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PAPER

β-Lactam-host defence peptide conjugates as antibiotic prodrug candidates targeting resistant bacteria†

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The first hybrid molecule of a *β*-lactam antibiotic and a host defence peptide and a method for the preparation of this type of molecule are reported. Conjugation of an antimicrobial peptide to a cephalosporin, through a cleavable linker, reversibly masks one of the activity determinants of the peptide. Its release from the *β*-lactam core can be selectively triggered by bacterial enzymes (*β*-lactamases) which mediate resistance to *β*-lactam agents. A prototypical cephalothin-bactenecin candidate was synthesised, using a copper(I)-catalysed azide–alkyne cyclo-addition reaction for the conjugation step. Enzymatic hydrolysis assays of this candidate were initially performed with a purified *β*-lactamase to confirm that the peptide can be released from the cephalosporin. The antimicrobial activity of the conjugate was then assessed against representative strains of bacteria and compared to the activities of its parent *β*-lactam and peptide components and to those of two analogous conjugates based on non-cleavable linkers. The results of these assays indicate that the conjugate has an activity distinct from its separate constituents and that the release of the peptide from the cephalosporin may contribute to its mechanism of action. Furthermore, the results of antimicrobial assays performed with an isogenic strain of bacteria expressing or not an extended-spectrum *β*-lactamase, suggest that antimicrobial peptide prodrug candidates targeting resistant bacteria could be generated from these hybrid antibiotics.

1. Introduction

Host defence peptides (HDPs) are multifunctional molecular effectors of innate immunity and are the first line of defence against infection in multicellular organisms. Their multiple functions in host defences, associated with their low susceptibility to classical mechanisms of drug resistance and low propensity to select resistant mutants, support the rationale of developing novel peptide-based therapeutics harnessing the effector mechanisms of innate immunity.[1] The development of systemic therapies based on antimicrobial peptides requires primarily solutions addressing the question of possible toxicity.[2] Several strategies have been explored to exploit the therapeutic potential of HDPs, including methods to improve their pharmacokinetic and/or pharmacodynamic properties, delivery or production.[3] A prodrug approach has also been proposed as a promising strategy to achieve clinical success with antimicrobial peptides.[4] This approach can indeed increase the therapeutic index of a pharmacologically active agent and overcome a barrier to drug targeting.[5] A prodrug modification is commonly performed by conjugating a promoiety to the parent drug through one of its activity determinants.[6] If the promoiety is eliminated *in vivo* by a chemical or enzymatic reaction only occurring at the target body site, selective delivery of the active agent can then be achieved.

The synthesis and activity of a *β*-lactam-HDP conjugate is reported herein. This dual-pharmacophore molecule constitutes the first reported candidate of a bioreversible derivative of a HDP which can be activated by a bacterial enzyme of antibiotic resistance.

2. Results and discussion

2.1. Prodrug design

HDPs are highly diverse in size and secondary structure. Their sequences are characterised by a net excess of positively charged residues (cationic peptides), a hydrophobic amino acid content approximating 50% and consequently by their ability to adopt amphipathic secondary structures.[2] The hydrophobicity and net

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charge of these peptides are important determinants of their antimicrobial activity and structure–activity relationships can be described in term of numbers of positive charges and lipophilic units and of sequence patterns.[7,8] The N^{α}-amino terminus is commonly free in natural and man-made HDPs and contributes to the net charge of the peptide sequence. Masking this group and reducing the net charge of the peptide is generally associated with a loss of activity in endogenous HDPs and their synthetic analogues.[9] Transiently modifying the N-terminus of a HDP with a negatively charged promoiety can therefore generate prodrug candidates of the parent peptide.

β-Lactams agents such as cephalosporins meet the requirements of a promoiety in prodrug design.[6] β-Lactam antibiotics have been in clinical use for more than 65 years and have proven to be safe, as well as their degradation products. They also allow the bioreversible modification of a pharmacologically active agent by conjugation of one of its functional groups at the 3′-position of the cephem core. Release of the 3′-substituent, *i.e.* the parent drug, is selectively triggered by cleavage of the β-lactam bond by bacterial enzymes.[10] They consist essentially of the enzymes of resistance to β-lactam antibiotics (β-lactamases) and include potentially the enzymes of bacterial cell-wall biosynthesis (penicillin-binding proteins or PBPs). The expression of β-lactamases is the most commonly acquired mechanism of antibiotic resistance, particularly in Gram-negative pathogens. It is conserved in multi-drug resistant (MDR) organisms which can be resistant against multiple agents, including those used as the last line of effective antibiotic treatment.[11] β-Lactamases, in particular extended-spectrum β-lactamases (ESBLs), are highly efficient catalysts and attractive enzymes for the activation of antibiotic prodrugs targeting MDR pathogens.[10]

Conjugation at a cephalosporin's 3′-position of a HDP through its N^{α}-amino terminus was therefore proposed to generate β-lactamase-dependent prodrugs of antimicrobial peptides. This conjugation masks an important pharmacophoric element of the peptide, but also reduces its net positive charge by 2 units, due to the presence of a carboxylate at the 4-position of the cephem nucleus. The structure of these prodrug candidates included also a self-immolative carbamate linker between the cephalosporin and peptide moieties, to facilitate the elimination of the 3′-primary amine substituent upon hydrolysis of the β-lactam bond (Fig. 1).

2.2. Conjugate synthesis

The synthesis of a cephalosporin-peptide conjugate is *a priori* based on a convergent synthetic route in which the β-lactam and peptidic components are prepared separately. A conventional approach involves their conjugation by selective formation of a carbamate linkage between the cephalosporin's 3′-hydroxyl and the peptide's N^{α}-amino terminus, requiring protection of all the other functional groups and therefore a final deprotection step. These steps have to be performed under conditions which meet the requirements of both the β-lactam and peptide chemistries. On one hand, a variety of α-amino acid side-chain functionalities are present in peptides. The guanidine and indole groups in particular are among the most common functional groups in HDPs, the optimisation of their antimicrobial activities generally yielding sequences enriched in arginine and tryptophan, respectively.

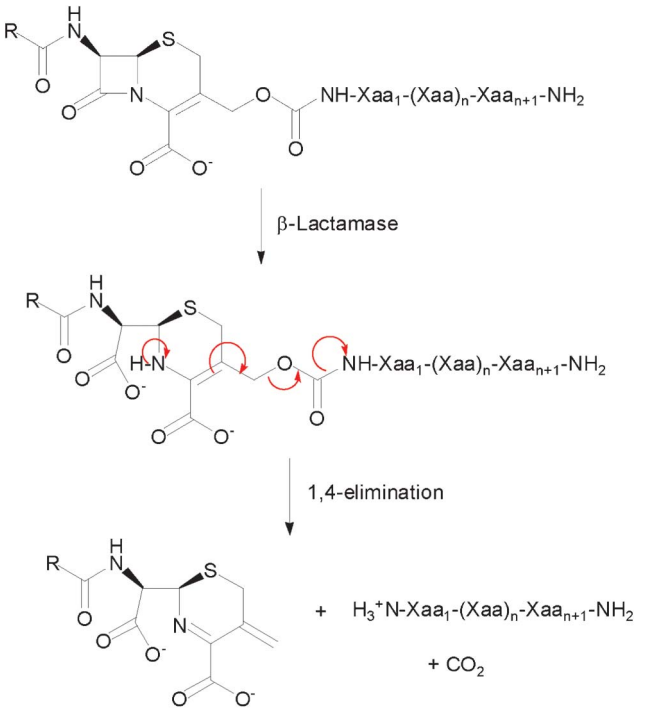


Fig. 1 General structure and reaction of a cephalosporin-host defence peptide conjugate as a β-lactamase-dependent prodrug.

Arginine is one of the most demanding residues in terms of acid concentration and reaction time for the deprotection of its guanidino group.[12] This reaction is in addition performed in the presence of nucleophilic reagents commonly named scavengers. On the other hand, the β-lactam ring of penicillins and cephalosporins is particularly labile in the presence of nucleophiles and at alkaline, but also acidic pHs.[13]

The cephalosporin and peptide candidates selected for the synthesis of a prototypical conjugate were cephalothin and Bac8c, respectively. Cephalothin **1**, containing a 3′-acetate substituent and a thienyl side-chain, is a first generation cephalosporin with a broad spectrum of activity. Bac8c is a peptide amide of sequence RIWVIWRR-NH_2, obtained by optimisation of the bovine dodecapeptide bactenecin.[14] The asset of this short 8-mer candidate is an activity against both Gram-negative and Gram-positive bacteria in the low micromolar range. Also, its number of residues, which remains close to the minimal length of a continuous epitope for an antigen,[15] should prevent its immunogenicity and limit its production cost. To impart metabolic stability to this candidate as well, preliminary studies were conducted to assess the antimicrobial activity of its enantiomeric sequence D-Bac8c. Typical Minimum Inhibitory Concentrations (MICs) of D-Bac8c-NH_2 against well characterised laboratory strains of Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus* were of 4.2 μM (Table 1), slightly higher than the reported MICs of 1.7 μM for L-Bac8c-NH_2 against different strains of the same organisms.[14] Homologous substitution of D-isoleucine with D-leucine was also attempted for economical reasons, yielding the amidated peptide **2** of sequence rlwvlwrr, named D-Bac8c(Leu^{2,5}). The 2 *E. coli* reference strains selected previously had similar susceptibilities to both all-D peptides (4.2 μM), while *S. aureus* (8325–4) showed higher

Table 1 Minimum inhibitory concentrations (μM)[a]

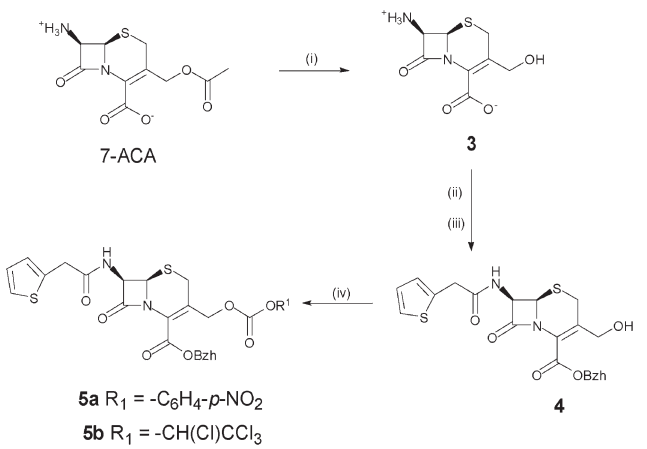
Strain	D–Bac8c riwviwrr–NH_2	D–Bac8c(Leu^{2,5}) rlwvlwrr–NH_2
S. aureus 8325-4[b]	4.2	2.1
E. coli 25922[c]	4.2	4.2
E. coli 35218[c]	4.2	4.2

[a] MIC experiments were carried out in triplicate. [b] NCTC strain. [c] ATCC strain.

susceptibility to D-Bac8c(Leu^{2,5}) (2.1 μM) than to D-Bac8c (Table 1). The activity of **2** against *E. coli* was also verified against 10 clinical isolates of ESBL-positive *E. coli*. A MIC of 4.2 μM was confirmed in 8 of these strains, whereas MICs of 2.1 μM and 8.4 μM were achieved against 2 individual strains.

