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Rapid Broad Spectrum Detection of Carbapenemases with a Dual Fluorogenic-Colorimetric Probe

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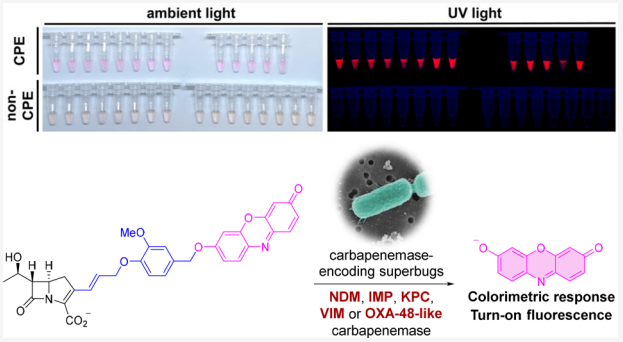
ABSTRACT: Carbapenems stand as one of the last-resort antibiotics; however, their efficacy is threatened by the rising number and rapid spread of carbapenemases. Effective antimicrobial stewardship thus calls for rapid tests for these enzymes to aid appropriate prescription and infection control. Herein, we report the first effective pan-carbapenemase reporter **CARBA-H** with a broad scope covering all three Ambler classes. Using a chemical biology approach, we demonstrated that the absence of the 1β-substituent in the carbapenem core is key to pan-carbapenemase recognition, which led to our rational design and probe development. **CARBA-H** provides a dual colorimetric-fluorogenic response upon carbapenemase-mediated hydrolysis. A clear visual readout can be obtained within 15 min when tested against a panel of carbapenemase-producing Enterobacteriaceae (CPE) clinical isolates that notably includes OXA-48 and OXA-181-producing strains. Furthermore, **CARBA-H** can be applied to the detection of carbapemenase activity in CPE-spiked urine samples.



INTRODUCTION

Carbapenems are one of the most effective broad-spectrum drugs-of-last-resort against bacterial infection. However, the emergence and dissemination of resistance toward carbapenems in recent decades have become a major public health issue worldwide.[1] Microbial production of carbapenemases, enzymes that catalyze the hydrolytic degradation of carbapenems, in addition to virtually all other β-lactams, represents the most clinically important resistance mechanism in carbapenem-resistant bacteria.[2]

Carbapenemases belong to the larger family of β-lactamases and are categorized based on their amino acid sequences and hydrolysis mechanisms under the Ambler classification.[2,3] Carbapenemases in Classes A (e.g., KPC) and D (e.g., OXA-48-like) are serine hydrolases, and those in Class B (e.g., NDM, IMP, VIM) are Zn-dependent metallo-β-lactamases. Over 200 unique carbapenemases have been identified to date, and the numbers are ever increasing.[2]

These carbapenemases are usually encoded on mobile genetic elements which greatly facilitate the transmission of the resistance, even across different bacterial species, through horizontal gene transfer.[2] Effective infection control programs are therefore key to curtail their spread in hospitals and dissemination into the community.[1] Patients infected by carbapenem-resistant bacteria may not show distinct symptoms, yet delay in effective treatment can lead to life-threatening complications like sepsis.[4,5] This poses a great challenge for clinicians in choosing the appropriate antibiotics from the hierarchy of therapeutic options. Rapid identification of carbapenemases therefore provides the bedrock for both infection control and appropriate antibiotic prescription, two main pillars in antimicrobial stewardship.

Despite their sensitivity in detecting certain carbapenemase-encoding genes, genotypic tests generally suffer from high cost, labor-intensive protocols and requirement of specialized instrumentation.[6] Furthermore, β-lactamases within the OXA-family that exhibit diverse carbapenemase activities may not be readily distinguished by polymerase chain reaction (PCR). Immunochromatographic assays, including RESIST-4 and CARBA 5, which do not require sophisticated instruments, have also been developed.[6] However, the scope of these assays are delimitated by recognizing particular fragment of DNA or protein sequence, and novel mutations may evade their detection. Together, these shortcomings urgently call for a reliable activity-based phenotypic assay.[7,8]

The only rapid phenotypic test for carbapenemase detection approved by Clinical & Laboratory Standards Institute (CLSI)

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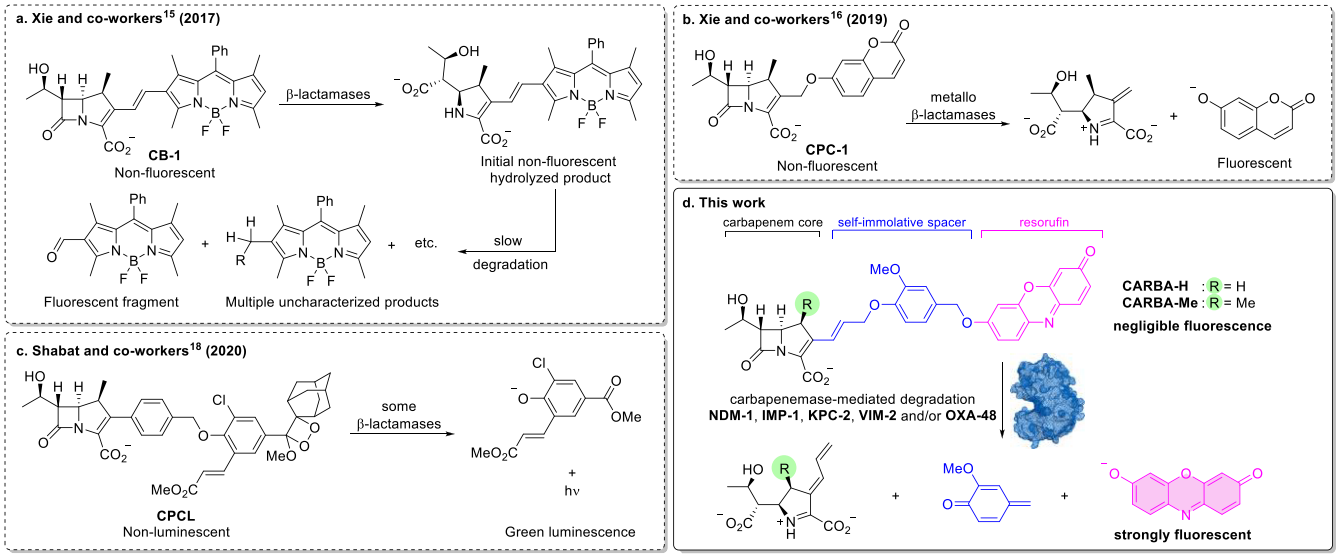

