less decay has not been investigated. Comparing the two molecules iLH_2 (**9**) has more degrees of freedom compared to LH_2 (**1**) due to rotation about an extra single bond (Fig. 2). This could allow more changes of the excited state geometry in the enzyme's active-site, causing greater radiation-less decay and a lower light output than LH_2 (**1**). In this study, we have investigated whether synthesis of a more rigid infraluciferin framework with the same number of rotational single bonds as LH_2 (**1**) affects bioluminescence light ouput.

To rigidify the infraluciferin structure, it was thought that a carbon bridge could be constructed to connect either of the heterocycle rings to the alkene linker (Fig. 2). It is known that substitution of the heteroatoms in the thiazoline ring to C or N lead to inert compounds that give little or no light,[12] Whereas modification of the benzothiazole heterocycle has been reported by Prescher for benzimidazole luciferin (**10**, $BILH_2$) to give bioluminescence emission more red-shifted (λ_{max} = 578 nm) than LH_2 (**1**), but again with a lower quantum yield.[19] Our hypothetical design structure **11** has incorporated this modification to allow a rigidifying linker in pyridobenzimidazole infraluciferin (**12**, $PBIiLH_2$), where X = N so that aromaticity and conjugation (with the corresponding ketone from oxidation) can be maintained in the final compound. Whilst not completely rigid, this structure has the same degrees of freedom and rotatable single bonds as LH_2 (**1**). Benzimidazole infraluciferin (**13**, $BIiLH_2$) would serve as a comparison to $PBIiLH_2$ (**12**) in the bioluminescence studies to test our hypothesis.

2. Results and discussion

2.1. Docking of luciferin analogues in Ppy-Luc

As these analogues scaffolds are different in size and shape than LH_2 (**1**), we have used docking studies to predict the interaction between the adenylated luciferin structures and the luciferase enzyme. For this purpose, the crystal structure of the *Photinus pyralis* luciferase enzyme, bound to the adenylate analogue 5′-O-[N-dehydroluciferyl)-sulfamoyl]- adenosine (LH_2-DLSA) (PDB accession number 4G36) was obtained.[20] GOLD[21] software version 5.4.1 was used for the docking studies. To test our system, we first performed the docking of the energy minimised structure of LH_2-DLSA at the ATP-binding site. Energy minimisation was carried out using the MM2 calculation, Chem3D Pro software version 13.0.2.3021. Calculations were ran on a PC with a 2.3 GHz-i5 processor and 8 GB RAM. The docking solutions were viewed using Discovery Studio Visualizer[22] with the protein surface coloured by hydrophobicity. The docking result for the crystallised ligand was overlayed with the co-crystal structure and the calculated exhaustive root mean squared deviation (RMSD) was 0.7453, indicating a good prediction ability of our docking protocol. We decided to carry out a rigid molecular docking due to close similarity in the binding site between different crystallographic structures.

Starting with the energy minimised structures of $PBIiLH_2$-DLSA and $BIiLH_2$-DLSA, the GOLD wizard was used to carry out the molecular modelling. The binding site was defined to within 6 Å of the LH_2-DLSA analogue and the docking was carried out using the slow method to identify six different solutions. By comparing the docking solutions to the binding mode of LH_2-DLSA, the pose of $PBIiLH_2$-DLSA and $BIiLH_2$-DLSA which gave the best overlay was selected for the basis of our docking model.

Both $PBIiLH_2$ (**12** Fig. 3A) and $BIiLH_2$ (**13** Fig. 3B) fitted into the narrow and deep substrate binding site. Several proposed favourable hydrophobic interactions and hydrogen bonds are shown in Fig. 3C and 3D. This preliminary study showed that it is structurally possible to locate the analogues in the luciferin binding site and gave us added confidence that the real molecules may bioluminesce.

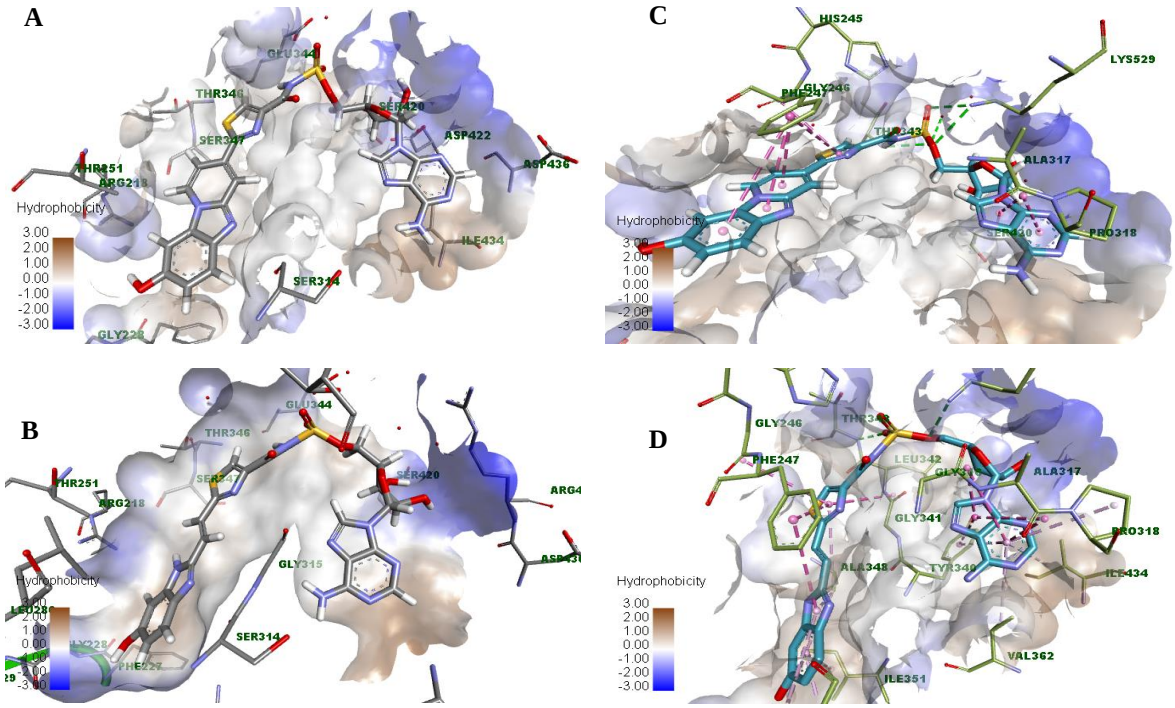
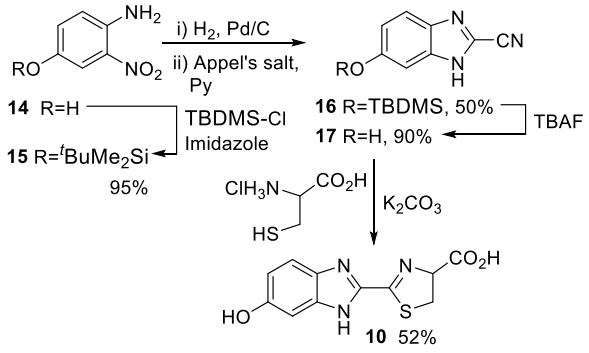


Fig. 3. - **A** and **B** - Pose prediction for $PBIiLH_2$ and $BIiLH_2$ in *PpyLuc* pocket; **C** and **D** - $PBIiLH_2$ and $BIiLH_2$ with predicted interactions; Colour code: Pink – Hydrophobic interactions; Green – hydrogen bonds

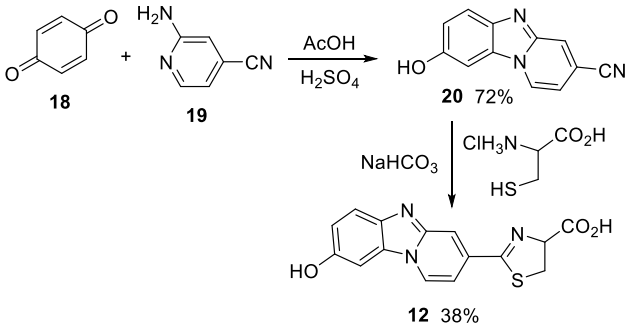
2.2. Synthesis of luciferin analogues

To circumvent possible epimerisation of the carboxylic acid forms of the infraluciferin analogues and accurate quantification of enantiopurity, all substrates were prepared as racemic mixtures using DL-cysteine. We have previously shown that DL-iLH2 (9) is a competent substrate for bioluminescence and that the L-enantiomer does not prohibit light production.[18] The synthesis of $BILH_2$ has been previously reported.[19, 23] The Prescher synthesis[19] was repeated, but in our hands the demethylation of the phenol ether was capricious and gave us poor yields of intermediate **17**. The route was modified by introducing a TBDMS protecting group on the phenol **14** with TBDMS-Cl to give **15** in 95% yield. The rest of the synthesis followed the Prescher route.[19] The nitro group in **15** was reduced by catalytic hydrogenation to give the crude unstable diamine product, which was immediately reacted with Appel's salt in dry pyridine to give the desired benzimidazole **16** in a 50% yield over 2 steps. The TBDMS protecting group was readily cleaved with TBAF at room temperature to obtain the key benzimidazole nitrile **17** in excellent yield (90%). This was then condensed with DL-cysteine hydrochloride to give racemic $BILH_2$ (**10**) in 52% yield (Scheme **2**) to compare against the racemic infraluciferin analogues.[24]



Scheme 2. Appel's salt=4,5-dichloro-1,2,3-dithiazol-1-ium chloride.

