Supplementary Material

Synthesis of an enantiopure thioester as key substrate for screening the sensitivity of penicillin binding proteins to inhibitors

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1. NMR spectra

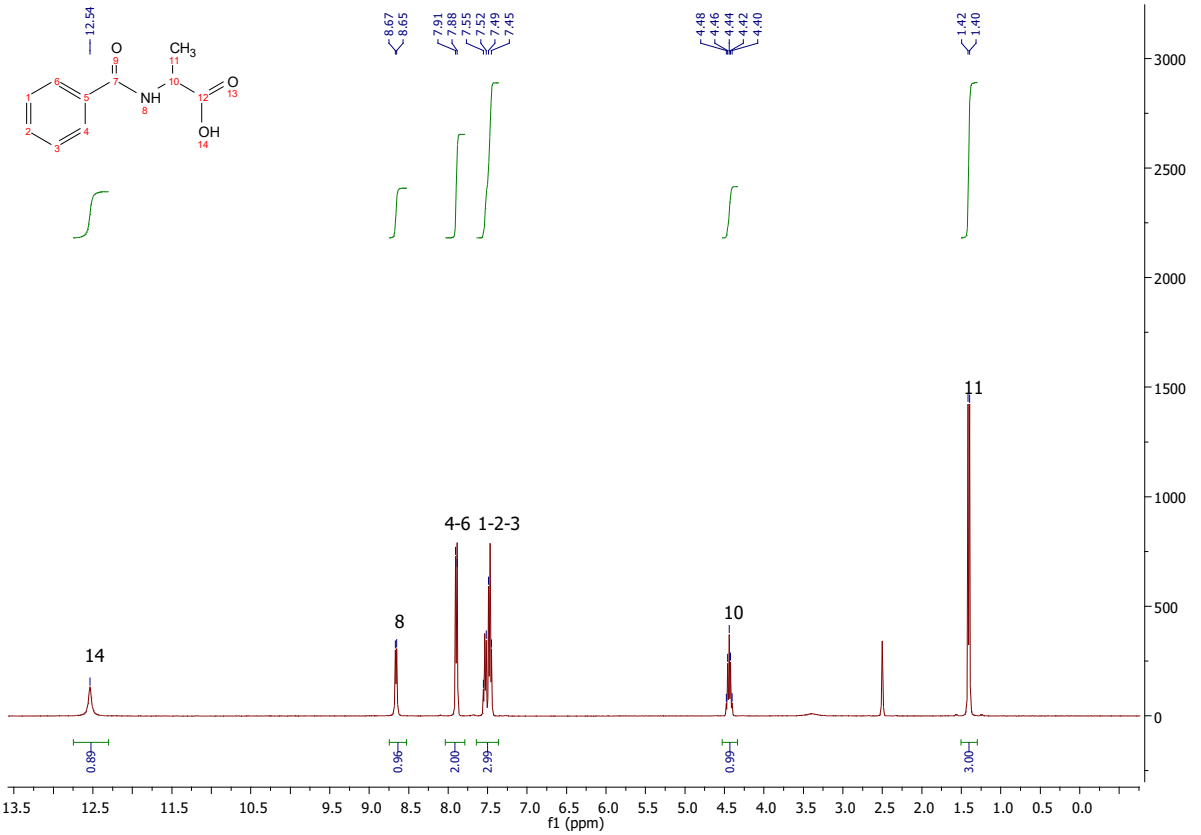