Figure 1. Previously reported carbapenem-based turn-on fluorogenic probes and the current design.

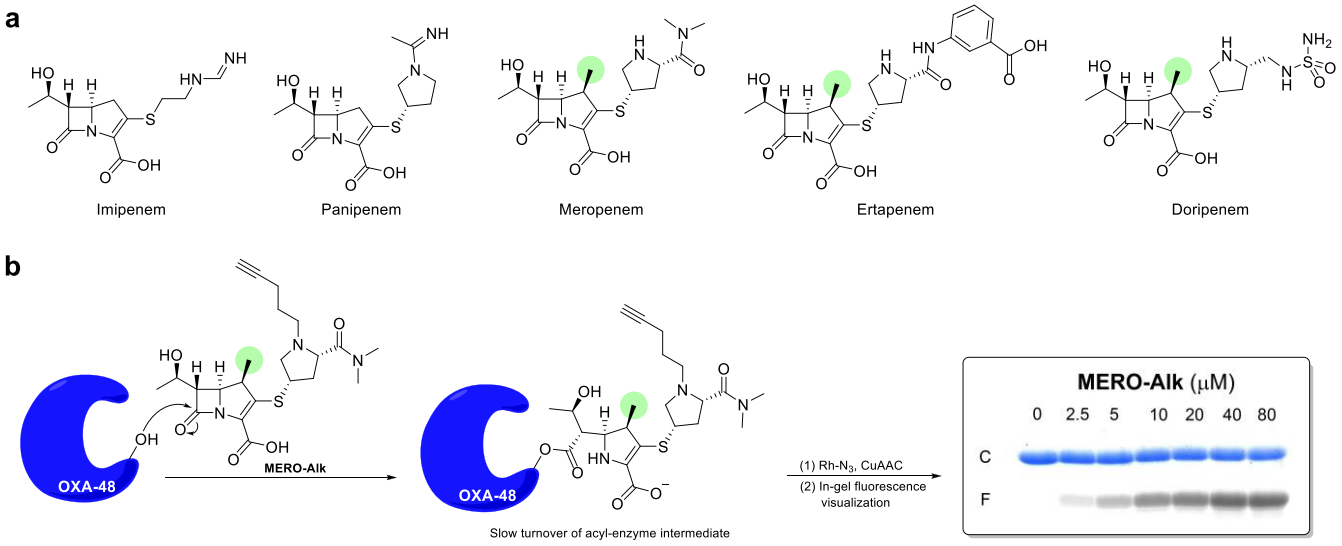

Figure 2. (a) Structures of some common carbapenem antibiotics. The 1β-methyl substituent was highlighted with a green background. (b) Recombinant OXA-48 (5 μM) was incubated with **MERO-Alk** at different concentrations, followed by CuAAC and SDS-PAGE analysis. Coomassie blue staining was indicated with **C** and in-gel fluorescence visualization was indicated with **F**.

to date is CarbaNP.[9,10] While nominally it can offer a result in 2 h, the pH-based colorimetric assay is highly insensitive. Notably, false-negative results have been reported in the detection of OXA-48 (sensitivity as low as 11%) and enzymes possessing lower carbapenemase activities like SME and GES.[7,9,10] Indeterminate results can also arise due to the subtlety of the color difference, thus invalidating the test in those circumstances. Since its identification, OXA-48 has emerged as one of the most clinically important carbapenemases,[11−14] yet none of the existing chemical probes could address the challenge of its detection.[9,10,15−19] In addition, certain carbapenem-derived fluorescent probes reported in the literature only responded to particular classes of carbapenemases[16] (Figure 1a−c), underscoring the fact that careful consideration must be put into probe design in order to cover the entire range of carbapenemases. Noting the above shortcomings, we endeavor to design a fluorogenic substrate that selectively turns on toward a broad spectrum of carbapenemases, encompassing the OXA-48-like carbapenemases in particular, and remains unresponsive toward non-carbapenemases.