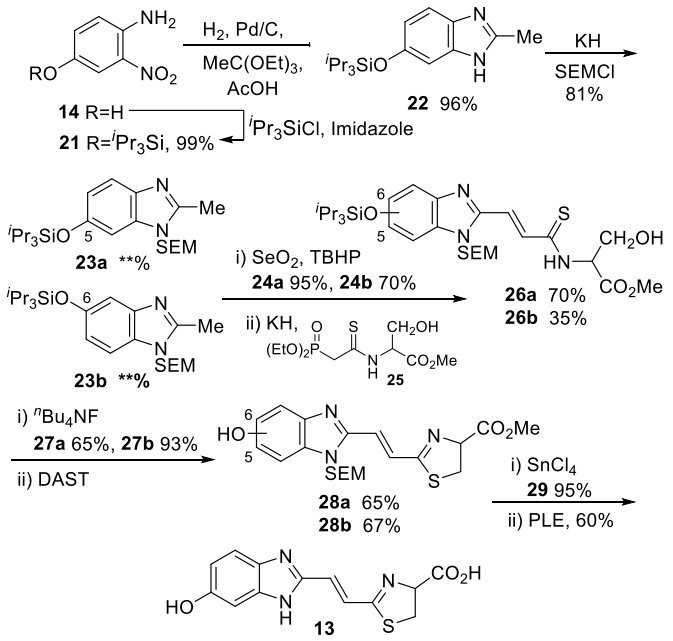
The conformationally restricted, $PBIiLH_2$ **12** was synthesised in two steps. As 2-aminopyridine is known to condense with 1,4-benzoquinone (**18**) in acidic conditions,[25] the pyridobenzimidazole core was synthesised in an analogous fashion by a one-pot condensation/oxidation reaction between 1,4-benzoquinone (**18**) and 2-amino-4-cyanopyridine (**19**), to give the nitrile **20** in 72% yield. DL-cysteine was then condensed with nitrile **20** under refluxing conditions,[26] after no product was obtained at room temperature, to give $PBIiLH_2$ **12** in 38% yield (Scheme **3**).



Scheme 3.

For the synthesis of $BIiLH_2$ (**13**) we followed a very similar route to our published infraluciferin (**9**) synthesis.[18] This required the key aldehydes **24a,b**, which we envisaged could be made from the oxidation of the corresponding methyl precursor **22**. The synthesis of benzimidazole **22** was achieved by following a literature route to an analogous compound[27] with the addition here of a protected hydroxyl group that could be unmasked in the final stages of the synthesis. Phenol **14** was protected with the triisopropylsilyl protecting group under standard conditions (Scheme **4**).[28] Several other protecting groups were also trialled (Boc, MEM, Tosyl) but these were incompatible with the various stages of the final synthetic route. The triisopropylsilyl-protected benzimidazole **22** was synthesised by a one-pot reduction and cyclisation[27] of **21** with H_2, Pd/C catalyst in the presence of triehtylorthoformate to give a single benzimidazole product by

NMR in 96% yield. The exact tautomeric form of the benzimidazole was not determined and we have drawn **22** as a representative structure. Oxidation of the aromatic methyl group to an aldehyde was attempted on **22** using selenium dioxide and TBHP,[29] but this did not work and gave unidentified products. Therefore **22** was protected using the SEM group as there is literature precedent of it being cleaved from sensitive biological substrates.[30] We observed that the SEM group protected each tautomer of the benzimidazole (**23a**:**23b**, 1:1) and these were separated in 41% and 40% isolated yields respectively. Both isomers were separated by column chromatography and each was carried forward in the synthetic route. Oxidation of **23a,b** was successful and key aldehydes **24a** (70%) and **24b** (96%) were obtained in good yields. Each aldehyde was reacted with key phosphonate **25** in a Horner-Wadsowrth-Emmons (HWE) reaction from our previously published route for infraluciferin (**9**).[17] Our reported Masamune and Rouche conditions (DBU and LiCl) [18,31] gave no product, in this case. A variety of bases were screened (NaH and K_2CO_3) and the use of KO^{t}Bu in the HWE reaction yielded the desired products **26a** (70%) and **26b** (35%) in modest yields. The triisopropylsilyl group was easily cleaved by TBAF at this stage, in good yields (**27a** 65%, **27b** 93%), as attempts at removing it at after the thiazoline had been constructed were unsuccessful. The thiazoline, especially in the π-extended analogues we have prepared is more prone to oxidation than luciferin. As a precaution all synthetic manipulations, workups and purifications when the thiazoline was formed were performed in degassed solvents. Cyclisation to the thiazoline (**28a** 65%, **28b** 67%) was achieved by treating thioamides **27a,b** with DAST at -78 °C. Finally, the SEM group was cleaved with Tin (IV) chloride[30] to give the methyl ester **29** in 95% yield. The ester was found to be unstable in base (LiOH) and so was hydrolysed with porcine liver esterase[17] to give $BIiLH_2$ (**13**). The ester and final compound $BIiLH_2$ (**13**) were again isolated as single benzimidazole tautomers by NMR, but the exact structures were not determined.



Scheme 4. SEMCl = 2-(trimethylsilyl)ethoxymethyl chloride; TBHP = tert-butyl hydroperoxide; diethylaminosulfur trifluoride; PLE = porcine liver esterase

2.3. Bioluminescence activity and spectra of analogues with wild-type and thermostable luciferases.

Wild-type (WT) *Photinus pyralis* firefly luciferase (Fluc), thermostable variants and also click beetle red were purified by nickel NTA chromatography and their basic properties were examined with compounds D-LH_2 (**1**), DL-$BILH_2$ (**10**), DL-iLH_2 (**9**), DL-$BIiLH_2$ (**13**) and DL-$PBIiLH_2$ (**12**). Specific activities were first collected by integrating light over the first 2 min of emission using the PhotonIMAGER Optima (PIO) small animal imager. Natural D-LH_2 (**1**) gave the highest activity with all enzymes, followed by DL-$BILH_2$ (**10**), which gave about 100-times lower activity with the majority of enzymes, except CBR, with which activity was 1000-times lower than with D-LH_2 (**1**) (Fig. 4). With x2 and x11 Flucs DL-iLH_2 (**9**) gave a similar level of activity as with the non-extended DL-$BILH_2$ (**10**), demonstrating the efficacy of engineered Flucderivatives when used with iLH_2 (**9**), since they are more active and have less decay of signal (SI Fig). Enzymes gave the lowest activity with DL-$BIiLH_2$ (**13**), which was ~10 000-times less bright than WT Fluc and the majority of the other enzymes with D-LH_2 (**1**), but ~250 000 times less active with CBR. However, the rigidified version DL-$PBIiLH_2$ (**12**) showed an enhancement in luminescence activity with WT Fluc compared to DL-iLH_2 (**9**) and also compared to DL-$BIiLH_2$ (**13**) with each enzyme: 3-fold with WT and x11 Flucs, 5-fold with x2 Fluc, 9-fold with x5 Fluc and 20-fold with CBR. The activity of CBR with DL-$PBIiLH_2$ (**12**) was of a similar level as with both DL-iLH_2 (**9**) and DL-$BILH_2$ (**10**). Therfore we can rationally improve the activity of different luciferases with an electronically modified extended infraluciferin backbone by making it more rigid and reducing its degrees of freedom.

The emission colours of enzymes with each of the analogues were assessed by acquiring spectra through successive 30nm pass-band filters in the PhotonIMAGER Optima (PIO). With D-LH_2 (**1**) enzymes displayed their expected different colours with WT, x5, x11, x2 and CBR giving λmax values ranging from 555nm (WT, x5, x11), 561nm (x2) and 614nm (CBR) (Table 1). As previously described the emission colours (Fig. 5) of enzymes with DL-iLH_2 (**9**) also showed a colour-shifting effect, producing λmax values red-shifted compared to with D-LH_2 (**1**) by 104nm for CBR and 124-142nm for the Flucs. Interestingly, a colour-shifting effect was also seen with DL-$BILH_2$, (**10**) with both x2 Fluc displaying a 23nm red-shift compared to WT, x5 and x11 and CBR displaying a 53nm shift, which is similar to the colours emitted with D-LH_2 (**1**), albeit CBR displayed a smaller blue-shifted peak at ~560nm.

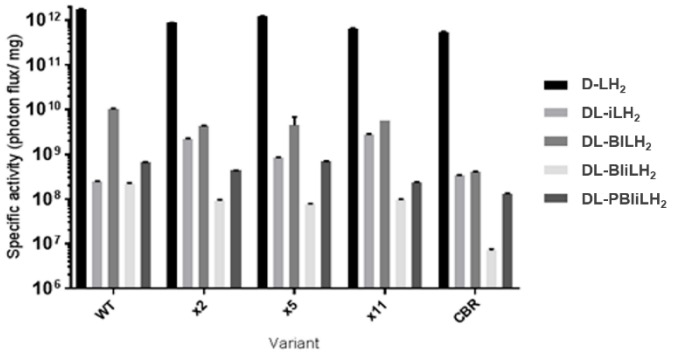


Fig. 4. - Specific activities of wild-type (WT) *Photinus pyralis* firefly luciferase and thermostable x2, x5 and x11 derivatives and click beetle red luciferase (CBR). Enzyme activities were measured immediately upon adding substrate for 2 min in the PhotonIMAGER Optima (PIO).

