



HAL
open science

Click and Release Chemistry for Activity-Based Purification of β -Lactam Targets

Saidbakhrom Saidjalolov, Emmanuelle Braud, Zainab Edo, Laura Iannazzo, Filippo Rusconi, Margaux Riomet, Antoine Sallustrau, Frédéric Taran, Michel Arthur, Matthieu Fonvielle, et al.

► **To cite this version:**

Saidbakhrom Saidjalolov, Emmanuelle Braud, Zainab Edo, Laura Iannazzo, Filippo Rusconi, et al.. Click and Release Chemistry for Activity-Based Purification of β -Lactam Targets. Chemistry - A European Journal, 2021, 27 (28), pp.7687-7695. 10.1002/chem.202100653 . hal-03281379

HAL Id: hal-03281379

<https://hal.sorbonne-universite.fr/hal-03281379v1>

Submitted on 8 Jul 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Click and release chemistry for activity-based purification of β -lactam targets

Saidbakhrom Saidjalolov,[†] Emmanuelle Braud,[†] Zainab Edoov,[‡] Laura Iannazzo,[†] Filippo Rusconi,[‡] Margaux Riomet,[§] Antoine Sallustrau,[§] Frédéric Taran,[§] Michel Arthur,^{‡*} Matthieu Fonvielle,^{‡*} Mélanie Etheve-Quellejeu^{†*}

[†] Université de Paris, UMR CNRS 8601, Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, F-75006, Paris, France.

[‡] INSERM, Sorbonne Université, Université de Paris, Centre de Recherche des Cordeliers (CRC), F-75006, Paris, France.

[‡] PAPPISO, Université Paris-Saclay, INRAE, CNRS, AgroParisTech, GQE - Le Moulon, F-91190, Gif-sur-Yvette, France.

[§] Université Paris Saclay, CEA, INRAE, Département Médicaments et Technologies pour la Santé (DMTS), SCBM, F-91191 Gif-sur-Yvette, France.

ABSTRACT: The β -lactams were the first antibiotics of natural origin to be used to treat bacterial infections and remain the cornerstone of antibiotherapy. In contrast to other drugs, such as those acting on the ribosome, β -lactams inhibit multiple targets with partially redundant functions, referred to as transpeptidases or penicillin-binding proteins (PBPs). These enzymes catalyze the essential cross-linking step of the polymerization of cell wall peptidoglycan. Due to the multiplicity of the targets, the understanding of the mechanisms of action of β -lactams and of resistance to these drugs requires the development of reliable methods to characterize the transpeptidases. Here, we describe an activity-based purification method of β -lactam targets based on click and release chemistry. We focused on β -lactams of the carbapenem class and obtained alkyne drugs with suitable properties with respect to the kinetics of acylation of a model target, the Ldt_{fm} L,D-transpeptidase, the stability of the resulting acylenzyme, and the reactivity of the alkyne for the cycloaddition of an azido probe containing a biotin moiety for affinity purification with streptavidin magnetic beads. The probe also contained an iminosynnone moiety that was developed as a bioorthogonal cleavable linker enabling in a single step a click and release reaction to elute and label the transpeptidase target using a TAMRA-cyclooctyne. We show that our purification scheme is specific for the presence of the Ldt_{fm} catalytic nucleophile and of a native β -lactam ring in the alkyne carbapenem. Specificity was also established by demonstrating the selective recovery of Ldt_{fm} from reconstituted protein pools.

Bacterial resistance is a major threat to the efficacy of antibiotics requiring innovative strategies for maintaining the efficacy of antimicrobial chemotherapy. Identification and characterization of the biological targets of drugs is a key step for their development and the study of the mechanisms of resistance. This is particularly the case for antibiotics belonging to the β -lactam family since these drugs have multiple targets in bacterial cells with partially overlapping functions.^{1,2} The essential targets of β -lactams are the high-molecular weight (HMW) penicillin-binding proteins (PBPs) that catalyze the last cross-linking step of peptidoglycan synthesis. This cell wall polymer is essential for sustaining the osmotic pressure of the bacterial cytoplasm. It consists of glycan chains linked to each other by short stem peptides (Figure 1A). The cross-linking reaction catalyzed by HMW PBPs, the D,D-transpeptidases, proceeds by the cleavage of the C-terminal D-Ala-D-Ala peptide bond of a stem pentapeptide and linkage of the penultimate D-

Ala to the third residue of a stem peptide carried by an adjacent glycan chain (4-3 cross-links) (Figure 1B). β -lactams are structure analogues of the D-Ala-D-Ala extremity of peptidoglycan precursors and act as suicide substrates of the HMW PBPs³ (Figure 1C). Acylation of the active site Ser of the PBPs results in a long-lasting covalent adduct and irreversible inactivation of the target at the time scale of a bacterial generation.

The cross-linking reaction is performed by multiple HMW PBPs in most bacteria, typically 5 to 6 in the main human pathogens.¹ In addition, β -lactams similarly inactivate low-molecular weight (LMW) PBPs that hydrolyze the D-Ala-D-Ala peptide bond of stem pentapeptides (D,D-carboxypeptidases) or the cross-links formed by the HMW PBPs (endopeptidases). A particular class of β -lactams, the carbapenems, also inactivate L,D-transpeptidases (Ldts) that form unusual cross-links connecting the third position

on both stem peptides (3→3 cross-links)⁴ (Figure 1B). Ldt inactivation results from acylation of their active-site Cys residue by the β-lactam ring of carbapenems (Figure 1C). Ldts are the main cross-linking enzyme in *Mycobacterium tu-*

*berculosis*⁵ and can be responsible for *in vitro* acquired resistance to β-lactams of the penam (penicillin) and cephem (cephalosporin) classes in other bacteria.⁶⁻⁷

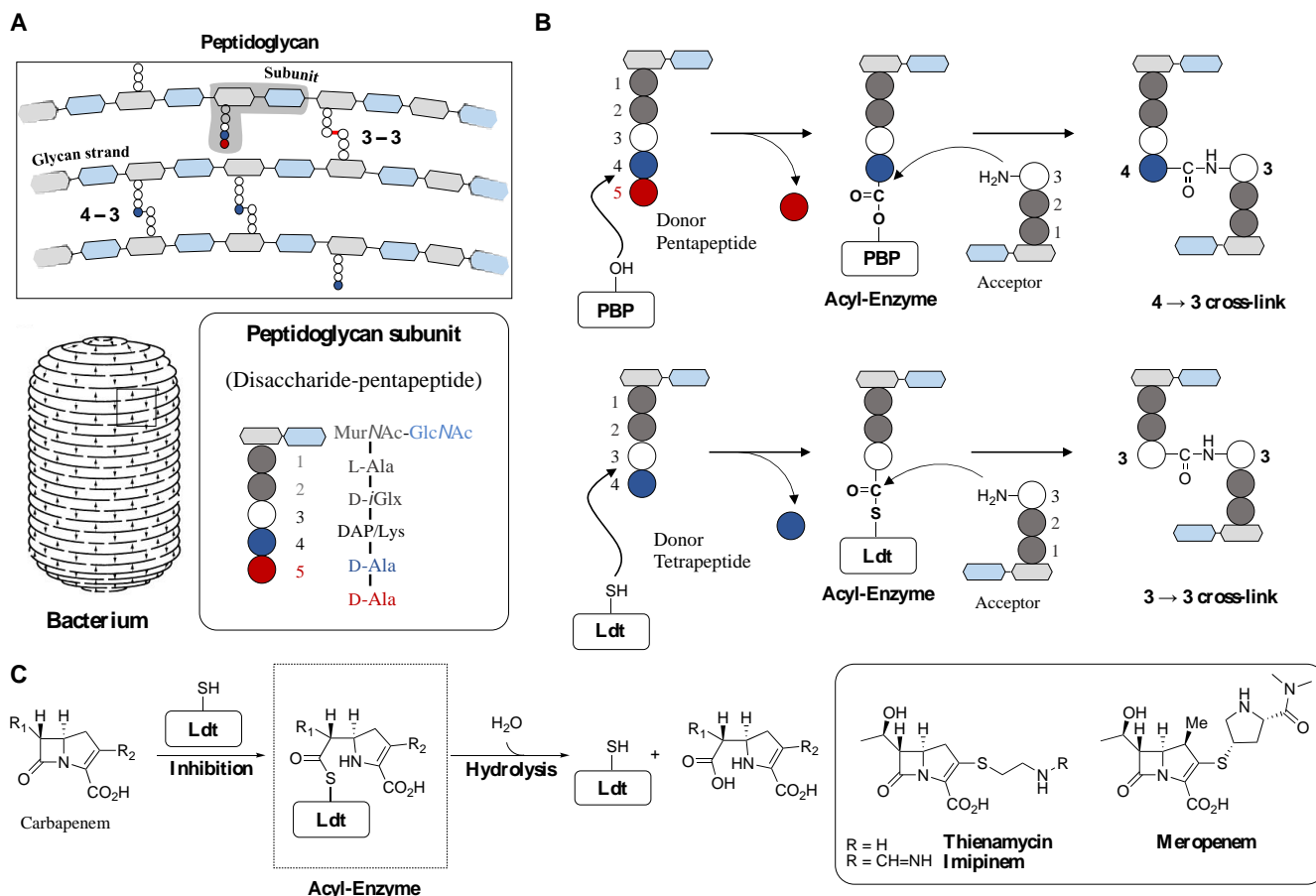


Figure 1. (a) Peptidoglycan structure. (b) Formation of peptidoglycan cross-links by PBPs and Ldts. (c) Inhibition of Ldts by carbapenems. The efficacy of Ldt inhibition depends upon slow hydrolysis of the acylenzyme. The inset shows the structure of carbapenems discussed in the text.

