Supporting Information

Fluorophore-Labeled β-Lactamase as a Biosensor for β-Lactam antibiotics: A Study of the Biosensing Process

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Protein expression and purification

The E166C mutant of the PenPC β-lactamase was expressed in the *Bacillus subtilis* strain 1A304 (ϕ105MU331)[1] in a 15-liter automated Biostat C fermenter (B. Braun, Germany). The bacterial strain was streaked on an agar plate containing 5 μg/mL chloramphenicol, and the plate incubated at 37 °C for 24 h. A few single colonies from the agar plate were inoculated into each of ten 1-liter incubation flasks containing 100 mL of sterile BHY medium (37 g/L Oxoid brain heart infusion broth and 5 g/L Oxoid yeast extract). They were then incubated at 37 °C with shaking at 300 rpm overnight (~11–12 h). The overnight culture (~1 liter) was added to the fermenter containing 7 liter of sterile BHY medium. The bacterial culture was incubated in the presence of 20% dissolved oxygen (DO) controlled by an air-flow rate of 1–2 vvm with stirring (300–1,000 rpm) at 37°C. The pH of the culture was maintained at 7.0 by adding 3 M NaOH to the fed medium. The fed medium for fed-batch fermentation contains 300 g/L glucose, 3.75 g/L $MgSO_4.7\ H_2O$, 75 g/L tryptone, 11.25 g/L K_2HPO_4, 5.625 g/L KH_2PO_4. When the culture density (OD_{600}) reached 12–13, the bacterial culture in the fermenter was heated from 37 °C to 50 °C and immediately cooled to 37 °C. It was then further incubated at this temperature with shaking at 300 rpm for another 6 h before the culture was centrifuged at 9,000 rpm and 4 °C for 25 min to collect the supernatant. The supernatant pH was adjusted to 7.0 with conc. HCl. The E166C mutant protein was extracted by mixing the supernatant with 1 kg of celite 545 (BDH Company, USA) for 20 min on an ice bath. After discarding the supernatant, the celite was washed with deionized water. The protein was collected by mixing the celite with 6 L of protein elution buffer (100 mM Tris-HCl, 2 M NaCl and 100 mM tri-sodium citrate, pH 7.0) and subsequently concentrated to 800 mL at 4 °C using an Amicon concentrator equipped with a piece of YM-1 membrane (Millipore, MW cut off = 1,000). The concentrated protein solution was exchanged with 20 mM NH_4HCO_3 (pH 8.0) and then freeze-dried. The protein powder (yield: ~600 mg) was stored at –80 °C.

Reference

(1) Leung, Y. C.; Robinson, C. V.; Aplin, R. T.; Waley, S. G. *Biochem. J.* **1994**, *299*, 671–8.

Mathematical studies of the enzyme kinetics of E166Cf

The Dynamic System Method. The enzymatic reaction scheme for E166Cf can be represented by:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\Leftrightarrow}} ES \xrightarrow{k_2} ES^* \xrightarrow{k_3} E + P$$

where E is the enzyme, S is an antibiotic substrate, ES is the non-covalent enzyme-substrate complex, ES^* is the acyl enzyme-substrate complex and P is the hydrolyzed product. The set of kinetic equations describing the concentration-time profiles of various reacting species are:

$$\frac{dS}{dt} = -k_{+1}(E)(S) + k_{-1}(ES), \quad (1)$$

$$\frac{d(ES)}{dt} = k_{+1}(E)(S) - k_{-1}(ES) - k_2(ES), \quad (2)$$

$$\frac{d(ES^*)}{dt} = k_2(ES) - k_3(ES^*), \quad (3)$$

$$\frac{dP}{dt} = k_3(ES^*), \quad (4)$$

which is a system of first order ordinary differential equations. Using the experimental conditions and the rate parameters determined for the binding of E166Cf to cefuroxime listed in Table 1:

	Experimental data
K_d (M)	0.91 mM
k_2(s^{-1})	1.9 s^{-1}
k_3 (s^{-1})	2.0 x 10^{-4} s^{-1}

and noting that $K_d = k_{-1}/k_{+1}$, the k_{+1} *and* k_{-1} values can be obtained by iteration and curve-fitting the theoretically calculated [ES*]/[E_{total}] profile to the experimental curve shown in Figure 4(e). The curve-fitting calculations were carried out by minimizing the difference between the calculated [ES*]/[E_{total}] values to the experimental data as a function of time, using the Matlab optimization toolbox. The values of k_{-1} and k_{+1} so obtained are:

Optimized kinetic parameters	**E166Cf**
k_{+1} ($M^{-1}s^{-1}$)	1.02 x 10^4
k_{-1} (s^{-1})	9.27

With the complete set of rate constants, k_{+1}, k_{-1}, k_2 and k_3, and the initial concentrations of *E* and *S* known, the concentrations of *S, E, ES, ES** and *P* as a function of time can be calculated by solving the set of differential equations (1)–(4) shown above.