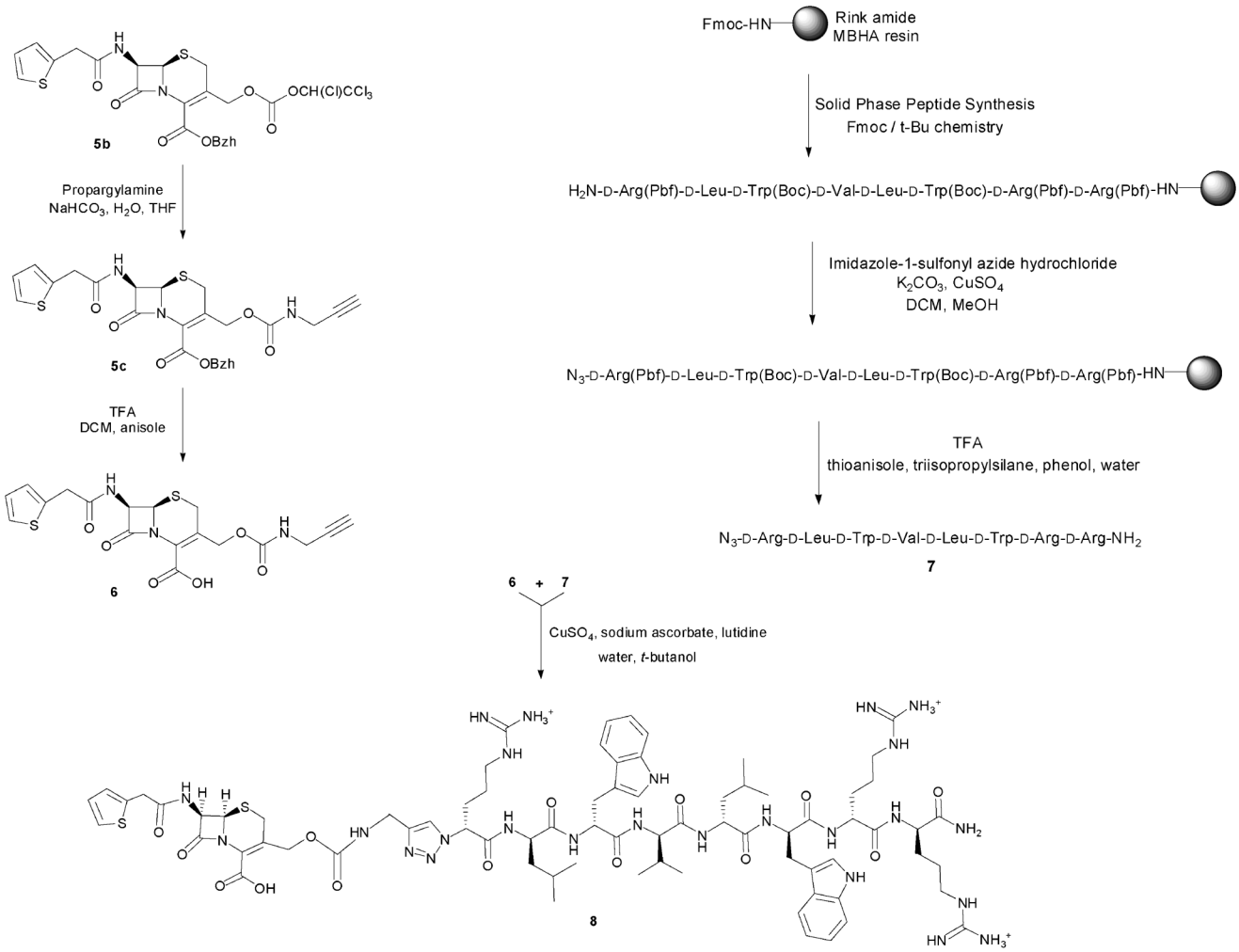
After selection of the β-lactam and peptide components, the cephalosporin synthon was assembled first and functionalised with an active carbonate at the C-3′ position of the cephem core for reaction with the N^{α}-amino terminus of a peptide sequence (Scheme 1). It was synthesised from commercially available 7-aminocephalosporanic acid (7-ACA), by 3′-deacetylation, followed by protection of the 7-amino and 4-carboxyl by thienylation and esterification by diphenyldiazomethane, respectively. The order of these protection steps was imposed by the lack of solubility of the zwitterionic deacetylated intermediate 7-AHCA **3** in organic solvents. Attempts to esterify the 4-carboxyl first resulted in the lactonisation of the 3-hydroxymethyl and 4-carboxyl groups or Δ^3/Δ^2 isomerisation of the cephem double bond. Functionalisation with a 3′-*p*-nitrophenyl- or 3′-tetrachloroethyl-carbonate was finally performed by reaction of **4** with the corresponding chloroformates.

The formation of a carbamate linkage between a cephalosporin and a peptide sequence was initially investigated by assessing the displacement of the active carbonate intermediates of cephalothin with a single α-amino acid. The methyl ester of alanine was reacted with **5a/5b**, with or without addition of 1 equivalent of pyridine and a catalytic or stoichiometric quantity of DMAP. In the absence of additives, partial formation of a carbamate product was only observed from the tetrachloroethyl-carbonate after extended reaction times (60% conversion in 24 h). In the presence of a catalytic quantity of DMAP, conversion to the desired carbamate occurred rapidly from the tetrachloroethyl-carbonate (100% conversion in 3 h), but only marginally from the *p*-nitrophenyl-carbonate even after 12 h of reaction. In both cases, the use of stoichiometric quantities of DMAP (1–2 equivalents) were associated with a degradation of the cephalosporin's β-lactam core.



Scheme 1 Synthesis of the cephalosporin synthon. (i) TBAOH, Et_3N, MeOH, H_2O; (ii) 2-thienylacetyl chloride, NaOH, H_2O, acetone; (iii) diphenyldiazomethane, AcOEt; (iv) *p*-nitrophenylchloroformate, pyridine, DMAP, DCM for **5a**; tetrachloroethylchloroformate, pyridine, DMAP, DCM for **5b**.

Displacement of the tetrachloroethyl-carbonate was then attempted directly with a model 6-mer, fully deprotected, peptide. Reaction of **5b** with the amidated sequence RWRWRW-NH_2, in the presence of 1 equivalent of pyridine and a catalytic amount of DMAP, induced however the degradation of the β-lactam ring without formation of the carbamate product. This was also the case when the single amino acid arginine was used as a nucleophile candidate to displace the tetrachloroethyl-carbonate. On the other hand the carbamates of the sterically unhindered amino acids glycine and alanine, protected as methyl or allyl esters, could be formed. In the latter case, deprotection of the α-carboxyl groups was performed with conservation of the β-lactam core's integrity. The combination of a catalytic quantity of $Pd(PPh_3)_4$ with 1 equivalent of toluene sulfinic acid in THF:H_2O (4 : 1) was found to provide the optimal deprotection conditions for this reaction. Finally, to complete the assembly of the peptide sequence by an approach similar to segment condensation, coupling to the 3′-carbamoylalanine derivative of cephalothin of a side-chain protected peptide was attempted in solution. A model sequence (H–Ahx–Ala–Gly–Arg(Pbf) –Pro–Val–Asp(O–*t*–Bu) –NH_2) containing common amino acids and protecting groups and modified with a N-terminal amino-hexanoyl spacer to reduce steric hindrance at the ligation site was assembled and cleaved protected from a Sieber resin. Coupling to the cephalosporin-amino acid conjugate was successfully carried out with DCC/HOBt coupling chemistry, as confirmed by mass spectrometry and ^{1}H NMR analyses. However, the final product could not be retrieved after attempting the side-chain deprotections by treatment with a cocktail of triisopropylsilane (5.5%), thioanisole (10.5%) and TFA (84%). Additional, independent, assays involving the treatment of cephalothin and a protected peptide with cleavage cocktails of varied composition, containing or not trifluoroacetic anhydride as a drying agent, also failed to identify a mixture of reagents which allowed at the same time complete deprotection of the arginine's guanidino group and maintenance of the β-lactam's integrity. Other protections of the guanidino group, requiring milder reaction conditions for their elimination, were therefore evaluated. The bis-allyloxycarbonyl (Alloc)-protection of arginine could be successfully removed from an isolated arginine, but yielded partially protected peptides for arginine-rich sequences such as Bac8c, even after repetitive treatment of the resin-bound peptide with tetrakis-(triphenylphosphine)-palladium(0) catalyst, acetic acid and morpholine. As the semi-permanent protection of arginine with super acid-sensitive groups is not currently available, the synthesis of a 4-methoxytrityl (Mmt) derivative of arginine was investigated. Starting from Fmoc–Arg–OH, preliminary protection of the carboxyl group as an allyl ester was required to selectively perform the bis-alkylation of the guanidino group with 4-methoxytriphenylmethyl chloride. The allyl ester was finally deprotected with palladium on charcoal to provide the



Scheme 2 Synthesis of the prodrug candidate cephalothin-D-Bac8c(Leu^{2,5}) **8**.

precursor Fmoc–Arg(Mmt)$_2$–OH. The application of this reagent in solid phase peptide synthesis was initially performed with a simple model sequence (Arg–Gly–Asp–Ser), assembled from a Sieber resin. However, coupling of this sterically hindered amino acid required its activation as an acid fluoride by reaction with TFFH, as even the PyBroP coupling chemistry remained inefficient. Furthermore, concentrations of TFA as high as 25%, incompatible with the stability of the β-lactam core, were required to deprotect the guanidino group.