Figure 1. δ_H **(400 MHz; d^6-DMSO) of (*R*)-2-benzamidopropanoic acid (*R*)-1**

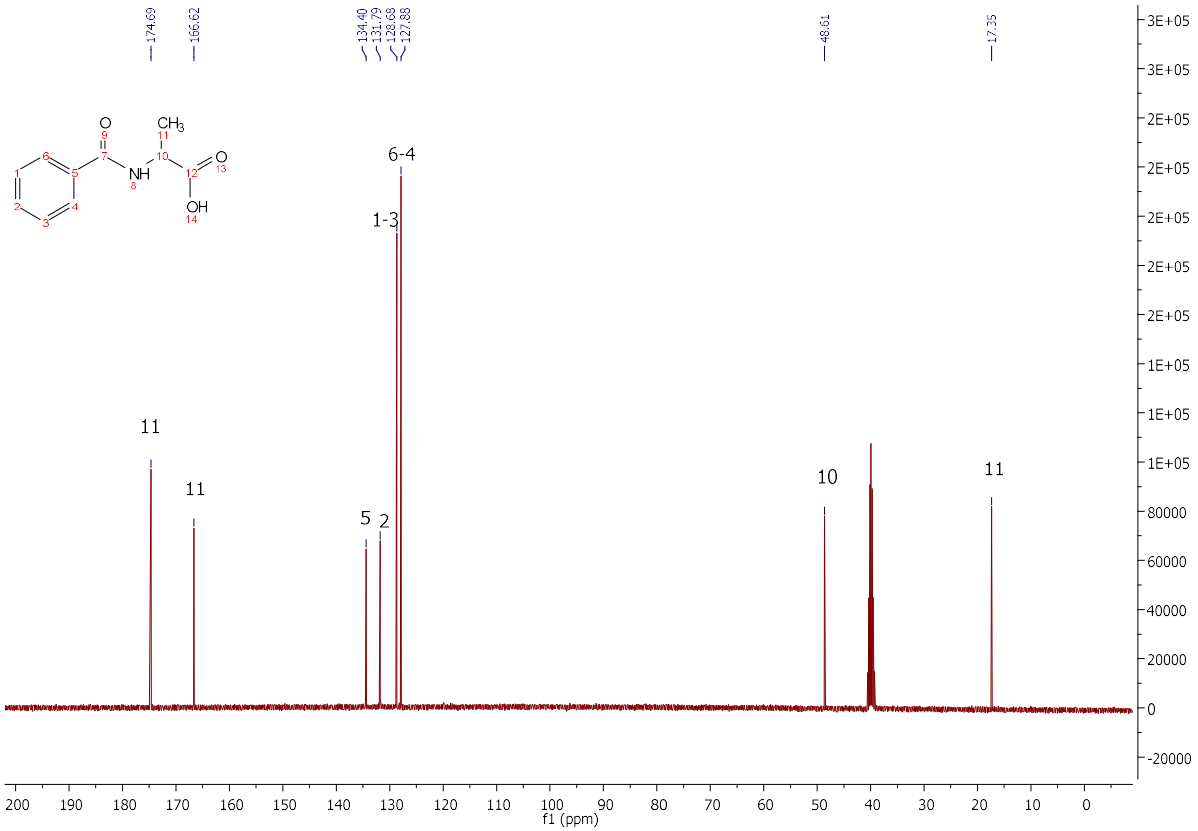


Figure 2. δ_C **(101 MHz, d^6-DMSO) of (*R*)-2-benzamidopropanoic acid (*R*)-1**

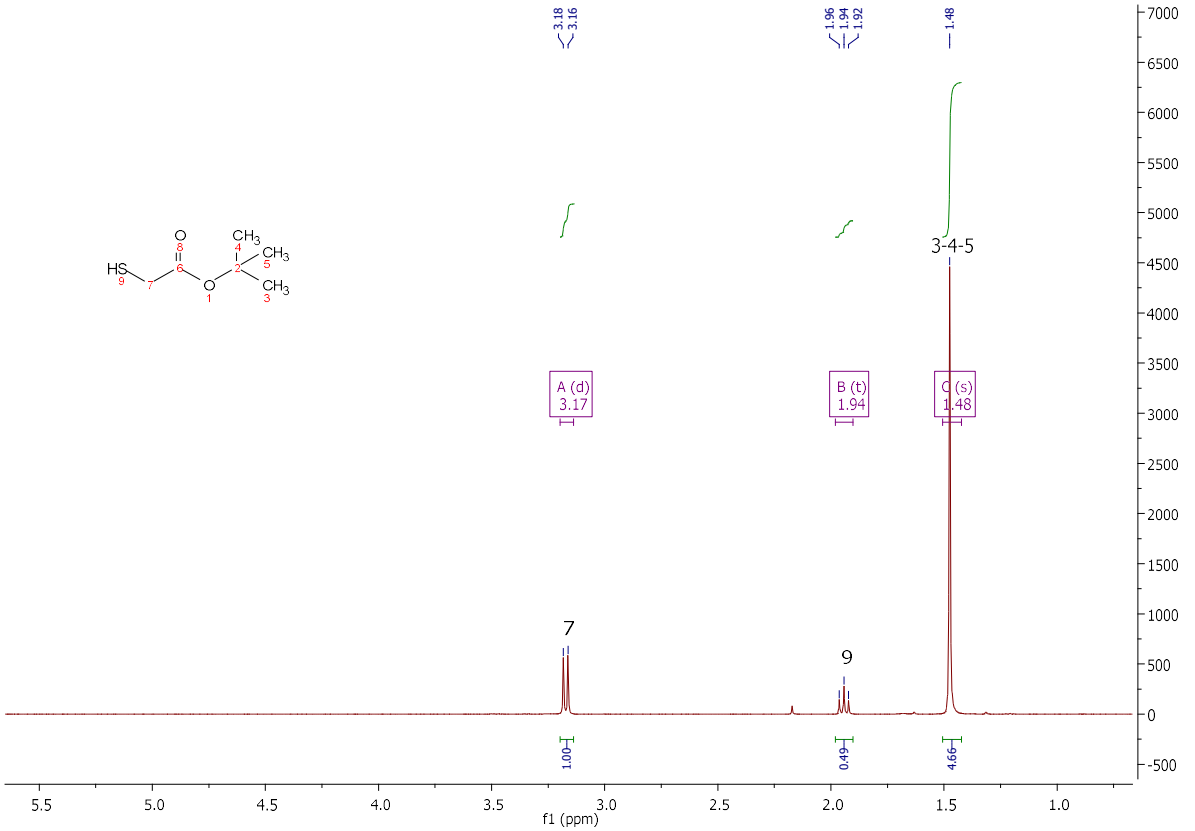


Figure 3. δ_H **(400 MHz; $CDCl_3$) of *tert*-butyl 2-mercaptoacetate 4**

