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Firefly inspired one-pot chemiluminescence system using *n*-propylphosphonic anhydride (T3P)

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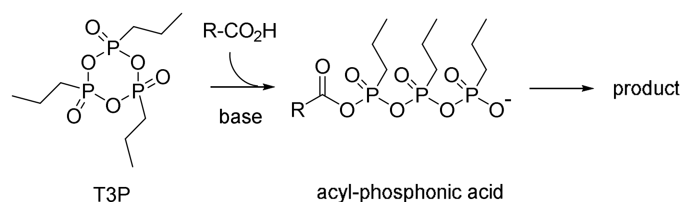
A simple reaction procedure for chemiluminescence of firefly luciferin (D-luc) using *n*-propylphosphonic anhydride (T3P) is reported. A luminescence photon is produced as the result of one-pot reaction, only requiring mixing with substrate carboxylic acid and T3P in the presence of mild organic base.

n-Propylphosphonic anhydride (T3P) is a cyclic anhydride formed by three *n*-propylphosphonic acid molecules. T3P is an easily handled reagent that has long shelf-life stability and does not have shock sensitivity, and it is commercially available as a 50% solution in DMF or ethyl acetate. This reagent also lacks toxicity and allergenic potential.[1] Normally, T3P is condensed with carboxylic acids to form highly reactive mixed anhydrides (acyl-phosphonic acids) (Scheme 1). These intermediates are converted to the final products by subsequent transformation processes. The use of T3P is now expanding to various fields in synthetic chemistry such as amide/peptide synthesis, nitrile synthesis, isonitrile synthesis, oxadiazole formation, 2-substituted 1,3-benzazole synthesis, dehydration, alcohol oxidation, alkene formation, and C–C bond formation reactions, among others.[2] These broad applications demonstrate the usefulness of this reagent.



Scheme 1. Structure of *n*-propyl phosphonic acid (T3P) and condensation with carboxylic acid to form an acyl-phosphonic acid intermediate.

In this communication, we report another application of this reagent in a chemiluminescence reaction. This study was inspired by the firefly bioluminescence reaction in which firefly luciferin (D-luc) is the substrate and firefly luciferase (EC 1.13.12.7) catalyzes ATP-dependent oxidation of D-luc. The firefly luciferase catalyzed bioluminescence reaction comprises three steps (Scheme 2).[3,4] Initially, the carboxyl moiety of D-luc is activated to form a mixed anhydride, D-luciferyl-adenylate (D-luc-AMP), as the result of reaction with ATP-Mg. This intermediate is subsequently oxidized by O_2 through several intermediates to give the electronically excited product oxyluciferin (oxyluc), AMP, and CO_2. Finally, the bioluminescence photon is produced when the excited oxyluc relaxes to its ground state. Although substrate D-luc and produced oxyluc are identical in many firefly species and/or their mutants, the emission color varies from green to red (530–635 nm).[5-10]

In the absence of luciferase, the intermediate D-luc-AMP is oxidized in the presence of base and proceeds through the same reactions as the second and third steps of the bioluminescence sequence, resulting in luminescence; this process is called chemiluminescence. To date, there have been two distinct chemiluminescence systems reported that use derivatives of D-luc, one using D-luc-AMP and the other esters of D-luc [11-13] In these systems, however, synthetic preparations of these substances are required and there is no simple example from substrate carboxylic acid as starting material. Specifically, for quantitative analysis of D-luc-AMP chemiluminescence, HPLC purification is essential, and chemiluminescence from the esters can only be obtained under strong basic conditions, such as with *t*-BuOK. Thus, it cannot be said that previously described systems are easily utilized all researchers.

