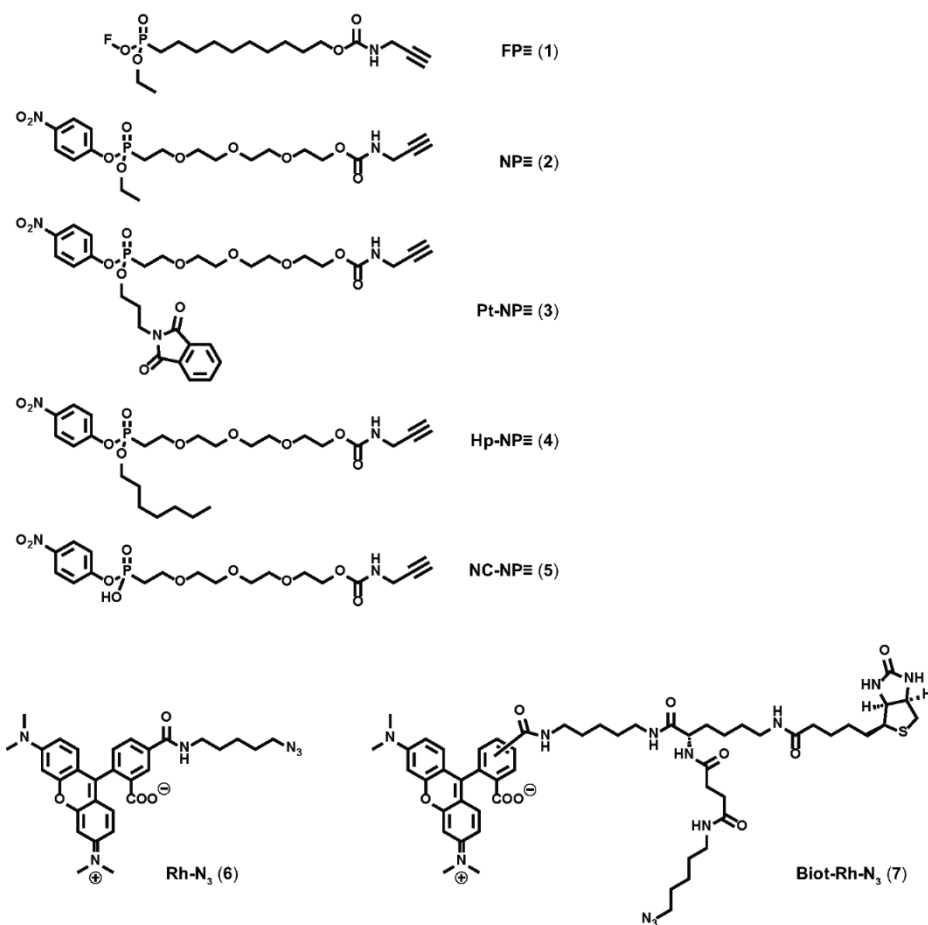
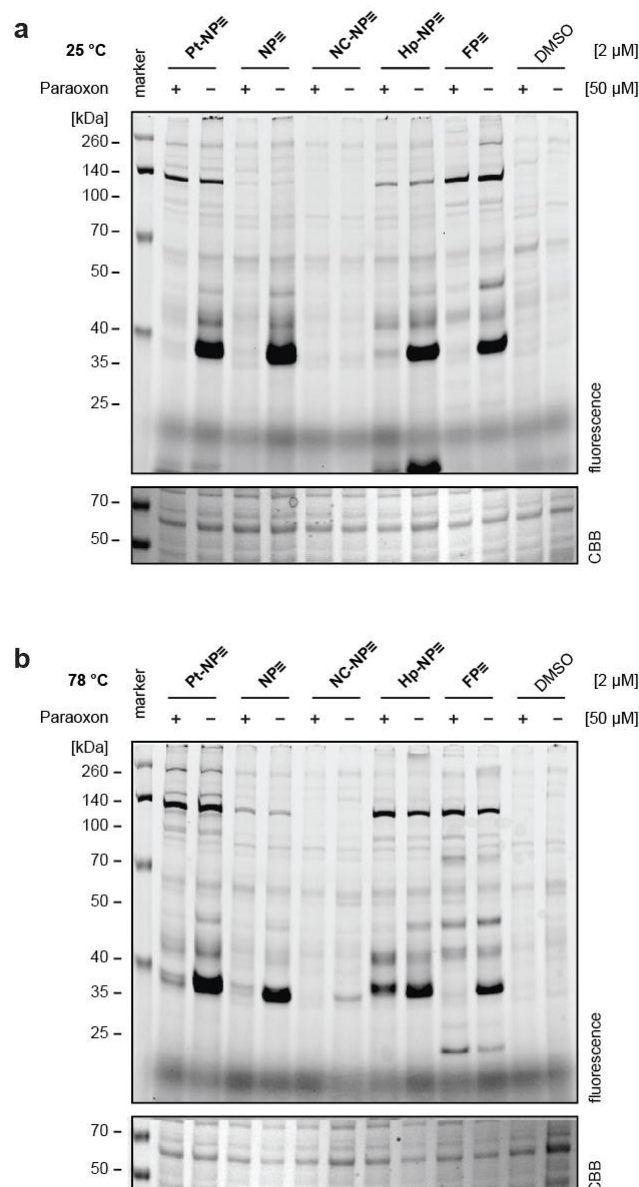