RESULTS AND DISCUSSION

Hypothesis and Preliminary Observation. The failure of OXA-48 detection by existing phenotypic tests points to a poor enzyme−substrate interaction, thus, we aimed to discover a molecular architecture that favors the binding and recognition. We noticed from the literature that OXA-48 exhibited at least 2 orders of magnitude higher catalytic efficiency (k_{cat}/K_M) toward hydrolysis of imipenem and panipenem than that of meropenem, ertapenem, and doripenem.[20,21] Although these carbapenems bear different side chains, we recognized a dichotomous feature in which a 1β-methyl substituent is present at the carbapenem core in the latter group but not in the former (Figure 2a). While the 1β-methyl substituent is crucial for the suppression of hydrolytic

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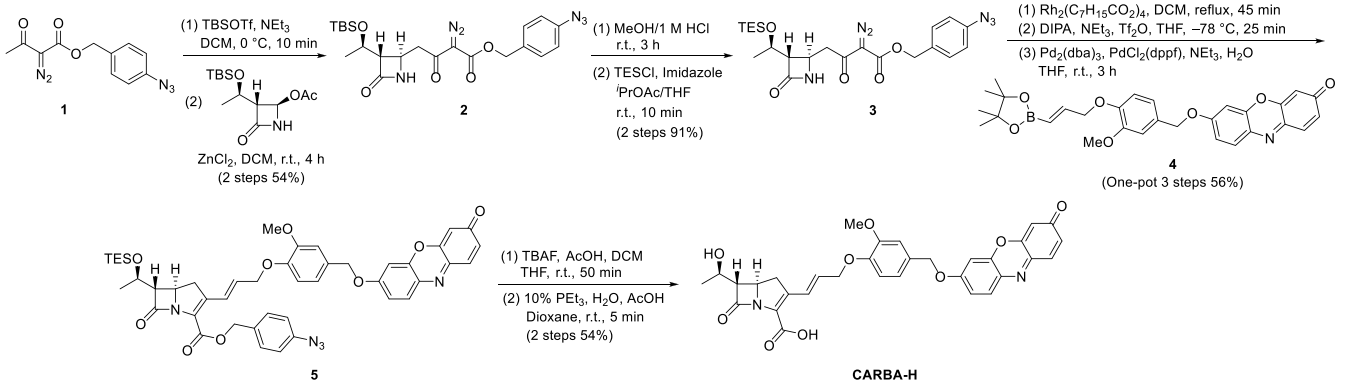
Scheme 1. Summary of Synthesis Toward CARBA-H



degradation of carbapenems by human peptidase DHP-1 in the renal brush border,[22,23] it may account for the failure of existing carbapenem-based sensors in detecting OXA-48. Meanwhile, Schofield and co-workers' molecular dynamic simulations provided *in silico* evidence that the 1β-methyl substituent in doripenem hindered the hydrolysis of the enzyme−substrate complex in OXA-1, a non-carbapenemase homologue of OXA-48.[24] We therefore hypothesized that the 1β-methyl substituent in carbapenems stabilizes the acyl-enzyme intermediate in OXA-48 and impedes the enzymatic turnover.

To provide experimental evidence, we prepared an alkyne-appended meropenem derivative, **MERO-Alk**, as a representative 1β-methyl bearing substrate for preliminary investigations (Figure 2b). If a stable covalent adduct is formed with OXA-48, the enzyme can be fluorescently labeled upon conjugation to an azido fluorophore using copper-catalyzed azide−alkyne cycloaddition (CuAAC). Accordingly, recombinant OXA-48 was incubated with different concentrations of **MERO-Alk** for 30 min, followed by treatment with rhodamine-azide (Rh−N_3) under CuAAC conditions, and the results were evaluated using in-gel fluorescent visualization. In line with our hypothesis, an increase in fluorescence was observed in the presence of **MERO-Alk** in a dose dependent manner, suggesting that **MERO-Alk** forms a covalent adduct with OXA-48 (Figure 2b).

To validate whether the adduct formation preferentially takes place with 1β-methyl substituted carbapenems, we performed competition assays with meropenem and ertapenem (both bearing a 1β-methyl group) and imipenem (without 1β-methyl group). Recombinant OXA-48 was preincubated with the aforementioned carbapenems (30 min) before the addition of **MERO-Alk**. In-gel visualization showed that the fluorescence signal can be depleted by meropenem and ertapenem but not by imipenem, strongly indicating that the covalent modification took place in the enzyme active site selectively with carbapenems featuring a 1β-methyl group (Figure S1a−c). Analogous labeling experiments were conducted with a panel of purified recombinant carbapenemases which are clinically relevant. Neither carbapenemases from other classes (i.e., Classes A and B) nor heat-denatured OXA-48 but only catalytically active OXA-48 could be fluorescently tagged, consistent with the notion that the covalent modification corresponds to the formation of the acyl intermediate and is specific toward OXA-48 (Figure S1d).

We further confirmed the acylation indeed took place at the active site by a tandem mass spectrometry approach. OXA-48 was incubated with **MERO-Alk**, followed by CuAAC with a commercially available acid-cleavable biotin-azide. The acylated OXA-48 was enriched by streptavidin−biotin enrichment followed by trypsin digestion. The modified peptide was cleaved and analyzed by LC-MS/MS, and the fragment search helped us identify that the modification took place in the active site (Figure S2). This mechanistic investigation uncovered a novel role of the 1β-methyl substituent in carbapenems and provided a rationale for our probe design.

In addition, we hope to integrate another structural feature in our design to achieve generality for pan-carbapenemase detection. It has been observed that the hydrolysis kinetics of certain β-lactamases are sensitive to substituent effects in the proximity of the β-lactam ring.[16,25−27] As different β-lactamases operate through different hydrolytic mechanisms, their active sites also exhibit significantly diverse structures.[28] Hence, we reasoned that by placing fluorophores, usually featuring extended π-systems that are electronically dissimilar to the polar side chains commonly found in carbapenems, further away from the core would allow probe recognition by a wider range of carbapenemases.