For fixed values of K_d, k_2 and k_3, the k_1 and k_{-1} values were found in the range of 10^3–10^8 $M^{-1}s^{-1}$ and 10^0–10^5 s^{-1}, respectively. This conforms with the literature k_1 values of protein–ligand interactions reported in the range of 10^5–10^8 $M^{-1}s^{-1}$,[1] the physically meaningful range for concentration–time profiles of various species, including *S, E, ES, ES** and *P,* in the hydrolytic reaction. However, we found the generated concentration–time profiles are insensitive to the individual values of k_1 and k_{-1} whenever the K_d value (0.91 mM) is fixed. The concentration–time profiles for the hydrolytic reaction between the E166Cf (0.5 μM) and the cefuroxime (5 μM) obtained are shown in Figure S9.

Reference

(1) Lu, W. P.; Sun, Y.; Bauer, M. D.; Paule, S.; Koenigs, P. M.; Kraft, W. G. *Biochemistry* **1999**, *38*, 6537-46.

Molecular modeling

Three structures of the fluorescein-labeled E166C mutant of the PenPC β-lactamase were constructed by homology modeling using the sequence builder of the CACHE software program (CACHE WorkSystem Pro 7.5.0.85), including the substrate-free E166Cf structure, the ES state of E166Cf with penicillin G non-covalently bound to the active site and the ES* state of E166Cf with penicillin G covalently bound to the hydroxyl group of the side chain of Ser70. The crystal structure of the *B. licheniformis* 749/C β-lactamase (PDB code: 4BLM) was used as the template for the substrate-free enzyme structure,[1] and the crystal structure of the acyl-enzyme intermediate of the *E. coli* RTEM-1 β-lactamase complexed with penicillin G (PDB code: 1FQG) used as the template for the ES* state.[2] The non-covalent ES state was built by superimposing unhydrolyzed penicillin G (extracted from PDB: 1UOF)[3] onto the ES* state. The E166C mutation and the conjugation of fluorescein-5-maleimide with the thiol group of the side chain of Cys166 in E166C were built by the CACHE program. The Ω-loop (including the manually built fluorophore) was treated with the default refinement procedure in the CACHE program to relax poor geometry. The refined models were then subjected to one round of simulated annealing using torsional molecular dynamics, followed by conjugate gradient minimization, using the CNS program.[4] The topology and parameter files for fluoresceine-5-maleimide and penicillin G used in the refinement were generated using the PRODRG server.[5] The solvent accessible areas (SAA) for the fluorescein label of E166Cf in the non-covalent ES and covalent ES* states were calculated on the basis of the final refined models using the NACCESS program.[6]

References

(1) Knox, J. R.; Moews, P. C. *J. Mol. Biol.* **1991**, *220*, 435–55.

(2) Strynadka, N. C.; Adachi, H.; Jensen, S. E.; Johns, K.; Sielecki, A.; Betzel, C.; Sutoh, K.; James, M. N. *Nature* **1992**, *359*, 700–5.

(3) Valegard, K.; Terwisscha Van Scheltinga, A. C.; Dubus, A.; Ranghino, G.; Oster, L. M.; Hajdu, J.; Andersson, I. *Nat. Struct. Mol. Biol.* **2003**, *11*, 95–101.

(4) Brunger, A. T.; Adams, P. D.; Clore, G. M.; DeLano, W. L.; Gros, P.; Grosse-Kunstleve, R. W.; Jiang, J. S.; Kuszewski, J.; Nilges, M.; Pannu, N. S.; Read, R. J.; Rice, L. M.; Simonson, T.; Warren, G. L. *Acta Crystallogr. D Biol. Crystallogr.* **1998**, *54 (Pt 5)*, 905–21.

(5) Schuttelkopf, A. W.; van Aalten, D. M. F. *Acta Crystallogr. D Biol. Crystallogr.* **2004**, *60(Pt 8)*, 1355–63.

(6) Hubbard, S. J.; Thornton, J. M. NACCESS, Computer Program, Department of Biochemistry and Molecular Biology, University College London, **1993**.

Figure S1. Transformed mass spectra acquired after incubation of E166Cf (0.5 μM) with cefuroxime (25 μM) in 20 mM ammonium acetate (pH 7.0) at different time intervals. Peaks A and B correspond to the free enzyme E and the covalent acyl enzyme-substrate complex ES*, respectively.