Herein, we hypothesize that T3P enables activation of D-luc to promote the chemiluminescence reaction, and because T3P is easy to used, it can be applied in a novel, simple, one-pot chemiluminescence system to overcome the drawbacks of other systems. The idea for a new chemiluminescence reaction was derived from the mechanism of amidation by T3P. In this case, the carboxylate initially attacks the T3P to form activated acyl-phosphonic acid. Then, the amine attacks this intermediate and a base picks up the excess proton to produce the amide. In this reaction pathway, the activated acyl-phosphonic acid intermediate is similar to D-luc-AMP (Scheme 2). D-luc has a carboxylic acid in the molecule, which should allow the first acyl-phosphonic acid formation step to proceed. Then, toward this D-luc-AMP like intermediate if an appropriate base would be present in the reaction mixture, the α-proton of carboxylate will be extracted easily and should produce light via the formation of dioxetanone intermediate after being reacted with O_2 molecule. Therefore, this chemiluminescence reaction will be completed only by mixing T3P, D-luc, and an organic base (e.g., triethylamine).

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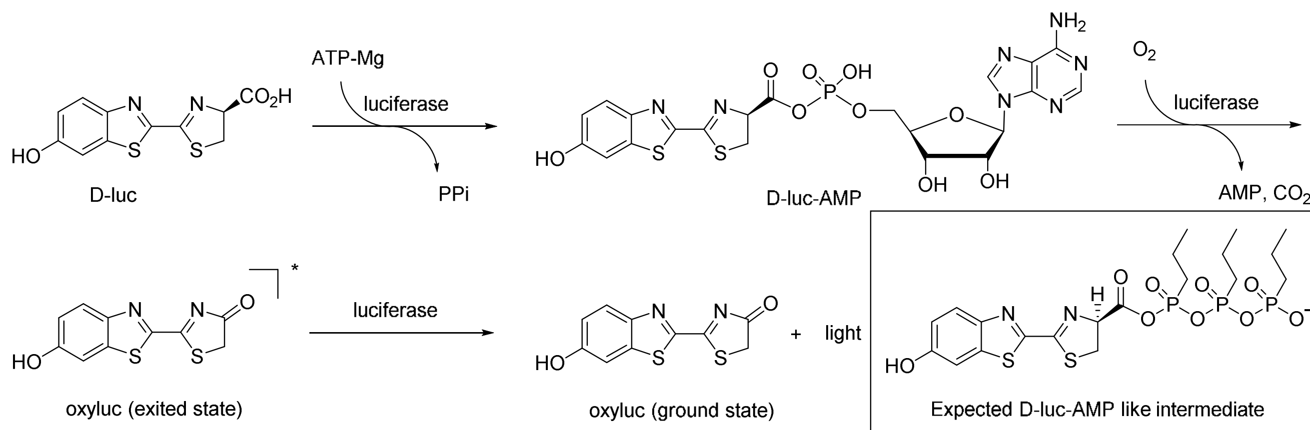

Scheme 2. Chemical conversion process of D-luc to oxyluc in the active site of firefly luciferase, and the structure of an expected D-luc-AMP-like intermediate formed as a result of D-luc and T3P conjugation.

To evaluate this hypothesis, a model reaction was performed. Briefly, a 1 mM D-luc solution in DMF was mixed with a 110 mM T3P solution in DMF in the presence of 100 mM triethylamine. The luminescence signal was detected using a CLX-101 luminometer (Toyobo). Figure 1 shows the time course of this chemiluminescence reaction. As expected, an obvious increase in counts occurred after T3P was added. Furthermore, no emission could be detected in the absence of base, which indicates the importance of a base in the promotion of the chemiluminescence reaction in this system. In case of a previous chemiluminescence system from D-luc esters, strong basic conditions are necessary for emission. T3P system, however, detectable light can be obtained with milder base. In addition, stable light was always recovered under more than 50 mM T3P concentration. The chemiluminescence intensity is on the par with D-luc-AMP chemiluminescence in BSA solution.[12] From the HPLC quantification, it was revealed that 60% of the starting D-luc substrate was consumed and some new peaks were detected after the reaction (supplemental data). A photograph of the solution in a test tube is provided to show the luminescence color of this reaction. D-luc emitted a red color under these conditions. It should be noted that because this system is free from the substrate chirality, the same result is obtained if L-luc is used in this reaction. The simplicity of this system will help to promote the mechanistic study of luminescence reactions similar to the firefly luciferase catalyzed bioluminescence reaction.

