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Bioluminescent coelenterazine derivatives with imidazopyrazinone C-6 extended substitution†

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Three novel coelenterazine (CTZ) derivatives with extension at the C-6 position of the imidazopyrazinone structure show significant bioluminescence emission with known *renilla* luciferase variants, indicating a promising method to develop CTZ derivatives with superior optical properties compared to hitherto reported compounds.

Bioluminescence-based assays such as gene reporter assays in cell cultures and *in vivo* imaging are known for their low background signals and high sensitivity.[1] The mechanism relies on an enzymatic oxidation reaction involving a bioluminescent substrate (luciferin) and an enzyme (luciferase).

The luciferin most widely applied in imaging assays is the firefly luciferin, which emits light at a relatively long wavelength (560 nm).[2–5] Unfortunately however, the process leading to light emission of firefly luciferin requires the presence of Mg^{2+} ions and ATP as co-factors, which potentially leads to complex assay protocols in bioanalysis. For this reason, the Mg^{2+} and ATP independent *renilla* luciferase (Rluc) enzyme in combination with coelenterazine (CTZ) (Fig. 1a) as the substrate has recently been used as a versatile reporter protein.[6] However, the Rluc/CTZ pair results in relatively short wavelength emission in the blue spectral region (480 nm) with a low intensity compared to other bioluminescent systems.[7,8] To overcome these limitations, there is a lot of interest in developing new CTZ derivatives.[9–14] But the design of novel CTZ derivatives resulting in better optical characteristics is challenging, since the detailed enzymatic recognition mechanism of the Rluc system is still mostly unknown.[15–17] In fact, most of the reported CTZ derivatives fail to emit bioluminescence, since their structural modifications prevent their enzymatic recognition.[5–9] The luminescence capacity of CTZ is due to its imidazopyrazinone structure. In previously published work, the effects on the bioluminescence properties of substitution at the C-2, C-5, C-6 and C-8 positions of the imidazopyrazinone core have been investigated.[9,10,14,18,19]

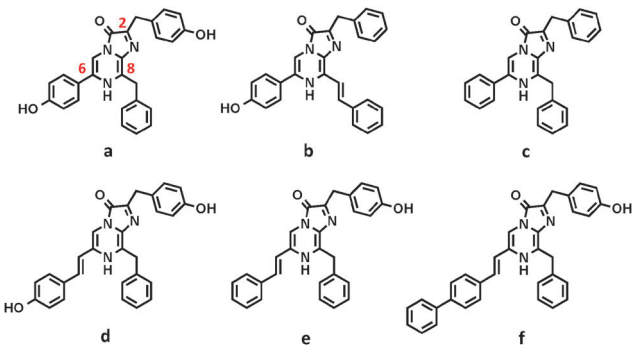

Fig. 1 The structure of native CTZ and CTZ derivatives.

Although it is generally said that the effect of substitution at the C-2 position on enzymatic recognition is relatively low, the benzyl group at the C-2 position is a prerequisite for the luminescence of imidazopyrazinone derivatives.[19] Few C-2 CTZ derivatives show luminescence properties superior to those of native CTZ, except in combination with aequorin. CTZ derivatives modified at the C-8 position have also been reported.[9] A compound having a styryl group at the C-8 position (Fig. 1b) showed approximately 120 nm red-shifted chemiluminescence emission compared to that of the native CTZ, but this structural change resulted in negligibly low bioluminescence in combination with Rluc.[20]

Different types of CTZ derivatives (*e*-CTZ and *v*-CTZ) have been obtained by forming a bridge between the C-5 carbon and the (*p*-hydroxy)-phenyl substituent at the C-6 position of the native CTZ.[14,21] This modification leads to more planar and rigid molecular structures with increased bioluminescence emission, however at the cost of molecular stability.[21]

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As mentioned above, native CTZ has a (*p*-hydroxy)-phenyl group in the C-6 position. Some CTZ derivatives with alternative substituents at this position have also been reported.[12,13,22,23] However, most of these studies have focused on the chemiluminescence properties.