As the synthetic routes requiring a final deprotection step were associated with an aminolysis of the β-lactam ring and/or limited conversions and reliability, an approach allowing the unequivocal attachment of a fully deprotected peptide to a cephalosporin, preserving the integrity of the β-lactam core and compatible with any peptidic functional group, was developed. The copper(I)-catalysed azide–alkyne cyclo-addition is an archetypal reaction of a click chemistry approach allowing the quantitative and selective ligation of two abiotic functional groups.[16] Applied to a cephalosporin as an acylated dipeptide mimic and a peptide, this reaction offers the additional advantage of producing a peptide bond isostere, the triazole ring, between these two ligated moieties.[17] To produce β-lactam-peptide conjugates, the roles of azide and alkyne partners in the *cyclo*-addition reaction were assigned to the peptide and the cephalosporin, respectively (Scheme 2). Substitution of the peptide's N^{α}-amino terminus with an azido group and functionalisation of the cephalosporin with a 3′-propargyl-carbamate group yield a 1,3-dipolar cycloaddition product in which the peptide component is modified with a N-terminal triazole-ε^2-amino acid. Release of the peptide from the cephalosporin upon hydrolysis of the β-lactam unmasks a primary amino group and restores the net charge of the native peptide sequence (Fig. 2).

The alkyne-modified cephalosporin **6** was prepared by reaction of the active tetrachloroethyl-carbonate **5b** with propargylamine, followed by deprotection of the benzhydryl ester under mild acidic conditions. For the azido-peptide, a simplified Bac8c sequence containing only one side-chain functionalised amino acid was initially selected to investigate its synthesis. Homologous substitution of tryptophan with 2-naphtylalanine was therefore performed at the positions 3 and 6 of the Bac8c sequence. Also, to limit the cost of these synthetic feasibility studies, Bac8c(2-Nal^{3,6}) was assembled from L-amino acids. The sequence was elongated by Solid Phase Peptide Synthesis (SPPS)[18] and N-terminally modified by a DiazoTransfer reaction directly applied on the polymer-supported peptide. The azido-peptide

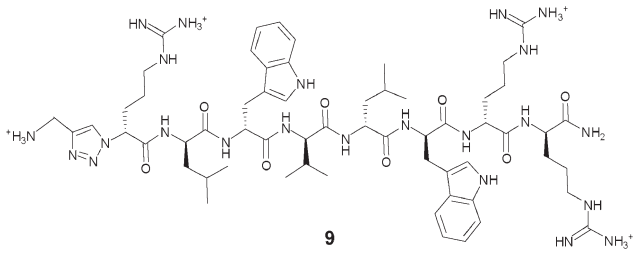


Fig. 2 Structure of D-Arg[1]-triazole-ε2- D-Bac8c(Leu[2,5]).

was then cleaved from the resin and deprotected by treatment with a cleavage cocktail composition of which was adapted to prevent reduction of the azide. This solid phase synthesis strategy for the preparation of the peptide fragment proved to be more expedient and efficient than a hybrid solid-solution phase approach, performed by converting the N-terminal α-amino acid (H-Arg(Pbf)-OH) into the corresponding α-azido acid and coupling the latter to the penultimate residue of a resin-bound peptide sequence. Finally, the copper(I)-catalysed cycloaddition of the alkyne-modified cephalosporin with the azido-peptide allowed the conjugation of the β-lactam and peptide fragments through a carbamate-1,4-triazole linkage. Ultimately, this synthetic method was successfully applied to the synthesis of the azido-D-Bac8c(Leu[2,5]) **7** and its conjugation to the 3′-propargylcarbamate cephalosporin **6**, to produce the prototypical prodrug candidate cephalothin-D-Bac8c(Leu[2,5]) **8** (Scheme 2). Characterisation of this conjugate was performed by ESI-MS and its integrity, namely the absence of Δ^3/Δ^2 isomerisation, was confirmed by ^{1}H NMR. Also, the characteristic coupling constants of the geminal protons at the 2-position were verified by C–H correlation 2-D NMR (ESI Fig. S1–S3†).

The active parent peptide of this prodrug candidate was synthesised separately as a Bac8c sequence modified with a N-terminal D-Arg-triazole-ε2-amino acid **9** (Fig. 2). Two independent synthetic routes, based on solid phase synthesis or on a hybrid solution/solid-phase synthesis were evaluated for this triazole-modified peptide. In the former case, a cyclo-addition of propargylamine and a resin-bound azido-peptide was performed. In the latter case, the synthesis and coupling of a Fmoc-protected D-Arg-triazole-ε^2-amino acid to a peptide sequence assembled up to the penultimate residue was carried out. Higher purities of the active parent peptide were obtained by the hybrid solution/solid-phase method as the solid phase approach yielded some unreacted peptide which could not be separated from the peptide triazole by RP-HPLC.

2.3. Activation assays and antimicrobial activity

Chemical and enzymatic hydrolysis assays of cephalothin-D-Bac8c(Leu[2,5]) were first undertaken to verify that the active parent peptide could be released from this prodrug candidate upon cleavage of the β-lactam bond. These experiments were carried out by monitoring the disappearance of the cephalosporin's β-lactam bond UV absorption[19] at 260 nm, in a sodium hydroxide solution for the base-promoted hydrolysis assay, or with a purified P99 enzyme from *Enterobacter cloacae* for the β-lactamase-mediated activation assay. No background hydrolysis of cephalothin-D-Bac8c(Leu[2,5]) was observed in buffer alone. The results of the assays with NaOH and P99 (ESI Fig. S4–S7†) show that the kinetics of the base-promoted and enzymatic hydrolyses are slower for the prodrug candidate than for cephalothin.

Also, the relative reductions in the absorbance upon hydrolysis were significantly lower for the prodrug candidate than for cephalothin, complete disappearance of the UV absorption being only obtained in the latter case. This was attributed to a residual absorption of the peptide at 280 nm and confirmed by performing wave-scans of cephalothin and the prodrug candidate **8** before and after (chemical) hydrolysis, and compared to the wave-scan of the peptide triazole **9** (ESI Fig. S8–S12†). While the absorption at 260 nm is completely abolished after hydrolysis of cephalothin, persistence of an absorption in the same region is observed with the prodrug candidate. This absorption is of similar intensity and wavelength than the one recorded in the wave-scans of the peptide triazole and is most likely associated with the absorption of the indole rings. Together, the results of these experiments indicate that the prodrug candidate can release its peptide component upon cleavage of the β-lactam ring.

The antimicrobial activity of the prodrug candidate **8** was then assessed against the strains of *E. coli* previously selected and against two strains of Methicillin-Resistant *Staphylococcus aureus* (MRSA). They were compared with the activities of its parent components, *i.e.* cephalothin **1** and D-Bac8c(Leu[2,5]) **2** and with the active parent peptide of the prodrug **9**. The Minimum Inhibitory Concentration (MIC) results presented in Table 2 show that the activities of the prodrug candidate against the strains selected are distinct from those of its parent cephalosporin and peptide components. In all cases, the parent peptide remained the most active of these 3 candidates. The MICs of the prodrug candidate were generally higher with the Gram-negative strains, although **8** can have MICs as low as 3 μM against clinical isolates expressing ESBLs (data not shown). Comparing the results of the prodrug candidate and its active parent peptide

Table 2 Minimum inhibitory concentrations of the prodrug candidate **8**, its parent components and controls (μM)[a]

Strain	Cephalothin **1**	D-Bac8c (Leu[2,5]) **2**	Prodrug **8**	Triazole peptide **9**	Control **10**	Control **11**
S. aureus 12493[b]	2.0	2.1	3.0–8.0	4.0–5.0	25.6	> 24.8[e]
S. aureus 44330[b]	2.0	1.0	1.5	2.0–4.0	12.8	> 24.8[e]
E. coli 25922[c]	31.6	2.1	12.0	5.0	> 25.6	> 24.8[e]
E. coli 35218[d]	119.0	1.0	8.0-16.0	32.0	⩾ 25.6	> 24.8[e]
E. coli BL21 $bla_{\text{CTX-M-15}}$ −	12.6	2.1	6.1	nd[f]	25.6	nd
E. coli BL21 $bla_{\text{CTX-M-15}}$ +	> 404.0	1.1	3.0	nd	25.6	nd

[a] MIC experiments were carried out in triplicate. [b] MRSA NCTC strain. [c] β-Lactamase-negative ATCC strain. [d] β-Lactamase-positive ATCC strain. [e] Highest concentration tested. [f] Not determined.

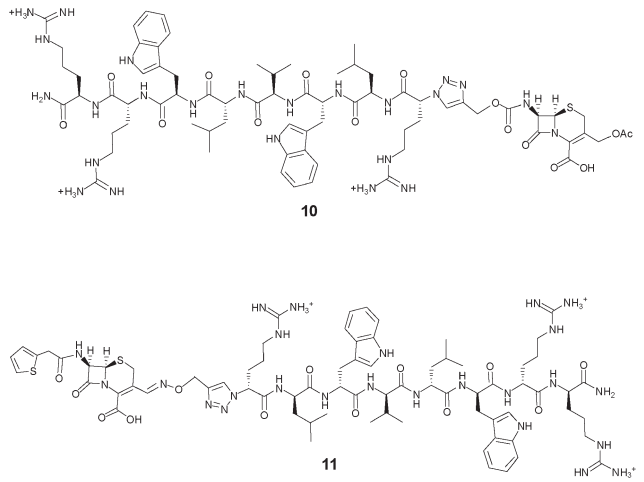


Fig. 3 Structures of the cephalosporin-D-Bac8c(Leu^{2,5}) conjugates based on non-cleavable linkers. In **10**, the peptide is conjugated at the 7-position of the cephem core; in **11**, the peptide is conjugated at the 3′-position of the cephem core through a stable oxime linker.

showed that *E. coli* had higher susceptibilities to the triazole-modified peptide **9** than to its cephalosporin conjugate **8**, while *S. aureus* had similar susceptibilities to both agents. Collectively, these results can be explained by a reduced uptake of the conjugated peptide through the outer bacterial membrane of Gram-negative organisms, as a consequence of a hindered N-terminus[9] which impedes the access of the conjugate to the β-lactamases. These enzymes are indeed expressed extracellularly in Gram-positive bacteria, but within the periplasmic space in Gram-negative bacilli.