Various analogues of D-luc have been also developed in recent years (Figure 2).[14-20] Because of the substrate specificity of firefly luciferase, however, the enzymatic activities toward these analogues are sometimes very low, and it is therefore difficult to measure the luminescence spectrum even with a highly sensitive detector. The bioluminescence signal from the commercially available substrate D-akalumine was approximately 5% of that of D-luc.[14] In the case of D-parablue, which is known as a blue luminescent analogue, the bioluminescence was only 0.01% of that of D-luc.[14] In addition, D-methoxyluc, whose phenolic hydroxyl group is replaced by a methoxy group, was an inert bioluminescence substrate. To evaluate the luminous potential of analogues with removing the substrate specificity of firefly luciferase, researcher must prepare the corresponding acyl-AMP intermediate for each analogue. By using the T3P chemiluminescence system, however, researchers the tedious preparation steps of acyl-AMP intermediates can be avoided, and therefore, various types of reaction conditions can be investigated quickly. This will promote systematic analysis of the relationship between the luminous color and the substrate structures and confirmation of the mechanism of the luminescence reaction toward substrates that are predicted to emit via the dioxetanone intermediate.

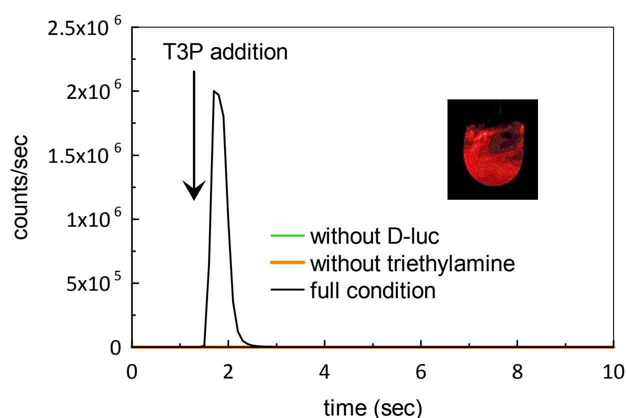

Figure 1. Time course of luminous counts using 100 mM triethylamine as base with a negative control, and a photo of luminescence color using D-luc.

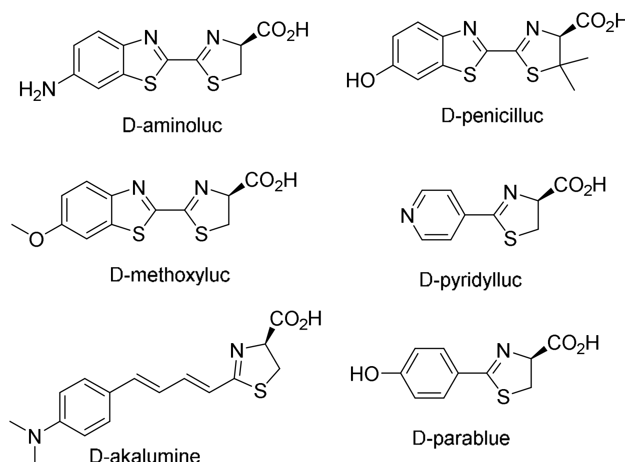

Figure 2. Structure of D-luc analogues that were used in this study

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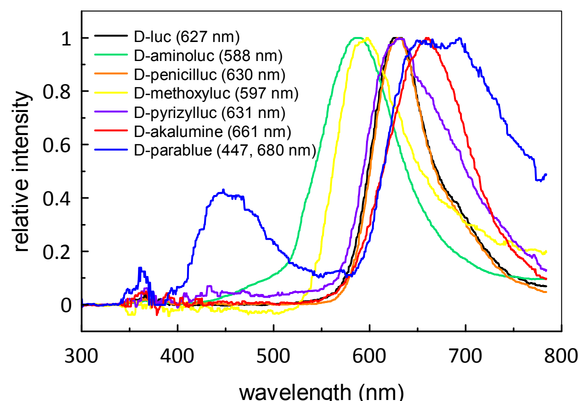

Figure 3. Emission spectra of D-luc and its analogues using triethylamine as base.