Historically, the targets of β-lactams have been identified by incubating membrane preparations with radiolabeled drugs followed by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.⁸ This method typically resolves a pattern of *ca.* 10 bands corresponding to proteins covalently labeled by the drug. Competitive binding with unlabeled drugs has been largely used to compare the reactivity of various β-lactams for the PBPs. For this approach, fluorescent β-lactams, such as bocillin, were developed to avoid the use of radioactive reagents.⁹⁻¹¹ Further refinement of the PBP labeling methods afforded single cell imaging of bacteria exposed to the drugs.¹² The key element of this application is the incorporation of a bioorthogonal active group in the β-lactam with minimal impact on drug penetration and on interaction of the drug with its multiple targets. Following labeling of the targets in the bacterium, the drug-target adducts were revealed by a bioorthogonal reaction with a functionalized dye.¹³⁻¹⁴ The most popular approach is the incorporation of

an alkyne group on the drug allowing copper-catalyzed cycloaddition (CuAAC) with an azido-containing dye.¹³⁻¹⁴ This approach enables detection of known PBPs but present limitations concerning the identification of unknown targets since it only provides information on the electrophoretic mobility of the protein-dye adduct. This limitation has been addressed by the use of biotinylated azido-containing dyes for streptavidin affinity pull-down purification followed by mass spectrometry analysis.¹⁵⁻¹⁶ Selective penicillin-binding protein imaging probes have also been described using fluorescent β-lactams,¹⁷ such as fluorescent cephalosporins.¹⁸ Of note, fluorescent β-lactams were also used as fluorogenic molecular probes for the detection of β-lactamases *in vitro* or in living bacteria.^{17, 19-20}

In this study, we explore a strategy for combined affinity purification and fluorescence labeling of carbapenem targets. We report synthetic routes to alkynylated carbapenems that effectively labeled a model L,D-transpeptidase (Ldt_{fm}) both with respect to the kinetics of acylation

and the stability of the resulting acylenzymes. The alkyne group of the enzyme-bound carbapenem was subsequently exploited for CuAAC attachment of a biotin-containing iminosydnone linker, recently developed as a bioorthogonal cleavable spacer (Figure 2).²¹⁻²² After binding to strep-

tavidin magnetic beads and purification, the iminosydnone mesoionic compound enabled the release and labeling of the captured carbapenem targets from the streptavidin support through a bioorthogonal click and release reaction,²¹ affording pure and fluorescent targets in a single step.

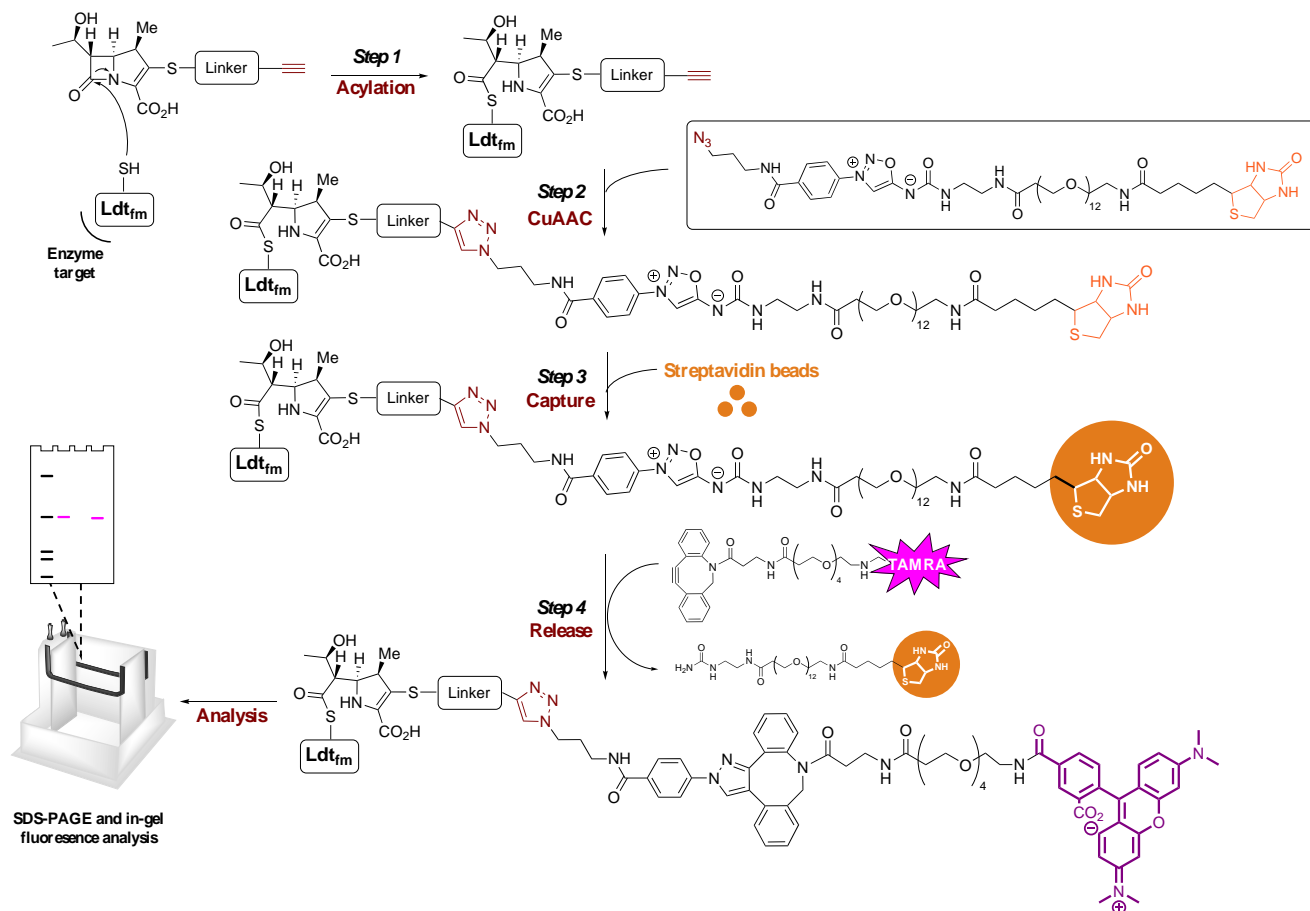


Figure 2. General strategy for activity-based purification of carbapenem targets.

RESULTS AND DISCUSSION

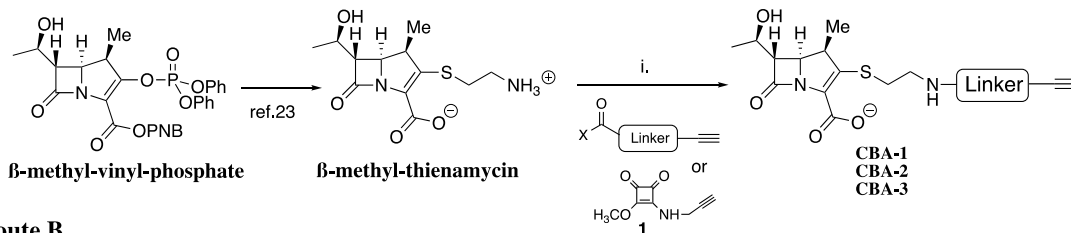
Synthesis of alkyne-carbapenems. We started our synthesis by using a post-functionalization strategy of the fully deprotected methyl-thienamycin (Route A, Scheme 1). Methyl-thienamycin was obtained in two steps (21% yield) from commercially available β -methyl vinyl-phosphate.²³ Methyl-thienamycin is renowned for its unstable chemical nature.²⁴ Indeed, strong acidic, basic, or nucleophilic conditions as well as exposure to light, high temperature, or prolonged storage in solution lead to β -lactam ring opening. Thus, the reaction conditions for functionalizing methyl-thienamycin should be mild ($6.0 < \text{pH} < 8.5$; $< 25^\circ\text{C}$) and should not involve prolonged reaction time (< 1 hour). Despite these limitations, three alkyne-carbapenems were obtained, **CBA-1**, **CBA-2**, and **CBA-3**, using methyl-thienamycin in the presence of alkyne linkers, which were commercially available (for **CBA-1** and **CBA-2**) or synthesized in this study (Compound **1**, Scheme 1). Alkyne-carbapenems **CBA-1**, **CBA-2**, and **CBA-**

3 were obtained with variable yields (49%, 67%, and 14%, respectively; Scheme 1, Route A).