Table 1. Primary and secondary bioluminescence peak wavelengths of enzymes with different compounds (λ/nm). Values acquired from smoothing (spline fitting) of spectra acquired in bandpasses in the PIO.

	D-LH_2 (**1**)	DL-$BILH_2$ (**13**)	DL-iLH_2 (**9**)	DL-$BIiLH_2$ (**10**)	DL-$PBIiLH_2$ (**12**)
WT	555	567	697	561	614
x2	561	590	691	572	596
x5	555	561	679	555	596
x11	555	561	685	561	608, 714
CBR	614	620, 555	720	620	620

(ketone *cf* Scheme 1) of iLH_2 (**9**). If PBIiLO (**30**) is

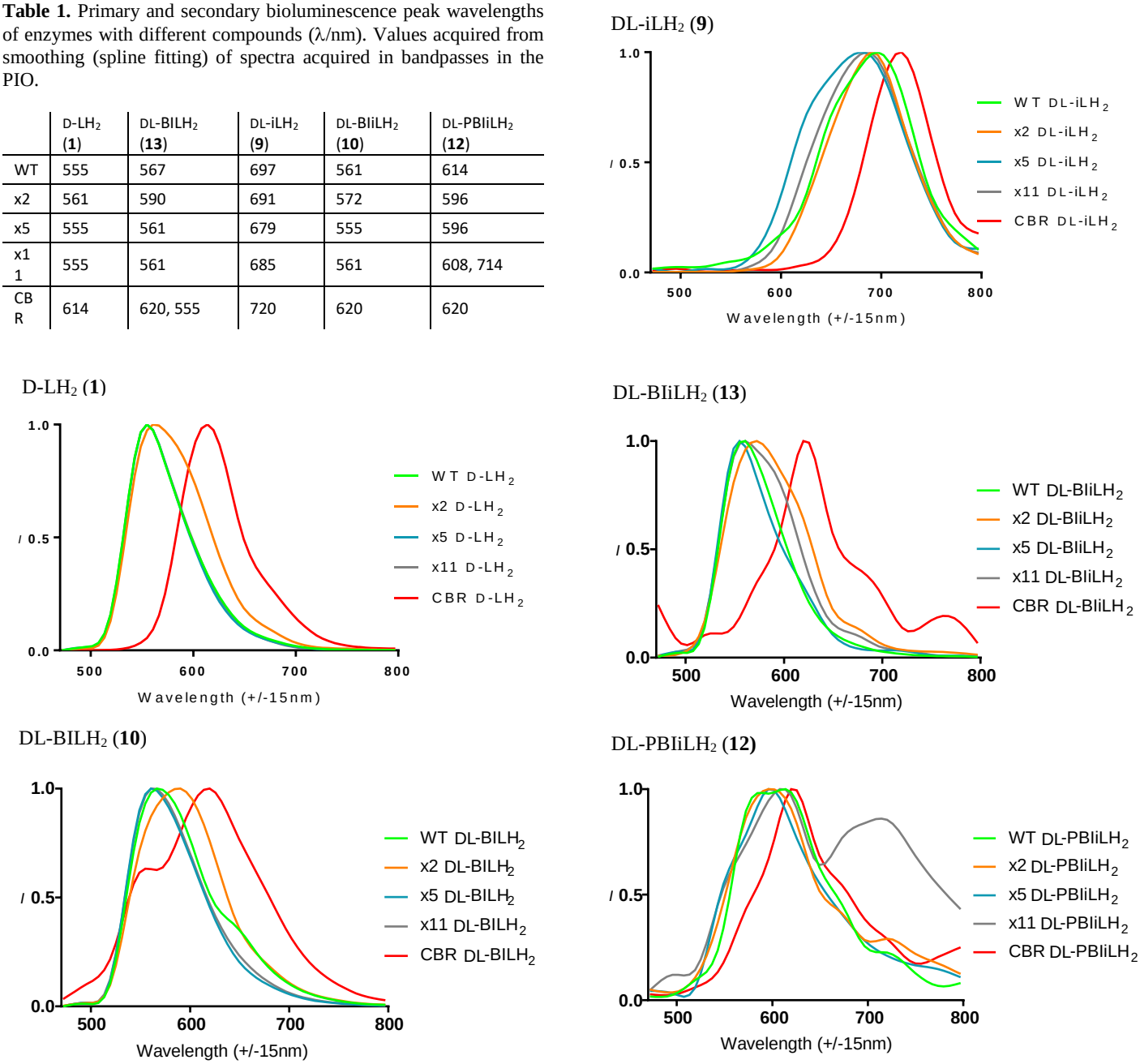


Figure 5. Bioluminescence spectra of WT, x2, x5 and x11 Flucs and CBR. Spectra acquired by integrating light for 1 min in different filters and fitted with a spline smoothing function in PRISM.

The situation was different for the extended electronically altered compound DL-$BIiLH_2$ (**13**) since the emission peak wavelengths of enzymes were markedly blue-shifted compared to those with DL-iLH_2 (**9**) and were more akin to those emitted with D-LH_2 (**1**) or DL-$BILH_2$ (**10**). This may be explained by twisting of the light emitting oxy form of $BIiLH_2$ in the active site of the luciferase. However, a red-shift observed with CBR indicated that a specific colour-shifting mechanism was also active for this compound. Emission colours with DL-$PBIiLH_2$ (**12**) were more red-shifted and remarkably, we detected a >100nm red-shifted secondary peak of emission x11 Fluc with DL-$PBIiLH_2$ (**12**). Although this peak was relatively unstable to time and temperature, it indicated the possibility that due to the increased rigidity of the light emitting oxy form it has access to relatively redshifted colour forms compared to the light giving oxidized form conformationally stabilized to be relatively planar around the only rotatable σ-bond in the active site of x11 Fluc, this red shifted peak might be explained by the stabilization of resonance forms accessible because of greater conjugation (Fig. 6). Bimodal bioluminescence emission is not unusual and has been explained by stablisation of each of the resonance forms of the anionic ketone of the oxidized species.[4,32] We speculate that x11 Fluc is able to stabilize the enolate form leading to red emission. Bioluminescene studies of x11 Fluc with DL-$PBIiLH_2$ (**12**) as a function of pH (Fig. 7) showed an increased bimodal emission with increasing pH. Recent reports suggest an acidic H-bonding network may stabilise the yellow-green emitting phenolate form of luciferins.[32] In the x11 Fluc/DL-$PBIiLH_2$ (**12**) system increasing the pH may remove this in favour of stabilization of the red emitting enolate form.

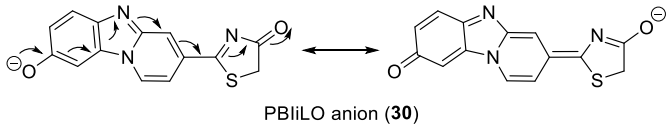


Figure 6. Potential resonance forms of light emitting PBIiLO (**30**) in its deprotonated form.

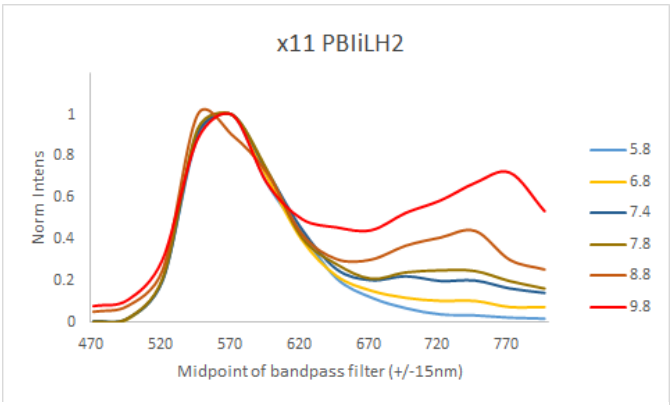


Fig. 7. Bioluminescence emission of x11 Fluc with DL-PBIiLH$_2$ (12) as a function of pH. Spectra acquired by integrating light for 1 min in different filters.

3. Conclusion

The synthesis of a rigid analogue PBIiLH$_2$ (**12**) of iLH$_2$ (**9**) has been achieved in two steps in an attempt to improve the brightness of π-extended analogues of LH$_2$ (**1**). Its bioluminescence activity was compared with its non-rigid counterpart BIiLH$_2$ (**13**) which in turn was compared to its non π-extended analogue BILH$_2$ (**10**). The results show that both π-extended analogues PBIiLH$_2$ (**12**) and BIiLH$_2$ (**13**) gave red shifted bioluminescence emissions with respect to LH$_2$ (**1**) with wild type and thermostable luciferases ranging from 6-159 nm. Most strikingly the rigid analogue PBIiLH$_2$ (**12**) gave a secondary emission of 714 nm with Fluc x11, comparable to the leading red shifted π-extended analogues **6a,b** and iLH$_2$ (**9**) reported.[11,16,17] Although the rigid π-extended analogue PBIiLH$_2$ (**12**) was not brighter than iLH$_2$ (**9**) or its parent benzimidazole luciferin BILH$_2$ (**10**), it did show enhanced brightness compared to its non-rigid analogue BIiLH$_2$ (**13**) with each enzyme tested. The greatest increase in activity was 20-fold with CBR which also gave similar brightness for all three luciferins iLH$_2$ (**9**), BILH$_2$ (**10**) and PBIiLH$_2$ (**12**). The concept of reducing the degrees in freedom in π-extended analogues of LH$_2$ (**1**) by synthetic rigidification has been demonstrated and we are investigating the refinement of this strategy on other analogues to investigate the brightness of red shifted luciferins.