There are only a few studies on the C-6 substituent effects on enzymatic recognition and on the bioluminescence properties of the resultant CTZ derivatives.[14] To fill this gap, we have decided to further investigate the influence of substituents at the C-6 position of native CTZ on the chemiluminescence (CL) and bioluminescence properties (BL). Compared to BL, the measurement of CL allows us to study the structure-related basic luminescence properties independent of any possible luciferin-luciferase mismatch.[24–26]

As the first new derivative, the styryl-substituted compound 6-pi-OH-CTZ was successfully synthesized (Fig. 1d). Its CL spectrum is shown in Fig. S1 (ESI†). In contrast to the significantly red-shifted C-8 styryl-substituted compound,[20] the spectral similarity between 6-pi-OH-CTZ and native CTZ shows that the C-6 substituent has little influence on the CL emission wavelength. This result is not really surprising, since it has been previously reported that even the complete absence of a substituent at C-6 still results in a CL emission peak at around 450 nm.[27] This finding has been generally attributed to a dihedral angle twist between the imidazopyrazinone core and the substituent at the C-6 position in various CTZ derivatives, which prevents an effective extension of π-electron conjugation from the imidazopyrazinone core.[28]

Next, the enzymatic recognition-dependent BL properties of 6-pi-OH-CTZ were investigated using native *renilla* luciferase (Rluc) and the widely known Rluc8 and Rluc8.6 variants.[15] Fig. 2 shows a comparison of the BL intensities and spectra of 6-pi-OH-CTZ and native CTZ. Fig. 2a indicates that 6-pi-OH-CTZ is recognized by Rluc8 and Rluc8.6, but not by Rluc. The BL intensity of the 6-pi-OH-CTZ/Rluc8 pair is 44% of that of the native CTZ/Rluc combination. On the other hand, the intensity of 6-pi-OH-CTZ with Rluc8.6 is 123% of that of the CTZ/Rluc pair and therefore it is a relatively bright derivative. As for the BL spectra with Rluc8.6, the emission wavelengths of 6-pi-OH-CTZ and native CTZ are very similar (Fig. 2b). However, with Rluc8, 6-pi-OH-CTZ shows approximately 50 nm red-shifted emission compared to that of CTZ (Fig. 2b). As a result, it can be concluded that CTZ derivatives with a π-conjugated substituent at the C-6 position can show significant BL emission with the known Rluc variants, Rluc8 and Rluc8.6.

In the case of BL, the spectral location of the emission band depends not only on the chemical structure of the CTZ derivative, but to a large extent on its interactions with proximal amino acid residues in the luciferase enzyme,[16] which remain to a large degree still unknown. Upon oxidation in the presence of Rluc, native CTZ is converted into the light emitting excited state coelenteramide, with its emission wavelength depending on the protonation state. It is hypothesized that the red-shifted emission of native CTZ and 6-pi-OH-CTZ with Rluc8.6 can be attributed to the excited state coelenteramide existing in its deprotonated pyrazine anion form inside the enzymatic pocket. This assumption is supported by the fact that CTZs having a hydroxyl group on the C-6 substituent (native CTZ, 6-pi-OH-CTZ) show a red-shift in their CL emissions upon addition of a base to the sample solution (Fig. S2 and Table S1, ESI†). Based on a previous report, the spectral shoulders near 400 nm observed in Fig. 2b are assumed to originate from the neutral species of coelenteramide[15] due to incomplete deprotonation of the hydroxyl group on the C-6 substituent.

As the next compound, 6-pi-H-CTZ (Fig. 1e) was designed and synthesized to evaluate the effect of the absence of the hydroxyl group in the C-6 substituent on the CL and BL properties. The measurement of the CL spectrum of 6-pi-H-CTZ (Fig. S1, ESI†) was carried out under the same conditions as in the case of 6-pi-OH-CTZ. Also for this substitution pattern, the emission peak of 6-pi-H-CTZ was found at 478 nm, almost identical to the native CTZ.

It has been reported that the replacement of the *p*-hydroxyl group on the 6-phenyl substituent of native CTZ can drastically decrease the capacity of BL emission.[11] Fig. 3 shows the results of BL measurements with 6-pi-H-CTZ. Emission was observed in combination with the Rluc8 and Rluc8.6 luciferases (Fig. 3a). The intensity of 6-pi-H-CTZ with Rluc8.6 is approximately 41% of that of native CTZ/Rluc. The BL spectra recorded in the presence of Rluc8 and Rluc8.6 are both significantly blue-shifted compared to those of native CTZ (Fig. 3b). This different BL spectral behaviour in comparison with 6-pi-OH-CTZ clearly indicates that the *p*-hydroxyl group of the C-6 substituent is involved in the enzymatic interaction of CTZs with the Rluc series luciferases. The absence of that hydroxyl group prevents the formation of the pyrazine anion upon enzymatic oxidation, most reasonably resulting in the neutral coelenteramide, which emits at a short wavelength. With the two CTZ derivatives 6-pi-OH-CTZ and 6-pi-H-CTZ, it has been demonstrated that compounds having a styryl group at the C-6 position show significant BL emission in combination with the known Rluc variants, Rluc8 and Rluc8.6. This is a clear indication that CTZ