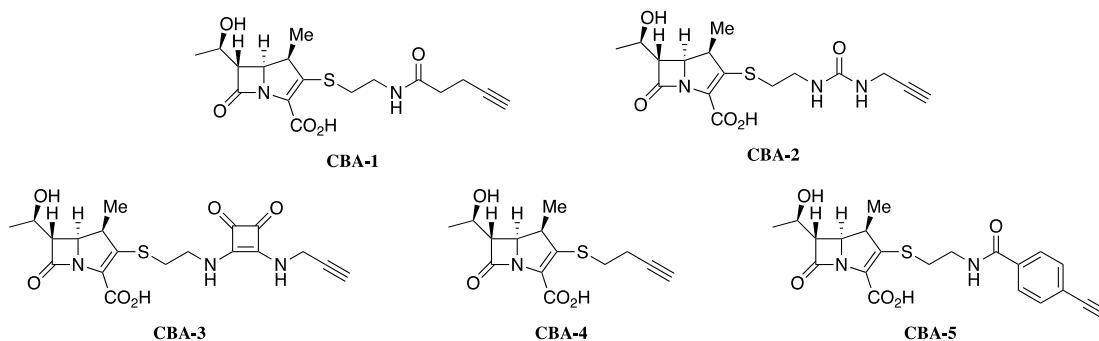
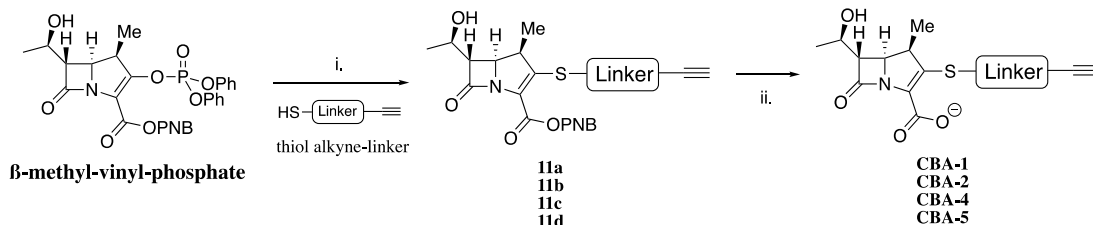
Ring opening could not be fully prevented and subsequent purification of the desired compounds free of their hydrolyzed products was cumbersome. For this reason, we explored an alternative approach starting from a protected carbapenem for direct addition of a thiol-alkyne onto the β -methyl vinyl-phosphate (Scheme 1, route B). Syntheses of the thiol alkyne linkers **3**, **6**, and **10** are described in the supporting information (SI, Section 2). This first step provided the intermediate carbapenems **11a**, **b**, **c**, **d** in 97%, 77%, 78%²³ and 54% yields, respectively. The critical step of the latter strategy was the final selective deprotection of the *para*-nitrobenzyl group (PNB), which was successfully achieved using zinc dust.²⁵ Minimal β -lactam ring opening provided by this approach facilitated purification of the final compounds (**CBA-1**, **CBA-2**, **CBA-4**, and **CBA-5** in 11%, 77%, 40%, and 43% yields, respectively). Comparison of the NMR spectra of **CBA-1** and **CBA-2** obtained by routes A and B (Supporting Scheme S5) supported the superiority of route B with respect to the purity of the final compound.

Acylation of L,D-transpeptidase Ldt_{fm} by alkyne-carbapenems. The alkyne function of compounds **CBA-1** to **CBA-5** is connected to the C₂ position of the carbapenem core *via* variable links, including an amide (**CBA-1**), a urea (**CBA-2**), a squaramide (**CBA-3**), an alkyl (**CBA-4**), or a benzamide (**CBA-5**). These compounds were synthesized to explore the impact of the linkers on the kinetics of target acylation and the stability of the resulting covalent adduct.

Route A



Route B



Scheme 1. Two strategies for alkyne carbapenems synthesis. Reaction conditions for route A: i. 4-pentynoic acid succinimidyl ester or *N*-(prop-2-yn-1-yl)-1*H*-imidazole-1-carboxamide²⁶ or compound **1**. Reaction condition for route B: i. thiol alkyne-linkers **3**, **6**, 3-butynyl ethanethioate or **10** and DIPEA in DMF, ii. Zn dust in THF:PB (1:2). Compounds were obtained by route A (**CBA-3**), route B (**CBA-4** and **CBA-5**), or both routes (**CBA-1** and **CBA-2**).

This was first investigated by mass spectrometry to detect Ldt_{fm}-carbapenems adducts (Figure 3). Incubation of Ldt_{fm} (6.5 μM) with alkyne carbapenems **CBA-1** to **CBA-5** (39 μM) for 15 min at 25 °C in 20 mM ammonium acetate (pH 7.2) led to full enzyme acylation, except for **CBA-3** (ca. 30% acylation). Stability of the Ldt_{fm}-carbapenems adducts was also monitored by mass spectrometry.

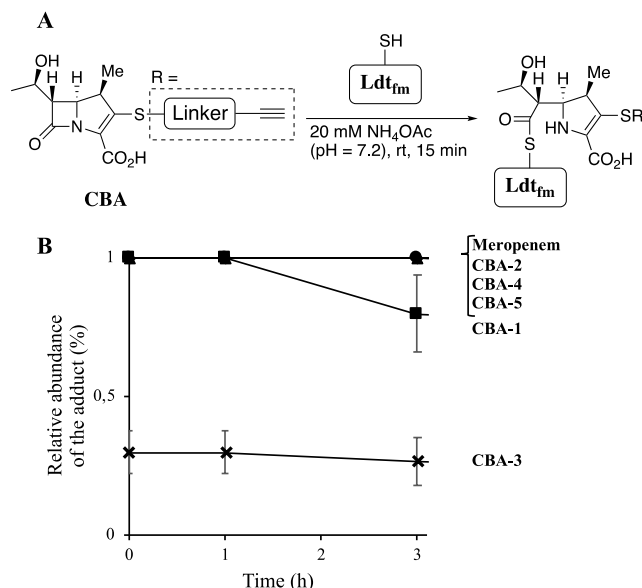


Figure 3. (a) Acylation of Ldt_{fm} by carbapenems. (b) Stability of Ldt_{fm} -carbapenem adducts determined by mass spectrometry (SI, Section 7.3).

