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Synthesis of α, β-unsaturated aldehydes as potential substrates for bacterial luciferases

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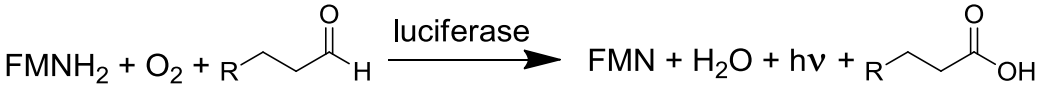
Abbreviations: DIBALH: diisobutylaluminum hydride; DMAP: 4-(dimethylamino)-pyridine; DMF: *N,N*-dimethylformamide; DCM: dichloromethane; THF: tetrahydrofuran

Abstract

Bacterial luciferase catalyzes the monooxygenation of long-chain aldehydes such as tetradecanal to the corresponding acid accompanied by light emission with a maximum at 490 nm. In this study even numbered aldehydes with eight, ten, twelve and fourteen carbon atoms were compared with analogs having a double bond at the α, β-position. These α, β-unsaturated aldehydes were synthesized in three steps and were examined as potential substrates *in vitro*. The luciferase of *Photobacterium leiognathi* was found to convert these analogs and showed a reduced but significant bioluminescence activity compared to tetradecanal. This study showed the trend that aldehydes, both saturated and unsaturated, with longer chain lengths had higher activity in terms of bioluminescence than shorter chain lengths. The maximal light intensity of (*E*)-tetradec-2-enal was approximately half with luciferase of *P. leiognathi*, compared to tetradecanal. Luciferases of *Vibrio harveyi* and *Aliivibrio fisheri* accepted these newly synthesized substrates but light emission dropped drastically compared to saturated aldehydes. The onset and the decay rate of bioluminescence were much slower, when using unsaturated substrates, indicating a kinetic effect. As a result the duration of the light emission is doubled. These results suggest that the substrate scope of bacterial luciferases is broader than previously reported.

1. Introduction

The "cold-light" phenomenon - the enzymatic production of light commonly known as bioluminescence - can be found in prokaryotes and eukaryotes.[1] The involvement of long-chain aliphatic aldehydes as substrates in bacterial bioluminescence has been known since 1953 when various chain lengths of the substrates were investigated by Strehler and Cormier.[2–4] In the 1960s, the role of these potential substrates was analyzed concerning the reaction velocity, the initial maximal intensity and the decay of luminescence.[5,6] In 1963 Spudich and Hastings tested the first unsaturated aldehyde, 2-decenal, and showed complete inactivity with this substrate.[7] Cormier *et al.* were the first to prove that long-chain aldehydes were definitely required for light production.[8,9] Further investigations focused on the determination of the stoichiometry, the quantum yield and the product of this bioluminescent reaction.[10–13] It took some more years to identify tetradecanal as the "natural" substrate for bacterial bioluminescence in 1974 by Shimomura.[14] By now it is known, that the luciferase catalyzes the monooxygenation of long-chain aliphatic aldehydes to the corresponding acids employing reduced flavin mononucleotide ($FMNH_2$) as redox cofactor (Scheme 1).[1]



R = $(CH_2)_nCH_3$; n = 4, 6, 8, 10

Scheme 1: **General reaction of bacterial bioluminescence.**

The initial step of the reaction is the binding of reduced FMN to luciferase. The enzyme-$FMNH_2$ complex reacts with molecular oxygen to form flavin-4a-hydroperoxide. This relatively stable enzyme-FMNHOOH complex subsequently reacts with a long chain aliphatic aldehyde to form a flavin-4a-peroxyhemiacetal intermediate.[15] Its slow decay results in the oxidation of the aldehyde to the corresponding acid and the free energy released during this reaction populates an excited state flavin-4a-hydroxide, which in turn serves as the light emitting molecule.[16]

Bacterial luciferases are heterodimeric enzymes consisting of an α-subunit and a β-subunit. The two subunits have a sequence identity of approximately 32 % and have evolved from a common ancestor.[17] The active site of the enzyme is exclusively on the α-subunit and also distant from the subunit interface. The exact role of the β-subunit is not clear, but deletion or mutation of this subunit reveals less or complete loss of activity. A mutation of βTyr151, for instance, has a negative effect on $FMNH_2$ binding. It seems that the β-subunit is responsible for high quantum yield and protein stability.[17,18]

Only two crystal structures of bacterial luciferases have been reported, where one of them elucidated the structure of the apo-LuxAB of *Vibrio harveyi* [17,19] and the other one revealed the apo-LuxAB of *Vibrio harveyi* soaked with FMN.[18] The isoalloxazine ring of the flavin shows a planar conformation and is held in place by mainly backbone contacts. The amino acids involved in the binding of the 5' phosphate are Arg107, Arg125, Glu175, Ser176 and Thr179 (Figure 1).[18,20] Both structures designate a TIM barrel fold $(\beta\alpha)_8$ for the enzyme. Both subunits have a loop between the β-strand 7 and α-helix 7. The α-subunit, in contrast to the β-subunit, has 29 additional amino acids and a stretch of disordered residues from Lys283 to Arg290. This loop region is the most conserved region of the luciferase sequence. It is highly protease-labile, but binding of FMN or polyvalent anions can prevent proteolytic inactivation.[17,18] Complete deletion of the loop results in reduction of total quantum yield by two orders of magnitude. It was hypothesized that the mobile loop has a lid-gating mechanism similar to other TIM-barrel enzymes.[21] This loop is in close proximity to the active center and seems to undergo conformational changes from an open or semi open state to a closed state after flavin binding and before reaction with oxygen.[17,18,21]

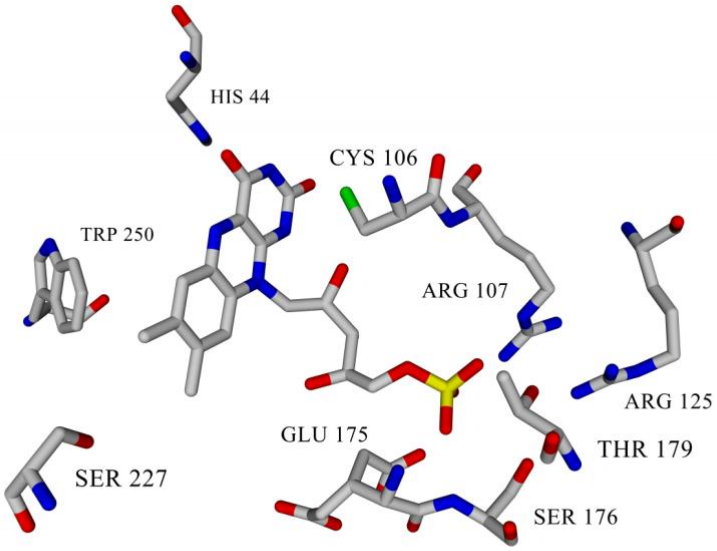


Figure 1: Graphical representation of FMN bound to luciferase of *V. harveyi*. Based on the crystal structure[18], FMN and a few key residues discussed in the text are depicted in stick confirmation with according labelling. General color code is used for the atoms. Residues Arg107, Arg125, Glu175, Ser176 and Thr179 are responsible for binding of the 5' phosphate group of FMN. His44, Ser227 and Trp250 might have a role in substrate binding and interaction.