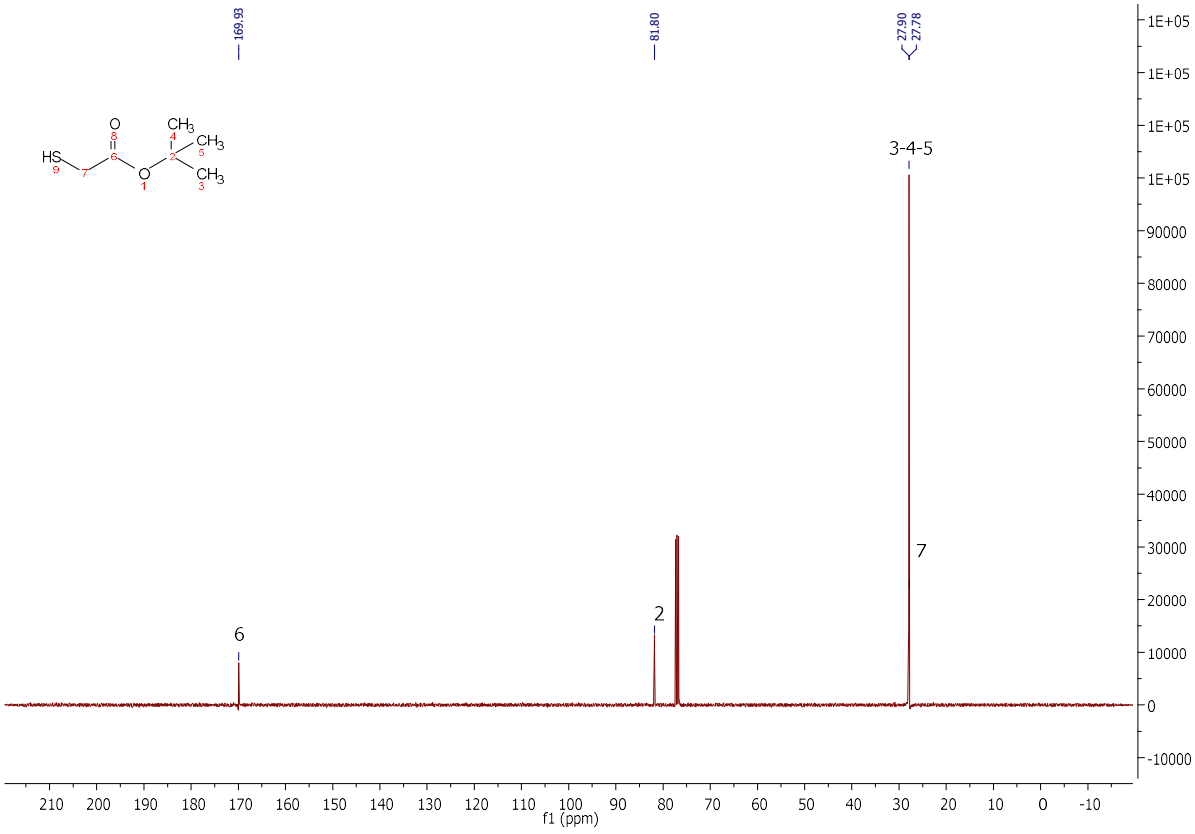


Figure 4. δ_C **(101 MHz; $CDCl_3$) of tert-butyl 2-mercaptoacetate 4**

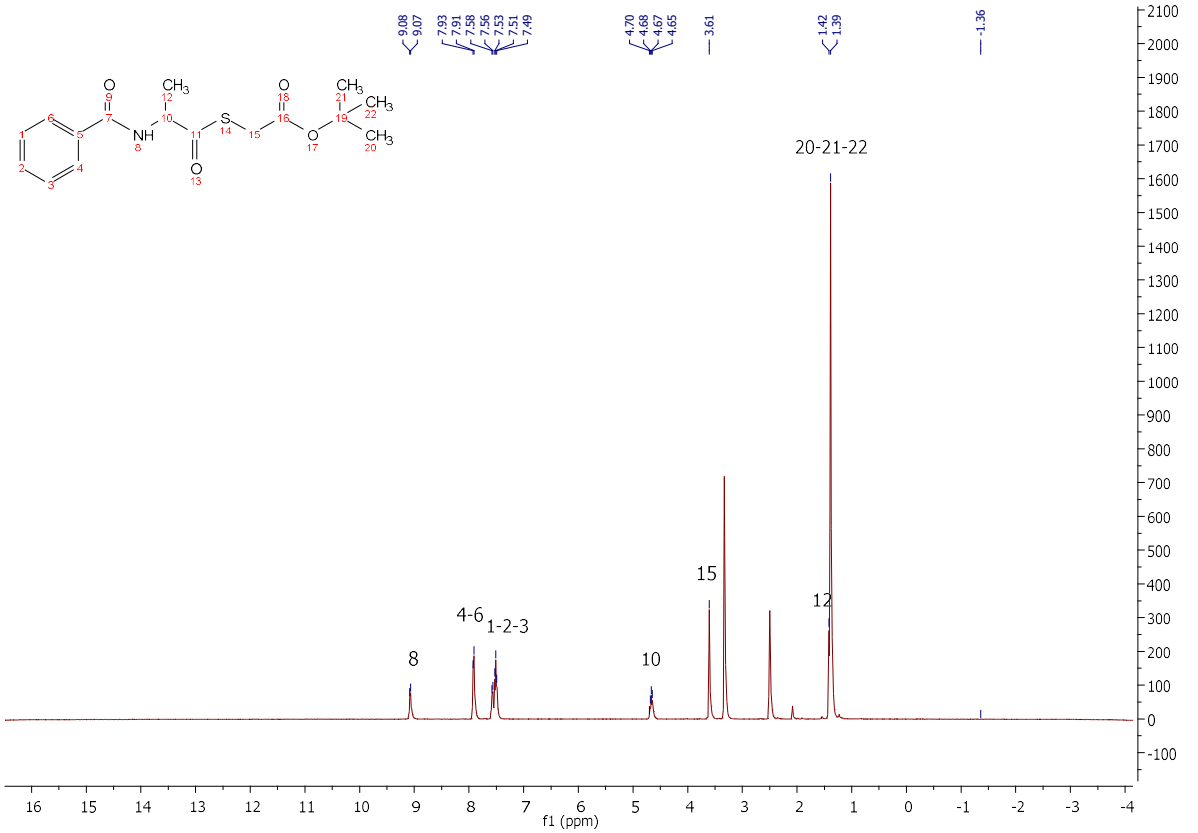


Figure 5. δ_H **(400 MHz; d^6-DMSO) of (*R*)-*Tert*-butyl 2-(2-benzamidopropanoylthio) acetate (*R*)-5**