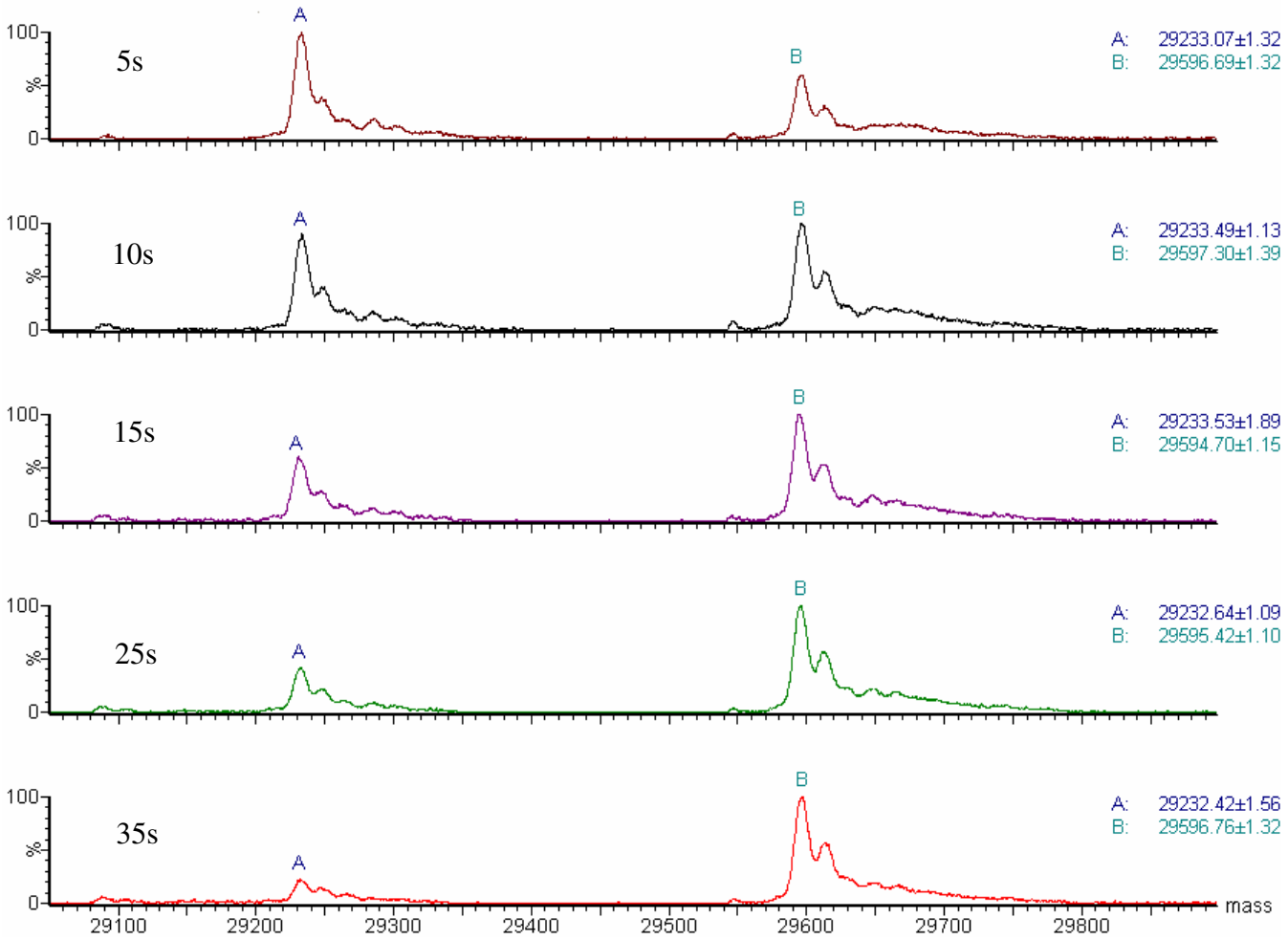
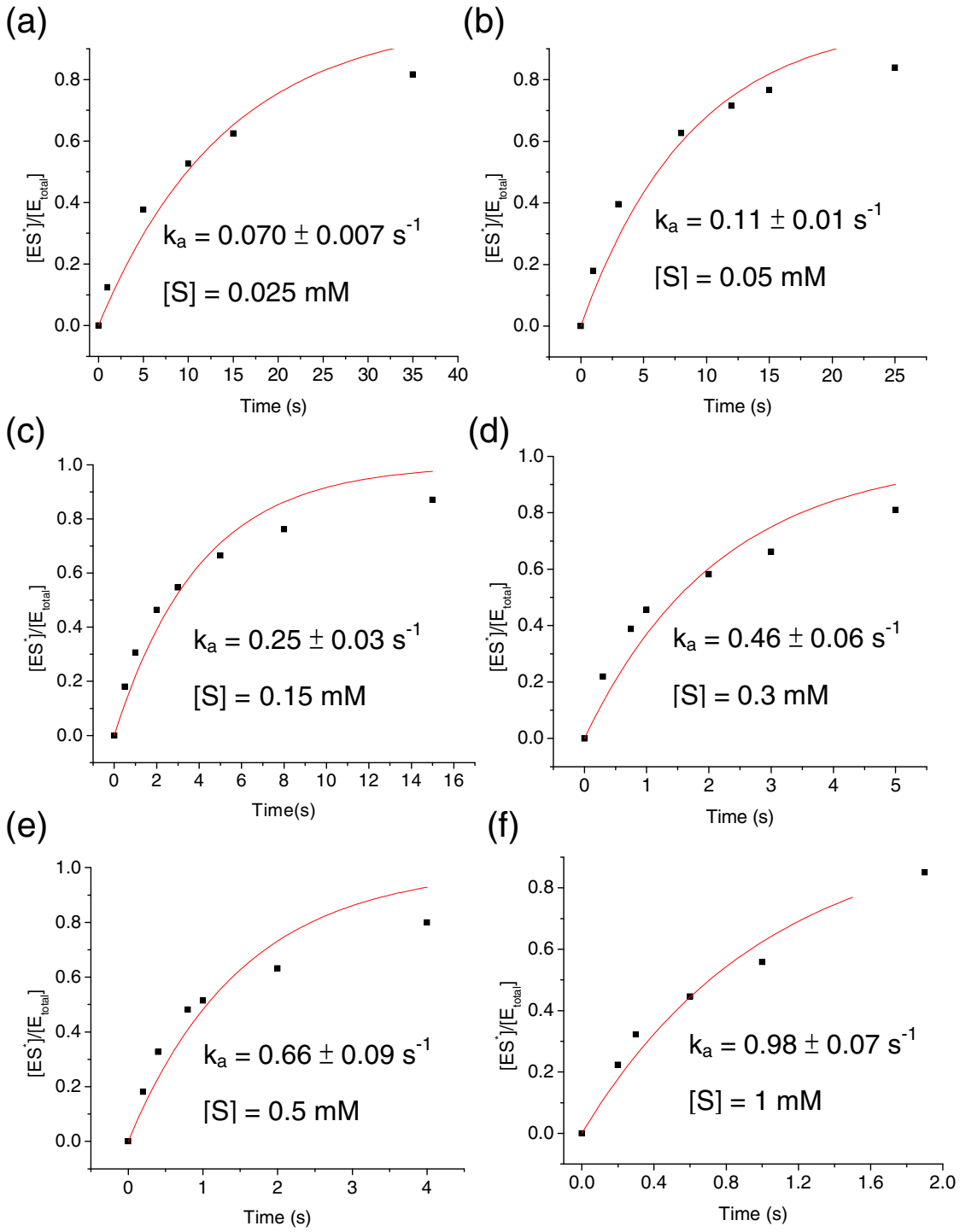