Currently, structural information on the spatial arrangement of FMN and the aldehyde substrate in the active site is lacking, however various mutagenesis and modelling studies were performed in the last years. It was assumed that the flavin binding pocket is large enough to accommodate $FMNH_2$, O_2 and long chain aldehydes.[22] In particular, two amino acids, Cys106 and Ser227, have attracted interest, because their exchange substantially affected enzymatic activity. The former amino acid apparently plays an important catalytic role as alkylation of its reactive thiol led to inactivation of the luciferase (Figure 1).[17,18] In addition it was found that the Cys106Val variant exhibits decreased aldehyde utilization and reduced stability of the flavin-4a-hydroperoxide intermediate.[20] On the other hand, replacement of Ser227 to phenylalanine in the α-subunit led to a steric effect in the well-characterized mutant AK-20 (Figure 1). Generally, replacement of Ser227 by large aromatic amino acids led to a 10-fold decreased binding affinity for aldehyde, smaller amino acids, e.g. alanine had no influence.[17,23] Modelling studies suggest that the bioluminescent reaction occurs on the *si*-face of the isoalloxazine ring facing the amino acid His44 (Figure 1). The distance of the C4a atom of flavin and the Nδ atom of His44 is approximately 7 Å. This distance leaves enough space for functional groups of the intermediates (peroxide, hydroxide) and aldehyde binding. Additionally, a spacious cavity is formed in the active site, which is surrounded by hydrophobic residues. Among those residues is Trp250, which was suggested to interact with the aldehyde substrate (Figure 1).[20,24] Despite these structural analyses, the exact structure of bacterial luciferases in complex with $FMNH_2$ and aldehyde

substrate is still unknown. A ^{13}C and ^{15}N NMR study by Vervoort *et al*. analyzed the differences of bound FMN and bound $FMNH_2$ to luciferase of *Vibrio harveyi*. There is clearly a change in electron densities, hydrogen bonding and planarity of the oxidized and reduced state of FMN. The N10 atom of $FMNH_2$ seems to be slightly out of the molecular plane.[25] All these indications prompt speculations about the reaction mechanism as well as the substrate scope.

In this study, α, β-unsaturated aldehydes with chain lengths of eight, ten, twelve and fourteen carbon atoms were synthesized. To investigate the mechanism of bacterial bioluminescence, the recombinant luciferases from *Photobacterium leiognathi*, *Vibrio harveyi* and *Aliivibrio fisheri* were chosen as model systems to test these potential substrates and analyze the substrate specificity of bacterial luciferases.

2. Materials and Methods

2.1. General experimental information

All commercially available reagents and solvents were purchased from Sigma-Aldrich, Alfa Aesar, Fisher Scientific, Acros Organics, Roth or VWR, and were used without further purification if not stated otherwise. When it was required, non-dry solvents were distilled before use. If reactions were performed under inert conditions, e.g. exclusion of water, oxygen or both, all experiments were carried out using established Schlenk techniques. Herein solvents were dried and/or degassed with common methods and afterwards stored under inert gas atmosphere (argon or N_2) over molecular sieves. In some cases, when explicitly mentioned, dry solvents were received from the listed suppliers. DCM (EtOH stabilized) was distilled first over P_4O_{10} to remove the stabilizer and then over CaH_2 under argon atmosphere and stored over 4 Å molecular sieves in an amber-colored 1000 mL Schlenk bottle.

All reactions were stirred with Teflon-coated magnetic stirring bars. Molecular sieves (Sigma Aldrich, beads with 8-12 mesh) were activated in a round-bottom flask with a gas-inlet adapter by heating them carefully in a heating mantle for approximately 12 h under high vacuum until complete dryness was obtained. These activated molecular sieves were stored at room temperature under argon atmosphere.

Temperatures were measured externally if not otherwise stated. Reactions that were carried out at -78 °C were cooled by keeping the reaction vessel immersed in a properly sized Dewar vessel containing acetone/dry ice.

Analytical thin layer chromatography (TLC) was carried out on Merck TLC silica gel 60 F254 aluminum sheets and spots were visualized by UV light (λ = 254 and/or 366 nm) or by staining with iodide, cerium ammonium molybdate (2.0 g $Ce(SO_4)_2$, 50.0 g $(NH_4)_6Mo_7O_{24}$ and 50 mL conc. H_2SO_4 in 400 mL water) (CAM) or potassium permanganate (0.3 g $KMnO_4$, 20 g

K_2CO_3, 5 mL 5 % aqueous NaOH in 300 mL H_2O) followed by the development of the stains in the heat. Flash column chromatography was performed on silica gel 0.035-0.070 mm, 60 Å (Acros Organics). A 30 to 100 fold excess of silica gel was used with respect to the amount of dry crude product, depending on the separation problem. The dimensions of the column were selected in such a way that the required amount of silica gel formed a pad between 10 cm and 25 cm. The column was equilibrated first with the solvent or solvent mixture, and the crude product diluted with the eluent was applied onto the top of the silica pad. In case when the crude product was insoluble in the eluent, the sample was dissolved in an appropriate solvent (EtOAc or DCM), and the equal amount of diatomaceous earth was added, followed by removal of the solvent under reduced pressure and drying the sample in vacuum, which was then directly loaded onto the top of the silica pad. The mobile phase was forced through the column using a rubber bulb pump.

2.2. General procedure GP-1 (Synthesis of α, β-unsaturated ethyl esters)

In a 100 mL single neck round-bottom flask equipped with a magnetic stir bar, 4-(dimethylamino)pyridine (122 mg, 1.00 mmol, 0.10 eq), mono-ethyl malonate (2.36 mL, 20.0 mmol, 2.0 eq) and saturated alkyl aldehyde (10.0 mmol, 1.0 eq) were dissolved in DMF (50 mL). The reaction mixture was stirred at room temperature for 42 h. Subsequently, the mixture was diluted with diethyl ether (50 mL), washed with saturated aqueous NH_4Cl (50 mL), saturated aqueous $NaHCO_3$ (50 mL), water (50 mL), and concentrated under reduced pressure. Flash chromatography (SiO_2, 5 % EtOAc in cyclohexane) afforded the desired unsaturated ethyl ester as a colorless liquid.

2.3. General procedure GP-2 (Synthesis of allyl alcohols)

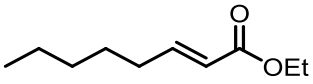
In a nitrogen-purged 100 mL Schlenk tube equipped with a magnetic stir bar, unsaturated ethyl ester (4.5 mmol, 1.0 eq) was dissolved in dry dichloromethane (18 mL), the vessel was sealed with a glass stopper and cooled to -78 °C in an acetone/dry ice bath. 1.0 M solution of diisobutylaluminum hydride in hexanes (10.8 mL, 10.8 mmol, 2.4 eq) was added dropwise via a syringe and a septum throughout 10 min. The reaction was stirred at -78 °C until TLC indicated quantitative conversion (3 h). The reaction mixture was quenched by the dropwise addition of MeOH (1 mL). Subsequently, the cooling bath was removed, saturated aqueous potassium sodium tartrate solution (18 mL) was added, and the mixture was stirred vigorously for 2 h. After phase separation the aqueous layer was extracted with dichloromethane (10 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Flash chromatography (SiO_2, 20 % EtOAc in cyclohexane) afforded the desired allyl alcohol as a colorless liquid.

2.4. General procedure GP-3 (Synthesis of α, β-unsaturated aldehydes)

In a nitrogen-purged 10 mL Schlenk tube equipped with a magnetic stir bar, manganese(IV) oxide (494 mg, 5.0 mmol, 5.0 eq) and activated 4 Å molecular sieves were suspended in dry dichloromethane (4 mL). Allyl alcohol (1.0 mmol, 1.0 eq) was dissolved in dry

dichloromethane (3.3 mL), added to the mixture in the Schlenk tube, which was sealed with a glass stopper. After stirring the mixture overnight at room temperature the dark brown reaction mixture was filtered through a compressed pad of diatomaceous earth. The pad was washed with dichloromethane (2 mL), and the filtrate was concentrated under reduced pressure. Flash chromatography (SiO_2, 10 % EtOAc in cyclohexane) afforded the desired unsaturated aldehyde as a pale yellow liquid.

2.5. Ethyl (*E*)-oct-2-enoate (3d)



Unsaturated ester **3d** was synthesized and isolated according to the general procedure GP-1 and its stated stoichiometry.

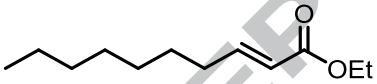
Yield: 940 mg (5.52 mmol, 55 %), colorless liquid.

R_f = 0.35 (cyclohexane/EtOAc 40:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 6.97 (dt, ^3J = 15.6 Hz, ^3J = 7.0 Hz, 1H), 5.81 (d, ^3J = 15.6 Hz, 1H), 4.18 (q, ^3J = 7.1 Hz, 2H), 2.25–2.14 (m, 2H), 1.52–1.40 (m, 2H), 1.36–1-25 (m, 7H), 0.82 (t, ^3J = 6.7 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 166.9 (s, 1C), 149.6 (s, 1C), 121.4 (s, 1C), 60.2 (s, 1C), 32.3 (s, 1C), 31.4 (s, 1C), 27.8 (s, 1C), 22.6 (s, 1C), 14.4 (s, 1C), 14.1 (s, 1C) ppm.