4. Experimental section

4.1. General

Experiments were carried out in flame dried glassware, in anhydrous solvents and under a positive flow of nitrogen or argon unless aqueous chemistry was involved. Temperatures of 0 °C were achieved using an ice-water bath. Cryogenic temperatures were obtained using a solid CO_2 – acetone bath (-78 °C). Commercially available solvents and reagents were used without further distillation. The petroleum ether used in purification procedures is the fraction that boils in the range of 40 – 60 °C. A solvent purification system (SPS) was used to obtain anhydrous solvents where needed. Thin layer chromatography (TLC) was carried out on Merck aluminium backed DC 60 F254 0.2 mm pre-coated plates. Visualisation of the plates was done under ultraviolet light and then stained with potassium permanganate solution where required. Flash column chromatography was performed on Gedran® silica gel 60, 40-63 μm.

All ^{1}H and ^{13}C NMR spectra reported were recorded on a Bruker Avance III 600 MHz spectrometer, unless stated otherwise. All ^{31}P NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer. The chemical shift (δ) for the resonances in spectra have been reported in parts per million (ppm) relative to the NMR solvent peaks, whilst coupling constants (J) are quoted in Hertz (Hz). ^{13}C NMR spectra were recorded as proton decoupled spectra at 151 MHz, and DEPT, COSY, NOESY and HSQC spectra were obtained to aid in complete assignment. A Perkin Elmer Spectrum 100 FT/IR with Spectrum 100 μATR machine was used to record IR spectra. Mass spectra were obtained on a ThermoMAT900 and an Accela LC- Finnigan LTQ instruments. Melting points are uncorrected and were recorded on Electrothermal IA9300 machine. iLH$_2$ and phosphonate **25** were synthesised as previously reported.[18]

4.2. Synthesis of luciferin analogues BILH$_2$ (10), PBIiLH$_2$ (12) and BIiLH$_2$ (13)

4.2.1. 4-(tert-butyldimethylsilyloxy)-2-nitroaniline (15)

To a solution of 4-amino-3-nitrophenol (**14**) (3.00 g, 19.6 mmol) and imidazole (2.66 g, 39.2 mmol) in DMF (20 mL) was added TBDMS-Cl (4.00 g, 26.5 mmol) at room temperature and stirred for 24 h. The reaction was extracted with Et_2O (3 x 20 mL), the organic layers combined, dried ($MgSO_4$), and filtered. After the volatile materials were removed *in vacuo*, the crude product was used without further purification. The product **15** was obtained as a red solid (5.00 g, 95%); mp 100 ºC; (lit.[34] 98 – 100 ºC); R_f = 0.80 (80% EtOAc/hexane); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 (1H, d, *J* = 8.0, Ar*H*), 6.97 (1H, d, *J* = 4.0, Ar*H*), 6.71 (1H, dd, *J* = 8.0, 4.0, Ar*H*), 0.98 (9H, s, SiC(C*H*$_3$)$_3$), 0.19 (6H, s, OSi(C*H*$_3$)$_2$). The data was in agreement with the literature.[33]

6-(tert-Butyldimethylsilyloxy)-1H-benzo[d]imidazole-2-carbonitrile (16)

To a solution of 4-(tert-butyldimethylsilyloxy)-2-nitroaniline (**15**) (1.75 g, 6.52 mmol) in MeOH (5 mL) was added 10% palladium on carbon (0.550 g) as a slurry in MeOH (5 mL) at room temperature. The solution was degassed with Ar, purged with H_2 3 times, and stirred for 7 h under a H_2 atmosphere (balloon). The mixture was filtered through celite and the filtrate concentrated in vacuo to obtain the diamine product in crude form as a purple oil. Without further purification, this was dissolved in anhydrous pyridine (20 mL) and Appel's salt (1.22 g, 5.85 mmol) added at 0 ˚C. The mixture was stirred at room temperature for 24 h. The mixture was concentrated under reduced pressure and the crude product was purified by flash column chromatography (20% EtOAc/hexane) to give **16** (1.66 g, 60%) as a pale yellow solid; mp 178 ºC; R_f = 0.60 (20% EtOAc/hexane); IR υ_{max} (solution in $CDCl_3$): 2953, 2930, 2892, 2239, 1626 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 (1H, d, J = 8.0, ArH), 7.05 (1H, s, ArH), 7.02 (1H, d, J = 8.0, ArH), 1.03 (9H, s, SiC(CH$_3$)$_3$), 0.25 (6H, s, OSi(CH$_3$)$_2$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 155.8 (C), 125.2 (CH), 121.3 (CH),112.7 (CH), 25.1 (CH_3), 19.0 (C), 4.4 (CH_3); HRMS (ESI$^+$) Calcd. for $C_{14}H_{20}N_3OSi$ [M + H]$^+$ 274.1376, found 274.1375.

2-Cyano-6-hydroxybenzimidazole (17)

To a solution of silyl ether **16** (0.200 g, 0.732 mmol) in THF (10 mL) was added 1M tetrabutylammonium fluoride (0.16 mL, 1.4 mmol) at 0 ˚C. The reaction was stirred for 2 h at room temperature. The reaction mixture was then concentrated under reduced pressure and the crude product purified by flash column chromatography (80% EtOAc/hexane) to give **17** (0.130 g, 90%)

as a yellow solid; mp 178 ºC; R_f = 0.25 (80% EtOAc/hexane); ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.51 (1H, d, *J* = 8.0, Ar*H*), 6.96 (1H, dd, *J* = 8.0, 4.0, Ar*H*), 6.92 (1H, d, *J* = 4.0 Hz, Ar*H*). The data is in agreement with the literature.[15]

*2-(6-hydroxy-1H-benzo[d]imidazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (**10**)*

To a solution of nitrile **17** (60.0 mg, 0.377 mmol) in MeOH (2 mL) and H_2O (1 mL) was added DL-cysteine hydrochloride monohydrate (70.1 mg, 0.399 mmol), K_2CO_3 (54.0 mg, 0.391 mmol) at room temperature and the suspension was stirred for 30 min. The mixture was acidified with 3 M HCl to pH = 3. The MeOH was removed *in vacuo*, and the remaining solution was extracted with EtOAc (3 x 100 ml). The organic layers were combined, dried ($MgSO_4$), filtered and concentrated under reduced pressure to obtain the crude product that was purified by flash column chromatography (100% EtOAc - 1% AcOH/EtOAc). The product **10** was obtained as a yellow solid (98 mg, 93%) (lit. [15] 82%). m.p. 203 ºC; ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.48 (1H, d, *J* = 8.9, Ar*H*), 6.94 (1H, d, *J* = 2.3, Ar*H*), 6.85 (1H, dd, *J* = 8.9, 2.3, Ar*H*), 5.27 (1H, app t, *J* = 9.0, C=NC*H*(COOH)CH_2), 3.71 (2H, d, *J* = 9.0, CH_2S). The data is in agreement with the literature.[15]

*4.2.2. 8-hydroxybenzo[4,5]imidazo[1,2-a]pyridine-3-carbonitrile (**20**)*

To a solution of 1,4-benzoquinone **18** (540 mg, 5.00 mmol) in AcOH (1.25 mL) was added a solution of 2-amino-4-cyanopyridine (290 mg, 2.50 mmol) in AcOH (0.75 mL). The mixture was diluted with H_2O (0.75 mL) and boiled for 5 mins until the mixture turned red. The mixture was cooled quickly to room temperature in an ice bath and then acidified with 6M HCl (1.25 mL) and diluted with H_2O (15 mL). The aqueous layer was extracted with Et_2O (3 x 15 mL), cooled to 0 °C and basified to pH 8 with solid Na_2CO_3. The crude, brown precipitate formed was filtered, dried under vacuum, and purified by flash column chromatography (10% MeOH/EtOAc) to give **20** as a yellow solid (379 mg, 1.81 mmol, 72%); mp 171-173 ºC; R_f 0.62 (10% MeOH/EtOAc); IR υ_{max} (solid) 3500 (br), 3086, 2229, 1641 cm^{-1}; ^{1}H NMR (600 MHz, Methanol-d_4) δ 8.77 (1 H, dd, *J* =7.1, ArC*H*), 8.05 (1 H, s, ArC*H*), 7.68 (1 H, d, *J* =8.9, ArC*H*), 7.43 (1 H, d, *J* =2.3, ArC*H*), 7.14 (1 H, dd, *J* =8.9, 2.3, ArC*H*), 7.02 (1 H, dd, *J* =7.1, ArC*H*); ^{13}C NMR (151 MHz, Methanol-d_4) δ 156.5 (*C*), 146.5 (*C*), 139.8 (*C*), 131.1 (*C*), 128.7 (*C*H), 124.9 (*C*H), 121.5 (*C*), 119.6 (*C*H), 118.6 (*C*H), 112.9 (*C*), 111.3 (*C*H), 97.2 (*C*H); m/z (ES$^-$) 208 (60%, [M-H]$^-$); HRMS for $C_{12}H_6N_3O$ [M-H]$^-$ calcd. 208.0511, found 208.0501.