Figure S2. Kinetic studies of E166Cf binding with cefuroxime analyzed by ESI-MS. (a)–(f) Time course of the reaction of E166Cf with different concentrations of cefuroxime [S]. The red lines represent the best fit of the experimental data to equation [1], from which the k_a values were obtained. (g) Plot of the k_a values as a function of cefuroxime concentrations. The red line represents the fit of the experimental data to equation [2], from which the K_d and k_2 values were obtained.



(g)

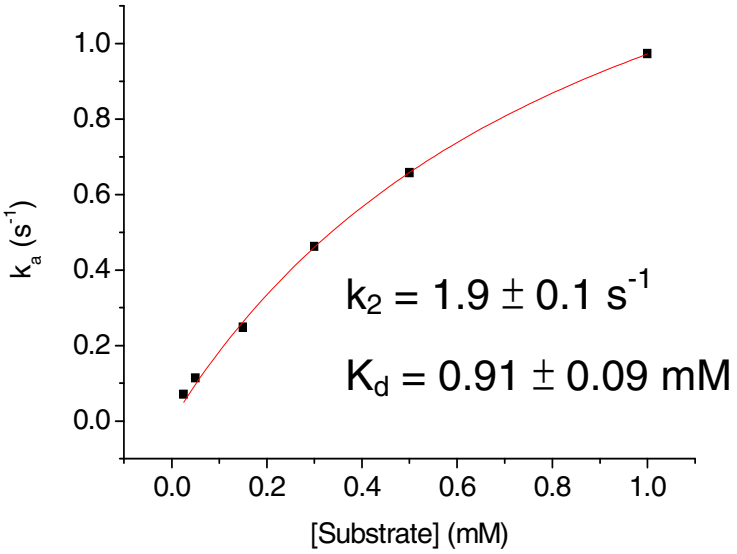

1.0
0.8
0.6
0.4
0.2
0.0
k_a (s^{-1})
0.0
0.2
0.4
0.6
0.8
1.0
[Substrate] (mM)
$k_2 = 1.9 \pm 0.1\ s^{-1}$
$K_d = 0.91 \pm 0.09$ mM

Figure S3. Kinetic studies of E166C binding with cefuroxime analyzed by ESI-MS. (a)–(e) Time course of the reaction of E166C with different concentrations of cefuroxime [S]. The red lines represent the best fit of the experimental data to equation [1], from which the k_a values were obtained. (f) Plot of the k_a values as a function of cefuroxime concentrations. The red line represents the fit of the experimental data to equation [2], from which the K_d and k_2 values were obtained.