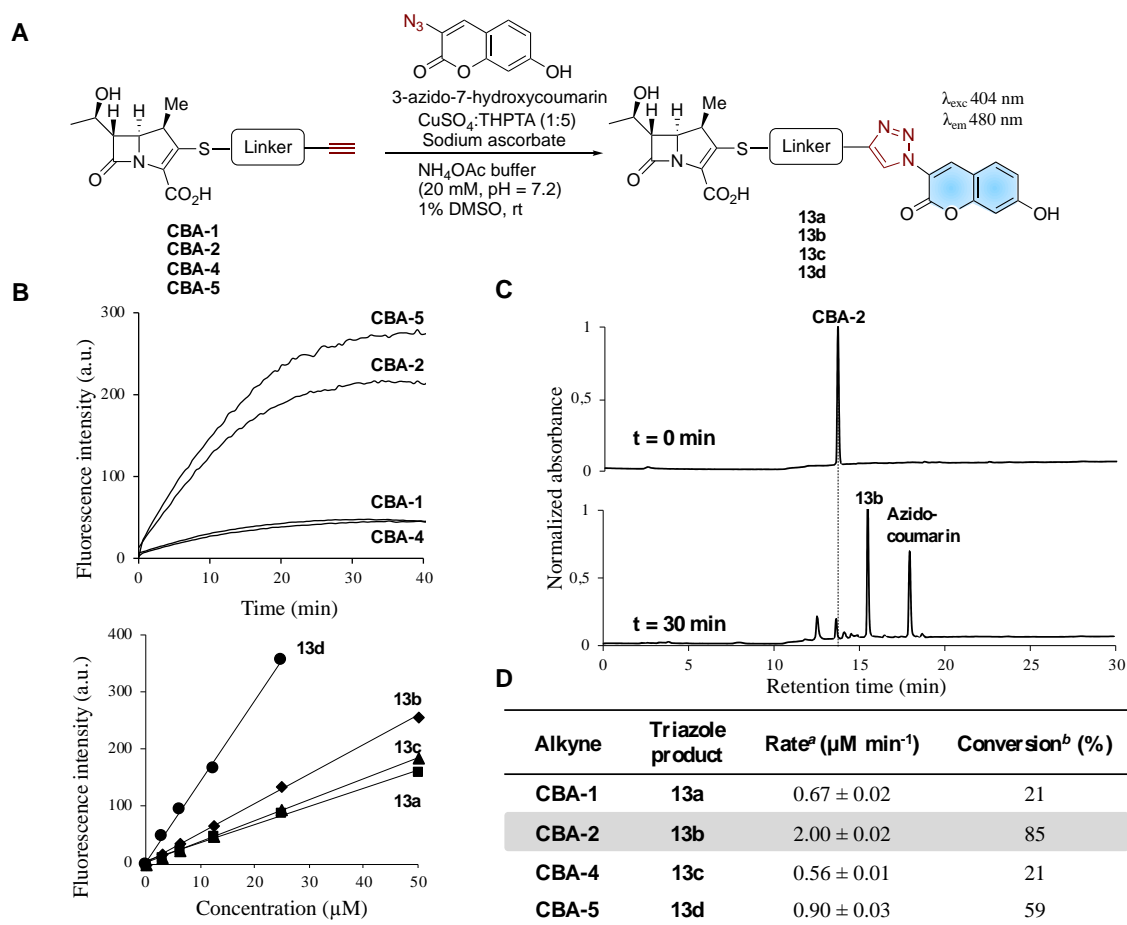


Figure 4. Impact of the linker of alkyne carbapenems on the efficiency of CuAAC. (a) CuAAC reaction with 3-azido-7-hydroxycoumarin. CuAAC performed with CBA-1, CBA-2, CBA-4, and CBA-5 provided compounds 13a, b, c, and d, respectively. (b) Upper panel: Fluorescence kinetics obtained with alkyne carbapenems CBA-1, CBA-2, CBA-4, and CBA-5. Lower panel: Calibration curves obtained with purified triazole compounds, 13a to 13d. Synthesis and characterization of these triazole compounds are reported

For this purpose, the Ldt_{fm} -carbapenem adducts were purified by gel filtration to remove the excess of unreacted carbapenem and incubated for 1 h and 3 h at 25 °C. This analysis showed that acylenzymes resulting from acylation of Ldt_{fm} by CBA-2, CBA-4, CBA-5, and the clinically-used carbapenem meropenem (Figure 1C) were stable (Figure 3). In addition, native Ldt_{fm} resulting from hydrolysis of the carbapenem- Ldt_{fm} thioester was not detected in the mass spectra. In contrast, partial hydrolysis *ca.* 20% was detected for CBA-1 since the ratio of peak intensities was in the order of 0.2 and 0.8 for native Ldt_{fm} and the Ldt_{fm} -CBA-1 acylenzyme, respectively. Since Ldt_{fm} was only partially acylated by CBA-3 this alkyne carbapenem was not selected for further studies.

Reactivity of alkyne-carbapenems in the Cu^{I} -catalyzed cycloaddition. The success of our click and release strategy relies on both the stability of the acylenzyme (above) and on the efficiency of the cycloaddition reaction (step 2; Figure 2). Kinetics of CuAAC were performed with a fluorogenic azido partner (3-azido-7-hydroxycoumarin)²⁷⁻²⁸ to compare the impact of the linkers present in the four alkyne carbapenems (Figure 4).

in the Supporting Information, Section 4 (c) HPLC analysis of **CBA-2** (top panel) and of the crude reaction products after 30 min of incubation (see Supporting Figure S1, S2, and S3 for the reactions involving **CBA-1**, **CBA-4**, and **CBA-5**). (d) Efficiency parameters. [a] Initial rate of CuAAC determined by fluorimetry (panel B). [b] Conversion (%) was determined by HPLC (panel C).

The rate of the cycloaddition reaction in the presence of CuSO_4 (at 25 μM), was higher for **CBA-2** ($2.00 \pm 0.02 \mu\text{M min}^{-1}$) than for **CBA-1**, **CBA-4**, and **CBA-5** (0.56 to 0.90 $\mu\text{M min}^{-1}$). Fluorescence reached a maximum after a 30 min reaction. To evaluate the yield of the reaction for each alkyne carbapenem, 7-hydroxy-3-azidocoumarin was allowed to react with alkyne carbapenems for 30 min and the progress of the reaction was followed by HPLC. The yield was the highest for **CBA-2** (85%), intermediate for **CBA-5** (59%), and the lowest for **CBA-1** and **CBA-4** (21%). The high reactivity of **CBA-2** might be accounted for by the chelating effect of the urea linker on the copper catalyst as observed for other chelating functions.²⁸ This alkyne carbapenem was selected for further experiments as the best candidate based on stability of the acylenzyme (Figure 3) and its efficiency in the cycloaddition reaction (Figure 4).

Kinetics of Ldt_{fm} acylation by alkyne carbapenem **CBA-2.** Our next objective was to evaluate the impact of the incorporation of a urea-linked alkyne function in **CBA-2** on the inactivation of Ldt_{fm} by this carbapenem. Kinetics based on variation in the intrinsic fluorescence of tryptophane residues of Ldt_{fm} upon acylation were monitored for

the reference carbapenem meropenem and **CBA-2** (Figure 5). **CBA-2** proved to be superior to meropenem both with respect to the rate constant k_1 for formation of the amine anion (0.426 ± 0.009 versus $0.168 \pm 0.001 \mu\text{M}^{-1} \text{min}^{-1}$) and the rate constant k_2 for formation of the acylenzyme from this intermediate (3.30 ± 0.05 versus $2.24 \pm 0.03 \text{min}^{-1}$) (Figure 5). In agreement with mass spectrometry analysis (Figure 3), hydrolysis kinetics obtained by spectrophotometry revealed that the acylenzymes formed with **CBA-2** and meropenem display a similar stability (half-lives 323 ± 2 and 504 ± 4 min, respectively). These values are higher than expected from the mass spectrometry analysis reported in Figure 3, which revealed that Ldt_{fm} remained fully acylated after 3 h of incubation. This discrepancy may indicate that the free unreacted carbapenems were not fully eliminated by the gel filtration step used in the mass spectrometry analysis (Figure 3) enabling re-acylation after acylenzyme hydrolysis. These results indicate that we have successfully functionalized the carbapenem core without impairing the efficiency of Ldt_{fm} acylation or the stability of the resulting adduct.