2.6. Ethyl (*E*)-dec-2-enoate (3c)



Unsaturated ester **3c** was synthesized and isolated according to the general procedure GP-1 and its stated stoichiometry.

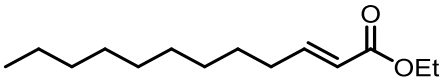
Yield: 1.480 g (7.46 mmol, 75 %), colorless liquid.

R_f = 0.35 (cyclohexane/EtOAc 40:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 6.96 (dt, ^3J = 15.6 Hz, ^3J = 7.0 Hz, 1H), 5.80 (d, ^3J = 15.6 Hz, 1H), 4.18 (q, ^3J = 7.1 Hz, 2H), 2.26–2.12 (m, 2H), 1.51–1.39 (m, 2H), 1.35–1-21 (m, 11H), 0.88 (t, ^3J = 6.7 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 166.9 (s, 1C), 149.6 (s, 1C), 121.4 (s, 1C), 60.3 (s, 1C), 32.3 (s, 1C), 31.9 (s, 1C), 29.3 (s, 1C), 29.2 (s, 1C), 28.2 (s, 1C), 22.8 (s, 1C), 14.4 (s, 1C), 14.2 (s, 1C) ppm.

2.7. Ethyl (*E*)-dodec-2-enoate (3b)



Unsaturated ester **3b** was synthesized and isolated according to the general procedure GP-1 and its stated stoichiometry.

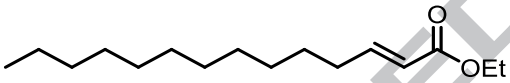
Yield: 1.235 g (5.46 mmol, 55 %), colorless liquid.

R_f = 0.36 (cyclohexane/EtOAc 40:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 6.96 (dt, ^3J = 15.6 Hz, ^3J = 7.0 Hz, 1H), 5.80 (d, ^3J = 15.6 Hz, 1H), 4.18 (q, ^3J = 7.1 Hz, 2H), 2.25–2.13 (m, 2H), 1.51–1.39 (m, 2H), 1.35–1-21 (m, 15H), 0.88 (t, ^3J = 6.7 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 166.9 (s, 1C), 149.6 (s, 1C), 121.3 (s, 1C), 60.2 (s, 1C), 32.3 (s, 1C), 32.0 (s, 1C), 29.6 (s, 1C), 29.5 (s, 1C), 29.4 (s, 1C), 29.3 (s, 1C), 28.2 (s, 1C), 22.8 (s, 1C), 14.4 (s, 1C), 14.2 (s, 1C) ppm.

2.8. Ethyl (*E*)-tetradec-2-enoate (3a)



Unsaturated ester **3a** was synthesized and isolated according to the general procedure GP-1 and its stated stoichiometry.

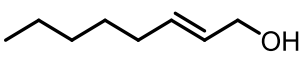
Yield: 1.733 g (6.81 mmol, 68 %), colorless liquid.

R_f = 0.38 (cyclohexane/EtOAc 40:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 6.96 (dt, ^3J = 15.6 Hz, ^3J = 7.0 Hz, 1H), 5.80 (d, ^3J = 15.6 Hz, 1H), 4.17 (q, ^3J = 7.1 Hz, 2H), 2.25–2.12 (m, 2H), 1.51–1.38 (m, 2H), 1.35–1-21 (m, 19H), 0.87 (t, ^3J = 6.7 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 166.9 (s, 1C), 149.6 (s, 1C), 121.3 (s, 1C), 60.2 (s, 1C), 32.3 (s, 1C), 32.0 (s, 1C), 29.8 (m, 2C), 29.7 (s, 1C), 29.5 (s, 1C), 29.4 (s, 1C), 29.3 (s, 1C), 28.2 (s, 1C), 22.8 (s, 1C), 14.4 (s, 1C), 14.2 (s, 1C) ppm.

2.9. (*E*)-Oct-2-en-1-ol (4d)



Allyl alcohol **4d** was synthesized and isolated according to the general procedure GP-2 and its stated stoichiometry.

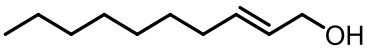
Yield: 449 mg (3.50 mmol, 78 %), colorless liquid.

R_f = 0.57 (cyclohexane/EtOAc 9:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 5.77–5.54 (m, 2H), 4.08 (br s, 2H), 2.03 (dt, ^3J = 6.9 Hz, ^3J = 6.6 Hz, 2H), 1.45–1.19 (m, 7H), 0.88 (t, ^3J = 6.7 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 133.8 (s, 1C), 129.0 (s, 1C), 64.0 (s, 1C), 32.3 (s, 1C), 31.5 (s, 1C), 29.0 (s, 1C), 22.7 (s, 1C), 14.2 (s, 1C) ppm.

2.10. (*E*)-Dec-2-en-1-ol (4c)



Allyl alcohol **4c** was synthesized and isolated according to the general procedure GP-2 and its stated stoichiometry.

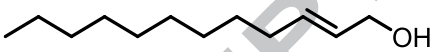
Yield: 605 mg (3.87 mmol, 86 %), colorless liquid.

R_f = 0.57 (cyclohexane/EtOAc 9:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 5.77–5.55 (m, 2H), 4.07 (br s, 2H), 2.03 (dt, ^3J = 6.9 Hz, ^3J = 6.5 Hz, 2H), 1.46–1.17 (m, 11H), 0.88 (t, ^3J = 6.7 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 133.7 (s, 1C), 129.0 (s, 1C), 64.0 (s, 1C), 32.3 (s, 1C), 32.0 (s, 1C), 29.3 (s, 3C), 22.8 (s, 1C), 14.2 (s, 1C) ppm.

2.11. (*E*)-Dodec-2-en-1-ol (4b)



Allyl alcohol **4b** was synthesized and isolated according to the general procedure GP-2 and its stated stoichiometry.

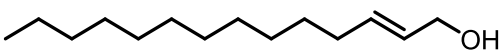
Yield: 617 mg (3.35 mmol, 74 %), colorless liquid.

R_f = 0.57 (cyclohexane/EtOAc 9:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 5.77–5.56 (m, 2H), 4.08 (br s, 2H), 2.03 (dt, ^3J = 6.9 Hz, ^3J = 6.6 Hz, 2H), 1.41–1.18 (m, 15H), 0.88 (t, ^3J = 6.7 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 133.8 (s, 1C), 129.0 (s, 1C), 64.0 (s, 1C), 32.4 (s, 1C), 32.1 (s, 1C), 29.8–29.6 (m, 2C), 29.5 (s, 1C), 29.4–29.2 (m, 2C), 22.8 (s, 1C), 14.3 (s, 1C) ppm.

2.12. (*E*)-Tetradec-2-en-1-ol (4a)



Allyl alcohol **4a** was synthesized and isolated according to the general procedure GP-2 and its stated stoichiometry.

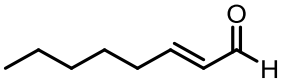
Yield: 641 mg (3.02 mmol, 67 %), colorless liquid.

R_f = 0.57 (cyclohexane/EtOAc 9:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 5.77–5.56 (m, 2H), 4.08 (br s, 2H), 2.03 (dt, ^3J = 6.9 Hz, ^3J = 6.6 Hz, 2H), 1.36–1.17 (m, 19H), 0.88 (t, ^3J = 6.7 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 133.8 (s, 1C), 129.0 (s, 1C), 64.0 (s, 1C), 32.4 (s, 1C), 32.1 (s, 1C), 29.9–29.7 (m, 3C), 29.7 (s, 1C), 29.5 (s, 1C), 29.4–29.2 (m, 2C), 22.8 (s, 1C), 14.3 (s, 1C) ppm.

2.13. (*E*)-Oct-2-enal (5d)



Unsaturated aldehyde **5d** was synthesized and isolated according to the general procedure GP-3 and its stated stoichiometry.