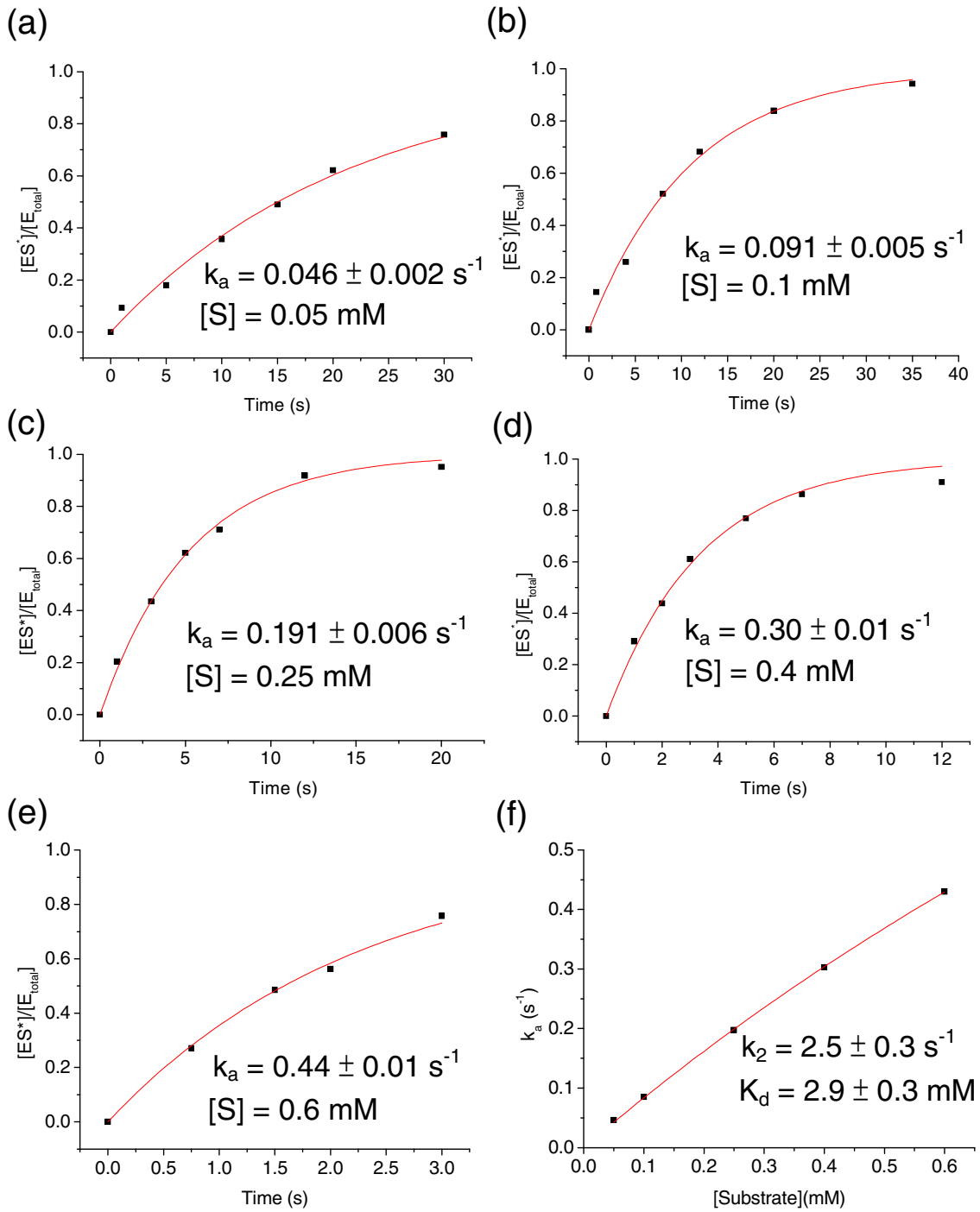


Figure S4. Kinetic studies of the deacylation of the covalent E166C- and E166Cf-cefuroxime complexes monitored by ESI-MS. The experimental data for the deacylation of the E166C-cefuroxime and E166Cf-cefuroxime complexes are shown in (a) and (b), respectively. The red lines represent the fit of the experimental data to equation [3], from which the k_3 values were obtained.

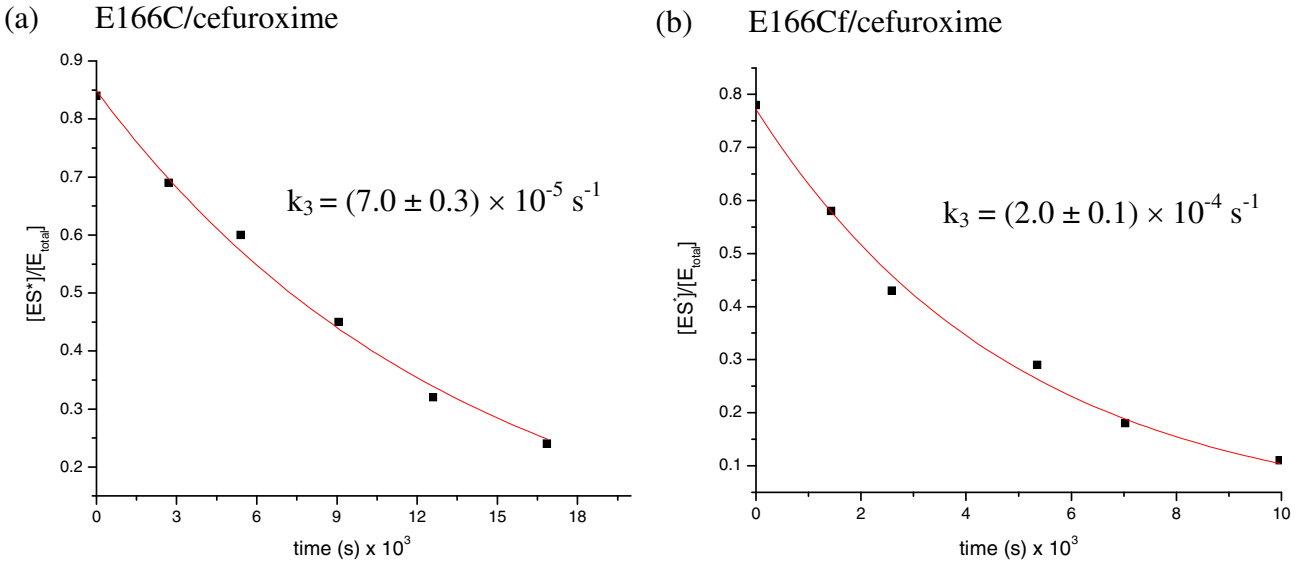


Figure S5. Kinetic studies of E166Cf binding with 6-APA analyzed by ESI-MS. (a)–(e) Time course of the reaction of E166Cf with different concentrations of 6-APA. The red lines represent the best fit of the experimental data to equation [1], from which the k_a values were obtained. (f) Plot of the k_a values as a function of 6-APA concentrations. The red line represents the fit of the experimental data to equation [2], from which the K_d and k_2 values were obtained.

