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Rapid and sensitive detection of acrylic acid using a novel fluorescence assay

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We report a fluorescence-based method for the detection of acrylic acid, a high value chemical precursor of acrylate esters and polymers. Our method generates a fluorescent pyrazoline product in the presence of acrylic acid using photoactivated 1,3-dipolar cycloaddition with a diaryltetrazole-based probe, and is capable of detecting, as little as 36 ppb of acrylic acid *in vitro* and *in vivo*. We anticipate that this probe will be useful in high throughput screens for biosynthetic production of acrylic acid from biomass and can replace existing chromatographic methods of detection such as GCMS and LCMS, which are low throughput, time-consuming and require sample preparation.

Acrylic acid is an important feedstock for the industrial production of a wide range of acrylate esters and polymers for use in plastics, latex, superabsorbent polymers, surface coatings, textiles, adhesives and sealants. The global demand for acrylic acid was more than USD $13.6 billion in 2012 and is expected to increase to USD $20.0 billion by 2018.[1] The key raw material in the production of acrylic acid is propylene, which is derived from petrochemical sources.[2, 3] In recent years, there has been great interest in producing acrylic acid from alternative, sustainable, biorenewable sources.[4-8] In the endeavour to produce green acrylic acid derived from biomass and waste products, the ability to detect acrylic acid with a sensitive and specific assay is critical.[9] A rapid, high-throughput fluorescence assay for acrylic acid will facilitate both strain engineering of microbial acrylic acid producers and engineering of relevant enzymes for improved acrylic acid production *in vivo*. In addition, a highly sensitive method of detection is also important for surveillance of acrylate contaminants from plastics, food products and environmental contamination in rivers or drinking water.[10, 11]

Current methods of detecting acrylic acid are chromatographic methods such as gas chromatography (GC)[10, 12, 13] and high pressure liquid chromatography (HPLC) coupled with mass spectrometry detection.[11, 14, 15] These chromatographic methods require tedious sample preparation and derivatization procedures for detection.[10, 13] In addition, they are low throughput and not suitable for large screens. Fluorescence assays, on the other hand, are easily adapted to high throughput screening formats. The rapid readout time is also advantageous compared to chromatographic or radioactivity detection methods.[16]

We have developed a highly sensitive and high throughput fluorescence assay for detection of acrylic acid. This method uses the photoinducible bio-orthogonal chemistry reported by Lin and colleagues, which involves a photoactivated 1,3-dipolar cycloaddition reaction between a diaryltetrazole and an alkene.[17-20] Upon photo-irradiation at 302 nm, the diaryltetrazole undergoes a cycloreversion reaction, generating a highly reactive nitrile imine dipole and releases N_2. This nitrile imine dipole then reacts with the alkene dipolarophile in a concerted manner to produce a pyrazoline cycloadduct, which is fluorescent. We synthesized seven diaryltetrazoles (Fig. 1) and tested their ability to detect acrylic acid. These compounds were selected based on their previous demonstrated ability to react with terminal alkenes. Compounds **5** and **6** have not been previously reported and were designed to incorporate electron-donating groups on the aryl rings, which have been shown to increase the rate of reaction.[19]

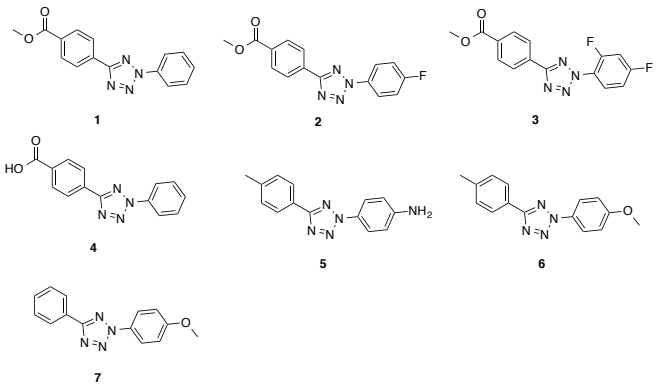


Fig 1. Chemical structures of the seven diaryltetrazole compounds synthesized.