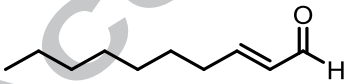
Yield: 103 mg (0.816 mmol, 82 %), pale yellow liquid.

R_f = 0.37 (cyclohexane/EtOAc 9:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 9.50 (d, ^3J = 7.9 Hz, 1H), 6.86 (dt, ^3J = 15.6 Hz, ^3J = 6.9 Hz, 1H), 6.11 (dd, ^3J = 15.6 Hz, ^3J = 7.9 Hz, 1H), 2.33 (dt, ^3J = 7.2 Hz, ^3J = 7.2 Hz, 2H), 1.58–1.42 (m, 2H), 1.37–1.26 (m, 4H), 0.98–0.85 (m, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 194.3 (s, 1C), 159.2 (s, 1C), 133.1 (s, 1C), 32.8 (s, 1C), 31.4 (s, 1C), 27.7 (s, 1C), 22.5 (s, 1C), 14.1 (s, 1C) ppm.

2.14. (*E*)-Dec-2-enal (5c)



Unsaturated aldehyde **5c** was synthesized and isolated according to the general procedure GP-3 and its stated stoichiometry.

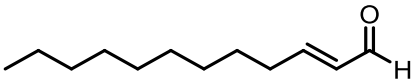
Yield: 122 mg (0.791 mmol, 79 %), pale yellow liquid.

R_f = 0.37 (cyclohexane/EtOAc 9:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 9.51 (d, ^3J = 7.9 Hz, 1H), 6.85 (dt, ^3J = 15.6 Hz, ^3J = 6.9 Hz, 1H), 6.11 (dd, ^3J = 15.6 Hz, ^3J = 7.9 Hz, 1H), 2.33 (dt, ^3J = 7.2 Hz, ^3J = 7.2 Hz, 2H), 1.57–1.44 (m, 2H), 1.40–1.19 (m, 8H), 0.88 (t, ^3J = 6.7 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 194.2 (s, 1C), 159.2 (s, 1C), 133.1 (s, 1C), 32.9 (s, 1C), 31.8 (s, 1C), 29.2 (s, 1C), 29.1 (s, 1C), 28.0 (s, 1C), 22.8 (s, 1C), 14.2 (s, 1C) ppm.

2.15. (*E*)-Dodec-2-enal (5b)



Unsaturated aldehyde **5b** was synthesized and isolated according to the general procedure GP-3 and its stated stoichiometry.

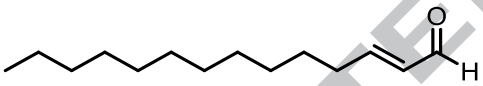
Yield: 136 mg (0.746 mmol, 75 %), pale yellow liquid.

R_f = 0.38 (cyclohexane/EtOAc 9:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 9.50 (d, ^3J = 7.9 Hz, 1H), 6.85 (dt, ^3J = 15.6 Hz, ^3J = 6.9 Hz, 1H), 6.11 (dd, ^3J = 15.6 Hz, ^3J = 7.9 Hz, 1H), 2.33 (dt, ^3J = 7.2 Hz, ^3J = 7.2 Hz, 2H), 1.57–1.43 (m, 2H), 1.39–1.18 (m, 12H), 0.88 (t, ^3J = 6.7 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 194.3 (s, 1C), 159.2 (s, 1C), 133.1 (s, 1C), 32.9 (s, 1C), 32.0 (s, 1C), 29.6 (s, 1C), 29.5 (s, 1C), 29.4 (s, 1C), 29.3 (s, 1C), 28.0 (s, 1C), 22.8 (s, 1C), 14.2 (s, 1C) ppm.

2.16. (*E*)-tetradec-2-enal (5a)



Unsaturated aldehyde **5a** was synthesized and isolated according to the general procedure GP-3 and its stated stoichiometry.

Yield: 159 mg (0.756 mmol, 76 %), pale yellow liquid.

R_f = 0.40 (cyclohexane/EtOAc 9:1 (v/v); staining: $KMnO_4$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 9.50 (d, ^3J = 7.8 Hz, 1H), 6.85 (dt, ^3J = 15.6 Hz, ^3J = 6.9 Hz, 1H), 6.11 (dd, ^3J = 15.6 Hz, ^3J = 7.9 Hz, 1H), 2.33 (dt, ^3J = 7.2 Hz, ^3J = 7.2 Hz, 2H), 1.57–1.44 (m, 2H), 1.38–1.20 (m, 16H), 0.88 (t, ^3J = 6.7 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 194.3 (s, 1C), 159.2 (s, 1C), 133.1 (s, 1C), 32.9 (s, 1C), 32.0 (s, 1C), 29.8 (s, 2C), 29.6 (s, 1C), 29.5–29.4 (m, 2C), 29.3 (s, 1C), 28.0 (s, 1C), 22.8 (s, 1C), 14.2 (s, 1C) ppm.

2.17. Instrumentation

UV/Vis absorption spectra were recorded with a Specord 210 spectrophotometer (Analytic Jena, Jena, Germany). The light emission was measured by a Berthold Technologies Centro LB 960 microplate Luminometer with Mikro Win version 4.16. Gel filtration was performed

using a Superdex-200 column (prep grade XK 16/100; GE Healthcare) with an Äktaexplorer 100 Pharmacia Biotech (GE Healthcare).

^{1}H-, ^{13}C-NMR spectra were recorded on a Bruker AVANCE III 300 spectrometer (^{1}H: 300.36 MHz; ^{13}C: 75.53 MHz). Chemical shifts were referenced to the residual proton and carbon signal of the deuterated solvent, respectively ($CDCl_3$: δ = 7.26 ppm (^{1}H), 77.16 ppm (^{13}C)). Signal multiplicities are abbreviated as s (singlet), bs (broad singlet), d (doublet), dd (doublet of doublet), t (triplet), q (quadruplet), p (pentet) and m (multiplet). Deuterated solvents for nuclear resonance spectroscopy were purchased from Euriso-top®.

2.18. Design, expression and purification of recombinant His-tagged proteins

LuxAB from *Photobacterium leiognathi* (ATCC 27561; PL_LuxAB) and YcnD from *Bacillus subtilis* were cloned into pET21a vector and transformed into *E. coli* BL21 (DE3) strain for expression as described previously.[26,27] LuxAB from *Vibrio harveyi* (ATCC 14126; VH_LuxAB) and *Aliivibrio fisheri* (ATCC 7744; AF_LuxAB) were cloned similarly. The genes for VH_LuxAB and AF_LuxAB were integrated into pET24b vector and transformed into *E. coli* Rosetta strain. Both constructs had an additional C-terminal octa-histidine tag. Heterologous expression cultures were grown at 37 °C in LB media containing kanamycin (50 µg/mL) and chloramphenicol (20 µg/mL) as selection markers until an OD (600 nm) of 0.6 was reached. The expression was induced with 0.1 mM IPTG and cells were further grown at 20 °C for 16 h. Cells were harvested by centrifugation (4400 g, 10 min, 4 °C) and the wet cell pellets were stored at -20 °C. The cell pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8), lysed by addition of lysozyme and sonication and after centrifugation the clear supernatant was loaded on 5 mL HisTrap FF/HP columns (GE Healthcare) for purification via Ni-NTA affinity chromatography. The columns were washed with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8) and the purified protein fractions were gained with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole, pH 8). After concentrating and buffer exchange to 45 mM Tris-buffer containing 40 mM MES and 20 mM L-malic acid pH 8, a subsequent gel filtration using a Superdex-200 column was performed. The concentration of the various proteins was determined spectrophotometrically at 280 nm using the extinction coefficients of 82,335 M^{-1} cm^{-1} (PL_LuxAB), 84,230 M^{-1} cm^{-1} (VH_LuxAB), 83,200 M^{-1} cm^{-1} (AF_LuxAB). In the case of YcnD the concentration was determined using an extinction coefficient of 12,190 M^{-1} cm^{-1} at 450 nm.