*2-(8-hydroxybenzo[4,5]imidazo[1,2-a]pyridin-3-yl)-4,5-dihydrothiazole-4-carboxylic acid (**12**)*

To a suspension of nitrile **20** (177 mg, 0.85 mmol) in EtOH (5 mL) were added D, L-cysteine (205 mg, 1.69 mmol) and $NaHCO_3$ (286 mg, 3.40 mmol) and the mixture heated at reflux for 48 h. The solvent was removed in vacuo and the remaining residue washed with ice-cold Et_2O (3 x 10 mL). The residue was then dissolved in ice cold H_2O (4.2 mL) and acidified with 2M HCl to pH 2. A precipitate was formed that was filtered and dried to give **12** as a tan solid (100 mg, 0.32 mmol, 38%); mp 279 °C; IR υ_{max} (solid state) 3253 (br), 3063, 2927, 1719; ^{1}H NMR (600 MHz, DMSO-d_6) δ 13.11 (1 H, s, CO_2*H*), 9.80 (1 H, br s, O*H*), 8.97 (1 H, d, J =7.1, ArC*H*), 7.84 (1 H, s, ArC*H*), 7.71 (1 H, d, J =8.7, ArC*H*), 7.59 (1 H, s, ArC*H*), 7.32 (1 H, d, J =7.1, ArC*H*), 7.10 (1 H, dd, J =8.8, 2.0, ArC*H*), 5.39 (1 H, t, J =8.9, C*H*CO_2H), 3.82 – 3.77 (1 H, m, CH_2S), 3.72 – 3.68 (1 H, m, CH_2S); ^{13}C NMR (151 MHz, DMSO-d_6) 171.6 (C), 167.1 (C), 153.7 (C), 145.0 (C), 138.2 (C), 131.8 (C), 129.4 (C), 126.8 (CH), 120.0 (CH), 118.0 (CH), 117.1 (CH), 107.8 (CH), 96.5 (CH), 78.5 (CH), 35.4 (CH_2S); m/z (ES$^+$) 314 (100%, [M+H]$^+$); HRMS for $C_{15}H_{12}N_3O_3S$ [M+H]$^+$ calcd. 314.0599, found 314.0587.

*4.2.3. 2-nitro-4-((triisopropylsilyl)oxy)aniline (**21**)*

To a solution of 4-amino-3-nitrophenol (1.00 g, 6.48 mmol) in CH_2Cl_2 (30 mL) was added imidazole (0.70 g, 10.3 mmol). The mixture was cooled to 0 °C, triisopropylsilyl chloride (1.78 mL, 8.32 mmol) added, the mixture allowed to warm to room temperature and stirred for 16 h. The resultant mixture was diluted with CH_2Cl_2 (30 mL), washed sequentially with a saturated solution of $NaHCO_3$ (20 mL), water (20 mL) and brine (20 mL), and then the organic layer dried ($MgSO_4$), filtered and concentrated under reduced pressure to give the crude compound as a bright red gelatinous solid which was purified by passing through a silica plug (50% EtOAc/hexane) to give **21** as a bright red crystalline solid (2.00 g, 6.48 mmol, 99%); mp 92-94 °C; Rf 0.80 (30% EtOAc/hexane); IR υ_{max} (solid state) 3500, 3346, 2939, 2861, 1561 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.60 (1 H, d, *J* = 2.9, ArC*H*), 7.03 (1 H, dd, *J* = 8.9, 2.9, ArC*H*), 6.71 (1 H, d, *J* = 8.9, ArC*H*), 5.81 (2 H, br s, NH_2), 1.27 – 1.23 (3 H, m, C*H*(CH_3)), 1.10 (18 H, d, CH(CH_3) *J* = 7.4); ^{13}C NMR (151 MHz, $CDCl_3$) δ 146.8 (*C*), 139.7 (*C*), 131.9 (*C*), 130.1 (*C*H), 119.7 (*C*H), 114.5 (*C*H), 18.0 (*C*H_3), 12.6 (*C*H); m/z (ES$^+$) 311 (100%, [M+H]$^+$); HRMS $C_{15}H_{27}N_2O_3Si$ [M+H]$^+$ calcd. 311.1791, found 311.1792.

*2-methyl-6-((triisopropylsilyl)oxy)-1H-benzo[d]imidazole (**22**)*

To a stirred solution of **21** (2.00 g, 6.45 mmol) in MeOH (25 mL) were added AcOH (7 drops), triethyl orthoacetate (2.36 mL, 12.9 mmol), and 10% palladium on carbon (0.200 g) as a slurry in MeOH (8 mL). The flask was evacuated and then purged with H_2 gas three times before leaving to stir under an atmosphere of H_2 overnight at room temperature. On completion the pale brown solution was filtered through Celite® and the Celite® washed with EtOAc (70 mL). The filtrate was concentrated under reduced pressure to yield a crude pale-brown oil which was purified by flash column chromatography (90% EtOAc/hexane) to give **22** as an orange oil (1.88 g, 6.16 mmol, 96%); Rf 0.26 (90% EtOAc/hexane); IR υ_{max} (neat) 3112 , 2940, 2863, 1630, 1588 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.90 (1 H, br s, N*H*), 7.36 (1 H, d, *J* = 8.6, ArC*H*), 7.03 (1 H, d, *J* =2.3, ArC*H*), 6.81 (1 H, dd, *J* =8.6, 2.3, ArC*H*), 2.58 (3 H, s, N=C*Me*), 1.29 – 1.21 (3 H, m, C*H*Me2), 1.09 (18 H, d, *J* =7.4, CH*Me*$_2$); ^{13}C NMR (151 MHz, $CDCl_3$) δ 152.1 (*C*), 151.2 (*C*), 138.9 (*C*), 134.0 (Ar*C*), 115.9 (*C*H), 114.9 (*C*H), 104.4 (*C*H), 18.1 (CH_3), 15.0 (CH_3), 12.8 (*C*H); m/z (ES$^+$) 305 (100%, [M+H]$^+$); HRMS $C_{17}H_{29}N_2OSi$ [M+H]$^+$ calcd. 305.2049, found 305.2043.

*2-methyl-6-((triisopropylsilyl)oxy)-1-((2-(trimethylsilyl)ethoxy)methyl)-1H-benzo[d]imidazole (**23a**) and 2-methyl-5-((triisopropylsilyl)oxy)-1-((2-(trimethylsilyl)ethoxy)methyl)-1H- benzo[d]imidazole (**23b**)*

Solid KH (30% dispersion in mineral oil, 0.738 g, 5.52 mmol) was cooled to 0 °C. Compound **22** (1.35 g, 4.45 mmol) was dissolved in THF (20 mL) and added drop-wise to the cooled KH over a period of 25 mins. Care was taken to ensure that the internal temperature of the reaction mixture did not exceed 5 °C. The mixture was stirred for 1 h at 0-5 °C before 2-(trimethylsilyl)ethoxymethyl chloride (0.982 mL, 5.52 mmol) was added drop-wise at 0 °C to the thick, sandstone coloured suspension. After stirring at room temperature for 19 h, the reaction mixture was cooled to 0 °C and water (15 mL) cautiously added drop-wise to quench the reaction. The aqueous layer was extracted with EtOAc (25 mL x 3). The organic layers were combined, dried ($MgSO_4$), filtered and concentrated under reduced pressure to yield the crude product as a brown oil. Purification by

flash column chromatography (30% EtOAc/petroleum ether) gave in order of elution compound **23a** as an off-white solid (0.766 g, 1.76 mmol, 40%), followed by compound **23b** as an off-white solid (0.795 g, 1.83 mmol, 41%).

Data for Compound **23a**: mp 58 – 59 °C; R_f 0.28 (40% EtOAc /petroleum ether); IR ν_{max} (neat solid) 2947 – 2867 (C-H), 1622 (C=N); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.20 (1 H, d, *J* =8.6, ArC*H*), 7.18 (1 H, d, *J* =2.2, ArC*H*), 6.85 (1 H, dd, *J* =8.6, 2.2, ArC*H*), 5.41 (2 H, s, NCH_2O), 3.50 (2 H, t, *J* =8.4, OCH_2CH$_2$Si), 2.62 (3 H, s, N=C*Me*), 1.30 – 1.26 (3 H, m, C*H*Me$_2$), 1.10 (18 H, d, *J* =7.5, CH*Me*$_2$), 0.88 (2 H, t, *J* =8.4, CH_2Si), -0.07 (9 H, s, Si*Me*$_3$); ^{13}C NMR (151 MHz, $CDCl_3$) δ 152.7 (*C*), 152.4 (*C*), 143.3 (*C*), 130.6 (*C*), 116.6 (*C*H), 109.5 (*C*H), 109.3 (*C*H), 73.0 (*C*H$_2$), 66.7 (*C*H$_2$), 18.3 (*C*H$_3$), 14.2 (*C*H$_2$), 13.0 (*C*H$_3$), -1.1 (*C*H$_3$); m/z (ES$^+$) 435 (100%, [M+H]$^+$), 421 (28%, [M$^+$ -CH$_3$]); HRMS $C_{23}H_{43}N_2O_2Si_2$ [M+H]$^+$ calcd. 435.2863, found 435.2858.