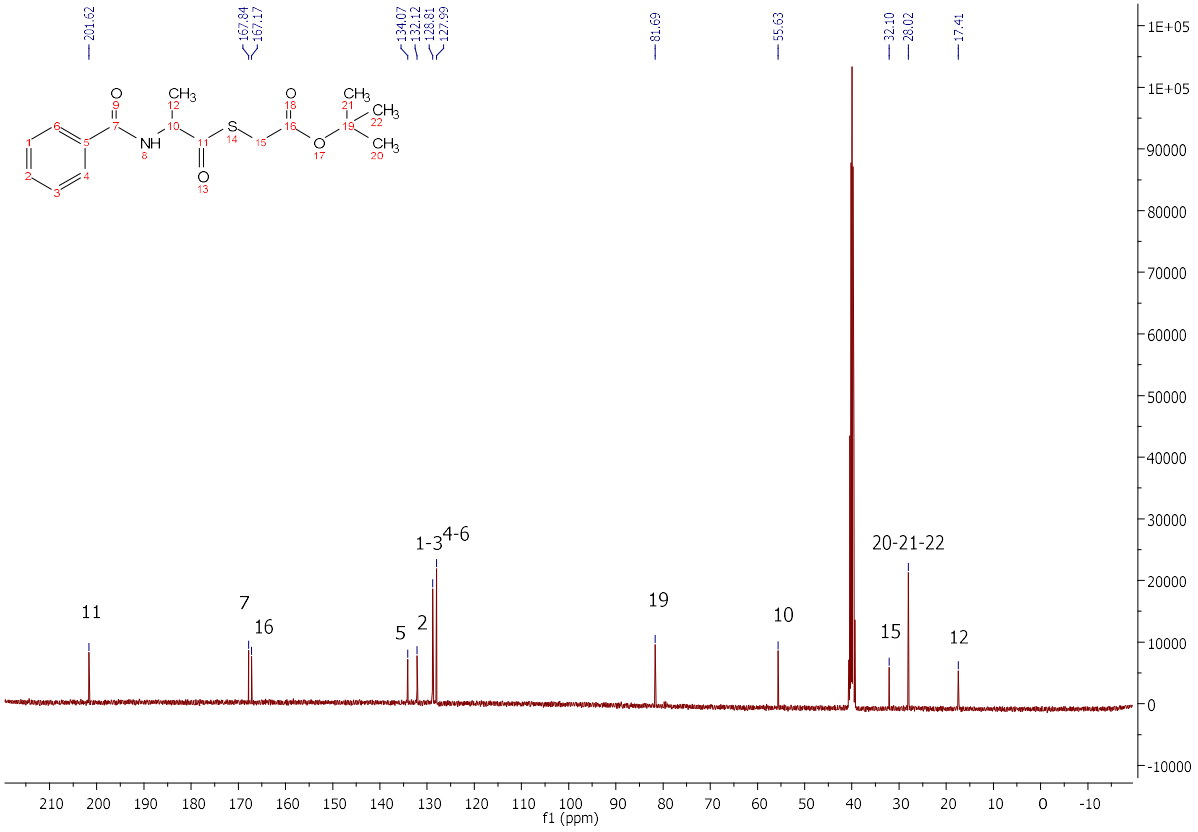


Figure 6. δ_C **(101 MHz, d^6-DMSO) of (*R*)-*Tert*-butyl 2-(2-benzamidopropanoylthio) acetate (*R*)-5**

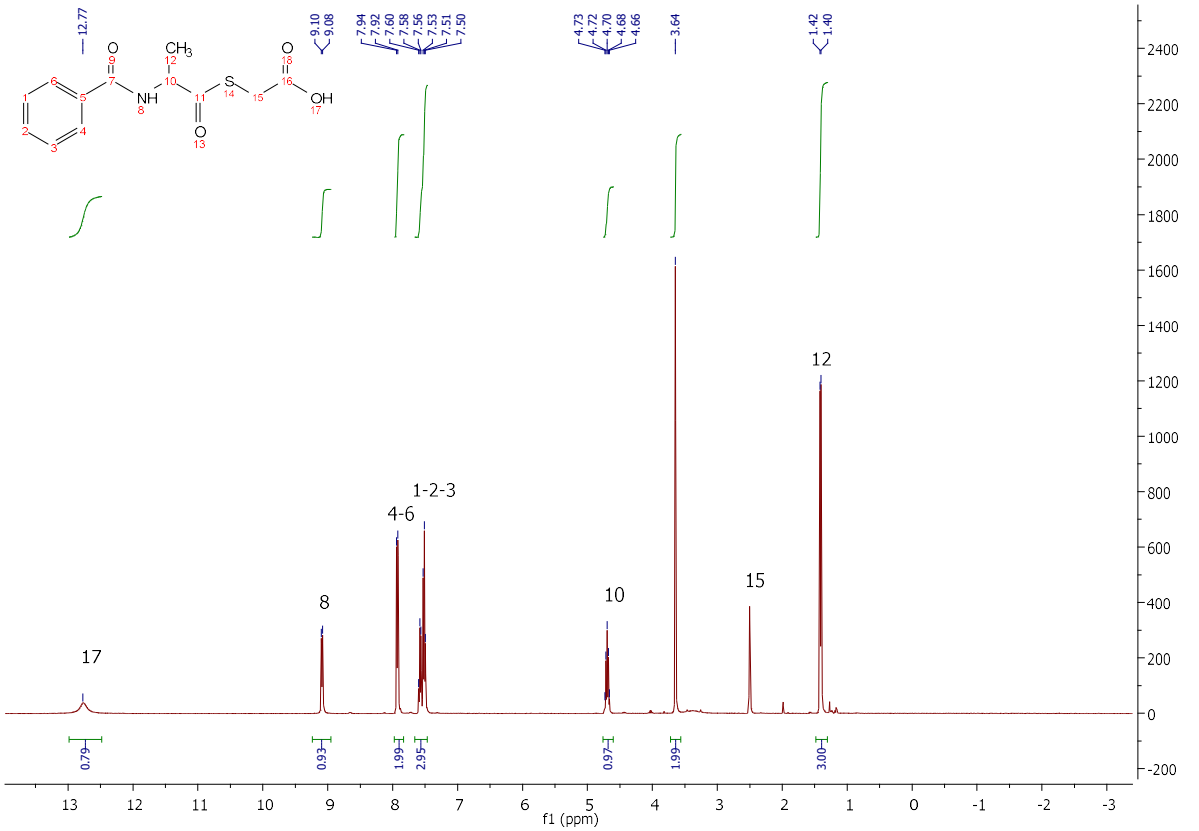


Figure 7. δ_H **(400 MHz; d^6-DMSO) of (*R*)-2-(2-benzamidopropanoylthio)acetic acid (*R*)-6**