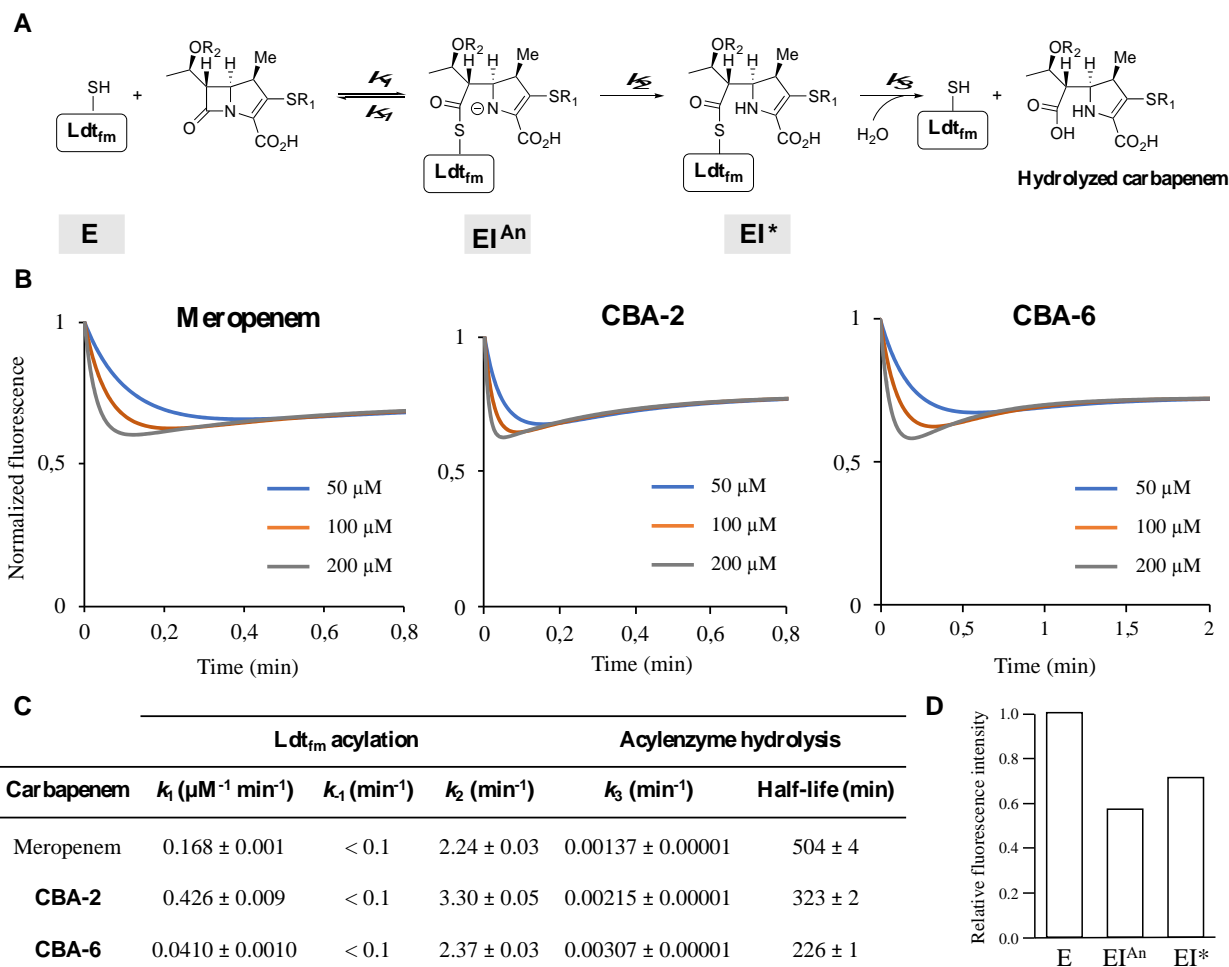


Figure 5. Kinetic parameters for Ldt_{fm} acylation by carbapenems. R₂ = H for **CBA-2** and a diazirine moiety for **CBA-6**. (a) Reaction scheme for the two-step acylation reaction. R₂ = H for **CBA-2** and a diazirine moiety for **CBA-6**. (b) Fluorescent kinetics obtained with three concentrations of carbapenem. (c) Kinetic parameters for the acylation reaction. (d) Relative fluorescence of the three forms of Ldt_{fm}. Biphasic fluorescence kinetics are accounted for by the low fluorescence intensity of the amine anion intermediate.²⁹⁻³⁰

Capture of Ldt_{fm} and release of the fluorescent protein adduct. **CBA-2** was used to explore the efficacy of step 3 (capture) and step 4 (release) of our click and release strategy (Figure 2). Ldt_{fm} (0.65 nmole) was acylated with 6 equivalents of **CBA-2** (Figure 2, step 1). Excess alkyne-carbapenem was removed by gel filtration (G25 microspin). The CuAAC reaction was performed with 10.8 equivalents of the azido-platform^{21, 22} leading to the **CBA-2**-Ldt_{fm} adduct (Figure 2, step 2). The platform provided a biotin handle for capture of the Ldt_{fm}-**CBA-2** adduct by streptavidin-coated magnetic beads (Figure 2, step 3). Following extensive washing, the click and release reaction was performed with 60 equivalents of the TAMRA fluorescent cyclooctyne (Figure 2, step 4). The latter reaction enabled elution and labeling of the Ldt_{fm} adduct in a single step. SDS-PAGE analysis (Figure 6A) showed that TAMRA-labeled Ldt_{fm} was successfully eluted from the streptavidin beads.

Assessing the specificity of the capture and release reactions. As a negative control, we used FemX, a tRNA-dependent aminocyl transferase that does not interact

with carbapenems. This protein was not recovered from streptavidin beads indicating that the procedure was specific to the Ldt_{fm} carbapenem-target (Figure 6B). The procedure was also specific in recovering Ldt_{fm} from a reconstituted mixture containing a whole protein extract from *E. faecium* (18.6 μg of protein) and 0.65 nmole of Ldt_{fm} (18.8 μg) (Figure S5 in Supporting information).

Specificity was further evaluated by preincubating Ldt_{fm} with meropenem to block the active-site Cys and prevent acylation by the **CBA-2** alkyne carbapenem. This pretreatment almost completely abolished recovery of fluorescent Ldt_{fm} from streptavidin beads (Figure 6C). In addition, we showed that recovery of TAMRA-labeled Ldt_{fm} required the native carbapenem core of **CBA-2** since preincubation of this compound with carbapenemase KPC-2, a hydrolytic enzyme of the β -lactamase family, abolished detection of the fluorescent protein band at 35 kDa (Figure 6D). Recovery of the TAMRA-labeled-Ldt_{fm} was also dependent upon acylation of the active-site Cys of the enzyme since no fluorescent band was observed for an Ldt_{fm} derivative devoid

of the Cys nucleophile following substitution of this residue by Ala.

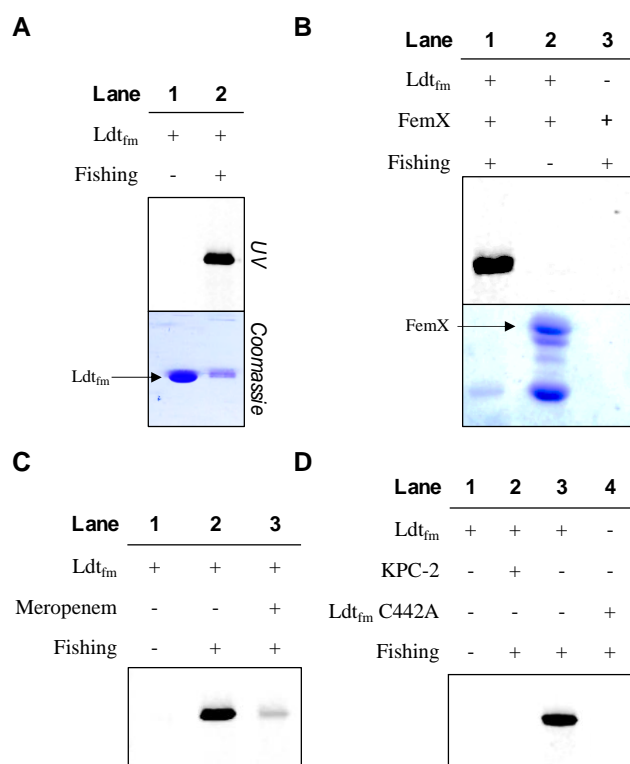


Figure 6. SDS-PAGE analyses of **CBA-2-Ldt_{fm}** adducts. (a) Click and release reactions applied to the Ldt_{fm} L,D-transpeptidase. Lane 1, 2 μg of native Ldt_{fm}; Lane 2, protein recovered from streptavidin beads. Proteins were detected by fluorescence (top panel) and Coomassie blue staining (bottom panel). (b) Assessing specificity by applying the click and release reactions to a mixture of equimolar amounts of Ldt_{fm} and FemX (Lane 1) and to FemX alone (Lane 3). Lane 2, native Ldt_{fm} and FemX proteins. (c) Assessing specificity by applying the click and release reactions to Ldt_{fm} (Lane 2) and to Ldt_{fm} preincubated with meropenem (Lane 3) to prevent acylation of the protein by the **CBA-2** alkyne carbapenem. Lane 1, native Ldt_{fm}. (d) Assessing specificity by applying the click and release reactions to native Ldt_{fm} (Lane 1), to **CBA-2** preincubated with carbapenemase KPC-2 (Lane 2) and to a derivative of Ldt_{fm} that cannot be acylated following replacement of the catalytic cysteine by alanine (Lane 4). Lane 3, click and release reactions applied to the Ldt_{fm} L,D-transpeptidase (as performed for panel A).

Bi-functionalization of the carbapenem scaffold with photoactivable and alkyne groups to improve acylenzyme stability. One possible limitation of the click and release procedure is the instability of the link connecting the drug to the target, a thioester bond in the case of Ldt_{fm}-carbapenem adducts. Indeed, the link is exposed to multiple conditions during the four steps of the click and release procedure (Figure 2), which lasts for ca. 2 hours in its current version. Thus, our next objective was to add a photoactivable group to the alkyne carbapenem in order to stabilize the drug-target adduct (Figure 7). Diazirines have been successfully used for photoaffinity labeling³¹ and for studies aimed at detecting a variety of interactions, such as

ligand-receptor,³² inhibitor-enzyme,³³ and protein-protein,³⁴ or for fishing of drug targets.³⁵⁻³⁶ The combined advantages of diazirines over other photoactivable cross-linking agents include their small size, long irradiation wavelength, short period of irradiation, and high stability in the presence of various nucleophiles.^{33, 37} Upon irradiation in the 330 to 380 nm range, diazirines form reactive carbenes, which can insert into C-H, N-H, and O-H bonds.³⁸

In our approach, synthesis of the bi-functionalized carbapenem started from the protected methyl-thienamycin,²³ which reacted with activated 3-(3-methyl-3*H*-diazirin-3-yl) propanoic acid³⁹ to give compound **14** in 92% yield (Figure 7A). Removal of the PNB group using hydrogenolysis catalyzed by platinum on carbon, followed by the functionalization of compound **15** by *N*-(prop-2-yn-1-yl)-1*H*-imidazole-1-carboxamide, afforded compound **CBA-6**.