Probe Design and Synthesis. To this end, we aspired to construct our carbapenemase probes from three key building blocks, namely, a fluorogenic unit, a self-immolative spacer, and a carbapenem core. Resorufin was selected as the fluorogenic unit, as it not only demonstrates a strong turn-on fluorescence upon uncaging but also exhibits long excitation and emission wavelengths, which minimize undesired interference from autofluorescent biological molecules.[29−31] In addition, upon release, it also provides a colorimetric change that can be captured by naked eye, thus precluding the need of extra instrumentation. A self-immolative allylic-hydroxybenzylic linker was used to bridge the fluorogenic unit and the carbapenem core, which should steer the fluorogenic unit away from the core and enable a broader carbapenemase recognition. To pin down the role of the 1β-methyl group as a stereochemical determinant for the selectivity in OXA-48, we designed two target molecules, namely, **CARBA-H** and **CARBA-Me**, with and without the 1β-methyl substituent in the carbapenem core, respectively (Figure 1d).

A modular synthetic strategy was adopted (Scheme 1), which allows for flexibility in the installation of alternative chromophores if other emission wavelengths or detection approaches are desired. The linker-fluorophore fragment **4** was prepared from propargyl alcohol through an 8-step sequence that terminates with the attachment of resorufin (Scheme S1). For the carbapenem fragment, we adopted the azidobenzyl ester as a carboxylic acid protecting group.[32,33] It provides

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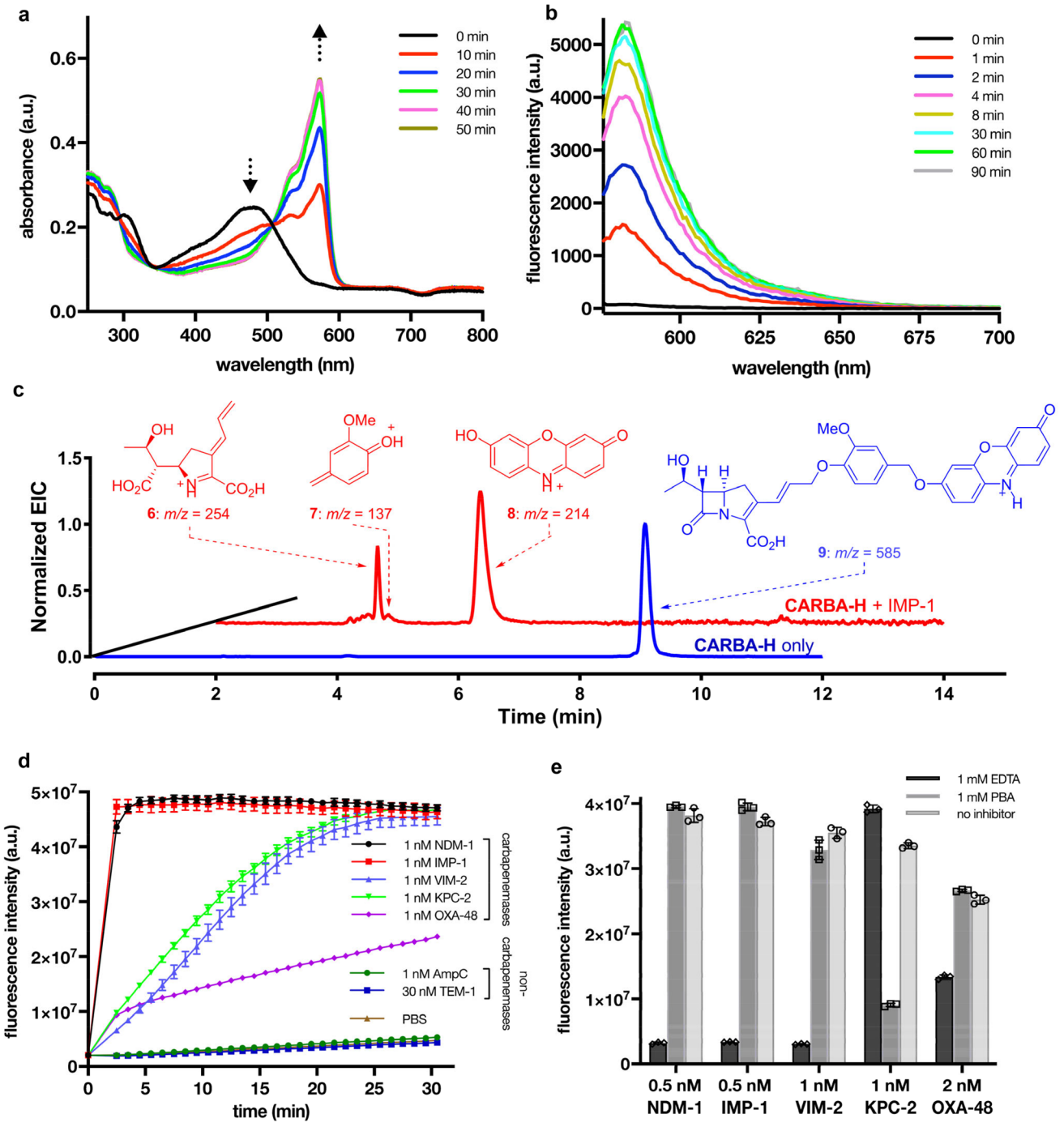

Figure 3. (a) Change in absorbance over time of **CARBA-H** (10 μM in PBS, pH 7.4) in the presence of IMP-1 (2 nM). (b) Fluorescence change over time of **CARBA-H** (1 μM in PBS, pH 7.4) toward IMP-1 (0.5 nM). λ_{ex} = 571 nm. (c) Extracted ion chromatogram of HPLC-ESI MS analysis. **CARBA-H** (15 μM) hydrolyzed by IMP-1 (10 nM) in NH_4OAc buffer (10 mM, pH 7.4), normalized to the largest peak. Observed peaks with selected mass-to-charge ratio (m/z) corresponding to the expected fragments were indicated. (d) Fluorescence change over time of **CARBA-H** (2 μM in PBS, pH 7.4) toward selected β-lactamases. λ_{ex} = 564 nm; λ_{em} = 629 nm. The error bars correspond to the standard deviation of the three replicates. (e) Fluorescence change of **CARBA-H** (2 μM in PBS, pH 7.4) in the presence of selected carbapenemases and β-lactamase inhibitors incubated for 30 min. λ_{ex} = 564 nm; λ_{em} = 629 nm. The error bars correspond to the standard deviation of the three replicates.