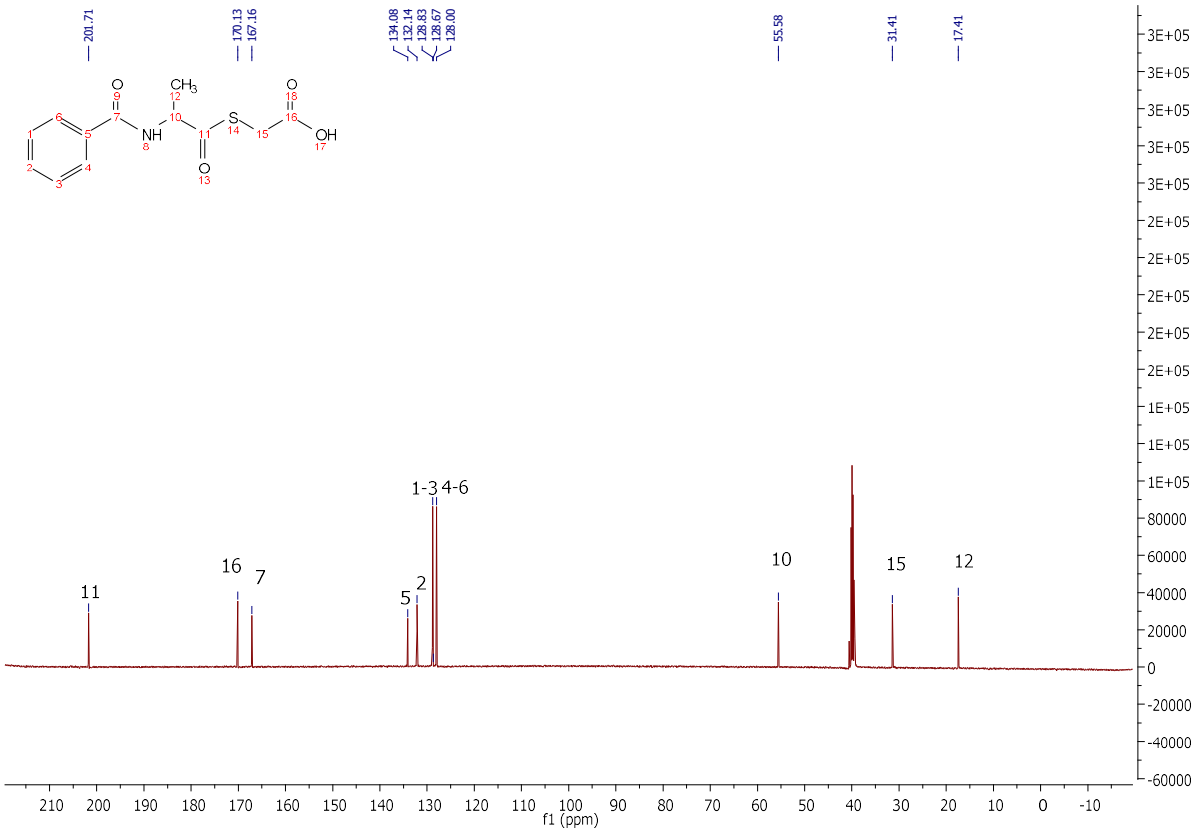


Figure 8. δ_C **(101 MHz, d^6-DMSO) of (*R*)-2-(2-benzamidopropanoylthio)acetic acid (*R*)-6**

2. Biological Experiments

In the literature[1–9], racemic 2-(2-benzamidopropanoylthio)acetic acid was often used for enzymatic studies. Here, we have conducted enzymatic kinetics of R39 from *Actinomadura,* PBP2x from a penicillin sensitive (PBP2xR6) and resistant (PBP2x5204) strain *from Streptococcus pneumoniae* with **(*R*)-6**.

First, the enzymatic hydrolysis of **(*S*)-6** was studied with R39, PBP2xR6 and PBP2x5204. As expected only spontaneous hydrolysis (15%) but no enzymatic hydrolysis was observed showing the strict specificity of all PBPs for the (*R*)-configuration in the penultimate position.

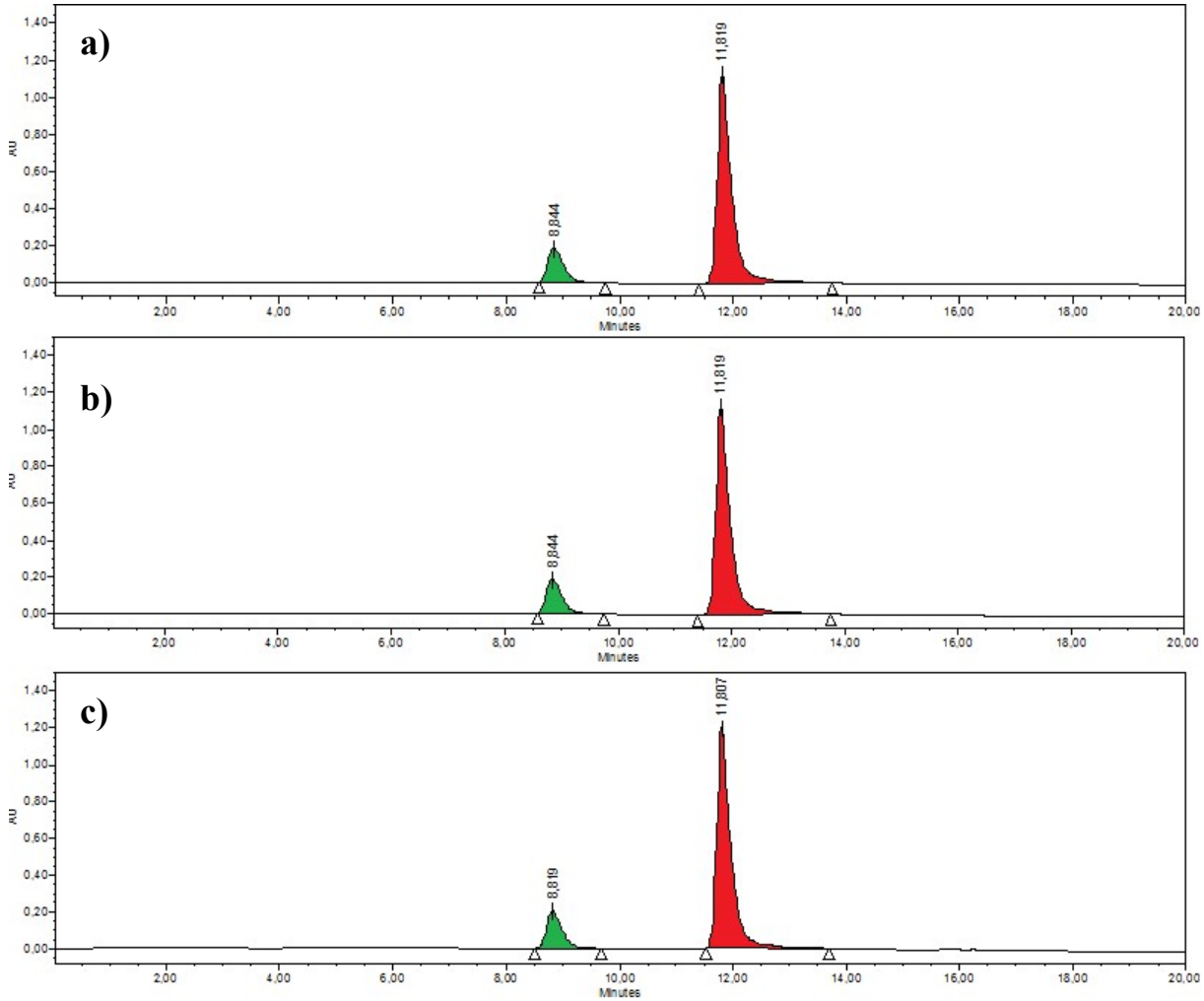
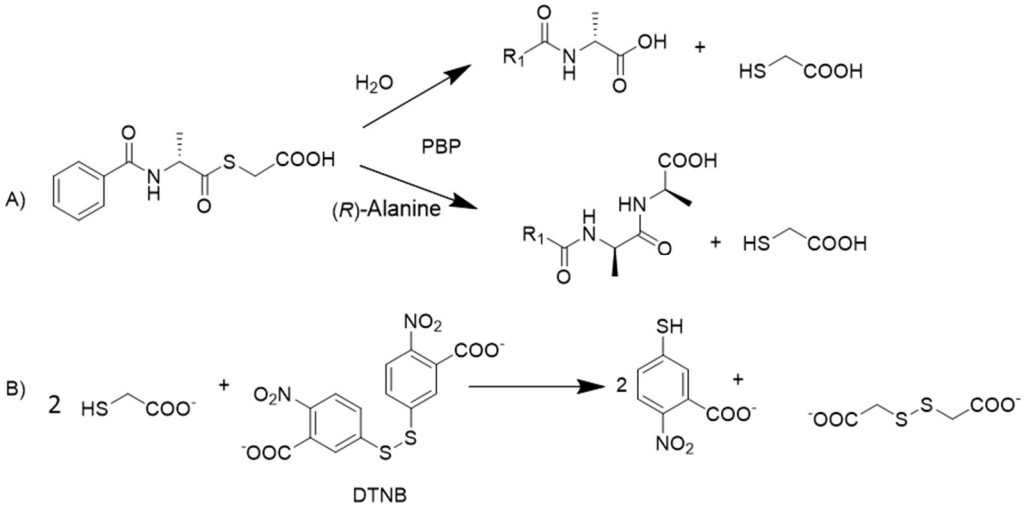