All seven compounds were reacted with acrylic acid and their fluorescence properties were characterized (Fig. S1). The results are summarized in Table 1. Compounds **1**, **2**, **3**, **6** and **7** gave moderate fluorescence increases of around 3-fold above the background when exposed to 10 mM of acrylic acid. **5** was not reactive to acrylic acid,

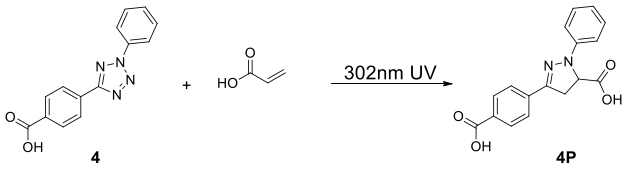
forming no fluorescent product. Interestingly, **4** gave the highest fluorescence turn-on signal (132-fold increase) upon photoactivation in the presence of 100 μM of acrylic acid. To explore the lowest concentration of acrylic acid that can be detected by **4**, we exposed **4** to less than 1 μM of acrylic acid and found that the fluorescence of the product formed was discernible even at 500 nM of acrylic acid. Thus, the lower limit of detection of acrylic acid for **4** was 36 parts per billion. This was achieved with just a one-minute photoactivation period using UV light at 302 nm (Fig. S2-S4). Importantly, this increase in fluorescence was not observed with lactic acid (10 mM, Fig. S4), a common starting material for bio-acrylic acid. Also, in control experiments where either of the reactants was omitted, no fluorescence turn-on signal was observed. In the absence of UV activation, the fluorescence turn-on was also not observed.

Compound[a]	$\lambda_{ex,\ max}$ (nm)	$\lambda_{em,\ max}$ (nm)	Fold increase in fluorescence	
			10 mM	100 μM
1	395	482	3.23	N.A.
2	392	487	3.38	1.12
3	380	482	3.17	1.12
4	380	520	5.15	132
5	N.A.	N.A.	N.A.	N.A.
6	371	450, 475	3.87	N.A.
7	368	456, 475	3.38	1.43

[a] Each compound (100 μM) was reacted with either 10 mM or 100 μM of acrylic acid. Photoirradiation (1 min) was carried out at 302 nm.

Table 1. Fluorescence properties of diaryltetrazole compounds upon reaction with acrylic acid.

A schematic of the reaction between **4** and acrylic acid is shown in Scheme 1. To determine the fluorescence response with increasing amounts of acrylic acid, **4** (100 μM) was added to 1 μM to 100 μM of acrylic acid. In the log-log plot (Fig 2), a linear response is observed between the fluorescence signal and concentration of acrylic acid.



Scheme 1. Schematic of the reaction between **4** and acrylic acid.

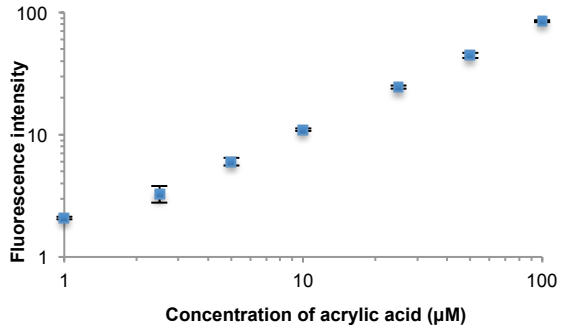
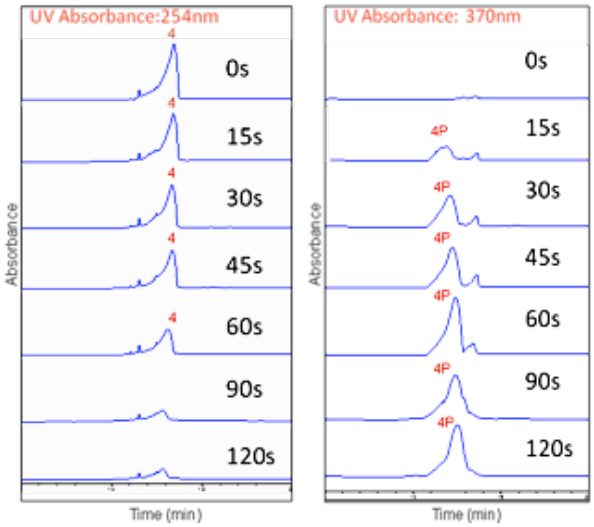


Figure 2. Log-log plot of the increase in fluorescence upon the addition of acrylic acid (1 μM to 100 μM) to 100 μM of **4** in water (n = 2 ± SD).