To assess the specificity of the activity of the prodrug candidate, analogs which recapitulate the hydrophobicity and net charge determinants of the antimicrobial activities of **8** were designed as controls.

In the first control **10** (Fig. 3), the separation of the peptide component from the β-lactam moiety cannot be triggered by the reaction of the latter with β-lactamases. The results presented in Table 2 show that this control has reduced antimicrobial activities against both organisms, in particular *S. aureus*, indicating that the release of the peptide component from the cephalosporin may contribute to the mechanism of action of the prodrug candidate. Another control **11** (Fig. 3) only differing from **8** by the introduction of a stable oxime linker in place of the self-immolative carbamate linker also had reduced antimicrobial activities against both organisms (Table 2).

Finally, to further investigate the mechanism of action of the cephalosporin-peptide conjugate **8**, isogenic strains of *E. coli* expressing or not the ESBL CTX-M-15[20] were created. The use of strains differing only by the presence of an ESBL gene directly indicates the contribution of the β-lactamase to the activity of a prodrug candidate.[21] The results reported in Table 2 show an activity differential of **8** against these isogenic strains consistent with a β-lactamase-dependent prodrug activity. Although these isogenic strains present different inherent susceptibilities to the parent peptide, the MICs of the non-cleavable control conjugate **10** containing the same peptide were identical against these strains, regardless of their β-lactamase status.

3. Conclusions

Prodrug candidates of HDPs activated by the main mechanism of antibiotic resistance can be synthesised by conjugation of non-ribosomal (β-lactam) and ribosomal (host defence) peptides through a carbamate-1,4-triazole linker. The results of the activation assays, antimicrobial testing and control experiments substantiate a β-lactamase-dependent prodrug activity of the cephalothin-D-Bac8c(Leu^{2,5}) candidate, but also indicate a contribution of the intact conjugate to its overall activity. This could be due to an insufficient modification by the cephalosporin of the net charge and/or hydrophobicity of the parent peptide. Shorter peptide sequences and/or sequences of lower net charges could be selected as active parent peptides to generate prodrug candidates with greater activity differentials between ESBL-positive and ESBL-negative strains. Alternatively, introducing a positive charge in the side-chain of the cephalosporin could be performed to reduce the hydrophobicity of the conjugate and potentially overcome its reduced uptake through the outer bacterial membrane of Gram-negative bacteria by restoring a cationic N-terminal end. Even if the parent peptide remains more active than its prodrug candidate, the latter possesses an activity which can potentially be controlled to prevent toxicity issues in systemic therapies and to target resistant Gram-negative *Enterobacteriaceae* producing ESBLs. These pathogens have been listed among the top 6 organisms to which novel antibiotics are urgently needed.[22]

4. Experimental

4.1. General methods

Material and reagents were purchased from commercial suppliers and used without further purification, unless stated otherwise. Protected amino acids were obtained from Novabiochem (Merck Biosciences, Hohenbrunn, Germany), Iris Biotech GmbH (Marktredwitz, Germany) and Senn chemicals (Dielsdorf, Switzerland). HBTU and resins for SPPS were purchased from Novabiochem; HOBt and PyBrop were from Iris Biotech GmbH. Solvents (NMP, DCM) for the peptide synthesiser were purchased from Applied Biosystems (Warrington United Kingdom). Other reagents and solvents were obtained from Sigma-Aldrich Ireland.

NMR spectra were recorded on a BRUCKER Avance 400 spectrometer. Samples were prepared in $CDCl_3$ (referenced to 7.26 ppm for ^{1}H and 77.0 for ^{13}C), DMSO-d_6 (referenced to 2.52 for ^{1}H and 40 for ^{13}C), DMF-d_7 (referenced to 2.74 ppm for ^{1}H and 75.46 for ^{13}C), MeOD (referenced to 3.31 ppm for ^{1}H and 49.00 for ^{13}C), $CDCl_3$/MeOD (referenced specified for each compound), D_2O (referenced to 4.79 ppm for ^{1}H) or CD_3CN (referenced to 1.94 ppm for ^{1}H and 118.26 for ^{13}C); normal TFA was used as reagent grade and referenced to 11 ppm for ^{1}H and 40 for ^{13}C. Infrared spectra (IR) were recorded as KBr discs using a Bruker Tensor27 FT-IR instrument. Absorption maximum (υ_{max}) was recorded in wave numbers (cm^{-1}) and only selected peaks are reported. Ultraviolet spectra (UV) were recorded in quartz cuvettes of 1cm length using abiochrom Libra S22 instrument. Electrospray ionization mass spectra were recorded on a Waters Micro mass Quattro LCMS for High Resolution Mass Spectrometry (HRMS) and on a Waters Micro

mass LCT for Low Resolution Mass Spectrometry (LRMS) at 80 eV. Flash chromatography was performed using silica gel 60 (0.040–0.063 mm, 230–400 mesh) or alumina oxide, activated, basic Brockmann 1, standard grade ≈ 150 mesh, 50 Å.

Peptide sequences were assembled from a Rink Amide MBHA resin, unless stated otherwise, by standard SPPS according to the Fmoc/*t*-Bu strategy with either HBTU/HOBt/DIEA coupling chemistry in NMP solvent (automated synthesis), or PyBrop/DIEA coupling chemistry in DMF solvent (manual synthesis). Automated syntheses were performed on an Applied Biosystems 433A synthesiser (Warrington, UK), on a 2.5×10^{-4} mol scale, using single coupling cycles with a 4-fold excess of Fmoc-amino acid derivatives to resin-bound peptide. Manual syntheses were carried out in a 5 mL syringe fitted with a teflon frit and a stopcock, on a 1.0×10^{-4} mol scale and a 3.5-fold excess of Fmoc-amino acid derivatives to resin-bound peptide. Chain elongation was monitored by the qualitative Kaiser Test and multiple coupling procedures were applied until a negative test was obtained. Coupling reactions were performed for 30 to 150 min. N^{α}–Fmoc-protecting groups were removed by treatment with 25% piperidine in DMF (5 mL) for 10 min followed by two additional treatments of 5 min each. The resin was then washed three times with DMF (5 mL, 3 min each).

Following chain assembly, the dry resin was treated with a cleavage cocktail consisting of 90% TFA, 2.5% triisopropylsilane, 2.5% thioanisole, 2.5% phenol and 2.5% water, unless stated otherwise. The reaction time was adjusted to the number of arginine residues present in the sequence, from a minimum reaction time of 2 h, incremented by 30 min for each arginine residue present in the sequence. After eliminating the resin by filtration, the peptide was precipitated from the filtrate with the minimum amount required of cold diethyl ether and isolated by centrifugation for five minutes at 2.8×10^{3} rpm. The peptide pellet was resuspended in diethyl ether and centrifuged twice. The peptide was allowed to dry before being dissolved in water for lyophilisation.

Chromatographic analyses and purifications were performed on a BioCAD SPRINT Perfusion Chromatography Workstation (*PerSeptive Biosystems*) using Gemini columns (Phenomenex, 110 Å, 5µ, C18, 4.6 mmd/250 mmL or 10 mmd/250 mmL, for the analytic or semi-preparative columns, respectively). Buffers used were mobile phase A: 0.1% TFA in water; mobile phase B: 0.1% TFA in acetonitrile, with a gradient of 2 to 65% B in 18 column volumes (analytical) or 5 column volumes (semi-preparative) with a flow rate of 1 ml min^{-1} (analytical) or 4 ml min^{-1} (semi-preparative) and single wavelength detection at 214 nm.

Mass spectrometry analyses performed by Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) were obtained on a Reflex Bruker spectrometer. Two type of matrix were used, 2,5-dihydroxybenzoic acid and α-cyano-4-hydroxy-cinnamic acid, dissolved in 30% acetonitrile and 70% water at a concentration of 5 mg mL^{-1} and 6 mg mL^{-1}, respectively.

4.2. Synthesis of the cephalosporin derivatives

(6*R*, 7*R*)-7-Amino-3-hydroxymethyl-3-cephem-4-carboxylic acid (7-AHCA, 3). To a suspension of (6*R*, 7*R*)-7-amino-3-acetoxymethyl-3-cephem-4-carboxylic acid (7-ACA) (2 g, 7.4 mmol) in a mixture of methanol (16 mL) and water (10 mL) was added triethylamine (1.1 mL, 8.8 mmol) over 10 min and at 5 °C, which resulted in the formation of a clear solution. Tetrabutylammomium hydroxide (40% solution in water, 6.68 g, 10 mmol) was then added to this solution at −10 °C over 10 min. The temperature was maintained at −10 °C for an additional 4.0 h. The mixture was then acidified to pH 4.5 by addition of formic acid or HCl 1 N at −10 °C and then allowed to warm up at room temperature. The precipitate was collected by filtration and washed with a mixture of $MeOH/H_2O$ (1/1, 10 mL) and then with acetone to give 7-AHCA (1.45 g, 6.3 mmol, 85%).

IR: ν_{max} (KBr)/cm^{-1}: 3376, 3184, 1798, 1614, 1548, 1410, 1348; δ_H (400 MHz, TFA), 3.08 (s, 1H, **H**-3′$_A$), 3.25 (s, 1H, **H**-3′$_B$), 4.41 (s, 2H, **H**-2), 4.67 (s, 1H, **H**-6), 4.81 (d, 1H, **H**-7); δ_C (100.6 MHz, TFA), 21.57, 52.50, 59.81, 72.09 (**C**-2, **C**-6, **C**-7, **C**-3′), 122.23, 143.21 (**C**-3, **C**-4), 157.50, 170.53 (2 × **C**=O). LRMS *m/z*: 229.05 (100%, M-H^+) .