Figure 1. a) spontanous **(*S*)-6** hydrolyzed (15%) in buffer B (10 mM sodium phosphate pH 7.0), **b) (*S*)-6** hydrolyzed (15%) catalysed by PBP2x5204 in buffer B (10 mM sodium phosphate pH 7.0), **c) (*S*)-6** hydrolyzed (15%) catalysed by PBP2xR6 in buffer B (10 mM sodium phosphate pH 7.0).

Thioester hydrolysis catalyzed by PBPs can be followed by measuring the absorbance at 250 nm (ε_{250nm}: -2.200 $M^{-1}cm^{-1}$) but the sensitivity of the assay can be increased by combining thiol formation with thiol oxidation by 4,4-dithiopyridine (DTP)[10] at 324 nm (ε_{324nm}: 20000 $M^{-1}cm^{-1}$) or 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) at 412 nm (ε_{412nm}: 14150 $M^{-1}cm^{-1}$) as shown for DTNB in **Scheme 1**. Screening experiments in our laboratory have shown that in the presence of DTNB less interference with testing compounds occurred. However, for detailed kinetic studies, DTP is still used.[9–11] Thioesters were often used as reporter substrates in the presence of (*R*)-alanine.[12] Here, the thioester is used according to hydrolysis and transpeptidation reactions, both yielding a thiol as shown in **Scheme 1**.



Scheme 1. A) Reactions of thioester, hydrolysis and transpeptidation, catalyzed by PBPs. B) Detection of thiol products by reaction with DTNB

Kinetic studies with R39 have shown that in the presence of (*R*)-alanine the K_m and the k_{cat} values were increased but that k_{cat}/K_m ratio remained unchanged.[9,12] Indeed, (*R*)-Alanine increases the overall rate k_{cat} of the reaction, which makes assay design easier. The influence of (*R*)-Alanine on the enzymatic hydrolysis catalyzed by R39, PBP2xR6 and PBP2x5204 is illustrated in **Figure 2**. The initial rates of **(*R*)-6** hydrolysis catalysed by R39 were increased in the presence of (*R*)-Alanine. No influence of (*R*)-Alanine was observed for the reaction catalysed by PBP2xR6 and PBP2x5204.(**Figure 2**) Thus, further experiments only were done in the presence of 100 mM (*R*)-Alanine with R39.

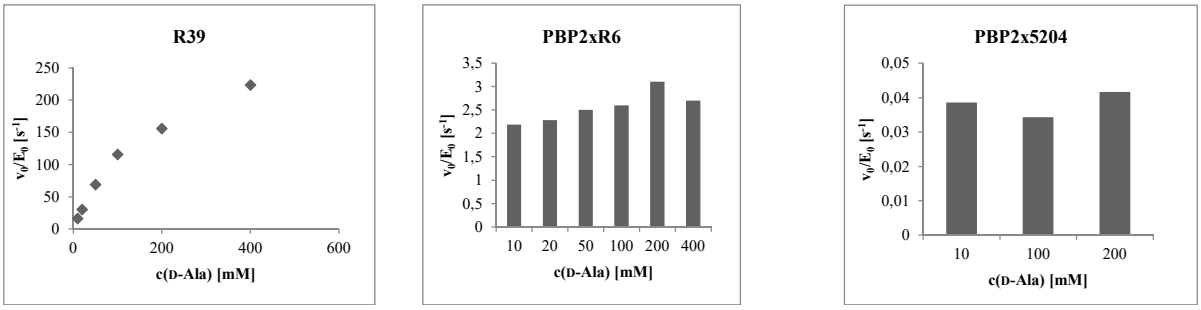


Figure 2. Variation of the initial rates of **(*R*)-6** hydrolysis with the concentration of (*R*)-alanine. (Note $v_0/E_0 > 0$ with R39, error bars ±5%)

K_m and k_{cat}–values were determined for R39 and PBP2xR6 using **(*R*)-6** and the racemate ***rac*-6** as substrates. In the presence of both substrates similar K_m and k_{cat}–values were found indicating that the presence of **(*S*)-6** has no influence on the enzymatic reaction (**Table 1**). Thus, ***rac*-6** can be used for enzymatic studies in the place of **(*R*)-6** with R39 and PBP2xR6 taking into account the concentration of **(*R*)-6** in the racemate mixture for the calculation. This concentration could be easily determined once the proportion of **(*R*)-6** was known by the chiral HPLC described above. *Km*-values of R39 are in agreement with values published in literature while for PBP2xR6 the *Km*-value was to be 2-fold smaller than the published one. [9,13] This may be due to the use of ***rac*-6** concentration for the calculation of the value published in literature: it is likely that it was not realised that racemisation had occurred and that the concentration of the racemic mixture was taken as the concentration of the pure isomer.