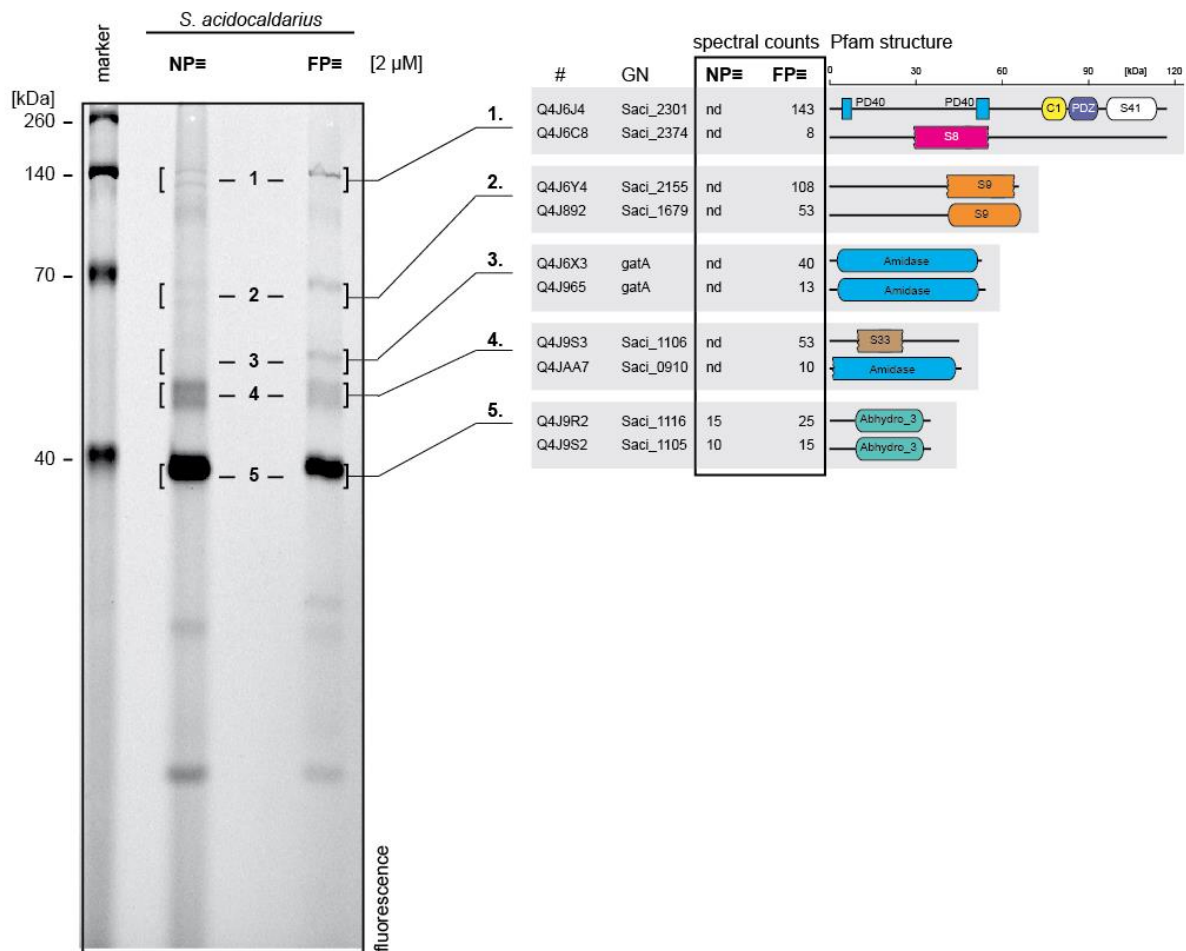
**Supplementary Figure 1.** Chemical structures of activity-based probes (ABPs) and of click reagents used in this study.