(6*R*, 7*R*)-3-Hydroxymethyl-7-(2-thiophen-2-yl-acetylamino) 3-cephem-4-carboxylic acid benzhydryl ester (4). 7-AHCA (1, 35 g, 5.89 mmol), finely grounded, was placed in suspension in water (10 mL) at 0 °C. Under vigorous stirring, a solution of 2N NaOH was slowly added until all the 7-AHCA dissolves. 2-Thienylacetyl chloride (0.860 mL, 7.04 mmol), dissolved in 2 ml of acetone, was then added dropwise. The pH was kept between 7.5 and 8.5 with 2 M, 1 M and 0.25 M solutions of NaOH (solutions of reduced concentrations are used as the reaction progresses) and the temperature maintained at 0 °C for an additional 1.0 h. The solution was then diluted with 17 ml of ethyl acetate, before acidification with a 1 M HCl solution to pH 2.5. The aqueous solution was extracted three times with ethyl acetate. The organic phases were combined and dried over $MgSO_4$. A solution of diphenyldiazomethane[23] was then added until the purple colour persists. After evaporating the solvent and triturating the solid residue was diethyl ether, the product was recovered as a white solid (1.75 g, 3.36 mmol, 57.3%).

IR: ν_{max} (KBr)/cm^{-1}: 3493, 3288, 1755, 1725, 1667, 1521, 1376, 1221; δ_H (400 MHz, $CDCl_3$), 2.62 (m, J = 9.6 and 4.4 Hz, 1H, O**H**), 3.54 (s, 2H, **H**-2), 3.85 (s, 2H, C$\mathbf{H}_2$ thienyl side-chain), 3.90 (dd, J = 12.8 and 9.6 Hz, 1H, **H**-3′$_A$), 4.39 (dd), J = 13.00 and 4.80 Hz, 1H, **H**-3′$_B$), 4.93 (d, J = 4.80 Hz, 1H, **H**-6), 5.89 (m, J =9.2 and 4.80 Hz, 1H, **H**-7), 6.46 (d, J = 9.2 Hz, 1H, N-**H**), 6.89 (s, 1H, C**H**$(Ar)_2$), 6.96-7.01 (m, 2H, Ar-**H** thiophene), 7.26-7.36 (m, 10H, Ar-**H**, benzhydryl), 7.42- 7.45 (m, 1H, Ar–**H** thiophene); δ_C (100.6 MHz, $CDCl_3$) 27.64, 37.10, 56.99, 59.00, 62.08, (**C**-2, **C**-3′, **C**-6, **C**-7, $\mathbf{C}H_2$ thienyl side-chain), 80.13 (**C**H$(Ar)_2$), 124.89, 126.14, 127.06, 127.48, 127.60, 127.96, 128.27, 128.49, 128.68, 133.32, 134.64 (**C**-3, **C**-4, (4 x **C**-Ar thiophene), (10 x **C**-Ar benzhydryl)), 138.92, 139.14 (2 × **C**-Ar benzhydryl), 161.72, 164.99, 170.09 (3× **C**=O). HRMS *m/z*: found 519.1034 [M-H^+], calcd for $C_{27}H_{23}N_2O_5S_2$ 519.1049; LRMS *m/z*: 519.12 (100%, M-H^+).

(6*R*, 7*R*)-3-(*p*-nitrophenoxycarbonyloxymethyl)-7-(2-thiophen-2-yl-acetylamino) 3-cephem-4-carboxylic acid benzhydryl ester (5a). Compound **4** (153.9 mg, 0.296 mmol) was dissolved in dichloromethane (9 ml). To this solution was added *p*-nitrophenylchloroformate (280 mg, 1.38 mmol), pyridine (24 µl, 0.296 mmol) and DMAP (3 mg, 0.0246 mmol). The reaction

mixture was stirred under nitrogen for 3 h at room temperature. It was then washed with water, dried and concentrated to yield the crude product (300 mg). This material was purified using column chromatography 70 : 30, hexane : ethyl acetate to yield a pale yellow solid (100 mg, 0.152 mmol, 51%).

δ_H (270 MHz, $CDCl_3$) 3.45 (d, J = 18.56 Hz, 1H, **H**-2_A), 3.65 (d, J = 18.80 Hz, IH, **H**-2_B), 3.89 (s, 2H, C**H**$_2$ cephalothin), 4.99 (d, J = 13.36 Hz, 1H, **H**-$3'_A$), 5.02 (d, J = 4.95 Hz, 1H, **H**-6), 5.25 (d, J = 13.11 Hz, 1H, **H**-$3'_B$), 5.87 (m, J = 4.95 and 9.15 Hz, 1H, **H**-7), 6.56 (d, J = 8.91 Hz, 1H, N-**H**), 6.94 (s, 1H, C**H**$(Ar)_2$), 6.82–7.04 (m, 2H, 2 Ar-**H** thiophene) overlapping with (m, 2H, **Ar-NO$_2$**), 7.22–7.42 (m, 10H, Ar-**H**), overlapping with (m 1H, Ar-**H** thiophene) and 8.10–8.32 (m, 2H, Ar-**H**-NO_2); δ_C (75.47 MHz, $CDCl_3$) 26.45, 36.96, 57.19, 59.17, 67.33 (**C**-2, **C**-3′, **C**-6, **C**-7, -CH_2 cephalothin), 80.24 (**C**H$(Ar)_2$), 121.60, 124.99, 125.34, 126.37, 126.96, 127.64, 127.68, 128.19, 128.23, 128.36, 128.49, 129.59, 133.97 (**C**-3, **C**-4, 4 × (**C**-Ar thiophene), 8 × (**C**-Ar), 6 × (**C**-Ar $ArNO_2$), 138.65, 138.81 (2 × **C**-Ar), 145.45, 152.02, 155.17, 160.43, 164.36, 171.15 (4 × **C**=O).

(6*R*, 7*R*)-3-(1,2,2,2-Tetrachloro-ethoxycarbonyloxymethyl)-7-(2-thiophen-2-yl-acetylamino) 3-cephem-4-carboxylic acid benzhydryl ester (5b). Compound **4** (0.4 g, 0.77 mmol) was dissolved in dichloromethane (8.8 ml). Tetrachloroethylchloroformate[24] (117.6 µl, 0.77 mmol), pyridine (62 µl, 0.77 mmol) and DMAP (4 mg, 0.033 mmol) were added and the resultant solution was stirred for 2.5 h. The reaction mixture was evaporated and the residue purified by flash chromatography (2/8 AcOEt/petroleum ether) to yield a solid (264 mg, 0.36 mmol, 47%).

IR: ν_{max} (KBr)/cm^{-1}: 3365, 1776, 1727, 1669, 1523, 1233; δ_H (400 MHz, $CDCl_3$), 3.37 (d, J = 18.56 Hz, 1H, **H**-2_A), 3.56 (d, J = 18.56 Hz, IH, **H**-2_B), 3.86 (s, 2H, C**H**$_2$ thienyl side-chain), 4.96–5.00 (m, 2H, **H**-$3'_A$ overlapping with **H**-6), 5.24–5.31 (m, 1H, **H**-$3'_B$), 5.89 (m, 1H, **H**-7), 6.28 (d, J = 9.2 Hz, 1H, N–**H**), 6.64 (s, 1H, C**H**Cl), 6.93 (s, 1H, C**H**$(Ar)_2$), 6.97–7.02 (m, 2H, Ar–**H** thiophene), 7.27–7.36 (m, 10H, Ar–**H** benzhydryl; 1H, Ar–**H** thiophene); δ_C (100.6 MHz, $CDCl_3$) 26.17, 37.11, 57.35, 59.16, 68.14, 68.21 (**C**-2, **C**-3′, **C**-6, **C**-7, CH_2 thienyl side-chain), 80.17 (**C**H$(Ar)_2$), 91.06 (**C**HCl), 96.89 (**C**Cl_3), 124.22, 124.26, 126.51, 126.56, 127.02, 127.69, 128.06, 128.23, 128.35, 128.52, 128.64, 134.50 (**C**-3, **C**-4, (4 × **C**-Ar thiophene), (10 × **C**-Ar benzhydryl)), 138.76, 138.90 (2 × **C**–Ar benzhydryl), 151.54, 160.35, 164.65, 169.98 (4 × **C**=O). Found C, 49.10; H, 3.45; N, 3.13%. $C_{30}H_{24}Cl_4N_2O_7S_2$ requires C, 49.33; H, 3.31; N, 3.84%. LRMS m/z: 311 (100%, M-H^+).

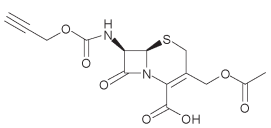
(6*R*, 7*R*)- 3-Prop-2-ynylcarbamoyloxymethyl -7-(2-thiophen-2-yl-acetylamino) 3-cephem-4-carboxylic acid benzhydryl ester (5c). Compound **5b** (111 mg, 152 µmol) was dissolved in 3 mL of THF, followed by $NaHCO_3$ 0.1 M (1.52 mL, 152 µmol) and propargylamine (8.36 mg, 152 µmol). The mixture was stirred during 1.5 h. The organic phase was diluted with DCM and subsequently extracted with water. Purification of the solid residue obtained by evaporation of the organic solvent was performed by flash chromatography with 65/35 Petroleum Ether/AcOEt to yield a white solid (66 mg, 110 µmol, 71%).