Capture and release of labeled Ldt_{fm} was performed as described above (Figure 2) with or without an additional photoactivation step between steps 1 and 2. Mass spectrometry analysis showed formation of the additional cross-link upon irradiation that resulted in the expected loss of two nitrogen atoms (-28.0 Da, see Table S1). **CBA-6** was functional in the click and release procedure (step 1 to 4 in Figure 2) but the irradiation did not improve recovery of labeled Ldt_{fm} (Figure 7C). This result suggests that hydrolysis of the thioester bond connecting **CBA-6** to Ldt_{fm} is not limiting to the recovery of fluorescent Ldt_{fm} after the final release step.

Conclusion

The targets of β-lactams are highly diverse and complex since they include both essential biosynthetic enzymes, the transpeptidases, as well as unessential hydrolases that participate in peptidoglycan metabolism.⁴⁰ Assessing the essential nature of these transpeptidases is critical for drug development since it determines the killing activity of the drug. This task remains highly challenging despite of decades of analyses of the mode of action and of the mechanisms of resistance to β-lactams. Since the enzymes have partially redundant functions, essentiality of the targets is defined by combinations of proteins rather than a single enzyme complement. These combinations differ both between and within bacterial species following the acquisition of the so-called “low-affinity” PBPs responsible for β-lactam resistance. In addition, the complexity of these combinations includes evolutionary unrelated enzymes,⁴¹⁻⁴⁴ including unidentified ones,⁴⁵ in particular in *M. tuberculosis*.^{43, 46} This context prompted us to develop here a new strategy for affinity purification of β-lactam targets. We functionalized the carbapenem core for affinity purification of β-lactam targets using a biotin-containing iminosydnone probe. First, we introduced at the carbapenem C₂ position an alkyne function connected by a series of five linkers. A urea linker was identified as the best candidate because (i) it did not impair the kinetics of target acylation and the stability of the resulting acylenzyme and (ii) it displayed the highest reactivity for copper-catalyzed cycloaddition of a fluorogenic probe. Second, we showed that the reaction of biotinylated Ldt_{fm} adducts captured on streptavidin magnetic beads with a TAMRA-cyclooctyne

enables release and fluorescence labeling of Ldt_{fm} in a single step. Third, we synthesized bi-functionalized carbapenems containing both an alkyne and a photoactivable diazirine to introduce a second stabilizing covalent bond between Ldt_{fm} and the carbapenem. The strategy was compatible with mass-spectrometry detection of protein adducts in addition to fluorescent labeling. These tools should facilitate the characterization of the complex set of

carbapenem targets, including the detection of unknown enzymes. Access to purification strategies based on acylation of active-site nucleophiles and photoactivation, alone or in combination offers specific advantages. Photoactivation provides very stable links but may decrease the specificity. Acylation of the nucleophile active-site is highly specific but the half-life of the adduct may compromise purification of certain targets.

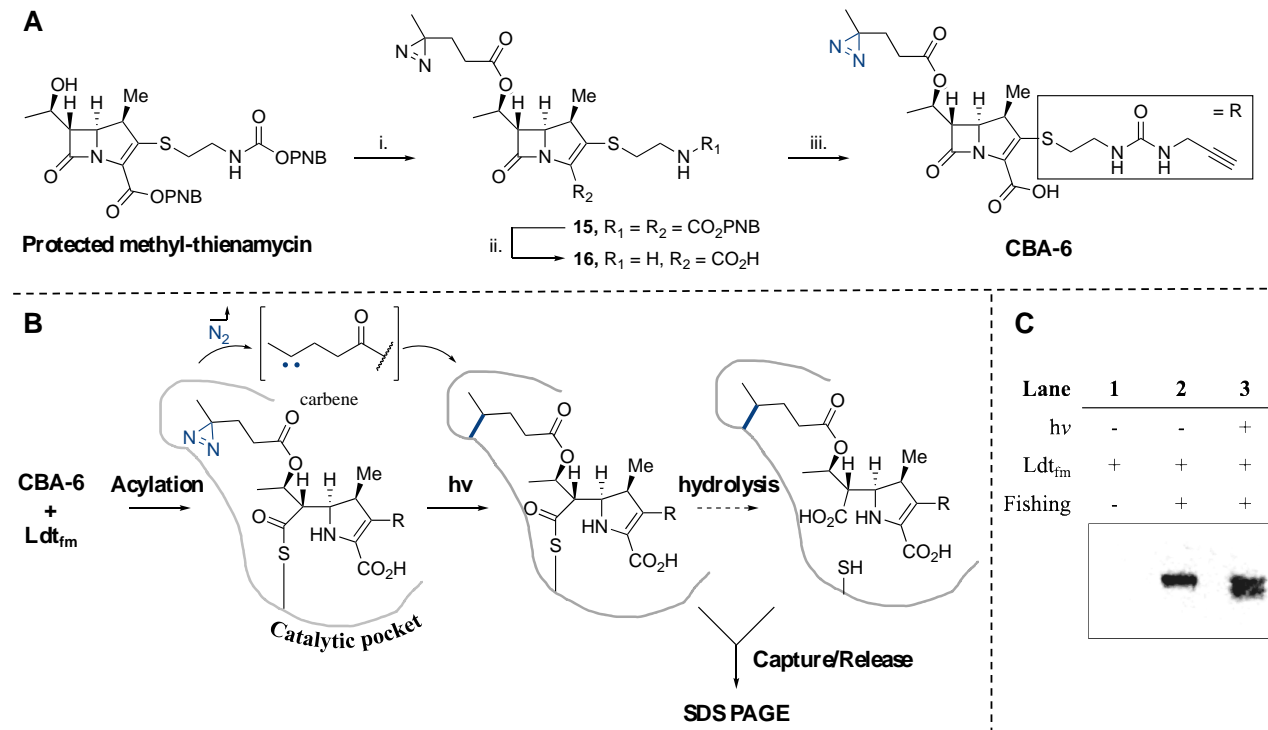


Figure 7. Synthesis and use of photoactivable carbapenem **CBA-6**. (a) Synthesis of **CBA-6**. i) 3-(3-methyl-3*H*-diazirin-3-yl) propionic acid, DMAP, EDC, -20 °C, 92%. ii) H_2 , 10% wt. Pt/C, THF/Buffer, 1h, 25%. iii) *N*-(prop-2-yn-1-yl)-1*H*-imidazole-1-carboxamide, DMF/ H_2O , $NaHCO_3$, 1h, 25 °C, 63%. (b) Acylation of Ldt_{fm} by **CBA-6** and photoactivation leading to formation of a second covalent bond stabilizing the adduct. The Ldt_{fm} -**CBA-6** interaction remains covalent even after hydrolysis of the Cys-**CBA-6** thioester bond. (c) SDS-PAGE of the click and release procedure applied to the Ldt_{fm} -**CBA-6** acylenzyme.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

AUTHOR INFORMATION

Corresponding Author

‡Mélanie Etheve-Quellejeu, melanie.etheve-quellejeu@u-paris.fr

Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, UMR 8601 CNRS, Université de Paris, 45, rue des saints-pères, Paris, F-75006, France.

‡Matthieu Fonvielle, matthieu.fonvielle@crc.jussieu.fr, INSERM UMR 1138, Sorbonne Universités, UPMC Univ Paris 06, Sorbonne Paris Cité, Université de Paris Centre de recherche des Cordeliers, Paris, F-75006, France

‡Michel Arthur, michel.arthur@crc.jussieu.fr, INSERM UMR 1138, Sorbonne Universités, UPMC Univ Paris 06, Sorbonne Paris Cité, Université de Paris Centre de recherche des Cordeliers, Paris, F-75006, France

Authors

Saidbakhrom Saidjalolov, Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, UMR 8601 CNRS, Université de Paris, 45, rue des saints-pères, Paris, F-75006, France

Emmanuelle Braud, Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, UMR 8601 CNRS, Université de Paris, 45, rue des saints-pères, Paris, F-75006, France

Zainab Edo, INSERM UMR 1138, Sorbonne Universités, UPMC Univ Paris 06, Sorbonne Paris Cité, Université de Paris Centre de recherche des Cordeliers, Paris, F-75006, France

Laura Iannazzo, Laboratoire de Chimie et Biochimie Pharmacologiques et Toxicologiques, UMR 8601 CNRS, Université de Paris, 45, rue des saints-pères, Paris, F-75006, France

Filippo Rusconi, PAPPSO, Université Paris-Saclay, INRAE, CNRS, AgroParisTech, GQE - Le Moulon, 91190, Gif-sur-Yvette, France.