Data for Compound **23b:** mp 59 – 60 °C; R_f 0.15 (40% EtOAc /petroleum ether); IR ν_{max} (neat solid) 2945 – 2867 (C-H), 1621 (C=N); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.49 (1 H, d, *J* =8.6, ArC*H*), 6.87 (1 H, d, *J* =2.2, ArC*H*), 6.81 (1 H, dd, *J* =8.6, 2.2, ArC*H*), 5.38 (2 H, s, CH_2), 3.50 (2 H, t, *J* =8.2, OCH_2CH$_2$Si), 2.61 (3 H, s, N=C*Me*), 1.29 – 1.25 (3 H, m, C*H*Me$_2$), 1.10 (18 H, d, *J* =7.5, CH*Me*$_2$), 0.88 (2 H, t, *J* =8.2, CH_2Si), -0.06 (9 H, s, Si*Me*$_3$); ^{13}C NMR (151 MHz, $CDCl_3$) δ 152.5 (*C*), 151.3 (*C*), 137.1 (*C*), 136.1 (*C*), 119.3 (*C*H), 115.8 (*C*H), 100.2 (*C*H), 72.8 (*C*H$_2$), 66.5 (*C*H$_2$), 18.1 (*C*H$_3$), 18.0 (CH$_2$), 14.0 (*C*H), 12.8 (*C*H$_3$), -1.4 (*C*H$_3$); m/z (ES$^+$) 435 (25%, [M+H]$^+$); HRMS $C_{23}H_{43}N_2O_2Si_2$ [M+H]$^+$ calcd. 435.2863, found 435.2837.

*6-((triisopropylsilyl)oxy)-1-((2-(trimethylsilyl)ethoxy)methyl)-1H-benzo[d]imidazole-2-carbaldehyde (**24a**)*

To a suspension of selenium dioxide (0.394 g, 3.55 mmol) in 1,4-dioxane (10 mL) was added 5.0 M tert-butyl hydroperoxide solution in decane (0.850 mL, 8.16 mmol) at room temperature and the suspension stirred at 90 °C for 30 mins. A solution of compound **23a** (0.796 g, 1.83 mmol) in 1,4-dioxane (4 mL) was added and the mixture was stirred at 110 °C for 5 h. The mixture was allowed to cool to room temperature and concentrated *in vacuo* to get a brown oil, to which sat. $NaHCO_3$ solution (15 mL) was added. The aqueous layer was extracted with CH_2Cl_2 (3 x 20 mL). The organic layers were combined, dried ($MgSO_4$), filtered and concentrated *in vacuo* to afford the crude compound as a brown oil which was purified by flash column chromatography (10% Et_2O/ petroleum ether) to give **24a** as a pale yellow oil (0.451 g, 1.23 mmol, 67%); R_f 0.64 (10% EtOAc/petroleum ether); IR ν_{max} (solution in $CHCl_3$) 2946, 2866, 1693, 1617 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.01 (1 H, s, C*H*O), 7.76 (1 H, d, *J* = 8.9, ArC*H*), 7.04 (1 H, d, *J* =2.3, ArC*H*), 7.01 (1 H, dd, *J* =8.9, 2.3, ArC*H*), 5.97 (2 H, s, NCH_2O), 3.54 (2 H, t, *J* = 8.2, OCH_2CH$_2$Si), 1.34 – 1.28 (3 H, m, C*H*Me$_2$), 1.12 (18 H, d, *J* =7.5, CH*Me*$_2$), 0.88 (2 H, t, *J* = 8.2, CH_2Si), -0.07 (9 H, s, Si*Me*$_3$); ^{13}C NMR (151 MHz, $CDCl_3$) δ 184.1 (*C*), 156.3 (*C*), 146.0 (*C*), 138.0 (*C*), 137.7 (*C*), 122.8 (*C*H), 119.6 (*C*H), 100.9 (*C*H), 73.3 (*C*H$_2$), 66.3 (*C*H$_2$), 17.9 (*C*H$_3$), 17.7 (*C*H$_2$), 12.6 (*C*H), -1.6 (*C*H$_3$); m/z (ES$^+$) 449 (65%, [M+H]$^+$) HRMS $C_{23}H_{41}N_2O_3Si_2$ [M+H]$^+$ calcd. 449.2656, found 449.2643.

*5-((triisopropylsilyl)oxy)-1-((2-(trimethylsilyl) ethoxy) methyl)-1H-benzo[d]imidazole-2-carbaldehyde (**24b**)*

Procedure as that for aldehyde **24a** on a 0.97 mmol scale to give **24b** as a pale yellow oil (0.420 g, 0.936 mmol, 96%); R_f 0.38 (10% EtOAc/petroleum ether); IR ν_{max} (solution in $CHCl_3$) 2947, 2867, 1693, 1618 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.07 (1 H, s, C*H*O), 7.48 (1 H, dd, *J* =8.9, 0.4, ArC*H*), 7.36 (1 H, dd, *J* =2.3, 0.4, ArC*H*), 7.13 (1 H, dd, *J* =8.9, 2.3, ArC*H*), 6.00 (2 H, s, NCH_2O), 3.55 (2 H, t, *J* =8.2, OCH_2CH$_2$Si), 1.33 – 1.29 (3 H, m, C*H*Me$_2$), 1.12 (18 H, d, *J* =7.5, CH*Me*$_2$), 0.88 (3 H, t, *J* =8.2, CH_2Si), -0.08 (9 H, s, Si*Me*$_3$); ^{13}C NMR (151 MHz, $CDCl_3$) δ 184.8 (*C*), 153.9 (*C*), 146.4 (*C*), 143.8 (*C*), 131.6 (*C*), 122.8 (*C*H), 112.3 (*C*H), 110.3 (*C*H), 73.4 (*C*H$_2$), 66.6 (*C*H$_2$), 18.0 (*C*H$_3$), 17.9 (*C*H$_2$Si), 12.7 (*C*H), -1.4 (*C*H$_3$); m/z (ES$^+$) 481 (100%, [M+CH$_3$OH+H]$^+$), 449 (4%, [M+H]$^+$); HRMS $C_{23}H_{41}N_2O_3Si_2$ [M+H]$^+$ calcd. 449.2656, found 449.2652.

*Methyl (E)-(3-(6-((triisopropylsilyl)oxy)-1-((2-(trimethylsilyl)ethoxy)methyl)-1H-benzo[d]imidazol-2-yl)prop-2-enethioyl)serinate (**26a**)*

A solution of phosphonate **25** (0.313 g, 1.00 mmol) in THF (5 mL) was cooled to 0 °C and solid KO^{t}Bu (0.150 g, 1.33 mmol) added. The mixture was allowed to warm to room temperature and stirred for 1 h. The mixture was cooled to 0 °C and a solution of aldehyde **24a** (0.301 g, 0.67 mmol) in THF (3 mL) added dropwise and the mixture stirred at room temperature for 3 h. The mixture was poured into brine (5 mL) and the organic layer diluted with EtOAc (10 mL). The aqueous layer was further extracted with EtOAc (2 x 10 mL). The organic extracts were combined and further washed with brine (10 mL) and then dried ($MgSO_4$), filtered and concentrated under reduced pressure to give the crude compound as a deep yellow oil which was purified by flash column chromatography (gradient elution: 20% EtOAc/ hexane – 50% EtOAc/hexane) to give **26a** as a deep yellow oil (0.212 g, 0.35 mmol, 35%); R_f 0.17 (50% EtOAc/petroleum ether); IR ν_{max} (solution in $CHCl_3$) 3245, 2944, 2863, 1739, 1615 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.09 (1 H, d, *J* =7.5, N*H*), 7.95 (1 H, d, *J* =14.7, *HC*=C), 7.61 (1 H, d, *J* =14.7, C=C*H*), 7.55 (1 H, d, *J* =8.6, ArC*H*), 6.93 – 6.89 (2 H, m, ArC*H*), 5.54 (2 H, s, NCH_2O), 5.48 – 5.44 (1 H, m, HN-C*H*-CO$_2$Me), 4.34 (1 H, dd, *J* =11.5, 2.7, CH_aH$_b$OH), 4.22 (1 H, dd, *J* =11.5, 2.9, CH$_a$H$_b$OH), 3.82 (1 H, br s, O*H*), 3.68 (3 H, s, O*Me*), 3.53 (2 H, t, *J* =8.1, OCH_2CH$_2$Si), 1.30 – 1.26 (3 H, m, C*H*Me$_2$), 1.12 (18 H, d, *J* =7.4, CH*Me*$_2$), 0.90 (2 H, t, *J* =8.1, CH_2Si), -0.07 (9 H, s, Si*Me*$_3$); ^{13}C NMR (151 MHz, $CDCl_3$) δ 193.9 (C), 170.3 (C), 154.2 (*C*), 148.4 (*C*), 137.2 (*C*), 136.4 (*C*H), 133.3 (*C*H), 127.2 (*C*H), 120.0 (*C*), 118.4 (*C*H), 100.5 (*C*H), 72.5 (*C*H$_2$), 66.8 (*C*H$_2$), 61.8 (C*H$_2$*), 60.5 (C*H*), 52.8 (*CH$_3$*), 18.1 (*CH$_3$*), 17.9 (*CH$_2$*), 12.8 (*C*H), -1.3 (*CH$_3$*); m/z (ES$^+$) 608 (100%, [M+H]$^+$); HRMS $C_{29}H_{50}N_3O_5SSi_2$ [M+H]$^+$ calcd. 608.3010, found 608.3010.