orthogonality toward other protecting groups, tolerates a wide range of transformations, and can be readily removed by Staudinger reduction. For **CARBA-H**, a Lewis acid ($ZnCl_2$)-mediated Mannich reaction yielded azetidinone **2** as a single diastereomer. Subsequent protecting group manipulations afforded precursor **3**. After rhodium-catalyzed ring closure and subsequent derivatization of the ensuing ketone to a vinyl triflate, fragments **3** and **4** were united utilizing a late-stage Suzuki coupling, catalyzed by a 1:1 mixture of Pd_2dba_3 and $Pd(dppf)Cl_2$ under the optimized conditions to provide **5**. **CARBA-H** was successfully obtained upon global deprotection, and the control probe **CARBA-Me** was synthesized in an analogous fashion (Scheme S2).

β-Lactamase Response toward CARBA-H and CARBA-Me. With the probes in hand, we first proceeded to measure the photophysical change of **CARBA-H** upon β-lactam ring

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opening by IMP-1, a typical carbapenemase. Over the course of reaction between **CARBA-H** (10 μM) and IMP-1 (2 nM), a decrease in absorbance at 480 nm was observed along with a simultaneous increase at 571 nm (Figure 3a). The new absorption maximum coincided with the reported value of resorufin.[31] A single isosbestic point at 507 nm implied that no intermediate was formed during the hydrolysis (Figure 3a). The presence of the expected fragmentation products was confirmed using HPLC-ESI MS (Figure 3c). Expectedly, the addition of IMP-1 resulted in the disappearance of signal corresponding to protonated **CARBA-H** (**9**), and the appearance of those corresponding to the protonated pyrrolidine **6**, quinonemethide **7** and resorufin **8**. For the fluorescence change, **CARBA-H** (1 μM) provided more than 60-fold fluorescence enhancement upon the addition of IMP-1 (0.5 nM) in only 8 min (Figure 3b). This compares very favorably with existing reports,[10,15−18] e.g., a much higher concentration (100 nM) of IMP-1 was required (*cf.* 0.5 nM IMP-1 for **CARBA-H**) to achieve a similar degree of enhancement with **CB-1** under otherwise identical conditions.[15] The pseudo-first order rate constant for the spontaneous hydrolysis reaction (PBS, pH 7.4) of **CARBA-H** was estimated to be 1.37×10^{-5} s^{-1} at room temperature, similar to that of carbapenems (Figure S3).[16,19,34,35]

We then evaluated the selectivity of the response of **CARBA-H** toward carbapenemases over other β-lactamases. Since NDM, IMP, VIM (Ambler Class B metallo-β-lactamases), KPC (Class A serine carbapenemases), and OXA-48-like carbapenemases (Class D serine carbapenemases) account for more than 99% of the carbapenemase-producing isolates,[36] a representative member from each of these families, namely, NDM-1, IMP-1, VIM-2, KPC-2, and OXA-48, were recombinantly expressed, purified, and utilized in this study to examine the carbapenemase selectivity and coverage of our probes (Figure S4). Two common non-carbapenemases, AmpC (Class C serine β-lactamase) and TEM-1 (Class A serine β-lactamase), were employed as negative controls. The fluorescence change of **CARBA-H** (2 μM) toward the aforementioned enzymes (at 1 nM concentration except 30 nM for TEM-1) was monitored over 30 min (Figure 3d). **CARBA-H** only gave significant fluorescence enhancement in the presence of carbapenemases (i.e., NDM-1, IMP-1, VIM-2, KPC-2, and OXA-48), but not with non-carbapenemases (i.e., AmpC and TEM-1) over the period.

To validate that the response of **CARBA-H** was due to bona fide carbapenemase activity, we tested the effect of phenylboronic acid (PBA), a KPC-specific inhibitor, and ethylenediaminetetraacetic acid (EDTA), a metallo-β-lactamase inhibitor, on the hydrolysis of **CARBA-H** by carbapenemases. In the presence of PBA, only KPC-2 activity was significantly blunted, and the enzymatic activities of NDM-1, IMP-1, VIM-2, and OXA-48 were not affected. Meanwhile, in the presence of EDTA, the activities of NDM-1, IMP-1, and VIM-2 were inhibited but remained unchanged for KPC-2 (Figure 3e). Interestingly, OXA-48 displayed a slightly decreased activity in the presence of EDTA, which is likely due to the increase in ion concentration.[37]

We also evaluated the performance of **CARBA-Me** against the same panel of carbapenemases (Figure S5). The fluorescence turn-on profiles for NDM-1, IMP-1, KPC-2, and VIM-2 were similar to that of **CARBA-H**; however, **CARBA-Me** did not yield a significant response toward OXA-48. While the detection limits of **CARBA-H** and **CARBA-Me** toward each carbapenemase can be improved upon prolonged incubation, we chose a 30 min time frame for evaluation purposes as it represents a reasonable duration for a rapid test. The detection limits were in picomolar range for both probes toward VIM-2 and KPC-2, and in subpicomolar range for IMP-1 and NDM-1 (Table 1, Table S1, Figures S6, S7). To our delight, while OXA-48 led to the slowest turn-on fluorescence among all the carbapenemases, the corresponding value is still in the picomolar range.