In this study, one fluorophosphonate (**FP≡**, **1**), three nitrophenol phosphonate probes (**NP≡** (**2**), **Pt-NP≡** (**3**) and **Hp-NP≡** (**4**)) as well as one non-reactive negative control compound **NC-NP≡** (**5**) were used. As click reporter, **Rh-N<sub>3</sub>** (**6**) and **Biot-Rh-N<sub>3</sub>** (**7**) were used. The synthesis of the activity-based probes is described in the Supplementary Methods section.



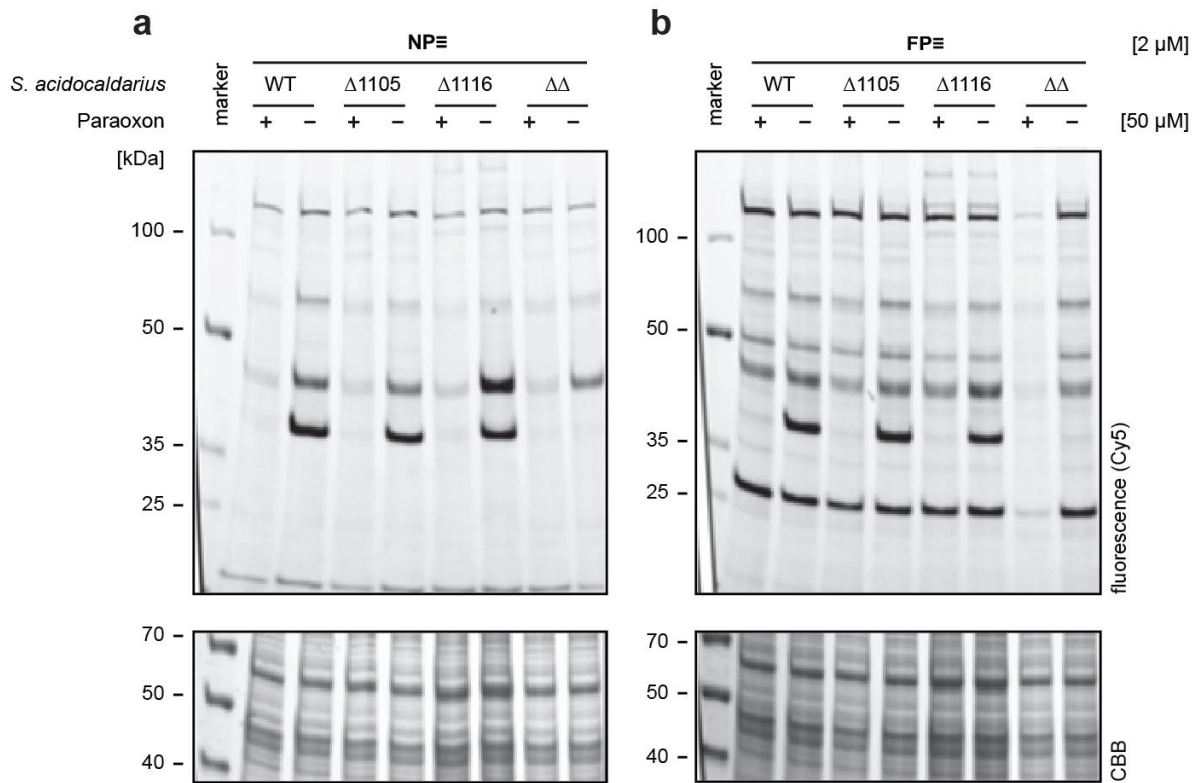
**Supplementary Figure 2.** *In vitro* ABPP with *S. acidocaldarius* MW001 lysates at 25 °C or 78 °C.

*S. acidocaldarius* MW001 lysates were treated for 60 min at either 25 °C (**a**) or 78 °C (**b**) with 2  $\mu$ M of the probe or an equivalent volume of DMSO either without or after pre-incubation with 50  $\mu$ M paraoxon for 10 min. After 2-step labeling with **Rh-Az** and gel separation labelled proteins were in-gel visualized by detecting fluorescence (Typhoon FLA 9000 scanner). CBB: Coomassie Brilliant Blue staining.