IR: ν_{max} (KBr)/cm^{-1}: 3314, 3291, 2913, 1791, 1695, 1645, 1538, 1250; δ_H (400 MHz, $CDCl_3$), 2.26 (t, J = 2.4 Hz, 1H, C**H** alkyne), 3.35 (d, J = 18.4 Hz, 1H, **H**-2_A), 3.53 (d, J = 18.4 Hz, 1H, **H**-2_B), 3.85 (s, 2H, CH_2 thienyl side-chain), 3.92 (m , 2H, CH_2 alkyne), 4.77 (d, J = 13.6 Hz, 2H, **H**-$3'_A$ overlapping with N–**H**), 4.96 (d, J = 4.8 Hz,1H, **H**-6), 5.03 (J = 13.6 Hz, 1H, **H**-$3'_B$), 5.87 (m, 1H, **H**-7), 6.34 (d, J = 9.2 Hz, 1H, N-**H**), 6.97 (s, 1H, C**H**$(Ar)_2$), 7.01 (m, 2H, Ar-**H** thiophene), 7.24–7.41 (m, 11H, 1 Ar–**H** thiophene, 10 × Ar–**H** benzhydryl); δ_C (100.6 MHz, $CDCl_3$) 26.40, 30.92, 37.11, 57.23, 59.07, 63.53 (**C**-2, CH_2 alkyne, CH_2 thienyl side-chain, **C**-6, **C**-7, **C**-3′),71.93, 79.70, (**C**-H alkyne, **C**-alkyne) 126.20, 126.46, 126.55, 127.17, 127.60, 127.64, 127.69, 128.02, 128.17, 128.25, 128.47, 128.53, 128.60, 134.59, 138.96, 139.19, 143.81 (**C**-3, **C**-4, (10 × **C**–Ar benzhydryl), (4 × **C**–Ar thiophene)),155.35, 160.69, 164.78, 170.07 (4 × **C**=O). Found: C, 62.48; H, 4.98; N, 5.95%. $C_{31}H_{27}N_3O_6S_2$ requires C, 61.88; H, 4.52; N, 6.98%.

(6*R*, 7*R*)- 3-Prop-2-ynylcarbamoyloxymethyl-7-(2-thiophen-2-yl-acetylamino) 3-cephem-4-carboxylic acid (6). Compound **5c** (66 mg, 0.11 mmol) was dissolved in 5 mL of dry DCM. Approximately 40 equivalents (483 µL) of TFA and 66 µL (0.6 mmol) of anisole were added at 0 °C and the reaction performed for 50 min. The reaction was followed by TLC using ethyl acetate as the mobile phase. The solution was then washed with a $KHSO_4$ solution, dried and evaporated. The crude product was then triturated with diethyl ether to yield a pale yellow solid (28 mg, 64 µmol, 58.3%).

IR: ν_{max} (KBr)/cm^{-1}: 3285, 1746, 1679, 1656, 1600, 1532, 1402, 1250; δ_H (400 MHz, $CDCl_3$/MeOD (0.450/0.3), (MeOD referenced to 3.49 ppm for ^1H and 48.17 ppm for ^{13}C). 2.53 (m, 1H, C**H** alkyne), 3.59 (d, J = 18.4 Hz 1H, **H**-2_A), 3.74 (d, J = 18.4 Hz, 1H, **H**-2_B), 3.97 (m, J = 20.8 and 15.6 Hz, 2H, CH_2 alkyne), 4.05 (s, 2H, CH_2 thienyl side-chain), 5.04 (d, J = 13.6 Hz, 1H, **H**-$3'_A$), 5.15 (m, 1H, **H**-6), 5.31 (d, J = 13.6, 1H, **H**-$3'_B$), 5.89 (s, 1H, **H**-7), 7.09 (m, 2H, Ar-**H** thiophene), 7.38 (m, 1H, Ar-**H** thiophene); δ_C (100.6 MHz, $CDCl_3$), 25.61, 29.98, 35.99, 57.23, 58.99, 63.32 (**C**-2, CH_2 thienyl side-chain, CH_2 alkyne, **C**-6, **C**-7, **C**-3′), 70.86, 79.24, (**C**-H alkyne, **C**-alkyne), 124.73, 125.32, 126.96 126.54, 135.26 (**C**-3, **C**-4, (4 × **C**-Ar thiophene)),156.28, 163.01, 164.55, 171.49 (4 × **C**=O). HRMS m/z: found 434,0473 [M-H^+], calcd for $C_{18}H_{16}N_3O_6S_2$ 434.0481; LRMS m/z: 434.03 (100%, M-H^+).

(6*R*, 7*R*)-3-Acetoxymethyl-7-prop-2-ynyloxycarbonylamino 3-cephem-4-carboxylic acid.



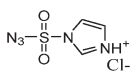
7-ACA (500 mg, 1.83 mmol) was dissolved in 4 mL of acetone and 2.5 mL of H_2O and the mixture was cooled to 0 °C. *N*,*N*-Diisopropylethylamine (300 µL, 0.22 mmol) was then added to the solution, followed by propargylchloroformate (214 µL, 0.22 mmol) dropwise. The pH was kept between 7.5 and 8.5 by addition of a molar solution of NaOH. After 1 h, the mixture was acidified with HCl 1 N, before extracting the product with diethyl ether and ethyl acetate. The combined organic phases were dried and evaporated yielding a white solid (400 mg, 1.13 mmol, 61.7%).

δ_H (400 MHz, DMSO), 2.04 (s, 3H, C**H**$_3$), 3.50 (d, J = 18.4 Hz, 1H, **H**-2$_A$), 3.56 (t, J = 2,4 Hz, 1H, CH alkyne) 3.63 (d, J = 18.4 Hz, 1H, **H**-2$_B$), 4.69 (m, 3H, **H**-3′$_A$ overlapping with C**H**$_2$ propargyl), 4.99 (d, J = 12.4 Hz, 1H, **H**-3′$_B$), 5.10 (d, J = 4.8 Hz, 1H, **H**-6) , 5.56 (dd, J = 4.8 Hz 1H, **H**-7), 8.58 (d, J = 11 Hz 1H, N–**H**), 13.85 (s, 1H, COO**H**). δ_C (100.6 MHz, $CDCl_3$) 20.55, 25.53, 52.35, 57.49, 60.87, 62.67, 77.61, 78.63 (**C**H$_3$, **C**-2, **C**H$_2$, **C**-6, **C**-7, **C**-3′, **C**H, CH$_2$–**C**), 123.63, 126.30, (**C**-3, **C**-4), 155.09, 162.83, 164.25, 170.19 (4 × **C**=O).

4.3. Synthesis of the peptides and their conjugates

4.3.1 Azido-peptides. The azido-peptide amide of Bac8c (2-Nal[3,6]) was prepared by performing a DiazoTransfer reaction on a fully assembled polymer-bound peptide sequence. An alternative route to this azido-peptide was also evaluated by coupling the N-terminal arginine modified as an azido acid to a resin-bound peptide sequence assembled up to the penultimate residue. In the former case, imidazole-1-sulfonyl azide hydrochloride was used as the DiazoTransfer reagent. In the latter case, azido arginine was prepared from H-Arg(Pbf)–OH or H-Arg(Mtr)–OH by reaction with triflyl azide in solution.

Synthesis by DiazoTransfer reaction with imidazole-1-sulfonyl azide hydrochloride. – Imidazole-1-sulfonyl azide hydrochloride



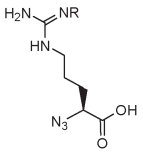
Sulfuryl chloride (3.72 mL, 46.2 mmol) was added dropwise to an ice-cooled solution of NaN_3 (3 g, 46.2 mmol) in 50 mL of acetonitrile and the mixture was stirred overnight at room temperature. Imidazole (6 g, 88 mmol) was added in portions to the iced-cooled mixture and stirred for 3 h. The mixture was diluted with EtOAc (100 mL), washed with H_2O (2 × 100 mL), saturated aqueous $NaHCO_3$ (2 × 100 mL), dried over MgSO4 and filtered. A solution of HCl was prepared by adding 5 mL (70 mmol) of acetyl chloride to chilled, freshly distilled, anhydrous EtOH. This solution was added dropwise with stirring to the filtrate chilled in an ice-bath, to give imidazole-1-sulfonyl azide hydrochloride as a white solid, which was filtered and washed with EtOAc (2.036 g, 9.7 mmol, 15%).

IR: ν_{max} (KBr)/cm^{-1}: 3111, 2502, 2169, 1915, 1588, 1414, 1160; δ_H (400 MHz, D_2O), 7.55 (s, 1H, **H**-2), 7.97 (s,1H, **H**-4), 9.27 (s,1H, **H**-5); δ_C (100.6 MHz, D_2O), 119.3, 124.08, 137.66 (**C**-3, **C**-2, **C**-5).

– DiazoTransfer reaction

0.33 mmol of resin-bound peptide (fully assembled sequence) was placed in a syringe and swelled during one hour in DCM/MeOH (4/1). Imidazole-1-sulfonyl azide hydrochloride (42 mg, 0.165 mmol) was then added, with K_2CO_3 (51.75 mg, 0.375 mmol) and some traces of $CuSO_4$, in a mixture of DCM/MeOH (4/1) and the mixture was agitated for 6 h. The resin was then isolated by filtration and washes were performed with DMF, DCM and MeOH. The reaction was repeated in THF/MeOH (4/1) as the solvent and the resin washed with DMF, DCM and THF after filtration. The reaction was monitored by the Kaiser test.

Synthesis by coupling of the α-azido acid of arginine. – Synthesis of azido arginine[25]



Sodium azide (1.78 g, 27.45 mmol) was added to a mixture of water (4.5 L) and DCM (7.5 mL) and the biphasic solution was cooled to 0 °C. Triflyl anhydride (0.93 mL, 5.55 mmol) was added slowly over 5 min maximum and the solution was then stirred for 2 h. The organic phase was kept aside and the aqueous phase was extracted with DCM (2 × 3.5 mL). The DCM fractions were combined, washed once with saturated $NaCO_3$ and used without further purification.