We studied the reaction between **4** and acrylic acid further by monitoring with HPLC from 0 to 120 seconds. Both the intermediate and product (**4P**) was observed within 15 seconds. The reaction was complete in 90 seconds at which time, the starting material **4** was almost completely used up (Fig. 3A). The formation of the fluorescent pyrazoline product, **4P**, was clearly observed through the large increase in fluorescence of the reaction mixture (Fig. 3B).

A



B

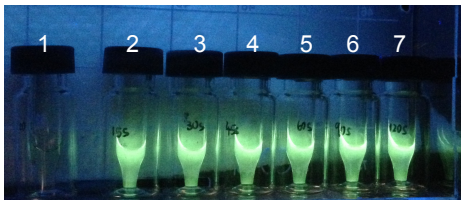


Fig. 3. Monitoring of the reaction between **4** with acrylic acid by HPLC and fluorescence. (A) HPLC chromatogram showing the disappearance of **4** within 90s (left) and formation of the product (right) (B) Fluorescence turn on of the pyrazoline product **4P** was observed within 15s of the reaction and increased from 15s to 120s (Vials 2-7). Vial 1 contained the reaction mixture without UV activation.

This large increase in fluorescence upon the reaction between **4** and acrylic acid is likely due to an internal charge transfer mechanism involving the benzoic acid functional group. To test this hypothesis, we carried out the reaction between **4** and acrylic acid in buffers from pH 1 to pH 13. The results in Figure 4 showed that the fluorescence of the pyrazoline product was strong between pH 6 to 12, with the highest fluorescence observed at pH 10. Negligible fluorescence was observed at other pH. The large increase in fluorescence intensity is thus dependent on the deprotonation of the carboxylic acid group on the aromatic pyrazoline fluorophore. At low pH, the protonated carboxylic acid is an electron-withdrawing group and could act as an electron acceptor in intramolecular photoinduced electron transfer (PET).[21] Intramolecular PET is a well-known mechanism that results in internal quenching of fluorescence and has been previously reported in other aromatic carboxylic acids.[22, 23]

At higher pH, the deprotonated carboxylate does not participate in photoinduced electron transfer. A large enhancement in fluorescence

and a red shift in emission wavelength were observed. This could be due to the interaction of the carboxylate with the π-conjugated pyrazoline fluorophore. The extension of π-conjugation leads to a lower energy excited state and thus, red-shifted emission and larger Stokes shift than the other pyrazoline products.[24] The rigidity of the pyrazoline core also plays an important role in its fluorescence enhancement.[25] Control experiments with only **4** showed that the probe was not fluorescent at all over the entire range of pHs tested.

A more commonly observed situation of intramolecular PET quenching occurs when the electron-rich species is an aromatic amine that is able to donate electrons to aromatic hydrocarbons.[22] This is seen in **5**, which contains an aromatic amine. It acts as a donor in PET, leading to fluorescence quenching of the pyrazoline chromophore.

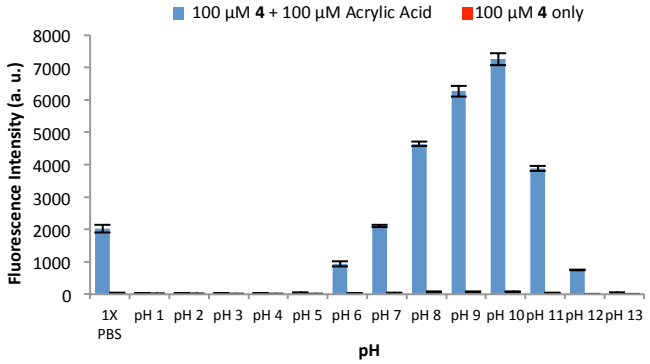


Fig. 4. Fluorescence results of **4**, with acrylic acid (100 μM) at various pH (pH 1-13). Red bars are control experiments with only **4** at different pH. Blue bars indicate the fluorescence of the product after reaction with acrylic acid. The left most bar showed the fluorescence intensity in 1x PBS (pH 7.0) buffer as used in previous experiments.

4 was then used in further experiments for detection of acrylic acid *in vitro* and *in vivo*. Acrylic acid standards were prepared in either minimal media or LB, the media used for microbial growth. Samples for GC analysis were extracted with ether, before they were analysed using both GC-MS and fluorescence. The detection limit for GC analysis was found to be 250 μM (see SI, Section 6 and Fig. S6), while 100 μM of extracted acrylic acid was readily detected using the fluorescence assay. This suggests that the fluorescence assay has a higher sensitivity than GCMS. Also, the fluorescence assay will not require organic extraction for aqueous samples, thus saving precious sample preparation time.