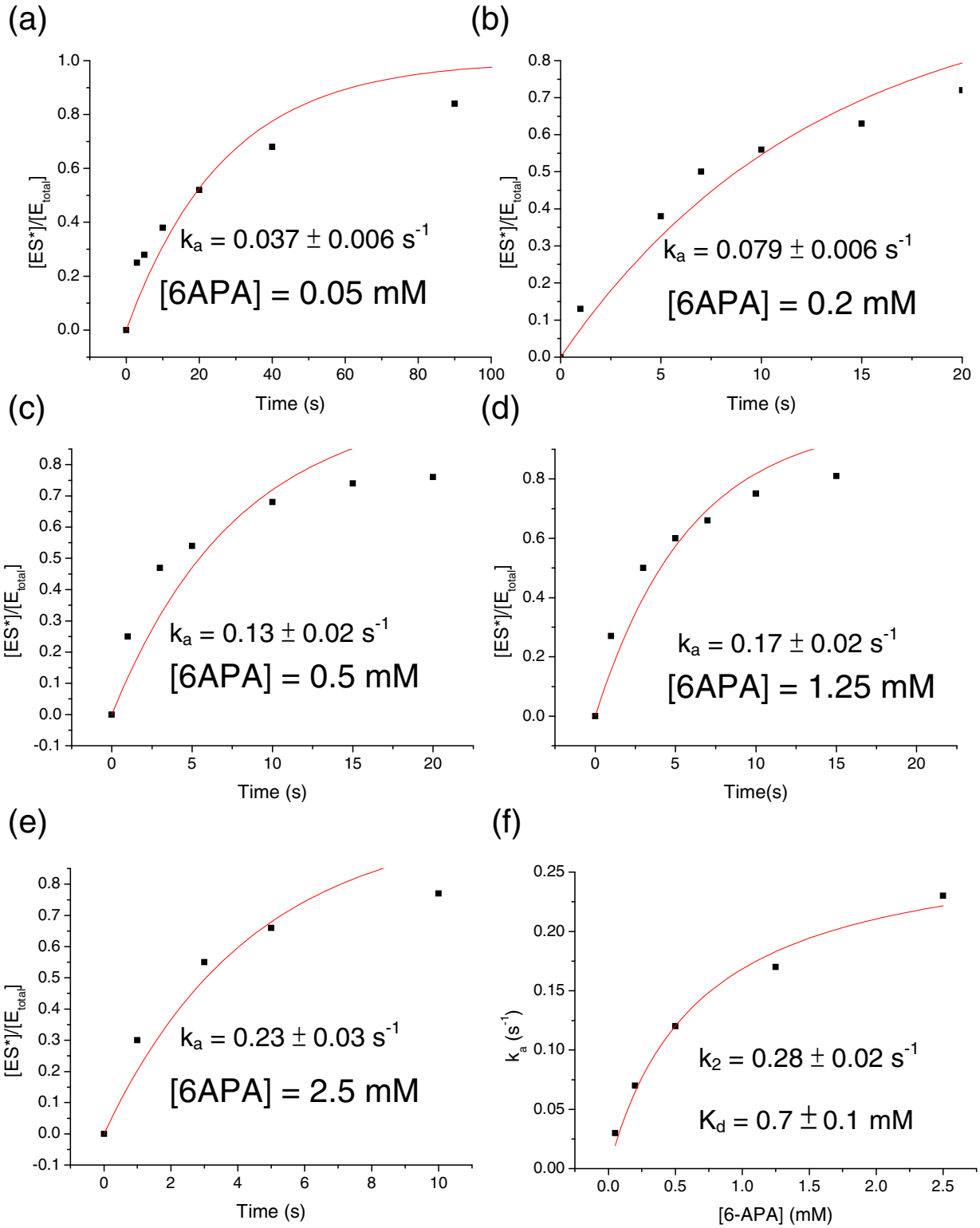


Figure S6. Kinetic studies of E166C binding with 6-APA analyzed by ESI-MS. (a)–(e) Time course of the reaction of E166C with different concentrations of 6-APA [S]. The red lines represent the best fit of the experimental data to equation [1], from which the k_a values were obtained. (f) Plot of the k_a values as a function of 6-APA concentrations. The red line represents the fit of the experimental data to equation [2], from which the K_d and k_2 values were obtained.