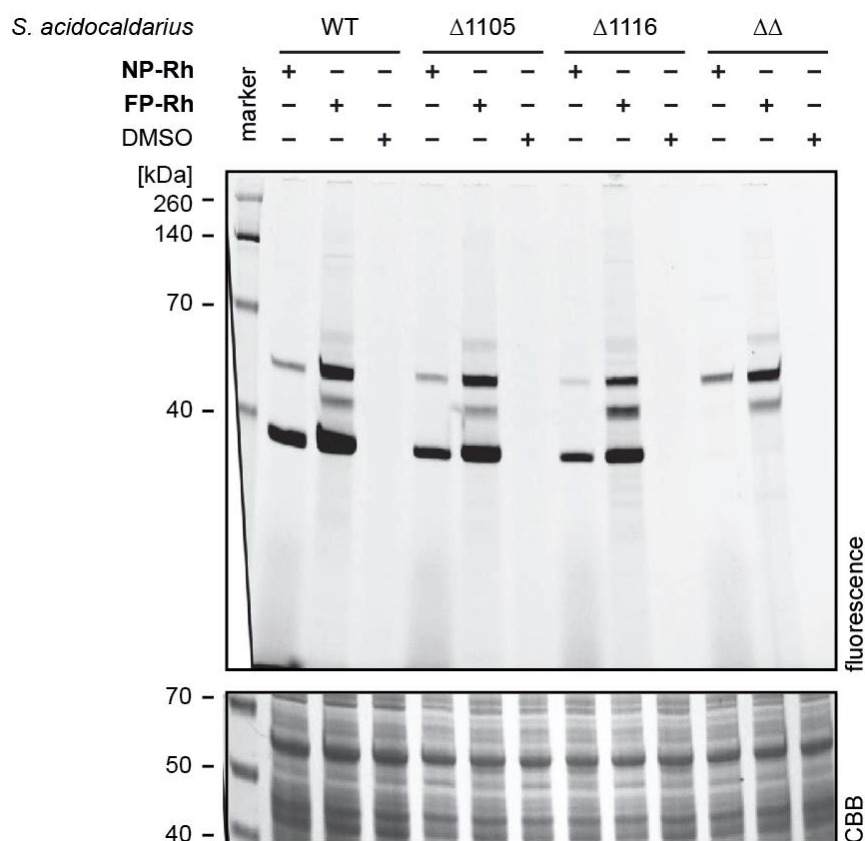
**Supplementary Figure 3.** Large scale *in vivo* profiling of *S. acidocaldarius* MW001 for MS-based target identification.

*S. acidocaldarius* growing at 78°C and pH 3.0 was treated for 30 min with 2 μM **NP** or **FP**. The cells were then collected by centrifugation, washed with culture medium and then lysed with 2% SDS. Labelled proteins were subsequently tagged with **Rh-Biot-Az** (using the 2-step labelling approach) and affinity purified on avidin-agarose beads. The pulled-down proteins were eluted from the beads by boiling in gel loading buffer, separated by SDS PAGE and visualized by in-gel fluorescence detection (Typhoon FLA 9000 scanner). The indicated gel regions were excised from the gel (red brackets), trypsinized and analyzed by LC-MS/MS. The identified serine hydrolases are indicated in the right panel with their corresponding Uniprot ID, gene name (GN), spectral counts and Pfam domain structure.