Table 1. Kinetic values of R39 and PBP2xR6. [§] In the presence of 100 mM (*R*)-Alanine, [#] without (*R*)-Alanine, * taking into account the concentration of **(*R*)-6** in the racemate mixture for the calculation, ** use of ***rac*-6** concentration for the calculation. All measurement were done at 30 °C.

PBP		***rac*-6**	**(*R*)-6**
R39[§]	k_{cat}	188 ± 19 s^{-1}	197 ± 13 s^{-1}
	K_m	0.19 ± 0.06 mM*	0.18 ± 0.04 mM
		0.34 mM± 0.06 Mm**	(lit.,[12] 0.17 mM)*
PBP2xR6[#]	k_{cat}	5.6 ± 1.3 s^{-1}	7.0 ± 0.5 s^{-1}
	K_m	2.7 ± 2 Mm*	2.6 ± 0.5 mM
			(lit.,[22] 5.6 ± 0.9 Mm) **

For PBP2x5204, initial rates for concentrations of **(*R*)-6** smaller than the *Km*-value were measured at 25 °C and a k_{cat}/K_m-value of 7.1 ± 0.7 M^{-1} s^{-1} was determined. As K_m was higher than 6 mM, high concentration of substrate was required involving a significant spontaneous hydrolysis. As spontaneous hydrolysis is more important with ***rac*-6** and increases the background absorbance, it is useful to use **(*R*)-6** for the measurement of initial rates. Moreover spontaneous hydrolysis is less important at 25 °C compared to 30°C used above. Under these conditions the ratio between spontaneous and enzymatic hydrolysis catalysed by PBP2x5204 was improved.

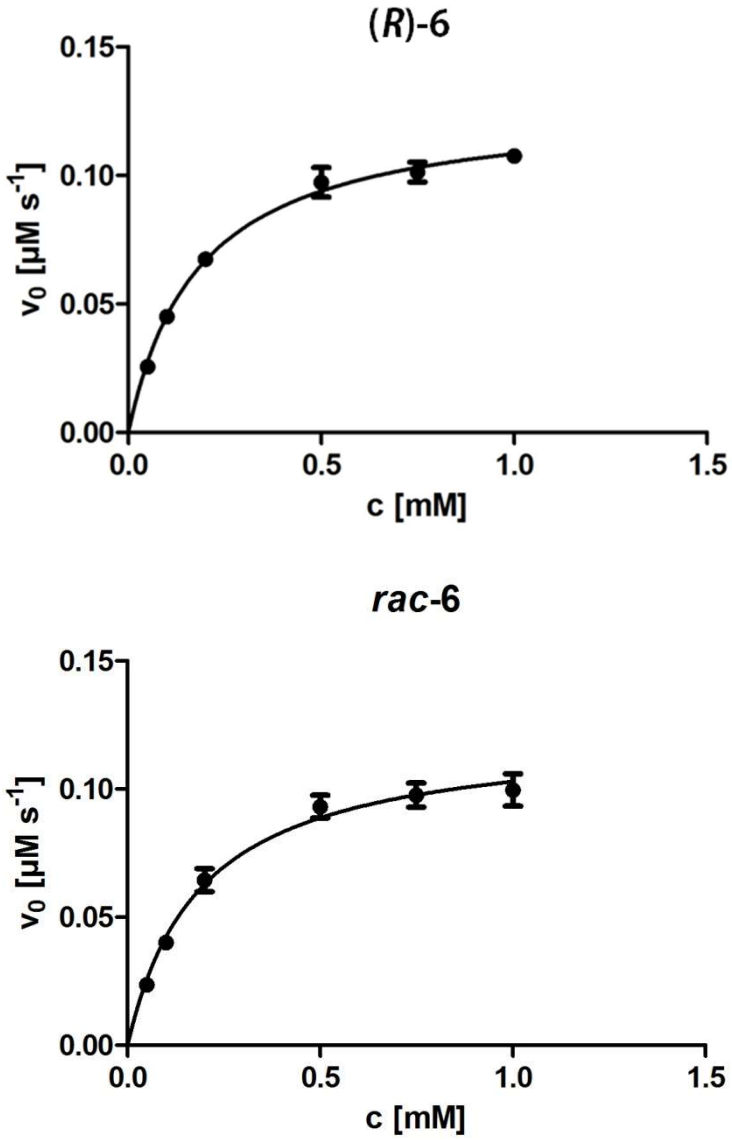


Figure 3. Michaelis-Menten-kinetics of the hydrolysis of **(*R*)-6** and **rac-6** catalysed by R39 from *Actinomadura* (0.7 nM) in the presence of 100 mM (*R*)-Alanine in 10 mM sodium phosphate buffer with 100 mM NaCl pH 7.2 at 30 °C.