*Methyl(E)-(3-(5-((triisopropylsilyl)oxy)-1-((2-(trimethylsilyl)ethoxy)methyl)-1H-benzo[d]imidazol-2-yl)prop-2-enethioyl)serinate (**26b**)*

Procedure as that for thioamide **26a** on a 0.96 mmol of phosphonate **25** gave **26b** as a deep yellow oil (0.206 g, 0.34 mmol, 53%); R_f 0.25 (40% EtOAc/petroleum ether); IR ν_{max} (solution in $CHCl_3$) 3183 (br), 2941, 2626, 1706, 1600 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.82 (1 H, d, *J* =7.5, N*H*), 7.96 (1 H, d, *J* =14.7, *H*C=C), 7.66 (1 H, d, *J* =14.7, C=C*H*), 7.32 (1 H, d, *J* =8.8, ArC*H*), 7.18 (1 H, d, *J* =2.3, ArC*H*), 6.97 (1 H, dd, *J* =8.8, 2.3, ArC*H*), 5.60 (2 H, s, NCH_2O), 5.45 (1 H, apt. dt, *J* =7.5, 3.1, HN-C*H*-CO$_2$Me), 4.27 (1 H, dd, *J* =11.5, 3.0, CH_aH$_b$OH), 4.22 (1 H, dd, *J* =11.5, 3.2, CH$_a$H$_b$OH), 3.75 (3 H, s, O*Me*), 3.54 (2 H, t, *J* =8.2, OCH_2CH$_2$Si), 1.33 – 1.28 (3 H, m, C*H*Me$_2$), 1.13 (18 H, d, *J* =7.5, CH*Me*$_2$), 0.91 (3 H, t, *J* =8.2, CH_2Si), -0.07 (9 H, s, Si*Me*$_3$); ^{13}C NMR (151 MHz, $CDCl_3$) δ 193.2 (*C*), 169.9 (*C*), 153.2 (*C*), 148.7 (*C*), 142.8 (*C*), 133.7 (*C*H), 130.1 (*C*), 126.5 (CH), 118.7 (*C*H), 110.2 (*C*H), 108.2 (*C*H), 72.1 (*C*H$_2$), 66.5 (*C*H$_2$), 61.5 (*C*H$_2$), 60.3 (*C*H), 52.5 (*C*H$_3$), 17.7 (*C*H$_3$), 17.4 (*C*H$_2$), 12.4 (*C*H), -1.8 (*C*H$_3$); m/z (ES$^+$) 608 (100%, [M+H]$^+$); HRMS $C_{29}H_{50}N_3O_5SSi_2$ [M+H]$^+$ calcd. 608.3010, found 608.3014.

*Methyl (E)-(3-(6-hydroxy-1-((2-(trimethylsilyl)ethoxy)methyl)-1H-benzo[d]imidazol-2-yl)prop-2-enethioyl)serinate (**27a**)*

A solution of 1.0 M solution of TBAF in THF was added to a solution of compound **26a** (0.055 g, 0.090 mmol) in THF (1.5 mL) at 0 °C. The mixture was left to stir at 0 °C for 10 mins and then quenched with a saturated solution of NH_4Cl (6 mL) at 0 °C, allowed to warm to room temperature, extracted with EtOAc (3 x 15 mL). The organic extracts were combined and washed with a saturated solution of NH_4Cl (3 x 20 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure to give the crude compound as red oil which was purified by flash-column chromatography (gradient elution: 90% EtOAc/petroleum ether – neat EtOAc) to give **27a** as a yellow oil (0.029 g, 0.064 mmol, 65%) as a mixture of E and Z isomers that inter-convert in solvent; R_f 0.31 (90% EtOAc/hexane); IR ν_{max} (solution in $CHCl_3$) 3242, 2953, 2895, 1739, 1621 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ δ 13.69 (0.6 H, br s, -N*H* for *Z*-isomer), 8.85 (1 H, br s, -N*H* for *E*-isomer), 7.82 (1 H, d, *J* =14.7, *H*C=C for *E*-isomer), 7.52 – 7.35 (2.4 H, m), 6.90 – 6.73 (4 H, m), 6.51 (0.6 H, d, *J* =13.5, *H*C=C for *Z*-isomer), 5.42 (2 H, s, NCH_2O; 1.6 H, HN-C*H*-CO_2Me), 5.34 (1.2 H, s, NCH_2O), 4.38 (1 H, dd, *J* =11.7, 3.3, CH_2OH), 4.22 (2.2 H, m, CH_2OH), 3.83 (1.8 H, s, O*Me* for *Z*-isomer), 3.75 (3 H, s, O*Me* for *E*-isomer), 3.50 (3.2 H, m, OC$H_2$$CH_2$Si), 2.55 (1 H, br s, -O*H*), 0.88 (5.4 H, m, CH_2Si, ArO*H*, CH_2O*H*, O*H*), -0.07 (14.4 H, overlapping singlets, Si*Me*$_3$); ^{13}C NMR (151 MHz, $CDCl_3$) δ 193.8 (C), 192.0 (C), 170.6 (C), 170.3 (C), 154.0 (C), 153.7 (C), 148.7 (C), 147.9 (C), 142.7 (C), 141.7 (C), 137.7 (CH), 133.9 (CH), 129.7 (CH), 129.1 (CH), 115.7 (CH), 113.7 (CH), 110.9 (CH), 110.4 (CH), 104.4 (CH), 104.0 (CH), 72.6 (*C*H_2), 72.3 (*C*H_2), 67.0 (*C*H_2), 66.9 (*C*H_2), 62.2 (*C*H_2), 62.0 (*C*H_2), 53.0 (CH_3), 53.0 (CH_3), 18.1 (*C*H_2), 17.8 (*C*H_2), -1.3 (CH_3); m/z (ES^+) 452 (55%, $[M+H]^+$); HRMS $C_{20}H_{30}N_3O_5SSi$ $[M+H]^+$ calcd. 452.1675, found 452.1686.

*Methyl (E)-(3-(5-hydroxy-1-((2-(trimethylsilyl)ethoxy)methyl)-1H-benzo[d]imidazol-2-yl)prop-2-enethioyl)serinate (**27b**)*

Procedure as that for **27a** on a 0.069 mmol gave to afford the title compound as a yellow oil (0.029 g, 0.064 mmol, 93%); R_f 0.17 (90% EtOAc/hexane); IR ν_{max} (solution in $CHCl_3$) 3252, 3037, 2949, 2850, 1737, 1620 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ 9.32 (1 H, s, N*H*), 7.81 (1 H, d, *J* =14.6, *H*C=C), 7.50 (1 H, d, *J* = 14.6, C=C*H*), 7.22 (1 H, d, *J* = 8.7, ArC*H*), 7.17 (1 H, s, ArC*H*), 6.89 (1 H, d, *J* = 8.7, ArC*H*), 5.45 (2 H, s, NCH_2O), 5.39 – 5.36 (1 H, m, HN-C*H*-CO_2Me), 4.20 – 4.11 (2 H, m, CH_2OH), 3.71 (3 H, s, O*Me*), 3.53 (2 H, t, *J* = 8.2, OC$H_2$$CH_2$Si), 2.76 (1H, br s, O*H*), 0.92 (2 H, t, *J* = 8.2, CH_2Si), -0.06 (9 H, s, Si*Me*$_3$); ^{13}C NMR (151 MHz, $CDCl_3$) δ 193.9 (C), 170.3 (C), 154.1 (*C*), 148.8 (*C*), 142.8 (*C*), 141.7 (*C*), 129.8 (CH), 126.6 (*C*H), 115.3 (*C*H), 111.0 (*C*H), 104.2 (*C*H), 72.4 (*C*H_2), 67.0 (*C*H_2), 60.7 (C*H*$_2$), 53.0 (CH_3), 17.8 (*C*H_2), -1.3 (CH_3); m/z (ES^+) 452 (65%, $[M+H]^+$); HRMS $C_{20}H_{30}N_3O_5SSi$ $[M+H]^+$ calcd. 452.1675, found 452.1678.

*methyl (E)-2-(2-(6-hydroxy-1-((2-(trimethylsilyl)ethoxy)methyl)-1H-benzo[d]imidazol-2-yl)vinyl)-4,5-dihydrothiazole-4-carboxylate (**28a**)*

A solution of compound **27a** (0.021 g, 0.047 mmol) in CH_2Cl_2 (1.0 mL) was cooled to -78 °C and diethylaminosulfur trifluoride (33 μL, 0.24 mmol) added. The mixture was stirred at -78 °C for 90 mins. The mixture was quenched with a sat. solution of $NaHCO_3$ (2 mL) at -78 °C, allowed to warm-up slowly to room temperature and extracted with CH_2Cl_2 (3 x 5 mL). The organic extracts were combined, dried ($MgSO_4$), filtered and concentrated under reduced pressure to give the crude compound as red oil which was purified by flash column chromatography (70% EtOAc/ hexane) to give **28a** as a deep yellow oil (0.013 g, 0.030 mmol, 65%); R_f 0.19 (70% EtOAc/ hexane); IR ν_{max} (solution in $CHCl_3$) 3163 (br), 2949, 2923, 1731, 1619 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ 7.64 (1 H, d, *J* =15.8, *H*C=C), 7.55 (1 H, d, *J* =8.7, ArC*H*), 7.24 (1 H, d, *J* =15.8, *H*C=C), 6.91 (1 H, d, *J* =2.0, ArC*H*), 6.87 (1 H, dd, *J* =8.7, 2.0, ArC*H*), 5.46 (2 H, s, NCH_2O), 5.26 (1 H, t, *J* =9.1, C*H*CO_2Me), 3.83 (3 H, s, O*Me*), 3.70 – 3.66 (1 H, m, CH_2S), 3.63 – 3.59 (1 H, m, CH_2S), 3.53 (2 H, t, *J* =8.1, OCH_2), 0.90 (2 H, t, *J* =8.1, CH_2Si), -0.06 (9 H, s, Si*Me*$_3$); ^{13}C NMR (151 MHz, $CDCl_3$) 171.1 (*C*), 169.3 (*C*), 153.7 (*C*), 148.9 (*C*), 130.3 (*C*), 129.7 (*C*), 129.2 (*C*H), 126.2 (*C*H), 114.9 (*C*H), 110.4 (*C*H), 104.7 (*C*H), 78.4 (C*H*), 72.6 (*C*H_2), 66.9 (*C*H_2), 53.1 (*C*H_3), 35.1 (*C*H_2), 17.8 (*C*H_2), -1.3 (*C*H_3); m/z (ES^+) 434 (55%, $[M+H]^+$); HRMS $C_{20}H_{28}N_3O_4SSi$ $[M+H]^+$ calcd. 434.1570, found 434.1573.