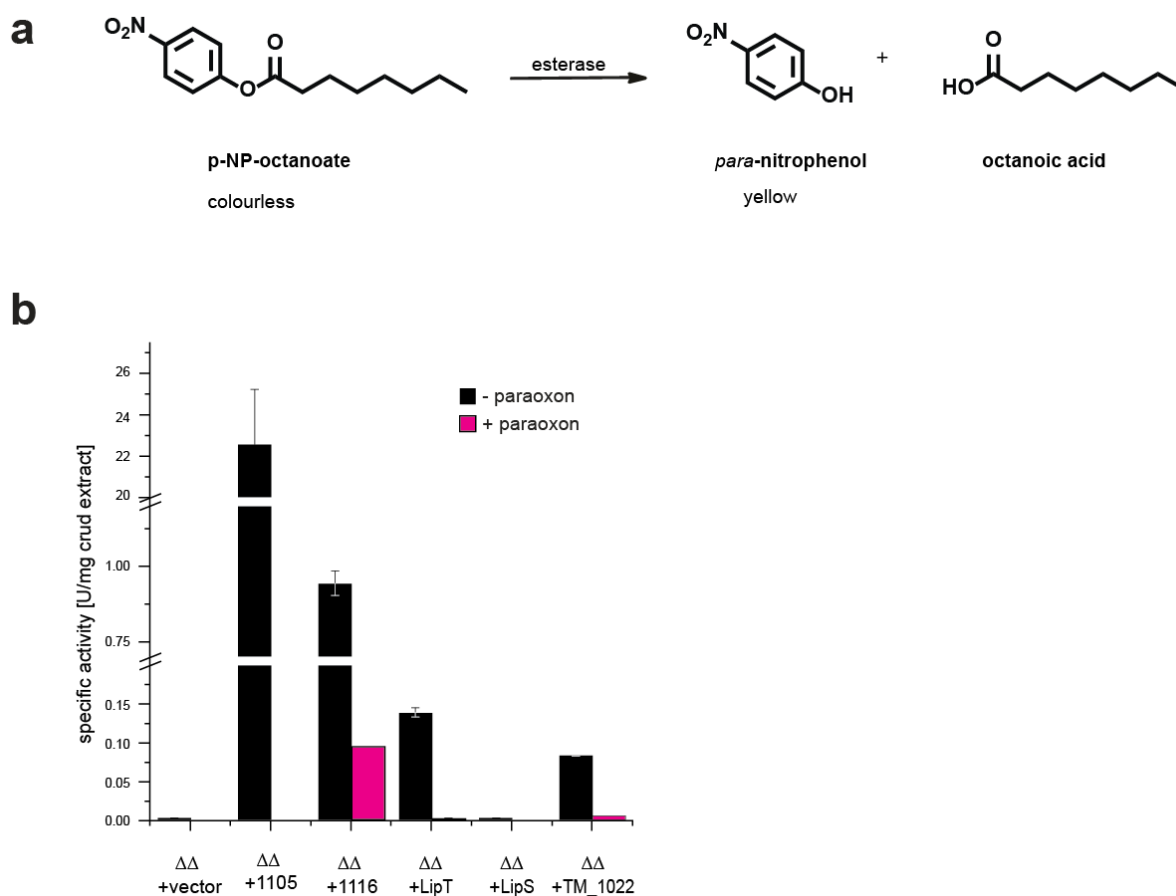
**Supplementary Figure 4.** *In vivo* labelling of serine hydrolases in *S. acidocaldarius* single and double esterase mutant strains.

Cultures of *S. acidocaldarius* MW001 as well as of an acetylcholinesterase 1116 knock-out mutant  $\Delta 1116$ , a lipase/esterase knock-out mutant  $\Delta 1105$  and a double-knockout mutant  $\Delta\Delta$  were incubated at 78 °C and pH 3.0 with 2  $\mu$ M **NP $\equiv$**  or **FP $\equiv$**  without or after preincubation with 50  $\mu$ M paraoxon for 10 min, followed by 2-step labeling with **Rh-Az**, gel separation and in-gel fluorescence detection. CBB = Coomassie Brilliant Blue stain.



**Supplementary Figure 5.** *In vitro* labelling of serine hydrolases in *S. acidocaldarius* single and double esterase mutant strains.

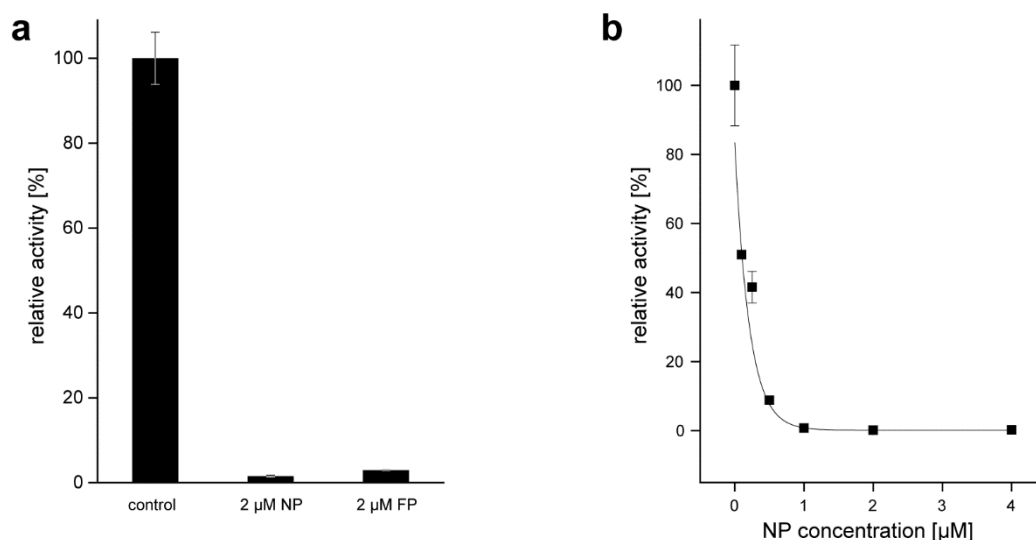
Lysates of *S. acidocaldarius* MW001 as well as of an acetylsterase 1116 knock-out mutant  $\Delta 1116$ , a lipase/esterase knock-out mutant  $\Delta 1105$  and a double-knockout (1105 and 1116) mutant  $\Delta\Delta$  were incubated at 78 °C and pH 3.0 with 2  $\mu$ M **NP** or **FP** or DMSO, followed by 2-step labeling with **Rh-Az**, gel separation and in-gel fluorescence detection.