2.19. *In vitro* assay

The *in vitro* assay was performed in 96 well white assay plates. For the assay all enzymes and substrates were prepared and/or diluted in 100 mM potassium phosphate buffer pH 7. The reaction mixture contained the enzyme luciferase with either 50 nM for *P. leiognathi* or 200 nM for *V. harveyi* or *A. fischeri*, respectively. YcnD and FMN were adjusted to 1.5 fold of the luciferase concentration, respectively. Additionally 500 nM NADPH and the substrate-buffer suspension were added to make up the final volume of 250 µL. The tested substrates

include even chain length (8 to 14 carbon atoms) saturated and unsaturated aldehydes, respectively. Due to the relatively low solubility of aldehydes in water, concentrated aldehyde suspensions were obtained by adding 5 µL of the substrate to 10 mL of the reaction buffer, respectively (concentration range of 1,94-3,30 mM; Supplementary Data).[5] The reaction was started by injecting NADPH to the reaction mixture (after a delay of 5 seconds) and the readings were subsequently taken every 0.01th of a second for a total of 90 seconds. The light was measured using the luminometer. The light emission was recorded as emission counts. The area under the curve was taken for calculation of the percentage and the total light emission of luciferase with tetradecanal (**6a**) as substrate was considered as 100 %. The data was calculated to 100 nM of luciferase concentration to compare the results with each other. The values for the conversion of luciferase of *P. leiognathi* are shown by means ± SD of seven individual measurements for the saturated aldehydes and four individual measurements for the unsaturated aldehydes, respectively. The values for the conversion of luciferase of *V. harveyi* and *A. fischeri* are shown by means ± SD of four individual measurements, respectively.

2.20. Molecular docking

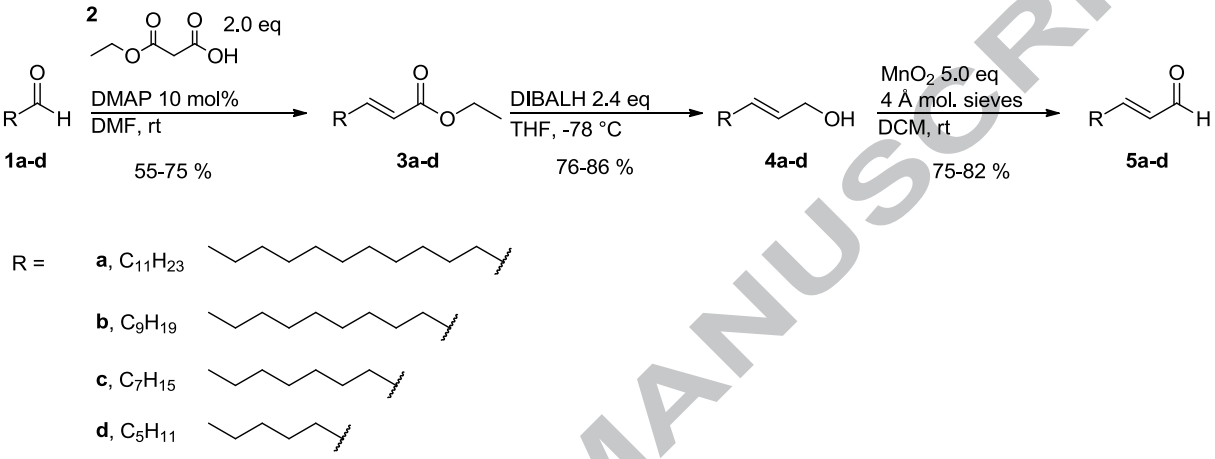
In silico molecular docking studies were performed using Yasara Structure 13.9.8.[28] The crystal structure of the luciferase/flavin complex of *Vibrio harveyi* was retrieved from the Protein Data Bank (PDB entry: 3FGC). Structure preparation and all following experiments were performed within Yasara Structure 13.9.8. All the crystallographic water molecules and the β-subunit of the luciferase were removed before molecular docking. Missing hydrogens were added to the molecules by using the clean mode of Yasara.

For the docking experiments the oxidized flavin structure was modified to the flavin-4a-hydroperoxide intermediate. Therefore the additional hydrogens and the two oxygens were attached to the molecule and refined by energy minimization using AMBER99 force field, while fixing Lys283 and Arg290 which connect the luciferase backbone with an unstructured and therefore missing loop.[18] The resulting crystal structure was utilized for docking the substrate molecules (**5a-d**, **6a-d**) in flexible mode into the rigid receptor using the plugin Autodock Vina in Yasara Structure 13.9.8.[29,30] The docking simulation cell was set to 15 Å around the flavin-4a-hydroperoxide intermediate and 500 docking runs with an RMSD cutoff of 2 Å were performed. The docked conformations for each substrate (**5a-d**, **6a-d**) were ranked according to the distance between the C1 atom of the substrate molecule and the terminal oxygen atom of the hydroperoxide functional group. The best-ranked docking pose for each substrate (**5a-d**, **6a-d**) was analyzed in Yasara Structure 13.9.8.

3. Results

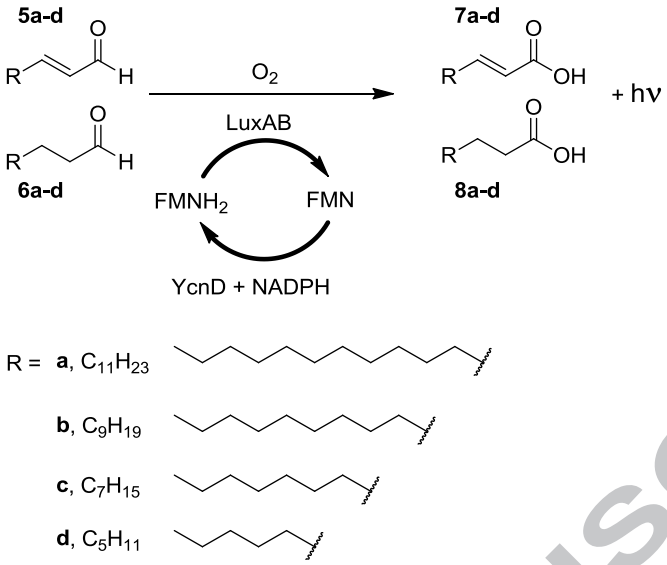
To obtain new insights into the activity and selectivity of luciferases, unsaturated aldehydes with various chain lengths were synthesized and analyzed. The substrate synthesis was

carried out in three steps (Scheme 2). The α, β-unsaturated aldehydes were synthesized starting from commercially available saturated aldehydes with two carbons less in chain length. The starting aldehydes **1a-d** were subjected to a DMAP-catalyzed Knoevenagel condensation with mono-ethyl malonate (**2**) to obtain the corresponding unsaturated esters **3a-d**.[31] **3a-d** were reduced to the corresponding allyl alcohols **4a-d** using 1.2 equivalents diisobutylaluminum hydride (DIBALH) in DCM at -78 °C and were subsequently oxidized with manganese(IV) oxide (MnO_2) to afford the desired α, β-unsaturated aldehydes **5a-d**.



Scheme 2: **Synthesis route for aliphatic, unsaturated aldehydes with different chain lengths (C8-C14).** Using DMAP-catalyzed Knoevenagel condensation the starting aldehydes **1a-d** were converted to the unsaturated ethyl esters **3a-d**, which were reduced to the corresponding allyl alcohols **4a-d** and were finally oxidized to the α, β-unsaturated aldehydes **5a-d**. The exact equivalents, solvents and temperature conditions are given and the yield for each step is given as percentage.

To test the newly synthesized substrates, an *in vitro* assay was developed (Scheme 3). Briefly, 50 nM recombinant luciferase of *P. leiognathi* (LuxAB) was used in a reaction mixture with 100 mM potassium phosphate buffer pH 7, 75 nM FMN, 75 nM YcnD, 500 nM NADPH and substrate-buffer suspensions of **5a-d** and **6a-d** (see Materials and Methods). YcnD, an NADPH-dependent oxidoreductase from *Bacillus subtilis*, reduces FMN to provide the cosubstrate $FMNH_2$.[27] The luciferase then oxidizes the various substrates to their corresponding acids using the enzyme-bound flavin-4a-hydroperoxide with concomitant emission of light. We assume that saturated aldehydes **6a-d** and unsaturated aldehydes **5a-d** are accepted in a similar way as substrates and converted to the corresponding acids. The photons, emitted during this reaction, were collected by a luminometer for 90 seconds and the areas under the light emission curve were compared to tetradecanal (**6a**).