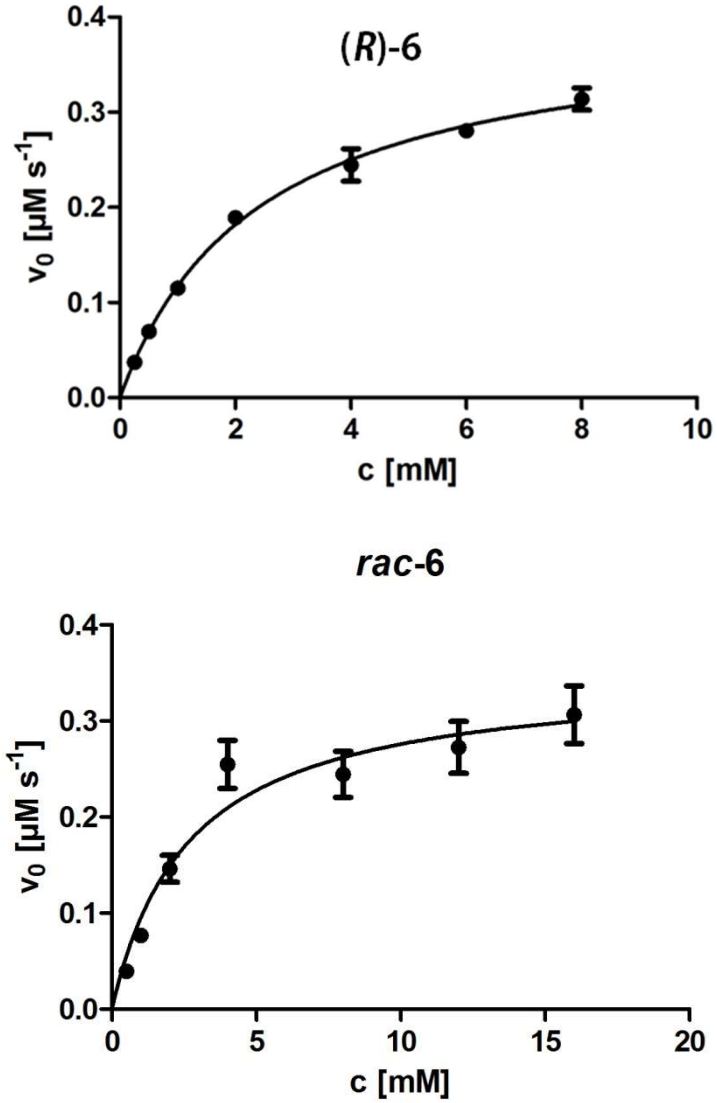


Figure 4. Michaelis-Menten-kinetics of the hydrolysis of **(*R*)-6** and **rac-6** catalysed by PBP2xR6 from a penicillin sensitive stain *from Streptococcus pneumoniae* (62 nM) in 10 mM sodium phosphate buffer pH 7.0 at 30 °C.

3. Development of screening assays with (*R*)-6

Classical inhibition studies of PBPs often rely on competition with fluorescently labeled ampicillin and are labor-intensive.[4,10,14] In 2010, Stefanova described a microtiter plate-based β-lactam binding assay for inhibition studies of PBPs based on a competition with binding of a biotin-ampicillin conjugate.[15] Two years later, Inglis developped a fluorescence polarization assay based on a competition with a boronic-acid, reversibly binding "tracer".[16] Protocols for microtiter plate-based thioester assays with **(*R*)-6** or ***rac*-6** were successfully developed in our laboratory and were used for inhibition studies with different PBPs with hundreds of compounds. Today, thioester assays in microtiter plates using **(*R*)-6** as substrate are available

for several PBPs like R39[1,3,8] and PBP2xR6[3,5] from penicillin sensitive strains and for PBP2x5204 from a penicillin-resistant *Streptococcus pneumoniae* strain.[3,5,7] These assays allowed a rapid screening of active compounds and are suitable for covalent binding (β-lactams), weakly binding and also for slow binding compounds.[17] Furthermore detailed kinetic studies using **(*R*)-6** as reporter substrate are possible and allowed to describe the inhibition by compounds which bind reversibly binding (boronic acid[9,18]), covalently (6-β-iodopenicillanate[6]) or by slow binding arylalkylidene rhodanine derivatives.[10] Thioesterase acitivities of other PBPs like PBP3 and PBP5 from *E. coli*[10] and R61 from *Streptomyces*[19,20] are well described in the literature and could be used for the development of microtiter plate assays in the future. Furthermore, **6** is commercially available and can be used for these studies after the determination of ee as described in this work.

Protocols for microtiter-plate assays for the screening of compound libraries for new inhibitors of PBPs

General

The experiments were performed by monitoring the degree of hydrolysis of **(*R*)-6** using microtiter 96 well plates and a Power Wave microtiter plate reader (Bio-Tek Instruments). Enzyme residual activity (RA) was determined after pre-incubation (60 min) of the PBPs in the presence of potential inhibitors at 25 °C. The initial rate of hydrolysis of **(*R*)-6** and the rate of spontaneous hydrolysis of **(*R*)-6** in the presence of the inhibitors was determined in the presence of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, ε_{412nm} = 14150 M^{-1} s^{-1}). Generally, all experiments were performed in triplicate only the activity of PBPs in absence of inhibitors (100% RA) was measured with six replicates on each plate.

Screening of "False Positives"

False positives (promiscuous inhibitors) can be detected by performing assays under the same conditions but in the presence of 0.01% Triton-X-100 *v/v*. As described in the literature, promiscuous inhibitors are slow binding, non-competitive inhibitors. In order to avoid a detailed kinetic study[10], it is possible to identify such compounds by performing tests in the presence of Triton-X-100.[21,22] Promiscuous inhibitors show no inhibition in the presence of Triton-X-100.

Preparation of solution of potential inhibitors:

Potential inhibitors are often solubilised in DMF. The final concentration of DMF in the assays is 1%.

Protocol for R39 from Actinomadura

Potential inhibitors were incubated with 3.5 nM R39 in 10 mM sodium phosphate buffer (pH 7.2) with 100 mM NaCl, 100 mM (*R*)-alanine and 0.01 mg/mL BSA for 60 min at 25 °C. After the pre-incubation RAs were determined by adding ***rac*-6** (1 mM) or **(*R*)-6** (0.5 mM) and DTNB (1 mM) and measuring the initial rate of hydrolysis of **(*R*)-6** at 412 nm (total test volume: 150 µL).

Protocol for PBP2xR6 from a penicillin sensitive strain from Streptococcus pneumoniae

PBP2xR6 (90 nM) enzyme was incubated in the presence of potential inhibitors in 10 mM sodium phosphate buffer (pH 7.0) and 0.01 mg/mL BSA for 60 min at 25° C. After the pre-incubation RAs were determined by adding ***rac*-6** (1 mM) or **(*R*)-6** (0.5 mM) and DTNB (1 mM) and measuring the initial rate of hydrolysis of **(*R*)-6** at 412 nm (total test volume: 150 µL).

Protocol for PBP2x5204 from a penicillin resistant strain from Streptococcus pneumoniae
PBP2x5204 (1.4 μM) enzyme was incubated in the presence of potential inhibitors in 10 mM sodium phosphate buffer (pH 7.0) and 0.01 mg/mL BSA for 60 min at 25° C. After the pre-incubation RAs were determined by adding **(*R*)-6** (2 mM) and DTNB (1 mM) and measuring the initial rate of hydrolysis of **(*R*)-6** at 412 nm (total test volume: 150 μL).

4. References

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