Margaux Riomet, Université Paris Saclay, CEA, INRAE, Département Médicaments et Technologies pour la Santé (DMTS), SCBM, F-91191 Gif-sur-Yvette, France.

Antoine Sallustrau, Université Paris Saclay, CEA, INRAE, Département Médicaments et Technologies pour la Santé (DMTS), SCBM, F-91191 Gif-sur-Yvette, France.

Frédéric Taran, Université Paris Saclay, CEA, INRAE, Département Médicaments et Technologies pour la Santé (DMTS), SCBM, F-91191 Gif-sur-Yvette, France.

Present Addresses

† If an author's address is different than the one given in the affiliation line, this information may be included here.

Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript. / ‡ These authors contributed equally.

Funding Sources

Ministère de l'Enseignement supérieur, de la Recherche et de l'Innovation for S. S. PhD grant.

Notes

Any additional relevant notes should be placed here.

ACKNOWLEDGMENT

ABBREVIATIONS

CuAAC, copper-catalyzed alkyne-azide cycloaddition; DIPEA, *N,N*-diisopropylethylamine; DMAP, *N*-dimethylamino-pyridine; DMF, *N*-dimethylformamide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HPLC, high performance liquid chromatography; KPC-2, *Klebsiella pneumoniae* carbapenemases; Ldt, L,D-transpeptidases; PB, phosphate buffer; PBPs, penicillin-binding proteins; TAMRA, 5-Carboxy-tetramethylrhodamine; THF, tetrahydrofuran; THPTA, tris(3-hydroxypropyltriazolylmethyl)amine.

REFERENCES

- Zapun, A.; Contreras-Martel, C.; Vernet, T., Penicillin-binding proteins and beta-lactam resistance. *FEMS Microbiol. Rev.* **2008**, *32*, 361-385.
- Gold, B.; Smith, R.; Nguyen, Q.; Roberts, J.; Ling, Y.; Lopez Quezada, L.; Somersan, S.; Warriar, T.; Little, D.; Pingle, M.; Zhang, D.; Ballinger, E.; Zimmerman, M.; Dartois, V.; Hanson, P.; Mitscher, L. A.; Porubsky, P.; Rogers, S.; Schoenen, F. J.; Nathan, C.; Aube, J., Novel Cephalosporins Selectively Active on Nonreplicating Mycobacterium tuberculosis. *J. Med. Chem.* **2016**, *59*, 6027-6044.
- Tipper, D. J.; Strominger, J. L., Mechanism of Action of Penicillins - a Proposal Based on Their Structural Similarity to Acyl-D-Alanyl-D-Alanine. *Prot. Natl. Acad. Sci. USA* **1965**, *54*, 1133.
- Mainardi, J. L.; Hugonnet, J. E.; Rusconi, F.; Fourgeaud, M.; Dubost, L.; Mouri, A. N.; Delfosse, V.; Mayer, C.; Gutmann, L.; Rice, L. B.; Arthur, M., Unexpected inhibition of peptidoglycan LD-transpeptidase from *Enterococcus faecium* by the beta-lactam imipenem. *J. Biol. Chem.* **2007**, *282*, 30414-30422.
- Lavollay, M.; Arthur, M.; Fourgeaud, M.; Dubost, L.; Marie, A.; Veziris, N.; Blanot, D.; Gutmann, L.; Mainardi, J. L., The peptidoglycan of stationary-phase Mycobacterium tuberculosis predominantly contains cross-links generated by L,D-transpeptidation. *J. Bacteriol.* **2008**, *190*, 4360-4366.
- Hugonnet, J. E.; Mengin-Lecreux, D.; Monton, A.; den Blaauwen, T.; Carbonnelle, E.; Veckerle, C.; Brun, Y. V.; van Nieuwenhze, M.; Bouchier, C.; Tu, K.; Rice, L. B.; Arthur, M., Factors essential for L,D-transpeptidase-mediated peptidoglycan cross-linking and beta-lactam resistance in *Escherichia coli*. *Elife* **2016**, *5*.
- Mainardi, J. L.; Legrand, R.; Arthur, M.; Schoot, B.; van Heijenoort, J.; Gutmann, L., Novel mechanism of beta-lactam resistance due to bypass of DD-transpeptidation in *Enterococcus faecium*. *J. Biol. Chem.* **2000**, *275*, 16490-16496.
- Preston, D. A.; Wu, C. Y. E.; Blaszczyk, L. C.; Seitz, D. E.; Halligan, N. G., Biological Characterization of a New Radioactive Labeling Reagent for Bacterial Penicillin-Binding Proteins. *Antimicrob. Agents Chemother.* **1990**, *34*, 718-721.
- Zhao, G. S.; Meier, T. I.; Kahl, S. D.; Gee, K. R.; Blaszczyk, L. C., BOCILLIN FL, a sensitive and commercially available reagent for detection of penicillin-binding proteins. *Antimicrob. Agents Chemother.* **1999**, *43*, 1124-1128.
- June, C. M.; Vaughan, R. M.; Ulberg, L. S.; Bonomo, R. A.; Witucki, L. A.; Leonard, D. A., A fluorescent carbapenem for structure function studies of penicillin-binding proteins, beta-lactamases, and beta-lactam sensors. *Anal Biochem.* **2014**, *463*, 70-74.
- Beatty, K. E., Fluorescent probes for investigating peptidoglycan biosynthesis in mycobacteria. *Curr. Opin. Chem. Biol.* **2020**, *57*, 50-57.
- Wright, M. H.; Sieber, S. A., Chemical proteomics approaches for identifying the cellular targets of natural products. *Nat. Prod. Rep.* **2016**, *33*, 681-708.
- Staub, I.; Sieber, S. A., Beta-lactams as selective chemical probes for the in vivo labeling of bacterial enzymes involved in cell wall biosynthesis, antibiotic resistance, and virulence. *J. Am. Chem. Soc.* **2008**, *130*, 13400-13409.
- Staub, I.; Sieber, S. A., Beta-lactam probes as selective chemical-proteomic tools for the identification and functional characterization of resistance associated enzymes in MRSA. *J. Am. Chem. Soc.* **2009**, *131*, 6271-6276.
- Romeo, E.; Ponzano, S.; Armirotti, A.; Summa, M.; Bertozzi, F.; Garau, G.; Bandiera, T.; Piomelli, D., Activity-Based Probe for N-Acylethanolamine Acid Amidase. *ACS Chem. Biol.* **2015**, *10*, 2057-2064.
- Chen, X.; Wang, Y.; Ma, N.; Tian, J.; Shao, Y.; Zhu, B.; Wong, Y. K.; Liang, Z.; Zou, C.; Wang, J., Target identification of natural medicine with chemical proteomics approach: probe synthesis, target fishing and protein identification. *Signal Transduct. Target Ther.* **2020**, *5*, 72.
- Ding, Y.; Li, Z.; Xu, C.; Qin, W.; Wu, Q.; Wang, X.; Cheng, X.; Li, L.; Huang, W., Fluorogenic Probes/Inhibitors of beta-Lactamase and their Applications in Drug-Resistant Bacteria. *Angew. Chem. Int. Ed.* **2020**, *132*, 2-19.
- Kocaoglu, O.; Calvo, R. A.; Sham, L. T.; Cozy, L. M.; Lanning, B. R.; Francis, S.; Winkler, M. E.; Kearns, D. B.; Carlson, E. E., Selective penicillin-binding protein imaging probes reveal substructure in bacterial cell division. *ACS Chem. Biol.* **2012**, *7*, 1746-1753.
- Das, S.; Ihssen, J.; Wick, L.; Spitz, U.; Shabat, D., Chemiluminescent Carbapenem-Based Molecular Probe for Detection of Carbapenemase Activity in Live Bacteria. *Chem. Eur. J.* **2020**, *26*, 3647-3652.
- Wang, J.; Xu, W.; Xue, S.; Yu, T.; Xie, H., A minor structure modification serendipitously leads to a highly carbapenemase-specific fluorogenic probe. *Org. Biomol. Chem.* **2020**, *18*, 4029-4033.
- Bernard, S.; Audisio, D.; Riomet, M.; Bregant, S.; Sallustrau, A.; Plougastel, L.; Decuypere, E.; Gabillet, S.; Kumar, R. A.; Elyian, J.; Trinh, M. N.; Koniev, O.; Wagner, A.; Kolodych, S.; Taran, F., Bioorthogonal Click and Release Reaction of Iminosydones with Cycloalkynes. *Angew. Chem. Int. Ed.* **2017**, *56*, 15612-15616.
- Decuypere, E.; Plougastel, L.; Audisio, D.; Taran, F., Sydnone-alkyne cycloaddition: applications in synthesis and bioconjugation. *Chem. Commun.* **2017**, *53*, 11515-11527.
- Iannazzo, L.; Soroka, D.; Triboulet, S.; Fonvielle, M.; Compain, F.; Dubee, V.; Mainardi, J. L.; Hugonnet, J. E.; Braud, E.; Arthur, M.; Etheve-Quellejeu, M., Routes of Synthesis of Carbapenems for Optimizing Both the Inactivation of L,D-Transpeptidase LdtMt1 of *Mycobacterium tuberculosis* and the Stability toward Hydrolysis by beta-Lactamase BlaC. *J. Med. Chem.* **2016**, *59*, 3427-3438.