To test this hypothesis, T3P was used to investigate chemiluminescence from D-luc analogues (Figure 2). Briefly, a 110 mM T3P solution in DMF was added to a 1 mM solution of D-luc or its analogues in DMF in the presence of 100 mM triethylamine. The emission spectrum was measured using an AB-1850 LumiFL-Spectrocapture (Atto) (slit width, 1 mm; spectral resolution, 0.5 nm). All spectra were corrected for the spectral sensitivity of the equipment and normalized (Figure 3). The peak wavelength of D-luc chemiluminescence (627 nm) showed good agreement with previous reports on D-luc-AMP or D-luc ester.[21-24] Other analogues showed various emission colors depending on their structures. These results indicate that the T3P chemiluminescence system is useful for evaluating the potential luminescence color of these substrates. The λmax of D-penicilluc (630 nm) and D-pyrizylluc (631 nm) is the same as that of D-luc. In the case of D-akalumine, the color is in the near infrared region (661 nm), and for D-aminoluc, it is bright orange (588 nm). These results agree with previous reports.[14,19] Although D-methoxyluc is unacceptable substrate for bioluminescence, it emitted luminescence photons, and the emission color was orange (597 nm) in this system. For D-parablue, two peaks were detected in the blue color region (450 nm) and near infrared region (approximately 680 nm), although its λmax was at 440 nm for the firefly luciferase catalyzed bioluminescence reaction. This may result from the solvent effect for D-parablue, although the reason is not explained properly why this phenomenon is observed only toward this substrate.

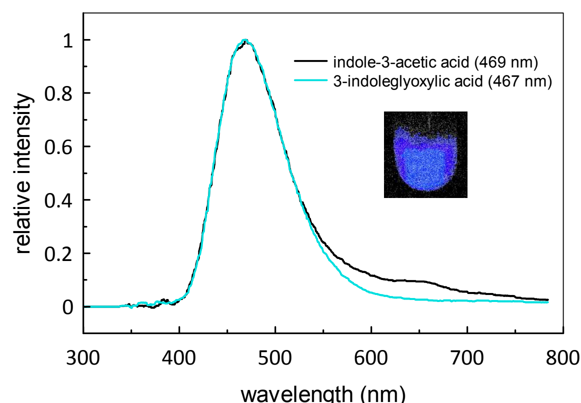

Figure 4. Emission spectra of indole-3-acetic acid and 3-indoleglyoxylic acid using DBU as base, and a photo of luminescence color of 3-indoleglyoxylic acid.

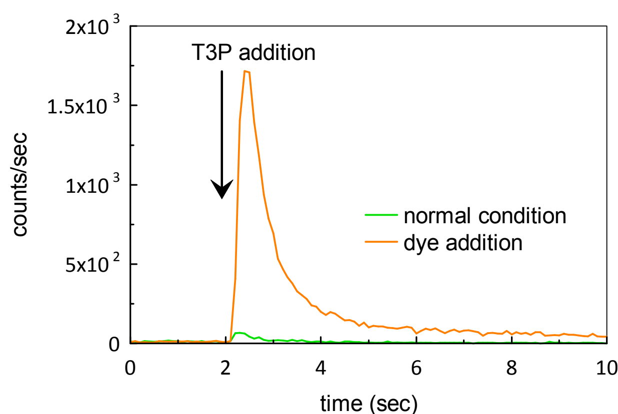

Figure 5. Time course of counts of T3P chemiluminescence reaction using 4-nitrophenylacetic acid and100 mM triethylamine (normal conditions) and with addition of 9,10-bis(phenylethynyl)anthracene (dye addition)