H-Arg(Pbf)-OH (1.18 g, 2.78 mmol) or H-Arg(Mtr)-OH (1.07 g, 2.78 mmol) was dissolved in a mixture of water (9 mL) and MeOH (18 mL) in presence of K_2CO_3 (577.5 mg, 4.19 mmol) and $CuSO_4$, $5H_2O$ (6.98 mg, 27.9 μmol). The triflyl azide solution (15 mL) was then added and the mixture stirred overnight at ambient temperature. After evaporation of the organic solvent, the aqueous phase was diluted with water (50 mL) and subsequently acidified to pH 6 with HCl 0.25 M and adjusted to pH 6.2 with a phosphate buffer (50 mL). This solution was then extracted with EtOAc. The aqueous phase was acidified to pH 2 with conc. HCl and extracted again with EtOAc. The organic phases were combined, dried over $MgSO_4$ and evaporated to give a white solid compound.

N_3-Arg(Pbf)-OH. δ_H (400 MHz, $CDCl_3$), 1.45 (s, 6H, C**H**$_3$ dimethyl-dihydrofuran), 1.61–1.90 (m, 4H, C**H**$_2$ arginine side chain), 2.07 (s, 3H, C**H**$_3$ tolyl), 2.45 (s, 3H, C**H**$_3$ tolyl), 2.51 (s, 3H, C**H**$_3$ tolyl), 2.94 (s, 2H, C**H**$_2$ dihydrofuran), 3.23 (s, 2H, C**H**$_2$ arginine side chain), 3.95 (m, 1H, C**H** arginine), 6.26–7.39 (m, 3H, N-**H** guanidinium); δ_C (100.6 MHz, $CDCl_3$), 12.50, 17.86, 19.31, 28.60, 29.72, (**C**-H$_3$ dimethyl-dihydrofuran, **Cn**H$_3$ tolyl, **C**-H$_3$ tolyl, **C**-H$_2$ arginine, **C**-H$_3$ tolyl), 43.13 (**C**-H$_2$ dihydrofuran), 60.48 (**C**-H$_2$ arginine), 61.88 (**C**-H arginine), 86.82, (**C**–N$_3$), 117.94, 125.04 (6 × **C**–Ar), 156.14 (**C**-guanidinium), 174.18 (**C**=O).

N_3-Arg(Mtr)-OH. δ_H (400 MHz, D_2O), 1.57–1.72 (m, 4H, C**H**$_2$ arginine side chain), 2.08 (s, 3H, C**H**$_3$ Ar), 2.53 (s, 3H, C**H**$_3$ Ar), 2.61 (s, 3H, C**H**$_3$ Ar), 3.19 (s, 2H, C**H**$_2$ arginine side chain), 3.79 (s, 3H, OC**H**$_3$), 3.95 (m, 1H, C**H** arginine), 6.50 (s, 1H, Ar-**H**), 6.26–6.90 (m, 3H, N-**H** Guanidinium); δ_C (100.6 MHz, $CDCl_3$), 11.92, 18.17, 22.70, 24.11, 29.37, 31.93 (3 × **C**–H$_3$ Ar, 3 × **C**-H$_2$ arginine), 43.13 (O-**C**H$_3$), 55.47 (**C**–H arginine), 86.82, (**C**–N$_3$), 111.91, (**C**–Ar–H), 125.18, 138.62 (5 × **C**–Ar), 156.23 (**C**-guanidinium), (**C**=O not observed).

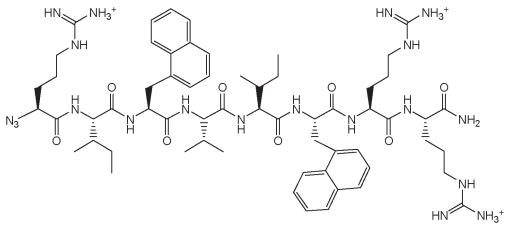
– Coupling of azido arginine

The resin-bound peptide sequence assembled up to the penultimate residue was placed in a syringe for manual synthesis. The resin was swelled in DMF for 30 min. 3 equivalents of N_3-Arg(R)-OH, DIC and HOBt were dissolved in DMF and added

to the resin. The coupling reaction was monitored by the Kaiser Test. Upon completion of the coupling reaction, the resin was washed with DMF and DCM.

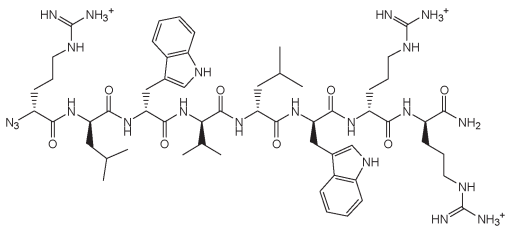
Isolation of the azido peptides. Azido peptides were isolated by treatment of the resin with a mixture of 3% triisopropylsilane, 3% water, 3% thioanisole and 3% phenol per ml of TFA for 3.5 h.

N_3-Arg-Ile-2-Nal-Val-Ile-2-Nal-Arg-Arg-NH_2.



LRMS *m/z*: 1231.9 (100%, $M+H^+$).

N_3-Arg-Leu-Trp-Val-Leu-Trp-Arg-Arg-NH_2 (7).

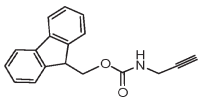


LRMS *m/z*: 1209.80 (100%, $M+H^+$).

4.3.2 D-Arg^1-triazole-ε2- D-Bac8c(Leu^{2,5}) (9). The D-Bac8c(Leu^{2,5}) peptide containing a N-terminal D-Arg-triazole-ε^2-amino acid was prepared by copper(I)-catalysed cyclo-addition of the azido peptide described in 4.3.1 with propargylamine. An alternative route to a triazole peptide was also evaluated by synthesising a protected D-Arg-triazole-ε^2-amino acid and coupling this moiety to a resin-bound peptide sequence assembled up to the penultimate residue using DIC/HOBt coupling chemistry. In the latter case, propargylamine was protected with a Fmoc group and reacted with N_3-Arg(Mtr)-OH (*vide supra*) by copper(I)-catalysed [3 + 2] cycloaddition to form the Fmoc-D-Arg(Mtr)-triazole-ε^2-amino acid.

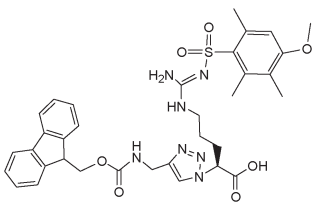
Solid phase synthesis of the triazole peptide. The resin of the azido peptide (0.05 mmol) was swelled during one hour in DMF. Propargylamine (0.15 mmol, 10 μL), CuI (9.5 mg, 0.05 mmol) and diisopropylethylamine (0.3 mmol, 52.1 μL) were then added. A mixture of DMF/MeOH (4/1) was then added and the mixture vortexed during 6 h. The coupling reaction was repeated in a mixture of THF/MeOH (4/1) as the solvent. The resin was then washed successively with DMF, MeOH and DCM. The peptide was cleaved and purified (92%) as described in paragraph 4.1. LRMS *m/z*: 1265.0 (100%, $M+H^+$).

Hybrid solution-solid phase synthesis of a triazole peptide. – *N*-Fmoc-propargylamine



Fmoc chloride (1.53 g, 5.9 mmol) was dissolved in 12 mL of THF at 0 °C. Propargylamine (0.427 mL, 6.2 mmol) was then added dropwise, followed by DIEA (1.084 mL, 6.2 mmol). The mixture was stirred for 3.5 h at RT. THF was then evaporated and the residual solid dissolved in ethyl acetate; the solution was washed with $KHSO_4$ and Na_2CO_3 solutions and finally with brine. The product was recovered by evaporation of the organic phase as a white solid (1.36 g, 4.9 mmol, 83.4%). δ_H (400 MHz, $CDCl_3$), 2.28 (t, 1H, J = 2.4 Hz, **H**-alkyne), 4.01 (m, 2H, C**H**$_2$ propargyl), 4.23 (t,1H, J = 6.4 Hz, C**H** Fmoc), 4.43 (d, 2H, J = 6.8 Hz, C**H**$_2$ Fmoc), 4.99 (s, 1H, N–**H**), 7.30–7.78 (m, 8H, Ar–**H**); δ_C (100.6 MHz, $CDCl_3$), 30.87, 47.13, 67.08 (**C**–H$_2$ alkyne, **C**–H Fmoc, **C**–H$_2$ Fmoc), 71.75, 79.60 (**C**–H alkyne, **C**-alkyne), 120.04, 125.04, 127.09, 127.76, (8 × (**C**–Ar)), 141.32, 143.79 (4 × **C**–Ar), 155.92 (**C**=O).

– Fmoc- D-Arg-triazole-ε^2-amino acid



N-Fmoc-propargylamine (195 mg, 0.70 mmol) and N_2=Arg(Mtr)–OH (290 mg, 0.70 mmol) were dissolved in 2.8 mL of acetonitrile. Lutidine (163 μL, 1.4 mmol), *N*,*N*-diisopropylethylamine (243 μL, 1.4 mmol) and sodium ascorbate (13.3 mg, 70 μmol) were then added. The solvent was then evaporated and the product dissolved in ethyl acetate. The organic phase was then washed with a solution of NH_4Cl/NH_4OH and HCl 0.5 N. TLC was performed with a mixture DCM/MeOH (8/2) as the mobile phase and purification on silica gel with a mixture of DCM/Petroleum Ether/MeOH (5/3/2) as the eluant. MW=689.76 g mol^{-1}; m= (200 mg, 0.3 mmol, 41.5%).

δ_H (400 MHz, MeOD), 0.78–0.93, (m, 2H, C**H**$_2$ arginine), 1.18–1.40 (m, 2H, C**H**$_2$ arginine), 2.00(s, 3H, C**H**$_3$ Ar), 2.53 (s, 3H, C**H**$_3$ Ar), 2.60 (s, 3H, C**H**$_3$ Ar), 3.12 (s, 2H, C**H**$_2$ arginine), 3.74 (s, 3H, OC**H**$_3$), 4.12 (t, 1H, J = 6.8 Hz, C**H** Fmoc), 4.30 (d, J = 7.2 Hz 2H, C**H**$_2$ Fmoc), 4.33 (s, 2H, C**H**$_2$ C^6), 5.03 (m, 1H, C**H** C^2), 4.43 (1H, C**H** arginine), 6.50 (s, 1H, Ar–**H** Mtr), 7.21–7.78 (m, 8H, Ar–**H** Fmoc), 7.82 (s, 1H, triazole); LRMS *m/z*: found 690.1 ($M+H^+$).