We next tested the use of **4** as a probe for acrylic acid *in vivo*. *E. coli* cells were grown to late log phase (OD_{600}~1.0), then treated with 100 μM acrylic acid for 10 minutes at 37 °C. Upon washing, cells were treated with 100 μM of **4** and incubated at 37 °C for 30 min in the dark. Cells were washed, pelleted, suspended in 1X PBS buffer and mounted on a slide. They were then exposed to UV light at 302 nm for 1 minute and imaged under a fluorescence microscope (using DAPI filters) after 2 hours of recovery at room temperature. The results in Fig. 5 showed that the bacterial cells were only fluorescent in the presence of acrylic acid, **4** and UV irradiation (top left). Control experiments omitting any one of these components did not yield detectable fluorescence. Importantly, **4** was able to enter the cells, and detect acrylic acid present within the bacteria cells. The reaction was bioorthogonal, as reported previously.[17, 18] Thus the detection reaction was able to proceed within the bacteria cell with minimal interference. Background fluorescence from both the cell only and probe only experiments were negligible.

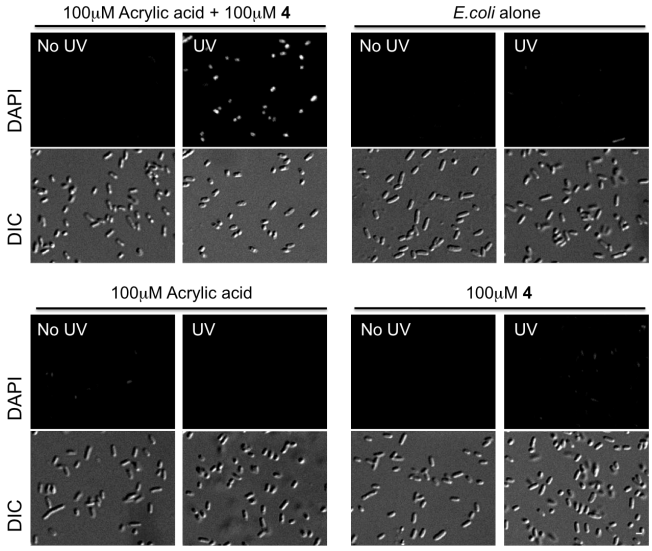


Fig. 5. Detection of acrylic acid in *E. coli* using **4**. (Scale bar indicates 10 μM).

Acrylic acid is produced as a metabolic intermediate in two bacterial species, *Clostridium propionicum* and *Megasphaera elsdenii*. In these microbes, the reduction of lactic acid to propionic acid proceeds *via* an acrylyl-CoA intermediate. 3-butynoic acid, an acyl CoA dehydrogenase inhibitor, has been shown to promote accumulation of acrylic acid in *M.elsdenii*.[26] To test the probe in *C. propionicum,* we first applied **4** to *C. propionicum* cell lysates. Cells were grown to mid-log phase (OD_{600}~0.4) in an anoxia chamber and treated with 0, 5 or 10 mM 3-butynoic acid. *E. coli* cells were used as a negative control. 500 μM of **4** was used to detect acrylyl-CoA or acrylic acid present in *C. propionicum* cell lysates. Only *C. propionicum* cells grown in 3-butynoic acid showed high fluorescence with **4** upon UV activation at 302 nm (Fig. 6). Control experiments indicated that whilst 3-butynoic acid appears to interact with **4** at higher (5 and 10 mM) concentrations (Fig. S7), this is not significant compared to the increase in fluorescence observed in *C. propionicum* cells grown in 3-butynoic acid. Furthermore, the concentration of 3-butynoic acid in the cell lysates (after removal of media) would be considerably low.

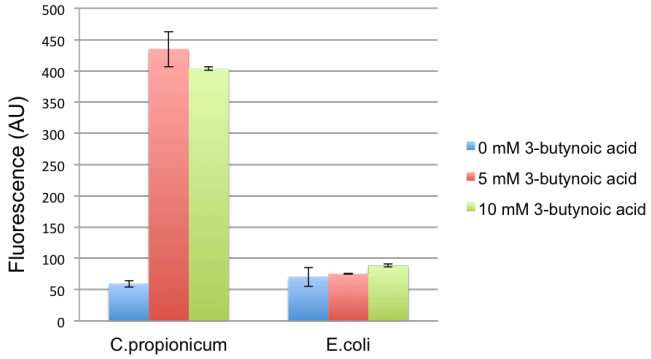


Fig. 6. Fluorescence signal of bacterial cell lysates from indicated cells grown in different concentrations of 3-butynoic acid and subsequently treated with 500 μM of **4**.