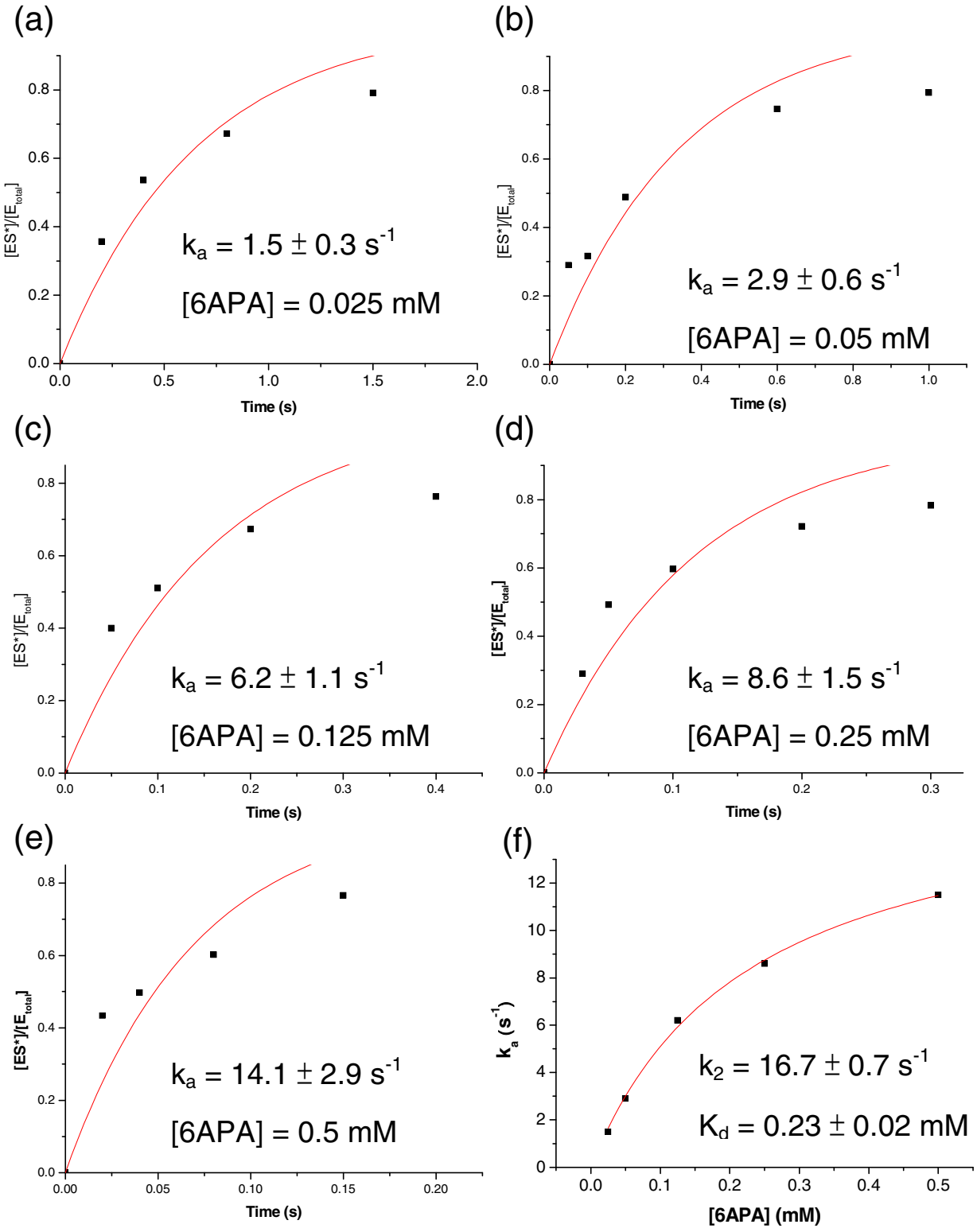
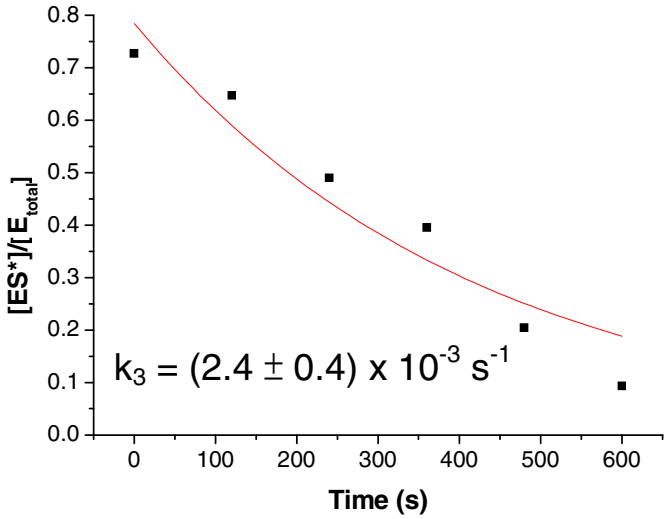


Figure S7. Kinetic studies of the deacylation of the covalent E166C- and E166Cf-6-APA complexes monitored by ESI-MS. The experimental data for the deacylation of the E166C- and E166Cf-6-APA complexes are shown in (a) and (b), respectively. The red lines represent the fit of the experimental data to equation [3], from which the k_3 values were obtained.