Table 1. Total emission counts with various concentrations of triethylamine or DBU base

entry	substrate	Total emission counts ($x10^6$ counts/sec)											
		Triethylamine (mM)						DBU (mM)					
		200	100	50	10	5	2	200	100	50	10	5	2
1	D-luc	7.7	6.8	5.8	2.0	2.0	3.1	11	6.3	8.1	3.3	3.2	3.5
2	D-aminoluc	1.7	2.6	3.7	0.48	0.24	0.24	0.5	0.47	0.52	2.3	4.0	2.6
3	D-penicilluc	19	22	14	1.6	0.33	0.012	5.8	3.3	6.5	5.4	5.3	12
4	D-methoxyluc	0.89	0.62	0.29	0.0023	N.D. [a]	N.D. [a]	0.88	0.77	0.96	0.27	0.16	0.086
5	D-pyridylluc	0.43	0.49	0.34	0.0054	0.00024	N.D. [a]	0.15	0.16	0.15	0.12	0.051	0.048
6	D-akalumine	3.6	6.1	4.9	0.19	0.02	N.D. [a]	0.27	0.33	0.28	1.3	1.4	2.3
7	D-parablue	0.26	0.14	0.062	0.00092	0.0025	N.D. [a]	0.60	0.60	0.52	0.28	0.18	0.19

[a]No emission was detected.

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All of the substrates were also reacted with the firefly luciferase from *Luciola cruciata* (Wako chemical) in the bioluminescence reaction. The emission spectrum was measured from the reaction mixture containing 1 mg of luciferase enzyme and 100 μL of substrate solution containing 1 mM D-luc or its analogues, 4 mM ATP-2Na, 8 mM $MgSO_4$, and 0.1 M Tris-HCl (pH 8.0). It is known that the bioluminescence color for D-luc and D-penicilluc is shifted to a shorter wavelength (approximately 550 nm) compared with that in the chemiluminescence reaction because of the interaction with enzyme.[21-24] Here, we confirmed that other substrates showed almost the same λmax in the two luminescence systems. These results indicate that excited oxidized substrates from D-luc and D-penicilluc interact with the enzyme and that other analogues do not. To understand the firefly reaction, it is important to know whether the analogues were able to interact with enzyme. Comparison of the results of the chemiluminescence and bioluminescence reactions should help to determine the state of substrate in the enzyme active site.

To investigate the influence of base, chemiluminescence activity under various basic conditions was comprehensively measured. Briefly, a 110 mM T3P solution in DMF was added to 1 mM of D-luc or its analogues in DMF and an appropriate amount of base (triethylamine or DBU). Total counts were measured for 3 seconds using a luminometer. Each assay was repeated 3 times. The left side of Table 1 summarizes the data from the T3P chemiluminescence reaction using triethylamine as base. In the case of D-luc (entry 1), obvious light was observed with less than 2 mM triethylamine, although detectable light was not obtained in the absence of base. Thus, the base is very important for the induction of light emission and may help the α-proton extraction from the acyl-phosphonic acid intermediate before attack by the O_2 molecule. The spectrum toward D-luc was unchanged by the base type and/or its concentration. The other entries in Table 1 show the results with an additional 6 luciferin analogues, which are shown in Figure 2. The strength of light emission is quite different from the various substrates. With 100 mM triethylamine, the strongest intensity was achieved from D-penicilluc and the lowest from D-parablue. There is a 100-fold difference in these values. Although stable light emission was obtained with more than 50 mM triethylamine, only feeble levels were detected with a low base concentration. Less than 10 mM triethylamine only produced very weak or no emissions with D-methoxyluc, D-pyridylluc, and D-parablue. A higher concentration of this base did not affect the total emission counts with these substrates At a concentration of no less than 50 mM triethylamine, it has utterly no effect on the activity. On the right side of Table 1, the results using DBU base are shown. In this case, a strong emission with all substrates was detected at a concentration of 2 mM. This may be due to the difference in pKa of these two bases. The stronger basicity of DBU would promote the proton extraction from the acyl-phosphonic acid intermediate, which was not accomplished by triethylamine. At a base concentration of more than 50 mM DBU, the total light counts were almost the same as when triethylamine was used, although the total intensity with D-penicilluc and D-akalumine was reduced approximately 10% when the same concentrations of DBU or triethylamine were used. Thus, the selection of base and/or its concentration is very important for obtaining sufficient light.