Table 1. Detection Limit (3S/k) of CARBA-H (2 μM in PBS, pH 7.4) Toward Selected Carbapenemases Where S Is the Standard Deviation of 6 Blank Samples[a]

Carbapenemase	3S/k
NDM-1	0.327 pM
IMP-1	0.333 pM
VIM-2	3.28 pM
KPC-2	4.43 pM
OXA-48	30.3 pM

[a] λ_{ex} = 564 nm; λ_{em} = 629 nm.

To gain a better understanding of the substrate-enzyme interactions between **CARBA-H**/**CARBA-Me** and our panel of carbapenemases, the Michaelis−Menten kinetics model was adopted. Our enzyme kinetics data showed that **CARBA-H** served as an excellent substrate for carbapenemases, explaining the rapid turn-on response (Table 2, Figure S8). For VIM-2, KPC-2, and OXA-48, the turnover numbers (k_{cat}/K_M) were found to be in the order of 10^6 M^{-1} s^{-1}, while those of NDM-1 and IMP-1 were even in the order of 10^7 M^{-1} s^{-1}. In general, the k_{cat}/K_M values for NDM-1, IMP-1, VIM-2, and KPC-2 toward **CARBA-H** and **CARBA-Me** are similar to that with imipenem and meropenem (Table 2, Table S2, Figure S9).[38−42] However, the corresponding values for the reaction between OXA-48 and **CARBA-Me** could not be determined, as the initial hydrolysis rate of **CARBA-Me** was found to be independent of OXA-48 concentration. The observation is again consistent with our preliminary protein labeling study in which 1β-methyl bearing substrates form a relatively stable acyl intermediate with OXA-48, thus rendering the Michaelis−Menten model unsuitable for the analysis.

Table 2. Michaelis-Menten Kinetics Data[a]

enzyme	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})
NDM-1	29.78 ± 0.81	1.66 ± 0.13	1.80×10^7
IMP-1	33.86 ± 0.71	1.20 ± 0.08	2.82×10^7
VIM-2	1.96 ± 0.05	1.18 ± 0.10	1.66×10^6
KPC-2	2.17 ± 0.07	0.93 ± 0.11	2.32×10^6
OXA-48	2.83 ± 0.18	0.66 ± 0.08	4.25×10^6

[a] k_{cat} and K_M values for **CARBA-H**.

Rapid Test Assays with Clinical Isolates. Encouraged by the results, we proceeded to apply **CARBA-H** to detect carbapenemase activities in clinical isolates of *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter aerogenes* (*Klebsiella aerogenes*), and *Citrobacter freundii*, which represent the most clinically relevant families of carbapenem-resistant Enterobacteriaceae.[1] Thirteen carbapenemase-encoding strains were used as positive controls (Figure 4). In addition to producers of KPC-2 (strain 1) and NDM-1 (strain 2), of which

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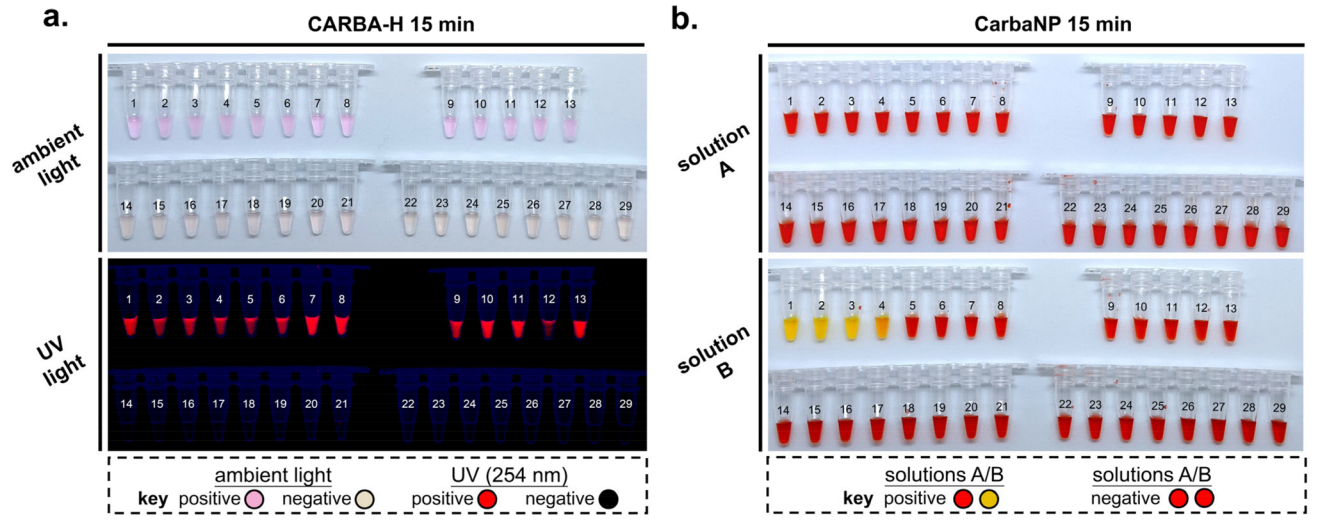

Figure 4. (a) Colorimetric and fluorogenic (UV 254 nm) response of **CARBA-H** (10 μM) (b) CarbaNP solution A and solution B, toward clinical isolates according to CLSI protocol. Images were taken after incubation for 15 min. The carbapenemases encoded in the clinical isolates were determined by PCR. Strain (1) *K. pneumoniae* (KPC-2); (2) *E. coli* (NDM-1); (3) *E. coli* (NDM-5); (4) *E. coli* (IMP-4); (5) *E. coli* (OXA-181); (6) *E. coli* (OXA-181); (7) *K. pneumoniae* (OXA-181); (8) *K. pneumoniae* (OXA-181); (9) *E. coli* (OXA-48); (10) *E. aerogenes* (OXA-48); (11) *E. aerogenes* (OXA-48); (12) *C. freundii* (OXA-48); (13) *K. pneumoniae* (OXA-48); (14−21) *K. pneumoniae* (non-CPE); (22−29) *E. coli* (non-CPE).