**Supplementary Figure 6.** Esterase enzyme activity in crude extracts from the constructed esterase deletion strain and cells overexpressing Saci\_1105, Saci\_1116, LipT, LipS and TM\_1022.

(a) Chemical reaction underlying the nitrophenol assay. UV spectroscopy is used to detect and quantify liberated *para*-nitrophenol. (b) Determination of esterase activity in crude extracts of *S. acidocaldarius* expression cultures. The following strains were tested: The  $\Delta\Delta$  strain (MW903) +empty overexpression vector as negative control ( $\Delta\Delta$ +vector);  $\Delta\Delta$  + pSVAmz\_saci1105 ( $\Delta\Delta$ +1105);  $\Delta\Delta$  + pSVA\_saci1116 ( $\Delta\Delta$ +1105),  $\Delta\Delta$  + pSVAmZ\_p\_lipT ( $\Delta\Delta$ +LipT),  $\Delta\Delta$  + pSVAmZ\_p\_lipS ( $\Delta\Delta$ +LipS) and  $\Delta\Delta$  + pSVAmZ\_p\_tm1022 ( $\Delta\Delta$ +TM\_1022). Esterase activity (black bars) was measured under standard conditions using pNP-octanoate (0.5 mM) and monitoring the release of *p*-nitrophenol at 35 °C (405 nm). For esterase inhibition (red bars) the residual activities were determined after incubation of crude extract with 0.25 mM paraoxon

(60 °C, 1 h). The error bars represent standard deviation of three independent measurements.



**Supplementary Figure 7.** Influence of the ABPP probes on esterase activity in crude extracts of *S. acidocaldarius* expression strain  $\Delta\Delta$  + pSVAmz\_saci1105 ( $\Delta\Delta$ +1105).

**(a)** Determination of relative esterase activity in crude extracts of  $\Delta\Delta$ +1105 after pre-incubation (30 °C, 1 h) in the presence and absence of 2  $\mu$ M of **FP** and **NP** probes.

**(b)** Concentration-dependent inhibition of esterase activity in crude extract of strain  $\Delta\Delta$ +1105 after pre-incubation in the presence of the indicated concentrations with the **NP** probe (30 °C, 1 h). Residual esterase activity was measured as described in Supplementary Fig. 6. The error bars represent standard deviation of three independent measurements.