*Methyl (E)-2-(2-(5-hydroxy-1-((2-(trimethylsilyl)ethoxy)methyl)-1H-benzo[d]imidazol-2-yl)vinyl)-4,5-dihydrothiazole-4-carboxylate (**28b**)*

Procedure as that for thiazoline **28a** on a 0.030 mmol scale to give **28b** as a yellow oil (0.010 g, 0.22 mmol, 67%); R_f 0.23 (90% EtOAc/petroleum ether); IR ν_{max} (solution in $CHCl_3$) 3279 (br), 2953, 2854, 1741, 1621 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ 7.68 (1 H, d, *J* = 15.8, *H*C=C), 7.30 – 7.26 (2 H, m, ArC*H*, C=C*H*), 7.23 (1 H, d, *J* =2.2, ArC*H*), 6.94 (1 H, dd, *J* = 8.7, 2.2, ArC*H*), 5.54 (2 H, s, NCH_2O), 5.26 (1 H, t, *J* = 9.1, C*H*CO_2Me), 3.83 (3 H, s, O*Me*), 3.68 (1 H, dd, *J* =11.1, 9.0, CH_2S), 3.60 (1 H, dd, *J* = 11.0, 9.1, CH_2S), 3.54 (2 H, t, *J* = 8.1, OCH_2), 0.92 – 0.88 (3 H, m, CH_2Si, O*H*), -0.06 (9 H, s, Si*Me*$_3$); ^{13}C NMR (151 MHz, $CDCl_3$) δ 171.1 (*C*), 169.3 (*C*), 153.5 (*C*), 149.0 (*C*), 143.8 (*C*), 130.5 (*C*), 129.2 (C), 126.3 (CH), 114.8 (*C*H), 110.4 (*C*H), 104.9 (*C*H), 78.5 (*C*H), 72.5 (*C*H_2), 66.9 (*C*H_2), 53.0 (*C*H_3), 35.1 (*C*H_2), 17.8 (*C*H_2), -1.3 (*C*H_3); m/z (ES^+) 434 (100%, $[M+H]^+$); HRMS $C_{20}H_{28}N_3O_4SSi$ $[M+H]^+$ calcd. 434.1570, found 434.1571.

*Methyl (E)-2-(2-(6-hydroxy-1H-benzo[d]imidazol-2-yl)vinyl)-4,5-dihydrothiazole-4-carboxylate (**29**)*

A solution of compound **28a** or **28b** (13 mg, 0.030 mmol) in CH_2Cl_2 (2 mL) was cooled to 0 °C and 1.0 M tin(IV) chloride solution in CH_2Cl_2 (0.15 mL, 0.15 mmol) **was** added. The mixture was stirred at 0 °C for 3 h, after which an additional volume of 1.0 M tin(IV) chloride solution in CH_2Cl_2 (0.15 mL, 0.15 mmol) added and left to stir at 0 °C for 1 h. The mixture was quenched at 0 °C with a 0.1 M solution of HCl (5 mL). The CH_2Cl_2 layer was separated and the aqueous layer was neutralised to pH=7 with a saturated solution of $NaHCO_3$ and extracted with EtOAc (3 x 10 mL). The organic extracts were combined, dried ($MgSO_4$), filtered and concentrated under reduced pressure to give the crude compound as a waxy solid which was purified by trituration with $CHCl_3$ (3 x 0.5 mL) and the residue dried to give the ester **29** as a brown solid (8.6 mg, 0.028 mmol, 95%); mp 175-176 °C; R_f 0.07 (2% MeOH/ EtOAc); IR ν_{max} (solid state) 3297 (br), 2923, 2853, 1719, 1639 cm^{-1}; ^1H NMR (600 MHz, MeOD-d_4) δ 7.63 (1 H, d, *J* = 9.0, ArC*H*), 7.59 (1 H, d, *J* = 16.5, *H*C=C), 7.26 (1 H, d, *J* = 16.5, *H*C=C), 7.16 (1 H, dd, *J* = 9.0, 2.2, ArC*H*), 7.08 (1 H, d, *J* = 2.2, ArC*H*), 5.42 (1 H, t, *J* = 9.1, C*H*CO_2Me), 3.83 (3 H, s, O*Me*), 3.78 (2 H, dd, *J* =12.6, 9.1, CH_2S); ^{13}C NMR (151 MHz, MeOD-d_4) δ 170.5 (*C*), 168.7 (*C*), 157.8 (*C*), 143.9 (*C*), 133.9 (*C*H), 133.1 (*C*), 125.2 (*C*), 122.7 (C*H*), 119.1 (*C*H), 115.9 (*C*H), 98.8 (*C*H), 79.4 (*C*H), 53.3 (*C*H_3), 36.1 (*C*H_2); m/z (ES^+) 304 (2%, $[M+H]^+$); HRMS $C_{14}H_{14}N_3O_3S$ $[M+H]^+$ calcd. 304.0756, found 304.0777.

(*E*)-2-(2-(6-hydroxy-1*H*-benzo[*d*]imidazol-2-yl)vinyl)-4,5-dihydrothiazole-4-carboxylic acid (**13**)

A solution of ester **29** (10.0 mg, 0.0330 mmol) was dissolved in phosphate buffer (10 mL, pH=7.8), and treated with PLE lyophilised powder (9.2 mg) and incubated at 37 °C for 24 h.

Solvent was removed *in vacuo* and the residue obtained suspended in a mixture of 1:1 MeOH/$CHCl_3$. The resultant precipitate was isolated by filtration and washed with a mixture of 1:1 MeOH/$CHCl_3$ (10 mL). The washings were combined and concentrated *in vacuo* to give **13** as a yellow solid (6.0 mg, 0.021 mmol, 63%); mp 190-191 °C; IR ν_{max} (solid state) 3400 (br), 3123, 2953, 1737, 1619 cm^{-1}; ^1H NMR (600 MHz, THF-d_8) δ 7.83 (1 H, d, *J* =8.8, ArC*H*), 7.36 (1 H, d, *J* = 16.1, *H*C=C), 7.33-7.28 (2 H, m, *H*C=C and ArC*H*), 6.99 (1 H, dd, *J* =8.8, 2.4, ArC*H*, 5.22 (1 H, t, *J* =8.9, C*H*CO_2H), 3.69 (1 H, d, CH_2S), 3.63 (1 H, d, CH_2S); ^{13}C NMR (151 MHz, THF-d_8) δ 171.6 (*C*), 166.7 (*C*), 160.1 (*C*), 148.0 (*C*), 136.9 (*C*), 133.7 (*C*H), 128.2 (*C*H), 124.2 (*C*H), 116.3 (*C*H), 106.1 (*C*H), 78.8 (*C*H), 34.6 (CH_2); m/z (ES^+) 242 (100%), 597 (10%, $[2M+Na]^+$); HRMS $[C_{26}H_{18}N_6O_6S_2+Na]^+$ calcd. 597.0627, found 597.0650.

4.3 Enzyme assays

To prepare the compounds for assays, D-LH_2 K^+ salt (Regis Tech. IL, USA), DL-$BILH_2$, DL-$PBILH_2$ and DL-iLH_2 were dissolved and made up to 10 mg/mL in TEM buffer (100 mM Tris-acetate, 2 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM magnesium sulphate ($MgSO_4$)).DL-$BILH_2$ (3.3 mg) was dissolved in 50 μL dimethylsulfoxide (DMSO) to aid solubility, before making up to 10 mg/mL in TEM buffer. These stocks were stored dark protected at -20°C. Subsequently, each compound was diluted to 600 μM as a working stock prior to its use. 200 μM compounds were assayed with 0.167 μM WT, x2, x5, x11 Flucs or click beetle red (CBR) in TEM buffer at pH 7.8. Reactions were initiated by the addition of 2 mM saturating ATP to obtain specific activities, kinetics and bioluminescence spectra using the PhotonIMAGER Optima at 29°C. Each substrate was assayed separately in separate plates and the assays were repeated starting from the 600 μM working stocks (stored at -20°C)

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Supplementary data

Supplementary data (NMR spectra, bioluminescence kinetics graphs) for this article can be found at

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33. One of the reviewers suggested that the increase in brightness may be due to the alkylation of the NH in $BIiLH_2$. We had no SEM alkylated **28a,b** left, so attempted the preparation of *N*-Me-$BIiLH_2$. We repeated the synthesis as in Scheme 4, alkylating with Me-I instead of SEM-Cl. Unfortunately the DAST cyclisation step was unsuccessful, yielding degraded products. This possibility will be explored at a later date and we thank the reviewer for their insightful comment.
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