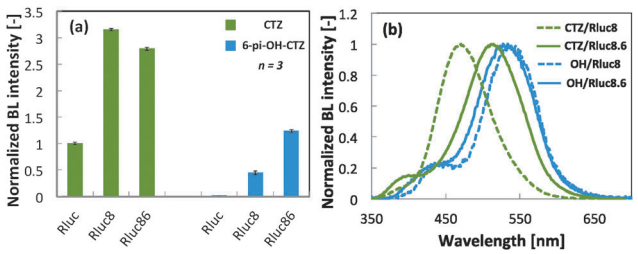

Fig. 2 Luminescence properties of 6-pi-OH-CTZ: (a) BL intensities; (b) BL spectra with Rluc8 and Rluc8.6.

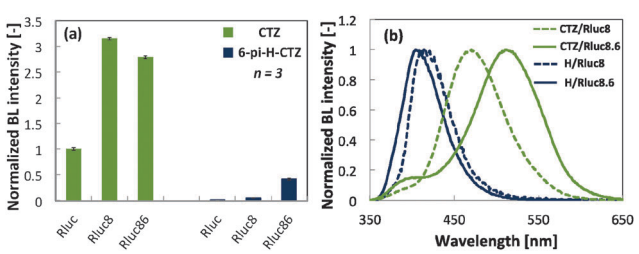

Fig. 3 Luminescence properties of 6-pi-H-CTZ: (a) BL intensities; (b) BL spectra with Rluc8 and Rluc8.6.

derivatives obtained by replacing the original (*p*-hydroxy)-phenyl substituent at the C-6 position undergo enzymatic recognition by Rluc8 and Rluc8.6. To the best of our knowledge, the styryl-groups are the largest examples of C-6 substituents reported so far, with the resulting CTZ derivatives still showing significant BL emission.

To further evaluate the possibility of extending the substituent at the C-6 position, 6-pi-Ph-CTZ having a bulky phenyl group in the *para* position of the 6-styryl group was designed and synthesized (Fig. 1f). Interestingly, the CL spectrum of 6-pi-Ph-CTZ (Fig. S1, ESI†) showed a 50 nm red-shifted emission compared to that of native CTZ. This seems to be in contradiction to the dihedral angle twist mentioned earlier. At this time, it is only possible to assume that the electronic properties of the additional phenyl group (*e.g.* its weak inductive electron withdrawing effect) result in an extension of the conjugated π-electron system by a reduction of the dihedral twisting at the C-6 position. The results of bioluminescence measurements are shown in Fig. 4a and b.

A comparison of BL intensities (Fig. 4a) shows that 6-pi-Ph-CTZ is recognized by Rluc8 and Rluc8.6, and in combination with Rluc8.6 its intensity reaches 94% of that of the native CTZ/Rluc pair. This result indicates that the structural modification at the C-6 position has little influence on the enzymatic recognition by Rluc8 and Rluc8.6. The corresponding bioluminescence spectra (Fig. 4b) show the emission peak of 6-pi-Ph-CTZ at 418 nm with Rluc8.6. This is again significantly blue-shifted in comparison to native CTZ, presumably because of the absence of a deprotonatable hydroxyl group. As a result, 6-pi-Ph-CTZ is the brightest short wavelength emitting CTZ derivative reported so far.

All of the three CTZ derivatives evaluated in this study having a styryl group on the C-6 position of the imidazopyrazinone core showed weak BL emission with Rluc8, but significantly stronger emission with Rluc8.6. In contrast to native CTZ (480 nm in Rluc8 and 526 nm in Rluc8.6), the three new derivatives show no differences in the BL emission wavelength between Rluc8 and Rluc8.6. Therefore, it can be assumed that C-6 styryl-substituted CTZ derivatives interact with different amino acid residues compared to native CTZ. A further hint in this direction is the fact that native CTZ also emits BL in combination with other related enzymes, such as *Gaussia* luciferase, whereas 6-pi-H-CTZ and 6-pi-OH-CTZ are selectively recognized by the Rluc8 and Rluc8.6 variants only (Fig. S3, ESI†).