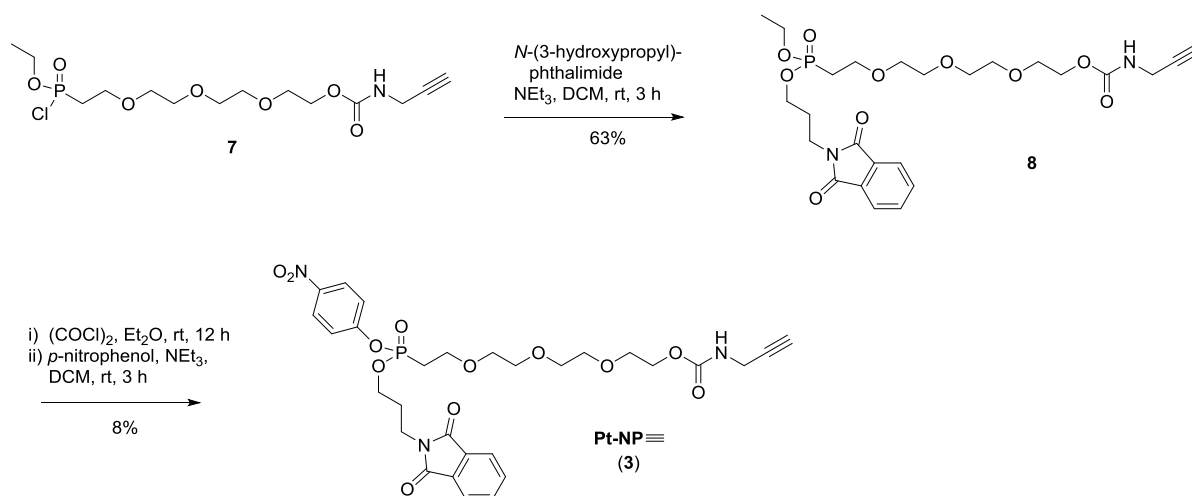


# Supplementary Methods

## Synthesis of ABPs and Click reporters

The **FP** probe was synthesized as reported Liu *et al.* [1]. The synthesis of the **NP** probe as well as of **Biot-Rh-N<sub>3</sub>** was published previously by Nickel *et al.* [2].

### Pt-NP (3)



As a starting material for the synthesis of **Pt-NP**, a chloro phosphonate derivative **7** (as a common intermediate in the synthesis of **NP**) was used. The synthesis of **7** is described in Nickel *et al.* [2].

To a solution of **7** (100 mg, 0.25 mmol) in DCM (10 mL) was added a solution of *N*-(3-hydroxypropyl)-phthalimide (103 mg, 0.5 mmol, 2 eq.) and triethylamine (173  $\mu$ L, 1.25 mmol, 5 eq.) in DCM (10 mL) and the resulting mixture was stirred at room temperature for 3 h. The solvent was removed in a N<sub>2</sub> stream and the residue was purified by column chromatography (CHCl<sub>3</sub>/MeOH 19:1) to yield 87 mg (0.16 mmol, 63%) of the desired product **8**.

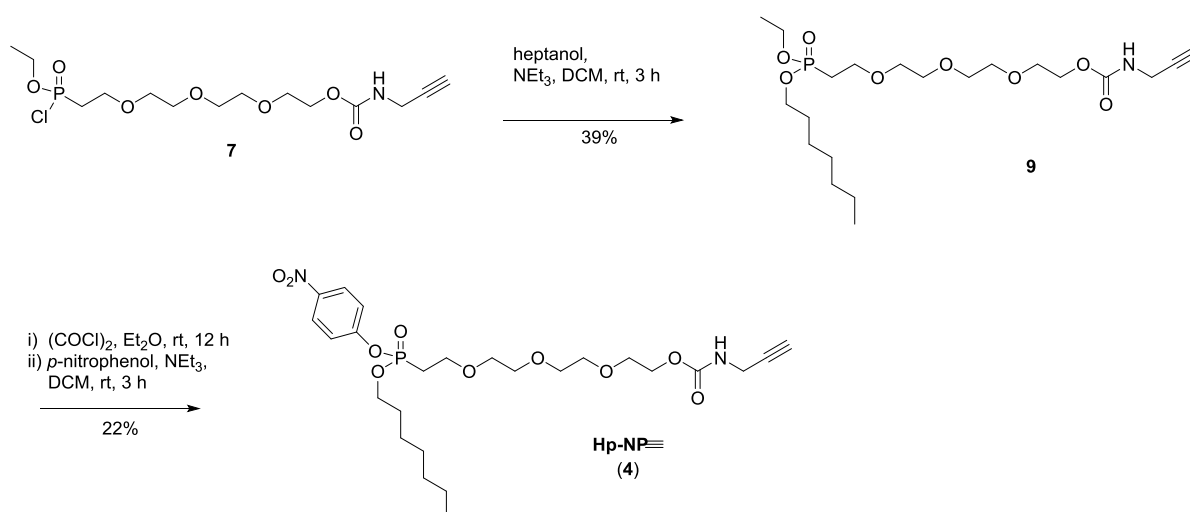
<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta$  = 7.84 (dd, *J* = 5.4, 3.1 Hz, 2H), 7.72 (dd, *J* = 5.4 Hz, 3.1 Hz, 2H), 4.28-4.20 (m, 2H), 4.13-4.08 (m, 4H), 3.98-3.93 (m, 2H), 3.81 (t, *J* = 7.0

Hz, 2H), 3.77-3.59 (m, 12H), 2.22 (t,  $J = 2.4$  Hz, 1H), 2.18-2.01 (m, 4H), 1.30 (t,  $J = 7.0$  Hz, 3H); MS ( $m/z$ ):  $[M+H]^+$  calcd. for  $C_{25}H_{36}N_2O_{10}P^+$ , 555.21; found 556.77  $[M+H]^+$  and 578.73  $[M+Na]^+$ .