Scheme 3: Schematic representation of the *in vitro* assay. The synthesized unsaturated aldehydes **5a-d**, as well as the four saturated aldehydes **6a-d**, were investigated in an *in vitro* assay. The oxidation reaction catalyzed by 50 nM luciferase (LuxAB), employing molecular oxygen (O_2) and reduced FMN ($FMNH_2$), results in long-chain aliphatic acids **7a-d** and **8a-d** and the emission of light (hν). For the reduced FMN a recycling system was established using the NADPH-dependent oxidoreductase YcnD from *Bacillus subtilis*. The light emission is measured by a luminometer and subsequently converted to total light emission in per cent for comparison and analysis.

As expected, tetradecanal (**6a**) showed the highest light emission and was set to 100 %. The other aldehydes showed lower activity with the luciferase (Figure 2). In the case of dodecanal (**6b**), decanal (**6c**) and (*E*)-tetradec-2-enal (**5a**), light emission was greater than 50 % in comparison to tetradecanal (**6a**). Octanal (**6d**) exhibited the lowest yield of the saturated aldehydes. The unsaturated aldehyde substrates **5b-d**, however, resulted in yields below 10 %. Comparing saturated aldehydes with each other, substrates with longer chain length emit more light than those with shorter chain length and therefore are apparently better substrates for luciferase. This tendency was already observed earlier[2,6], however a clear comparison and definite values were not reported. The same tendency was found for the unsaturated aldehydes, where total light emission decreases with shorter chain length. Thus, saturated and unsaturated aldehydes exhibit a similar chain length dependency but are clearly accepted as substrates for bacterial luciferase.

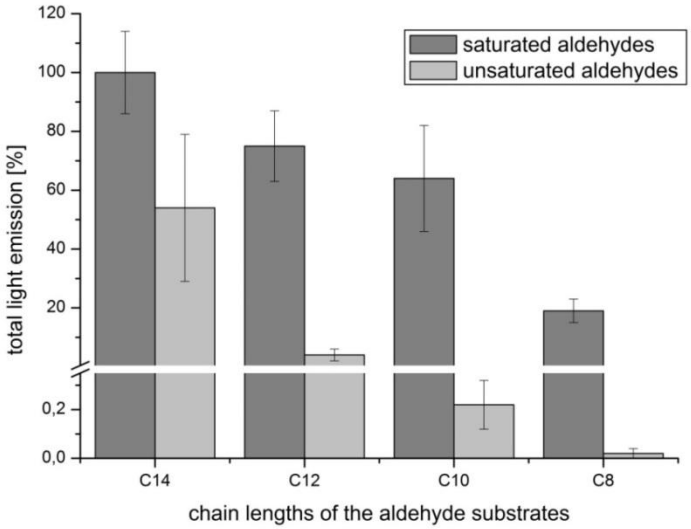


Figure 2: Comparison of saturated and unsaturated aldehydes as potential substrates for the luciferase of *P. leiognathi*. The conversion of unsaturated aldehydes **5a-d** (light grey) and saturated aldehydes **6a-d** (dark grey) by the luciferase during the *in vitro* assay (Scheme 3) can be determined by the total light emission of the reaction measured by the luminometer. The counts of the light emission were converted to percent. The total light emission (as percentage) was plotted against the substrates (here differentiated by their chain lengths). The production of light by the conversion of tetradecanal (**6a**) was set to 100 %. The values are shown by means ± SD of seven individual measurements for the saturated aldehydes and four individual measurements for the unsaturated aldehydes, respectively.

Next, we analyzed the time course of light emission for unsaturated and saturated aldehydes. As an example, the kinetics of light emission with tetradecanal (**6a**) and (*E*)-tetradec-2-enal (**5a**) as substrates are shown in Figure 3. Generally, the onset as well as the decay of the light emission is faster with saturated aldehydes. In the case of **6a** a maximum light emission is reached after ca. 7 s whereas light emission with **5a** peaks at ca. 10 s. On the other hand, light emission lasted much longer for the unsaturated aldehyde **5a** than for the saturated aldehyde **6a**. A similar kinetic behavior was observed for all other saturated/unsaturated aldehyde pairs. This result indicates that the rate-limiting step (or steps) leading to the population of the excited state luciferin (presumably the flavin-4a-hydroxide[32]) is slowed down when unsaturated aldehydes are used as substrates.

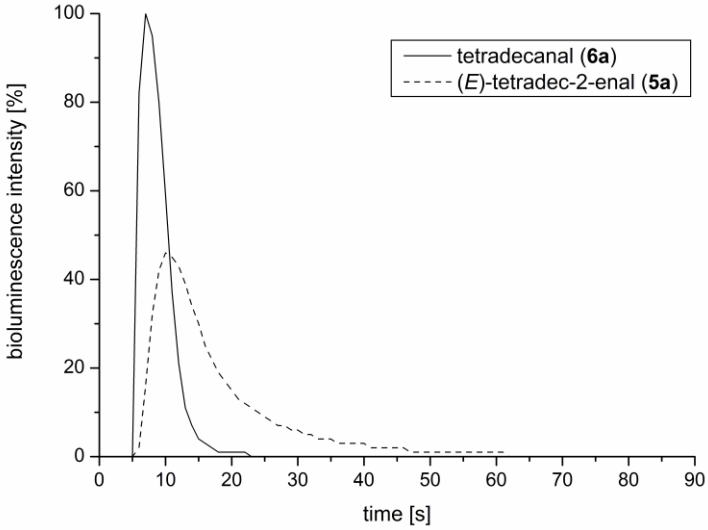


Figure 3: Time course of the total light emission of tetradecanal (6a) and (*E*)-tetradec-2-enal (5a). The light emission during the conversion of the substrates tetradecanal (**6a**, solid line) and (*E*)-tetradec-2-enal (**5a**, dashed line) was measured as a function of time by the luminometer. The counts of light emission were plotted as per cent, setting the maximal bioluminescence intensity of tetradecanal (**6a**) as 100 %, against the time (in seconds). This is a representative figure of a single measurement.

Tetradecanal (**6a**) and (*E*)-tetradec-2-enal (**5a**) were chosen as substrates for the *in vitro* assay with luciferases from different genera (*P. leiognathi, V. harveyi, A. fisheri*) as depicted in Figure 4. The assay conditions were adopted for *V. harveyi* and *A. fisheri*. For the latter, a luciferase concentration of 200 nM was used and the concentrations for FMN and YcnD were set to 300 nM, respectively. Because light emission was highest with the luciferase from *P. leiognathi* it was used as a reference point, i.e. set to 100 %. *V. harveyi* and *A. fisheri* accepted both substrates but showed a much lower activity than *P. leiognathi*. Comparison of these two substrates with various luciferases confirms the previous results by depicting a decline of light emission with unsaturated aldehydes. Nevertheless, (*E*)-tetradec-2-enal (**5a**) is a substrate for various bacterial luciferases.

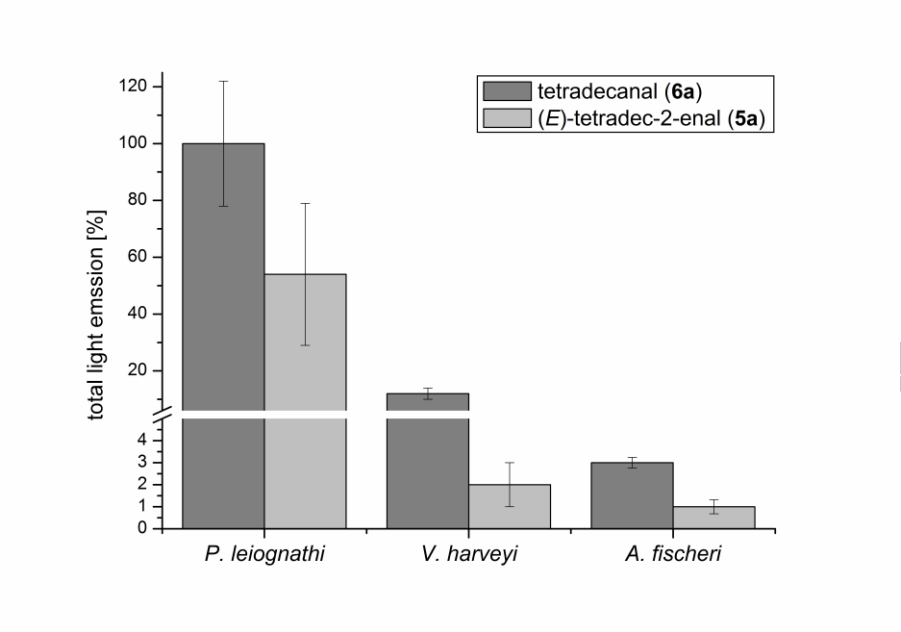


Figure 4: Comparison of the total light emission for luciferases from *P. leiognathi*, *V. harveyi* and *A. fisheri*. Three luciferases of different genera were compared with each other by analyzing the total light emission (as percentage) during conversion of the two potential substrates tetradecanal (**6a**, dark grey) and (*E*)-tetradec-2-enal (**5a**, light grey) in *in vitro* assays. The values are shown by means ± SD of four individual measurements, respectively.