To test the use of **4** in live *C. propionicum,* cells were grown in media containing 5 mM 3-butynoic acid to accumulate acryly-CoA and/or acrylic acid and processed as described above for *E. coli*. Positive control cells included cells treated with exogenous acrylic acid and **4** to observe positive fluorescence. The results in Fig. 7A

showed that cells were fluorescent in both the control experiment where cells were treated with 500 μM of acrylic acid and 500 μM of **4** (first panel) and in *C. propionicum* cells treated with 500 μM of **4** alone (last panel). This indicates the production of acrylic acid and/or intermediates in these cells, and sensitive detection by the probe. Cell lysates of the above treated cells were used to measure fluorescence signal in a more quantitative manner using a fluorescence plate reader (Fig. 7B). The results correlated with the semi-quantitative imaging experiments.

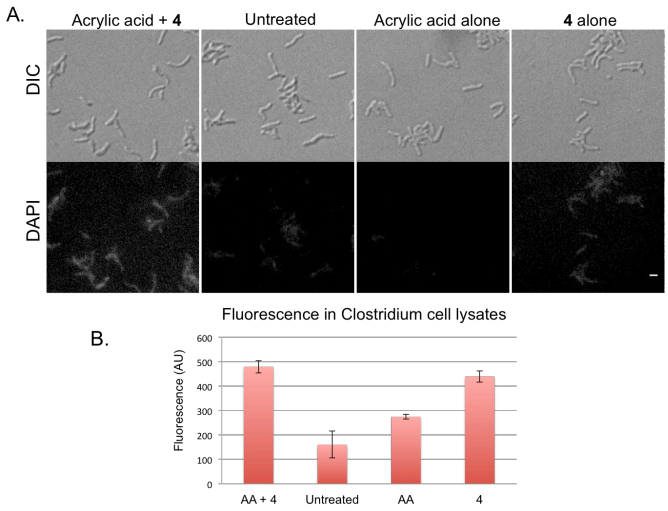


Fig. 7. (A) Detection of acrylic acid and/or intermediates present in *Clostridium propionicum* cells grown in media containing 5mM 3-butynoic acid either untreated, treated with acrylic acid, **4** or both. (Scale bar indicates 2 μM) (B) Cell lysates from the same experiment were used to quantitatively measure the fluorescence signal using a fluorescence plate reader.

Conclusions

In conclusion, we demonstrated the novel application of using a diaryltetrazole-based probe to generate a fluorescent product upon its reaction with acrylic acid. Traditional methods of detecting acrylic acid using gas chromatography and liquid chromatography techniques are time-consuming, require specific technical expertise and instrumentation and are not suitable for high throughput screening applications. On the other hand, fluorescence assays can be easily adapted for high throughput screening using multi-well plates.

In our assay, we detect acrylic acid in a photoactivated reaction that is completed within 2 minutes, which enables rapid detection. In comparison with GCMS, our fluorescence-based assay provides significant reduction in hands-on time because it does not require sample preparation, extraction and derivatization. Hence, our method of detection can potentially be used in portable optical devices. This will be highly useful for real-time monitoring of acrylic acid and acrylates in the environment..

A fluorescence assay capable of *in vitro* and *in vivo* detection will also be useful in approaches based on enzyme evolution to produce bioacrylic acid. It can be incorporated into high throughput screening workflows that rely on fluorescence microplate readers, flow cytometry methods and cellular assays, which is not compatible with methods based on chromatographic analysis.

Notes and references

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† Electronic Supplementary Information (ESI) available: Synthetic details and characterization of compounds, fluorescence data of compounds, GCMS data. See DOI: 10.1039/c000000x/

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Graphical abstract

A novel fluorescence detection assay for acrylic acid, an important target for biomass production, was developed. This fluorescence assay is based on a photoactivated 1,3-dipolar cycloaddition reaction, and was demonstrated for both *in vitro* and *in vivo* applications.