For the expansion of the application of T3P chemiluminescence, we tried two additional substrates, indole-3-acetic acid and 3-indoleglyoxylic acid, which are used as detection substrates in commercially available ELISA kits. In the presence of H_2O_2 under basic conditions, it was predicted that these two substrates would emit light via the dioxetanone intermediates, as occurs during firefly luciferin emission.[25-27] Luminescence was detected when DBU was used as base, but the light emission was not adequate when triethylamine was used as base (Table 2). The spectra of these two substrates were measured, and the λmax for both was approximately 470 nm under the DBU base condition. The luminescence color was also confirmed from the photo (Fig. 4). These results agree well with previous experiments and therefore indicate that this system has the potential to expand the general chemiluminescence reaction by way of dioxetanone intermediates.

For the T3P chemiluminescence reaction, carboxylic acids have to contain the chromophore to emit a photon. 4-Nitrophenylacetic acid did not have a significant luminescence signal under triethylamine base conditions, although it is very likely to form the dioxetanone intermediate with T3P because its structure is similar to luminous indole-3-acetic acid (Fig. 5). However, when a fluorescent dye, 9,10-bis(phenylethynyl)anthracene, was added to the normal T3P chemiluminescence reaction mixture, a slight but obvious increase of luminescence signal was obtained. Thus, even substrates that do not have a luminous chromophore in the molecule can emit a photon by transferring the excited energy to the appropriate fluorescent dyes.

Conclusions

We introduced a novel one-pot chemiluminescence system using T3P. This system is applicable to a series of carboxylic acid substrates such as D-luc and indole-3-acetic acid derivatives that would be oxidized via the dioxetanone intermediate. Handling is very easy, only requiring mixing of the substrate, T3P, and mild organic base to achieve the emission. With appropriate base selection, sufficient light intensity can be obtained. The peak wavelength also showed good agreement with the results of other reported methods. Although detail product assignments are not performed yet, this system might be useful to obtain the color structure relationship of D-luc derivatives and is free from the limitation of the substrate specificity of firefly luciferase catalyzed bioluminescence reactions. In addition, in the presence of a fluorescent chromophore, even nonluminous carboxylic acid can produce light. Elucidation of the conditions to allow emission of light in other organic or aqueous solvent is now underway.

Table 2. Total emission counts with indole-3-acetic acid and 3-indoleglyoxylic acid

entry	substrate	Total emission counts (x10^6 counts/sec)					
		Triethylamine (mM)			DBU (mM)		
		100	50	10	100	50	10
1	indole-3-acetic acid	0.0076	0.0055	0.0079	0.30	0.30	0.094
2	3-indoleglyoxylic acid	0.21	0.31	0.11	1.4	1.7	1.1

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Notes and references

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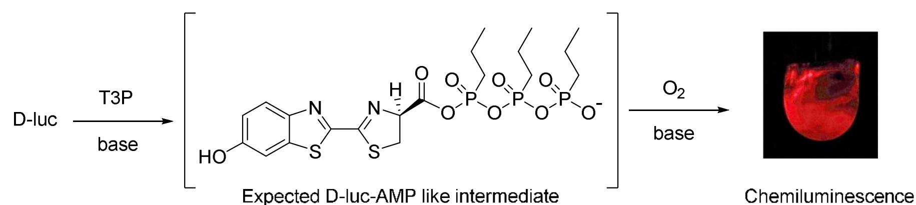
Graphical abstract

A simple one-pot chemiluminescence reaction using *n*-prorpylphosphonic anhydride (T3) toward firefly luciferin and its derivatives are developed.



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