To evaluate whether the binding of the substrate within the active site might influence the enzyme activity and maximal light intensity, a preliminary docking study was performed. Based on the crystal structure with bound FMN[18], the intermediate state of flavin-4a-hydroperoxide was predicted and the various substrates were docked into the active site. After energy minimization, the structure with the flavin-4a-hydroperoxide in the active site was used for further docking studies with the substrates used in this study*, i. e.* **5a-d** and **6a-d**. The docking results obtained with the saturated and unsaturated aldehydes indicate similar distances of the C1 atom of the respective aldehyde and the distal oxygen atom of the flavin-4a-hydroperoxide (Supplementary Data).

In Figure 5, an overlay of the two docking results with the substrates tetradecanal (**6a**) and (*E*)-tetradec-2-enal (**5a**) is depicted. It appears that the two substrates **5a** and **6a** adopt similar conformations and orientations, except for the position of the oxygen atom of the aldehyde that points in opposite directions. The distance between the C1 of the aldehyde and the distal oxygen of the flavin intermediate is around 3.6-3.7 Å (Supplementary Data).

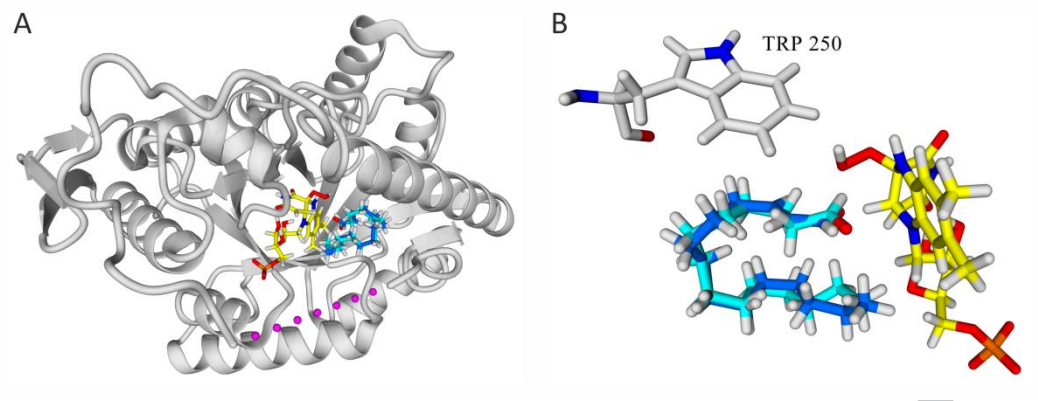


Figure 5: Docking of tetradecanal (6a) and (*E*)-tetradec-2-enal (5a) into the active site of the luciferase of *V. harveyi* with bound flavin-4a-hydroperoxide intermediate. A: Crystal structure of the α-subunit of the luciferase of *V. harveyi* with modelled flavin-4a-hydroperoxide intermediate is depicted in yellow. The unstructured loop is displayed as pink dots. The two substrates tetradecanal (**6a**, cyan) and (*E*)-tetradec-2-enal (**5a**, blue) are docked into the active site of the luciferase. **B**: Zoom into the active site and overlay of tetradecanal (**6a**, cyan) and (*E*)-tetradec-2-enal (**5a**, blue). The flavin-4a-hydroperoxide intermediate (yellow) is in close proximity to the substrates **6a** and **5a**, having a distance between the distal oxygen of flavin-4a-hydroperoxide to C1 of 3,637 Å and 3,740 Å, respectively.

Thus, our docking results support our experimental findings that unsaturated aldehydes are accepted substrates and indicate that luciferases have a broader substrate range as previously assumed. The different orientation observed for the aldehyde function may be a first hint why unsaturated aldehydes show a substantial difference in kinetics as compared to their saturated counterparts. The hydrophobic pocket, lined for example by Trp250 (Figure 5) within the active site does not allow binding of bulkier or larger substrates, however, replacement of amino acids in the active site of luciferase may help to engineer the putative substrate binding pocket for other aldehyde substrates. Clearly, further structural and computational methods need to be applied to enhance our understanding of the mechanism and substrate scope of bacterial luciferases.

4. Discussion

Four different compounds, namely (*E*)-tetradec-2-enal (**5a**), (*E*)-dodec-2-enal (**5b**), (*E*)-dec-2-enal (**5c**) and (*E*)-oct-2-enal (**5d**), were successfully synthesized and analyzed as potential substrates for recombinant luciferases from three different genera. Spudich and Hastings showed in 1963 that 2-decenal (referring to (*E*)-dec-2-enal (**5c**)) is completely inactive in the production of light with the luciferase of *Achromobacter fisheri*. On the contrary, this compound was found to be a potent competitive inhibitor in bioluminescence with decanal in the reaction inhibited by decenal. Strangely enough, they have reported similar quantum yields for the reaction with saturated and unsaturated substrate.[7] Additionally, Lei and coworkers proposed an inhibitory effect of a postulated “luciferase-aldehyde dead end

complex". In this case the sequence of substrate binding (aldehyde and $FMNH_2$) to the luciferase seems to be essential for activity or inhibition.[33] Thus, it was assumed that all α, β-unsaturated aldehydes exert an inhibitory effect and were therefore not considered as possible substrates. In contrast to that, we show here that unsaturated aldehydes are accepted as substrates by various recombinant luciferases from the genera *Photobacterium*, *Vibrio* and *Aliivibrio*, although the light emission yield was lower with the unsaturated aldehydes.

In light of our observations, the previously observed inhibitory effect[7] seems to be a kinetic one. The time course of the light emission is strongly influenced by the various substrates. Unfortunately, substrate-buffer suspensions had to be used for the assays; therefore, it was not possible to conduct more detailed kinetic measurements. Studies with organic co-solvents were attempted, but led to denaturation of the luciferases (data not shown). The solubility of the aldehydes corresponds to the aldehyde chain length according to molar solubility values (Supplementary Data). Octanal (**6d**), for instance, should presumably give better results than tetradecanal (**6a**), as its solubility in aqueous buffer is higher. However, the reverse dependency was observed, as aldehydes with longer chain length are more efficient in light emission in our *in vitro* assay system.

As mentioned in the introduction, structural information on the active site of luciferase is still scarce in particular in regard of the positioning of the aldehyde substrate. Current mechanistic considerations are based on the crystal structure of luciferase of *V. harveyi* with bound FMN.[18] Modelling studies suggested several amino acids that may play an important role for binding or interacting with the aldehyde substrate, as for example His44 and Trp250. Additionally, a spacious hydrophobic cavity was postulated as potential substrate binding position.[20] Nevertheless, a structure of the ternary complex of luciferase, $FMNH_2$ and aldehyde is still lacking leading to speculations concerning substrate binding and the reaction mechanism.