**8** (87 mg, 0.16 mmol) was dissolved in DCM (10 mL) and oxalylchloride (274  $\mu$ L, 3.2 mmol, 20 eq.) was added. The resulting mixture was stirred overnight at room temperature. The solvent was removed in a  $N_2$  stream and the residue was dried in high vacuum. The residue was taken up in DCM (10 mL) and a solution of nitrophenol (45 mg, 0.32 mmol, 2 eq.) and triethylamine (111  $\mu$ L, 0.8 mmol, 5 eq.) in DCM (10 mL) was added. The resulting mixture was stirred at room temperature for 3 h. The solvent was removed in a  $N_2$  stream and the residue was first purified by a silica gel column chromatography ( $CHCl_3/MeOH$  19:1) and then again by reverse chromatography on a self-packed C18 column (60% aq. acetonitrile) to yield 8 mg (0.012 mmol, 8%) of the desired product **Pt-NP $\equiv$**  (**3**).

$^1H$  NMR (400 MHz,  $CDCl_3$ ):  $\delta$  = 8.22 (d,  $J = 8.3$  Hz, 2H), 7.85-7.83 (m, 2H), 7.74-7.72 (m, 2H), 7.40 (d,  $J = 8.5$  Hz, 2H), 4.27-3.62 (m, 20H), 2.36-2.07 (m, 5H); MS ( $m/z$ ):  $[M+H]^+$  calcd. for  $C_{29}H_{35}N_3O_{12}P^+$ , 648.20; found 647.03  $[M+H]^+$  and 669.03  $[M+Na]^+$ .

### Hp-NP $\equiv$ (**4**)



To a solution of **7** (100 mg, 0.25 mmol) in DCM (10 mL) was added a solution of heptanol (71  $\mu$ L, 0.5 mmol, 2 eq.) and triethylamine (173  $\mu$ L, 1.25 mmol, 5 eq.) in DCM

(10 mL) and the resulting mixture was stirred at room temperature for 3 h. The organic phase was washed with water, dried over  $\text{Na}_2\text{SO}_4$  and evaporated to dryness. The residue was purified by column chromatography (DCM/MeOH 9:1) to yield 45 mg (0.10 mmol, 39%) of the desired product **9**.

**9** (45 mg, 0.10 mmol) was dissolved in DCM (10 mL) and oxalylchloride (172  $\mu\text{L}$ , 2.0 mmol, 20 eq.) was added. The resulting mixture was stirred overnight at room temperature. The solvent was removed in a  $\text{N}_2$  stream and the residue was dried in high vacuum. The residue was taken up in DCM (10 mL) and a solution of nitrophenol (28 mg, 0.2 mmol, 2 eq.) and triethylamine (69  $\mu\text{L}$ , 0.50 mmol, 5 eq.) in DCM (10 mL) was added. The resulting mixture was stirred at room temperature for 3 h. The solution was washed with water, dried over  $\text{Na}_2\text{SO}_4$  and reduced to dryness. The residue was purified by column chromatography (DCM/MeOH 50:1) to yield 20 mg (0.04 mmol, 40 %) of the desired product **Hp-NP $\equiv$  (4)**.

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  = 8.34 (d,  $J$  = 9.0 Hz, 2H), 7.55-7.49 (m, 2H), 4.52-4.17 (m, 4H), 3.93-3.76 (m, 4H), 3.74-3.64 (m, 10H), 2.60 (s, 1H), 2.47-2.42 (m, 2H), 1.78-1.61 (m, 2H), 1.39-1.31 (m, 8H), 0.93 (t,  $J$  = 6.9 Hz, 3H).

MS (m/z):  $[\text{M}+\text{H}]^+$  calcd. for  $\text{C}_{25}\text{H}_{40}\text{N}_2\text{O}_{10}\text{P}^+$ , 559.24; found 559.02  $[\text{M}+\text{H}]^+$  and 581.24  $[\text{M}+\text{Na}]^+$ .

## Supplementary References

1. Liu Y, Patricelli MP, Cravatt BF (**1999**) Activity-based protein profiling: the serine hydrolases. *Proc. Natl. Acad. Sci. USA* 96: 14694-14699.
2. Nickel S, Kaschani F, Colby T, van der Hoorn RA, Kaiser M (**2012**) A para-nitrophenol phosphonate probe labels distinct serine hydrolases of *Arabidopsis*. *Bioorg. Med. Chem.* 20: 601-606.