24. Papp-Wallace, K. M.; Endimiani, A.; Taracila, M. A.; Bonomo, R. A., Carbapenems: past, present, and future. *Antimicrob. Agents Chemother.* **2011**, *55*, 4943-4960.
25. Kumagai, T.; Abe, T.; Fujimoto, Y.; Hayashi, T.; Inoue, Y.; Nagao, Y., Mild and Chemoselective Cleavage of P-Nitrobenzyl Esters and p-Nitrobenzyloxycarbonyl Amines with Zinc Dust. *Heterocycles* **1993**, *36*, 1729-1734.
26. Rillahan, C. D.; Schwartz, E.; Rademacher, C.; McBride, R.; Rangarajan, J.; Fokin, V. V.; Paulson, J. C., On-chip synthesis and screening of a sialoside library yields a high affinity ligand for Siglec-7. *ACS Chem. Biol.* **2013**, *8*, 1417-1422.
27. Kislukhin, A. A.; Hong, V. P.; Breitenkamp, K. E.; Finn, M. G., Relative performance of alkynes in copper-catalyzed azide-alkyne cycloaddition. *Bioconjug. Chem.* **2013**, *24*, 684-9.
28. Bevilacqua, V.; King, M.; Chaumontet, M.; Nothisen, M.; Gabillet, S.; Buisson, D.; Puente, C.; Wagner, A.; Taran, F., Copper-chelating azides for efficient click conjugation reactions in complex media. *Angew. Chem. Int. Ed.* **2014**, *53*, 5872-5876.
29. Bhattacharjee, N.; Triboulet, S.; Dubee, V.; Fonvielle, M.; Edo, Z.; Hugonnet, J. E.; Etheve-Quellejeu, M.; Simorre, J. P.; Field, M. J.; Arthur, M.; Bougault, C. M., Negative Impact of Carbapenem Methylation on the Reactivity of beta-Lactams for Cysteine Acylation as Revealed by Quantum Calculations and Kinetic Analyses. *Antimicrob. Agents Chemother.* **2019**, *63*, e02039-18.
30. Triboulet, S.; Edo, Z.; Compain, F.; Ourghanlian, C.; Dupuis, A.; Dubee, V.; Sutterlin, L.; Atze, H.; Etheve-Quellejeu, M.; Hugonnet, J. E.; Arthur, M., Tryptophan Fluorescence Quenching in beta-Lactam-Interacting Proteins Is Modulated by the Structure of Intermediates and Final Products of the Acylation Reaction. *Acs Infect. Dis.* **2019**, *5*, 1169-1176.
31. Blencowe, A.; Hayes, W., Development and application of diazirines in biological and synthetic macromolecular systems. *Soft Matter* **2005**, *1*, 178-205.
32. Dubinsky, L.; Jarosz, L. M.; Amara, N.; Krief, P.; Kravchenko, V. V.; Krom, B. P.; Meijler, M. M., Synthesis and validation of a probe to identify quorum sensing receptors. *Chem. Commun.* **2009**, *47*, 7378-7380.
33. Dubinsky, L.; Krom, B. P.; Meijler, M. M., Diazirine based photoaffinity labeling. *Bioorg. Med. Chem.* **2012**, *20*, 554-570.
34. Durek, T.; Zhang, J.; He, C.; Kent, S. B., Synthesis of photoactive analogues of a cystine knot trypsin inhibitor protein. *Org. Lett.* **2007**, *9*, 5497-5500.
35. Hill, J. R.; Robertson, A. A. B., Fishing for Drug Targets: A Focus on Diazirine Photoaffinity Probe Synthesis. *J. Med. Chem.* **2018**, *61*, 6945-6963.
36. Hulse, J. J.; Cognetta, A. B.; Niphakis, M. J.; Tully, S. E.; Cravatt, B. F., Proteome-wide mapping of cholesterol-interacting proteins in mammalian cells. *Nat. Methods* **2013**, *10*, 259-264.
37. Hatanaka, Y.; Sadakane, Y., Photoaffinity labeling in drug discovery and developments: chemical gateway for entering proteomic frontier. *Curr. Top. Med. Chem.* **2002**, *2*, 271-288.
38. Brunner, J., New Photolabeling and Cross-Linking Methods. *Annu. Rev. Biochem.* **1993**, *62*, 483-514.
39. Ahad, A. M.; Jensen, S. M.; Jewett, J. C., A traceless Staudinger reagent to deliver diazirines. *Org. Lett.* **2013**, *15*, 5060-5063.
40. Zapun, A.; Contreras-Martel, C.; Vernet, T., Penicillin-binding proteins and beta-lactam resistance. *FEMS Microbiol. Rev.* **2008**, *32*, 361-385.
41. Mainardi, J. L.; Hugonnet, J. E.; Rusconi, F.; Fourgeaud, M.; Dubost, L.; Mouti, A. N.; Delfosse, V.; Mayer, C.; Gutmann, L.; Rice, L. B.; Arthur, M., Unexpected inhibition of peptidoglycan LD-transpeptidase from *Enterococcus faecium* by the beta-lactam imipenem. *J. Biol. Chem.* **2007**, *282*, 30414-30422.
42. Lavollay, M.; Arthur, M.; Fourgeaud, M.; Dubost, L.; Marie, A.; Veziris, N.; Blanot, D.; Gutmann, L.; Mainardi, J. L., The peptidoglycan of stationary-phase *Mycobacterium tuberculosis* predominantly contains cross-links generated by L,D-transpeptidation. *J. Bacteriol.* **2008**, *190*, 4360-4366.
43. Gupta, R.; Lavollay, M.; Mainardi, J. L.; Arthur, M.; Bishai, W. R.; Lamichhane, G., The *Mycobacterium tuberculosis* protein Ldt(Mt2) is a nonclassical transpeptidase required for virulence and resistance to amoxicillin. *Nat. Med.* **2010**, *16*, 466-469.
44. Magnet, S.; Bellais, S.; Dubost, L.; Fourgeaud, M.; Mainardi, J. L.; Petit-Frere, S.; Marie, A.; Mengin-Lecreulx, D.; Arthur, M.; Gutmann, L., Identification of the L,D-transpeptidases responsible for attachment of the Braun lipoprotein to *Escherichia coli* peptidoglycan. *J. Bacteriol.* **2007**, *189*, 3927-3931.
45. Lopez Quezada, L.; Smith, R.; Lupoli, T. J.; Edo, Z.; Li, X.; Gold, B.; Roberts, J.; Ling, Y.; Park, S. W.; Nguyen, Q.; Schoenen, F. J.; Li, K.; Hugonnet, J. E.; Arthur, M.; Sacchetti, J. C.; Nathan, C.; Aube, J., Activity-Based Protein Profiling Reveals That Cephalosporins Selectively Active on Non-replicating *Mycobacterium tuberculosis* Bind Multiple Protein Families and Spare Peptidoglycan Transpeptidases. *Front. Microbiol.* **2020**, *11*, 1248.
46. Kumar, P.; Arora, K.; Lloyd, J. R.; Lee, I. Y.; Nair, V.; Fischer, E.; Boshoff, H. I.; Barry, C. E., 3rd, Meropenem inhibits D,D-carboxypeptidase activity in *Mycobacterium tuberculosis*. *Mol. Microbiol.* **2012**, *86*, 367-381.

Authors are required to submit a graphic entry for the Table of Contents (TOC)

Click and release chemistry for activity-based purification of β -lactam targets