5. Conclusion

Bacterial bioluminescence is a fascinating phenomenon and the structure-function relationships responsible for the population of an excited state remains a scientific challenge even after decades of research. Here, we have demonstrated that the scope of substrates utilized by bacterial luciferases is not as limited as previously thought. In this study, the α, β-unsaturated aldehydes with chain length of 8, 10, 12 and 14 carbon atoms were synthesized in a three step synthesis approach. To elucidate the conversion of these potential substrates, an *in vitro* assay was developed. The four synthesized, unsaturated aldehydes **5a-d**, as well as their saturated analogs **6a-d** were analyzed with luciferases from three different genera (*P. leiognathi*, *V. harveyi*, *A. fisheri*). The results indicate, that all of them are accepted by the enzymes and show reasonable to low light emission. Comparing the different potential substrates, tetradecanal (**6a**) exhibits the highest light emission yield, while three other

substrates (**5a**, **6b**, **6c**) reached only about 54-75 % of the best performing tetradecanal (**6a**). This study comprises the first comparison of these eight aldehydes (**5a-d**, **6a-d**) as substrates for bacterial luciferases. Having a closer look at the time course of light emission, the different kinetics in the onset as well as decay of light emission for tetradecanal (**6a**) and (*E*)-tetradec-2-enal (**5a**) were evident. Also, we have shown that luciferases from other bioluminescent bacteria show a similar pattern with regard to yield and kinetics of light emission. In summary, all eight substrates **5a-d** and **6a-d** were accepted by the luciferase leading to the conclusion that further investigations on substrate specificity and compatibility will lead to new insights in to bacterial bioluminescence.

Acknowledgments

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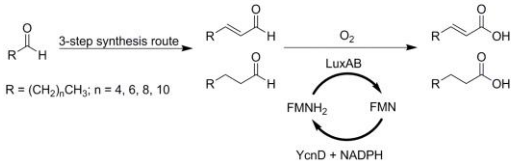
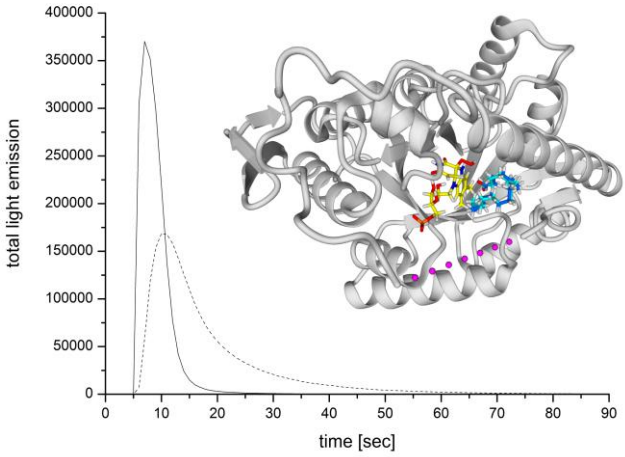
References

(1) Dunlap, P. In *Encyclopedia of Microbiology*; Schaechter, M., Ed.; Elsevier: Oxford, 2009; pp 45–61.
(2) Strehler, B. L.; Cormier, M. J. *J. Biol. Chem.* **1954**, *211*, 213–225.
(3) Strehler, B. L. *J. Am. Chem. Soc.* **1953**, *75*, 1264.
(4) Strehler, B. L.; Cormier, M. J. *Arch. Biochem. Biophys.* **1953**, *47*, 16–33.
(5) Hastings, J. W.; Gibson, Q. H. *J. Biol. Chem.* **1963**, *238* (7), 2537–2554.
(6) Hastings, J. W.; Spudich, J.; Malnic, G. *J. Biol. Chem.* **1963**, *238* (9), 3100–3105.
(7) Spudich, J.; Hastings, J. W. *J. Biol. Chem.* **1963**, *238* (9), 3106–3108.
(8) Cormier, M. J.; Totter, J. R. *Biochim. Biophys. Acta* **1957**, *25*, 229–237.
(9) Cormier, M. J.; Eley, M.; Abe, S.; Nakano, Y. *Photochem. Photobiol.* **1969**, *9*, 351–358.
(10) Lee, J. *Biochemistry* **1972**, *11* (18), 3350–3359.
(11) Shimomura, O.; Johnson, F. H.; Kohama, Y. *Proc. Natl. Acad. Sci. USA* **1972**, *69* (8), 2086–2089.
(12) McCapra, F.; Hysert, D. W. *Biochem. Biophys. Res. Commun.* **1973**, *52* (1), 298–304.
(13) Lee, J.; Murphy, C. L. *Biochemistry* **1975**, *14* (10), 2259–2268.
(14) Shimomura, O.; Johnson, F. H.; Morise, H. *Proc. Natl. Acad. Sci. USA* **1974**, *71* (12), 4666–4669.
(15) Macheroux, P.; Ghisla, S.; Hastings, J. W. *Biochemistry* **1993**, *32*, 14183–14186.
(16) Dunlap, P. In *Bioluminescence: Fundamentals and Applications in Biotechnology - Volume 1*; Thouand, G., Marks, R., Eds.; Springer Berlin Heidelberg: Berlin, Heidelberg, 2014; Vol. 144, pp 37–64.
(17) Fisher, A. J.; Raushel, F. M.; Baldwin, T. O.; Rayment, I. *Biochemistry* **1995**, *34*, 6581–6586.

(18) Campbell, Z. T.; Weichsel, A.; Montfort, W. R.; Baldwin, T. O. *Biochemistry* **2009**, *48* (26), 6085–6094.
(19) Fisher, A. J.; Thompson, T. B.; Thoden, J. B.; Baldwin, T. O.; Rayment, I. *J. Biol. Chem.* **1996**, *271* (36), 21956–21968.
(20) Lin, L. Y.-C.; Sulea, T.; Szittner, R.; Vassilyev, V.; Purisima, E. O.; Meighen, E. A. *Protein Sci.* **2001**, *10* (8), 1563–1571.
(21) Campbell, Z. T.; Baldwin, T. O.; Miyashita, O. *Biophys. J.* **2010**, *99* (12), 4012–4019.
(22) Baldwin, T. O.; Christopher, J. A.; Raushel, F. M.; Sinclair, J. F.; Ziegler, M. M.; Fisher, A. J.; Rayment, I. *Curr. Opin. Struct. Biol.* **1995**, *5*, 798–809.
(23) Chen, L. H.; Baldwin, T. O. *Biochemistry* **1989**, *28*, 2684–2689.
(24) Li, Z.; Meighen, E. A. *Biochemistry* **1995**, *34*, 15084–15090.
(25) Vervoort, J.; Muller, F.; O'Kane, D. J.; Lee, J. *Biochemistry* **1986**, *25*, 8067–8075.
(26) Bergner, T.; Tabib, C. R.; Winkler, A.; Stipsits, S.; Kayer, H.; Lee, J.; Malthouse, J. P.; Mayhew, S.; Müller, F.; Gruber, K.; Macheroux, P. *Biochim. Biophys. Acta BBA - Proteins Proteomics* **2015**, *1854* (10), 1466–1475.
(27) Morokutti, A.; Lyskowski, A.; Sollner, S.; Pointner, E.; Fitzpatrick, T. B.; Kratky, C.; Gruber, K.; Macheroux, P. *Biochemistry* **2005**, *44* (42), 13724–13733.
(28) de Groot, B. L.; van Aalten, D. M. F.; Scheek, R. M.; Amadei, A.; Vriend, G.; Berendsen, H. J. C. *Proteins Struct. Funct. Genet.* **1997**, *29*, 240–251.
(29) Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J. *J. Comput. Chem.* **1998**, *19* (14), 1639–1662.
(30) Trott, O.; Olson, A. J. *J. Comput. Chem.* **2009**, 455–461.
(31) List, B.; Doehring, A.; Fonseca, M. T. H.; Wobser, K.; van Thienen, H.; Torres, R. R.; Galilea, P. L. *Adv. Synth. Catal.* **2005**, *347* (11-13), 1558–1560.
(32) Kurfürst, M.; Ghisla, S.; Hastings, J. W. *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 2990–2994.
(33) Lei, B.; Cho, K. W.; Tu, S.-C. *J. Biol. Chem.* **1994**, *269* (8), 5612–5618.

Graphical abstract





Highlights

- α, β-unsaturated aldehydes were synthesized in a three-step approach
- (un-)saturated aldehydes were investigated as potential substrates for luciferases
- saturated aldehydes showed higher light emission than unsaturated aldehydes
- light emission increases with longer aldehyde chain length, i.e. C14>C12>C10>C8
- unsaturated aldehydes display slower kinetics of light emission