the enzymatic activities toward **CARBA-H** were carefully characterized, we included strains that produce NDM-5 (strain 3) and IMP-4 (strain 4), homologues of NDM-1 and IMP-1, respectively, to demonstrate the pan-carbapenemase coverage of our probe. Moreover, 9 strains (from 4 different organisms) that express OXA-181 (strains 5−8) and OXA-48 (strains 9−13) were employed to showcase the unique ability of **CARBA-H** to detect the OXA-48-like family. The presence of the carbapenemase-encoding genes in these isolates were cross validated by PCR, and 16 non-carbapenemase-producing Enterobacteriaceae (non-CPE) isolates (strains 14−29) were used as negative controls.

While the detection can be carried out with live bacteria as carbapenemases are often secreted (Figure S10a), we sought to lyse the bacteria in order to release the carbapenemases from the periplasmic space of the bacteria for rapid detection purposes. We first investigated the efficiency of some nondenaturing detergents, namely Triton X-100, Tween-20, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesul-fonate (CHAPS), and B-PER, as additives in PBS buffer. Accordingly, bacteria were incubated with **CARBA-H** in the detergent-containing buffer solutions, and the efficiency of bacterial lysis was determined by the degree of color change after 15 min (Figure S10b-S11). CHAPS was found to provide the most rapid color change and hence was selected for the subsequent rapid test assay. Thus, clinical isolates were lysed in PBS buffer containing 0.5% CHAPS for 15 min before **CARBA-H** treatment (Figure S12). The carbapenemase-encoding strains (Figure 4a; strains 1−13) induced marked color change from pale orange to deep pink, visible under ambient light, within 15 min. In contrast, the color of the negative controls remained unchanged (Figure 4a; strains 14−29). The fluorescence turn-on could also be conveniently visualized with the aid of a hand-held UV lamp. Again, only the CPE strains provided intense fluorescence signals (Figure 4a; strains 1−13 vs 14−29). A consistent observation was obtained after prolonged incubation (120 min; Figure S13a), indicating the fluorescent probe and fluorophore are stable in the complex biological system as a reliable rapid test. A head-to-head comparison was made by performing the CarbaNP test in parallel. In contrast to **CARBA-H**, CarbaNP did not provide any positive color change for the bacteria expressing Class D carbapenemases (strains 9−13) within 15 min (Figure 4a). Even with prolonged incubation for up to 120 min, the maximum incubation time as recommended by CLSI for CarbaNP, CarbaNP did not provide a distinct color change, corresponding to false-negative or inconclusive results for those isolates (Figure S13c). Moreover, due to the high sensitivity, even OXA-232, a known Class D carbapenemase with notably low carbapenemase activity, can be clearly detected (Figure S13a, strains 30−32). For completeness, a direct comparison against **CARBA-Me** was also performed (Figure S13b). As expected, after 120 min, **CARBA-Me** provided the colorimetric and fluorogenic responses toward KPC, NDM, and IMP-encoding strains but variable results toward the strains encoding the OXA-48-like carbapenemases. Together, our study clearly showcased the superior performance of **CARBA-H** over existing protocols and probe designs.

Urine Test with Clinical Isolates. To demonstrate potential clinical applicability of **CARBA-H**, we set out to detect carbapenemase activity directly in urine samples. Urinary tract infection (UTI) was reported to be leading healthcare-associated infection among hospitalized adults and in critical care units, and the second or third most common type of nosocomial infection in intensive care units.[43,44] As a proof of concept, we mimicked urine samples from UTI patients by spiking urine from healthy subjects with known amounts of well-characterized carbapemenase-producing clinical isolates, particularly with the inclusion of OXA-48, OXA-181, and OXA-232-producing strains. Remarkably, **CARBA-H** (0.25 μM) provided significant fluorescence enhancement in all CPE-spiked (5×10^6 cfu) urine samples, in the presence of 0.5% CHAPS, within 2 h. The turn-on response could be readily detected by a microplate reader, with a clear differentiation against the background from non-CPE *E. coli* and *K. pneumoniae* isolates (Figure 5). This assay potentially obviates the need for bacterial culture and creates new opportunities for rapid detection of UTI by CRE, as the

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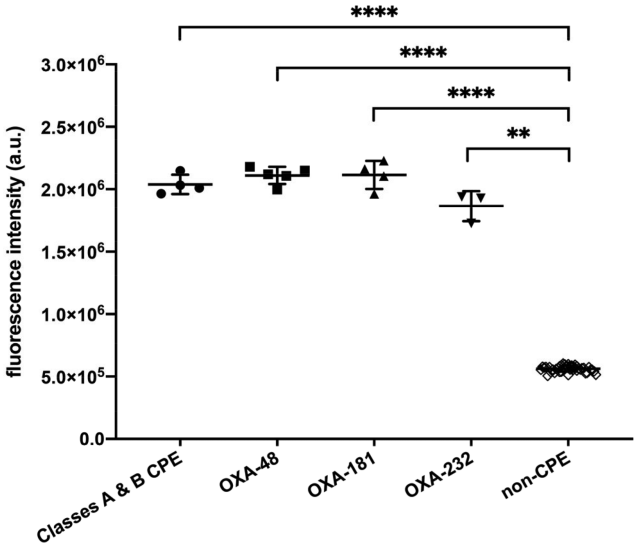