Measurements of the bioluminescence emission time profiles revealed that all three CTZ derivatives show flash-type luminescence

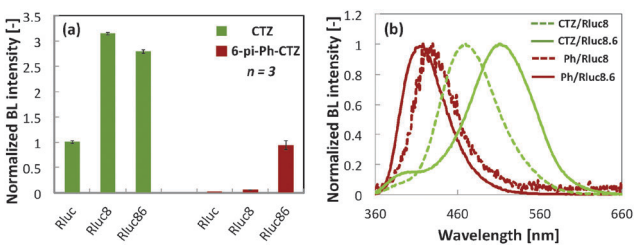

Fig. 4 Luminescence properties of 6-pi-Ph-CTZ: (a) BL intensities; (b) BL spectra with Rluc8 and Rluc8.6.

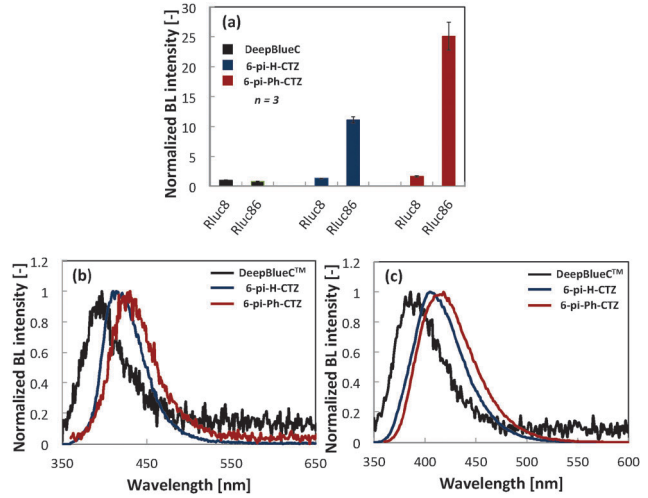

Fig. 5 Comparison of BL properties: (a) BL intensity; (b) BL spectra with Rluc8 and (c) BL spectra with Rluc8.6.

with emission kinetics and half-life times of an order similar to native CTZ (Fig. S4–S8, Table S2, ESI†).

An example of a commercially available CTZ derivative, emerging from structural modifications at both C-2 and C-6 (missing hydroxyl substituent) positions of the imidazopyrazinone core, is the emission wavelength blue-shifted DeepBlueC™ (Fig. 1c).[29] DeepBlueC™ is used as a donor in BL resonance energy transfer (BRET)-based applications with green fluorescent protein (GFP) as the acceptor, because its emission peak at around 400 nm minimally interferes with the emission of the GFP acceptor. Typical applications are BRET studies to image protein–protein interactions.[30] However, the BL emission intensity of DeepBlueC™ is only 3.7% compared to that of native CTZ. Since the BL emission of 6-pi-H-CTZ and of DeepBlueC™ is in a similarly short wavelength range, a comparison of the BL properties of the two compounds was performed (Fig. 5). In terms of BL intensities, 6-pi-H-CTZ and 6-pi-Ph-CTZ with Rluc8.6 showed approximately 11-fold and 25-fold stronger emission than DeepBlueC™ with Rluc8 (Fig. 5). Therefore, 6-pi-H-CTZ and 6-pi-Ph-CTZ are promising bright blue-shifted CTZ derivatives.

In BL, the environmental effects of the enzyme active site on the light emitting species are very high. Although many details still remain unknown, the emission wavelength of native CTZ with various luciferases is significantly altered by the surrounding environment determined by amino acid residues due to factors such as hydrophobicity and pH changes.

In this work, three types of novel CTZ derivatives with styryl substituents at the C-6 position have been successfully developed. All of them showed strong BL emission in combination with the Rluc8 and Rluc8.6 *renilla* luciferase variants, with brightest emission observed in the case of the 6-pi-Ph-CTZ/Rluc8.6 pair. With 11-fold and 25-fold stronger emission at comparable wavelengths and similar bioluminescence emission half-life times (Fig. S6–S8, Table S2, ESI†), 6-pi-H-CTZ and 6-pi-Ph-CTZ are useful bright blue-shifted alternatives to DeepBlueC™, which is commonly applied in BRET-based assays in combination with GFP.

It has been demonstrated for the first time that an extension of the imidazopyrazinone core of native CTZ by comparably large substituents can result in Rluc variant substrates with significant BL emission. We believe that the knowledge gained through this study is useful for the continued elucidation of the enzymatic recognition mechanism and contributes to the development of novel CTZ derivatives with superior optical properties, in particular regarding C-6 substituted derivatives, which have been mostly out of focus, so far.

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