(a) E166C/6-APA



(b) E166Cf/6-APA

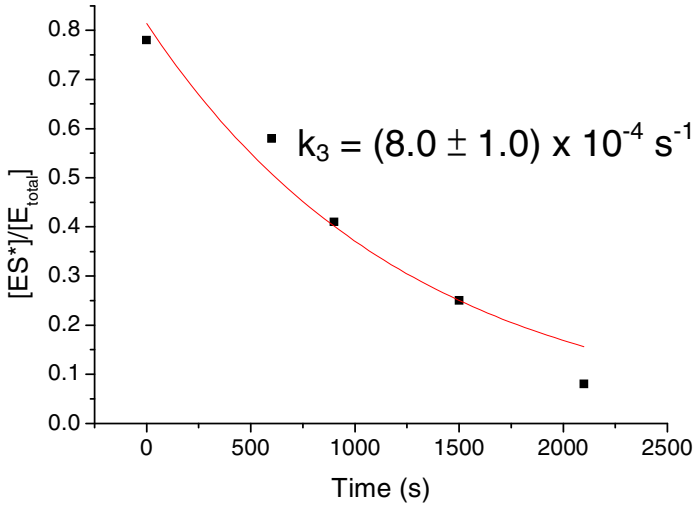


Figure S8. Steady-state fluorescence measurements of E166Cf with penicillin G. The fluorescence signals of E166Cf with and without 10 μM penicillin G are shown in cyan and blue, respectively. [E166Cf]: 0.12 μM; buffer: 50 mM potassium phosphate (pH 7.0). The inset shows the fluorescence of free fluorescein (without the thiol-reactive maleimide linker) with the unlabeled E166C mutant (0.12 μM) in the presence of 10 μM penicillin G in 50 mM potassium phosphate buffer (pH 7.0). Note that E166Cf exhibits stronger fluorescence with the antibiotic, whereas free fluorescein gives no observable fluorescence change in the presence of the antibiotic and the unlabeled E166C mutant over the same time interval under similar experimental conditions.

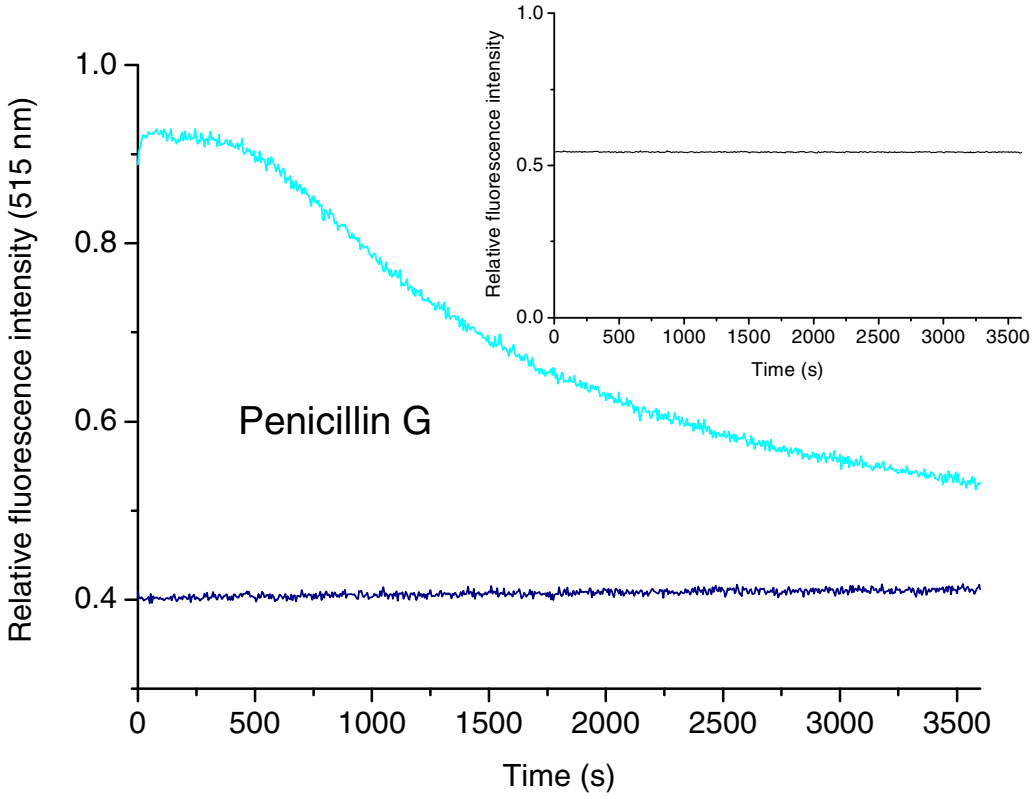


Table S1. MALDI mass spectral data for the peptide fragments of E166C and E166Cf produced by trypsin digestion.

Peptide fragment	Measured mass (error in ppm)	Theoretical mass	E166C	E166Cf
H24–K31	963.514 (-2.2)	963.512	N.A.	√
L119–R128	1028.576 (-0.2)	1028.573	√	√
I159–R164	721.378 (-16.4)	721.366	√	√
F165–R178	1577.767 (-2.6)	1577.763	√	√*
[F165–R178] + fluorescein (+H_2O)	2022.817 (12.9)	2022.843	N.A.	√
A192–R204	1373.756 (-1.8)	1373.754	N.A.	√
I206–K212	920.496 (-5.4)	920.491	√	√
A223–K234	1257.647 (0)	1257.647	√	√
S237–R244	855.403 (-8.7)	855.395	√*	√
N245–R256	1280.675 (0)	1280.675	√	√

N.A.: not available

* weak signal

Figure S9. Concentration-time profiles of various states of E166Cf with cefuroxime obtained by mathematical simulation. E, ES, ES*, S and P represent free E166Cf, the non-covalent E166Cf-cefuroxime complex, the covalent E166Cf-cefuroxime complex, unhydrolyzed cefuroxime and hydrolyzed cefuroxime, respectively. To show the concentration profiles of ES and S on the same concentration scale, the concentration values for ES and S were multiplied by a factor of 50 and 1/10, respectively.