Figure 5. Fluorescent response of **CARBA-H** (0.25 μM; λ_{ex} = 564 nm; λ_{em} = 629 nm) toward carbapenemase in a panel of 71 Enterobacteriaceae clinical isolates spiked in urine (5×10^6 cfu), was determined upon incubation at 37 °C for 2 h. Strain (1) *E. coli* (IMP-4); (2) *K. pneumoniae* (KPC-2); (3) *E. coli* (NDM-1); (4) *E. coli* (NDM-5); (5) *C. freundii* (OXA-48); (6) *E. aerogenes* (OXA-48); (7) *E. aerogenes* (OXA-48); (8) *E. coli* (OXA-48); (9) *K. pneumoniae* (OXA-48); (10) *E. coli* (OXA-181); (11) *E. coli* (OXA-181); (12) *K. pneumoniae* (OXA-181); (13) *K. pneumoniae* (OXA-181); (14) *E. coli* (OXA-232); (15) *K. pneumoniae* (OXA-232); (16) *K. pneumoniae* (OXA-232); (17−24) *K. pneumoniae* (non-CPE); (25−71) *E. coli* (non-CPE). The error bars correspond to the standard deviation of the two replicates. Statistical significance was calculated using the unpaired two-tailed Student's *t* test with Welch's correction (** $p < 0.01$, **** $p < 0.0001$).

necessary bacterial count is readily achievable by routine centrifugation of patient urine samples as per traditional urinalysis[45] (typical UTI patients are reported to have a bacterial count exceeding 1×10^5 cfu/mL in their urine).[44,46] This could further reduce the time for culturing bacteria and minimize delay for the golden treatment period. The compatibility of the fluorogenic carbapenemase detection using **CARBA-H** in urine samples highlights the judicial choice of the reporting fluorophore (Figure S14) and demonstrates the scope for extending this strategy for use in other biological specimens.

DISCUSSION

The success in the development of **CARBA-H** as a fluorescent probe for broad spectrum carbapenemase detection hinges upon our rational design with two key features, i.e., an effective self-immolative spacer and the lack of the 1β-methyl substituent at the carbapenem core.

The allylic-hydroxybenzylic self-immolative spacer in our probe design is crucial in providing high sensitivity and a broad scope for carbapenemase detection. A **CARBA-H** analog without the self-immolative spacer was also prepared as a control (Figure S15 and Scheme S3). As expected, the molecule displayed poor response toward KPC-2 and suffered from poor aqueous stability. At the outset of the project, we also briefly explored the use of a phenyl spacer, which was later found to be utilized in a recent carbapenem-based probe design.[18] However, this strategy resulted in incomplete fluorophore release during carbapenemase mediated hydrolysis (Figures S16 and Scheme S4), leading to a drastic decrease in sensitivity. In contrast, the small allylic spacer in **CARBA-H** and **CARBA-Me** provided a complete and immediate fluorophore uncaging upon carbapenem ring opening (Figure S17), resulting in maximum chromophore release suited for rapid detection. This highlights that, in the context of small molecule-based chemosensors, extra consideration should be made in linker selection for future probe design to maximize signal generation.

Through a chemical biology approach, we have uncovered a crucial role of 1β-methyl group in carbapenems during OXA-48 mediated hydrolysis. Recent reports by Chen[47] and Vakulenko[48] on crystal structures of OXA-48 and its covalent adducts with several carbapenems shed light on the enzymatic mechanisms, yet these structural studies could not offer ready explanation for the slow hydrolysis kinetics with 1β-methyl substituted carbapenems.[49] Meanwhile, Schofield and co-workers showed that the presence of 1β-methyl group led to a detour in the carbapenem degradation pathway with OXA-48.[24] In this study, we revealed that this substituent is crucial in slowing down the deacylation process, and this discovery provides new insight for antibiotic therapy and drug discovery. Clinical research has shown that patient infected with certain carbapenemase-producing bacteria (e.g., OXA-48-encoding bacteria) could be effectively treated with coadministration of two carbapenem antibiotics (i.e., double-carbapenem therapy).[50,51] To explain the observed synergistic effect, we postulated that one of the carbapenems act as a suicidal inhibitor for OXA-48, and the other targets the bacterial cell wall synthesis.[50,51] While there is almost no molecular evidence to back up such treatment, our finding provided strong rationale for this therapy from a mechanistic perspective.

CONCLUSION

We have developed **CARBA-H** successfully as the first broad spectrum off-on fluorogenic carbapenemase probe. Through direct comparison on the performance of **CARBA-H** with **CARBA-Me** and a series of mechanistic studies, we have provided clear evidence that the 1β-methyl substituent strongly affects the OXA-48 enzyme hydrolytic kinetics. **CARBA-H** exhibit excellent sensitivity toward the 5 clinically most relevant carbapenemases, including OXA-48, of which the detection limit was determined to be in the pico-molar range.

We have applied our probe **CARBA-H** to an optimized rapid test for carbapenemase detection in clinical isolates. The absence of the 1β-methyl substituent is essential for pan-carbapenemase recognition, where carbapenemase encoding strains, including OXA-48, OXA-181, and OXA-232, could be successfully detected. Reliable chromogenic and fluorogenic readouts could be easily discerned by naked eye within 15 min, demonstrating its outstanding performances compared to currently existing assays. Our success in establishing an operationally simple protocol for detecting carbapenemase activities from clinical isolates should accelerate the development of an affordable point-of-care device, for antimicrobial resistance tests performed without specialized equipment available only in hospitals.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c00462.

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Notes

The authors declare the following competing financial interest(s): The results reported in this manuscript were included in a PCT Application (No. PCT/CN2019/106645) filed by D.Y. and C.-W.M.

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