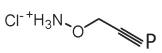
4.3.3 Cephalothin-D-Bac8c(Leu^{2,5}) (8). A azide-alkyne *cyclo*-addition reaction was performed by using one molar equivalent of the fully deprotected azido-peptide described in 4.3.1 (14.5 mg, 12 μmol) and 2 molar equivalents of (6*R*, 7*R*)- 3-prop-2-ynylcarbamoyloxymethyl -7-(2-thiophen-2-yl-acetylamino) 3-cephem-4-carboxylic acid **6** (10.5 mg, 24 μmol). Three different

solutions were prepared containing respectively 0.4 equivalent of $CuSO_4 \cdot 5H_2O$, 0.8 equivalents of sodium ascorbate and 7 equivalents of lutidine in a mixture of water and *t*-butanol (1 : 1). 160 μL of the first two solutions and 240 μL of the last one were taken and added along with 600 μL of *tert*-butanol. The mixture was stirred at 30 °C for 4 to 12 h. The product was precipitated by addition of diethyl ether and isolated by centrifugation. The residue dissolved in water for HPLC purification, performed as described above, except 0.08% formic acid was added to the buffers in place of 0.1% TFA and dual wavelength UV detection (214 and 260 nm) was performed. Collected fractions containing the conjugate were lyophilised (5.9 mg, 3.5 μmol, 29%; purity 91%). NMR analysis was recorded on a BRUCKER AC 600 and LRMS analysis was performed at 35 eV. LRMS *m/z* found 822.4 $(M+2H)^{2+}$.

4.3.4 Control 10. The control conjugate formed by attachment of the peptide at the 7-position of the cephem core was prepared by an azide-alkyne *cyclo*-addition reaction, performed by using one molar equivalent of the fully deprotected azido-peptide (7,5 mg, 6.2 μmol) described in 2b and 2 molar equivalents of (6*R*, 7*R*)-3-acetoxymethyl-7-prop-2-ynyloxycarbonylamino 3-cephem-4-carboxylic acid (*vide supra*) (4,6 mg, 13 μmol), dissolved in 225 μL of *t*-butanol. 75 μL of a solution containing 0.4 eq. of $CuSO_4 \cdot 5H_2O$ in *t*-butanol, 75 μL of a solution containing 0.8 eq. of sodium ascorbate in *t*-butanol and 75 μL of a solution containing 7 eq. of lutidine in *t*-butanol were then added to this solution. The mixture was stirred at 30 °C for 4 to 12 h. Precipitation of the product was achieved by addition of 2 mL of diethyl ether and HPLC purification was performed as in 2d (purity 97.9%, yield 0.275 mg 0.17 μmol, 2.8%). LRMS *m/z*: found 1564.0 $(M+H^+)$.

4.3.5 Control 11. The control conjugate based on an oxime linker was synthesised by oxidation of the 3′-hydroxyl group of compound **4** to an aldehyde, followed by formation of an oxime by reaction with *O*-prop-2-ynyl-hydroxylamine. This alkyne-modified cephalosporin intermediate was subjected to an azide-alkyne *cyclo*-addition reaction with the azido-peptide described in 4.3.1.

– *O*-prop-2-ynyl-hydroxylamine hydrochloride



To *N*-(propargyloxy)phthalimide (400 mg, 1.98 mmol), dissolved in 2 ml of diethylether, was added 0.1 mL of hydrazine hydrate (3.2 mmol). The mixture was stirred for 10 min, then 1.5 mL of diethylether was added. The stirring was maintained during another 30 min. A solution of ethereal HCl was then added dropwise. The product was filtered and used without further purification. Starting material occasionally remaining was eliminated during the next step. δ_H (400 MHz, CD_3CN), 2.04 (t, *J* = 2,4 Hz, 1H, terminal alkyne), 4.91 (d, *J* = 2.4 Hz, 2H, CH_2); δ_C (100.6 MHz, CD_3CN), 62.19 (C–H_2), 74.11, 79.62 (C alkyne, C–H alkyne).

– (6*R*, 7*R*)- 7-(2-thiophen-2-yl-acetylamino)-3-formyl-3-cephem-4-carboxylic acid benzhydryl ester

The reaction was carried out in a solid phase reaction vessel. Compound **4** (100 mg, 0.192 mmol) was dissolved in 3 mL of dry DCM; 3 equivalent of IBX resin (0.576 mmol) were then added and the mixture was stirred overnight. The resin was removed by filtration and subsequently washed with 3 × 5 mL of DCM. The DCM fractions were combined and evaporated, yielding quantitatively the product which can be used directly without purification. The resin can be reused after oxidation. δ_H (400 MHz, $CDCl_3$), 3.26 (d, *J* = 18.8 Hz, 1H, **H**-2_A), 3.86 (s, 2H, C$\mathbf{H}_2$ thienyl), 3.97 (d, *J* = 18.8 Hz, IH, **H**-2_B), 5.03 (d, *J* = 5.6 Hz, 1H, **H**-6), 5.99 (m, 1H, **H**-7), 6.37 (d, *J* = 9.2 Hz, 1H, N–**H**), 6.64 (s, 1H, C**H** thienyl), 6.97–7.01 (m, 2H, 2 × =C**H** thienyl), 7.06 (s, 1H, C**H**$(Ar)_2$), 7.26–7.37 (m, 10H, Ar–**H**), 9.63 (s, 1H, **H**-3′); δ_C (100.6 MHz, $CDCl_3$) 22.29, 37.05, 58.91, 59.84 (**C**-2, C$\mathbf{H}_2$ thienyl, **C**-6, **C**-7), 80.86 (**C**H$(Ar)_2$), 123.87, 126.34, 127.09, 127.30, 127.60, 127.71, 127.92, 128.10, 128.31, 128.51, 128.64, 128.69, 128.73, 130.10, 132.47, 134.35, 138.09, 138.41, 138.44 (**C**-3, **C**-4, 4 × (=**C**) thienyl, 8 × (**C**-Ar)) 159.63, 165.01, 169.98 (3 × **C**=O) , 187.76 (**C**-formyl).

– (6*R*, 7*R*)-7-(2-thiophen-2-yl-acetylamino)-3-(prop-2-ynyloxyimino-methyl)-3-cephem-4-carboxylic acid benzhydryl ester

6*R*, 7*R*)- 7-(2-thiophen-2-yl-acetylamino)-3-formyl-3-cephem-4-carboxylic acid benzhydryl ester (90 mg, 0.188 mmol) was dissolved in 6 mL of dry acetonitrile. The solution was chilled to 0 °C and *O*-prop-2-ynyl-hydroxylamine hydrochloride (18 mg, 0.17 mmol) was then added. The mixture was stirred overnight and the solvent was then evaporated to recover the oxime product (107.6 mg, 0.19 mmol, quantitative). δ_H (400 MHz, $CDCl_3$), 2.47 (t, *J* = 2.4 Hz, 1H, C**H** alkyne), 3.49 (d, *J* = 18.4 Hz, 1H, **H**-2_A), 3.86 (s, 2H, C$\mathbf{H}_2$ thienyl), 4.05 (d, *J* = 18.4 Hz, 1H, **H**-2_B), 4.67 (d , *J* = 2.4 Hz, 2H, C$\mathbf{H}_2$ propargyl, 5.02 (d, *J* = 4.8 Hz,1H, **H**-6), 5.91 (m, 1H, **H**-7), 6.33 (d, *J* = 9.2 Hz, 1H, N–**H**), 6.97 (s, 1H, C**H**$(Ar)_2$), 7.01 (m, 2H, =C**H** cephalothin), 7.26–7.41 (m, 11H, =C**H** cephalothin, 10 × Ar–**H**), 8.34 (s, 1H, **H**-3′); δ_C (100.6 MHz, $CDCl_3$) 22.52, 36.06, 56.91, 58.61, 61.18 (**C**-2, $\mathbf{CH}_2$ thienyl, **C**-6, **C**-7, $\mathbf{CH}_2$ propargyl), 73.93, 78.99, (**C**-alkyne, **C**-H alkyne), 121.80, 125.18, 126.03, 126.48, 126.61, 126.71, 126.98, 127.21, 127.29, 127.46, 127.62, 133.49, 137.70, 137.98, (**C**-3, **C**-4, 10 × (**C**–Ar), 4 × (=**C**) thienyl), 146.29 (**C**-3′), 159.18, 163.65, 168.92 (3 × **C**=O).

– Control **11**

The azide-alkyne cyclo-addition reaction was performed by using one molar equivalent of the fully deprotected azido-peptide described in 2b (10.4 mg, 8.6 μmol) and 2 molar equivalents of compound (6*R*, 7*R*)- 7-(2-thiophen-2-yl-acetylamino)-3-(prop-2-ynyloxyimino-methyl)-3-cephem-4-carboxylic acid benzhydryl ester (9.8 mg, 17 μmol). Three different aqueous solutions were prepared containing respectively 0.4 equivalent of $CuSO_4 \cdot 5H_2O$ (20 mg/2.5 mL), 0.8 equivalents of sodium ascorbate (31.6 mg/2.5 mL) and 7 equivalents of lutidine (170 μL/2.5 mL) in a mixture of water. 107 μL of each solution were withdrawn and added along with 321 μL of *tert*-butanol. The mixture was stirred at 30 °C for 4 to 12 h and then evaporated. The residue was dissolved in water for HPLC purification, performed as described above, with 0.08% formic acid added to the buffers in place of 0.1% TFA. Collected fractions containing the control, deprotected, peptide (deprotection of the benzhydryl ester occurs during HPLC purification) were lyophilised (0.168 mg, 0.1 μmol, 1.2%; purity 75.23%). LRMS *m/z*: found 1615.1 $(M+H^+)$.

4.4. Activation assays

These assays were performed at pH 7.25 in a 10 mM PBS buffer, by monitoring with a UV-spectrophotometer the disappearance of the cephalothin's β-lactam bond at 260 nm. The chemical hydrolysis assay was carried out in a 0.41 M NaOH solution using a 0.140 mM solution of **8**. β-Lactamase-mediated reactivation assays were performed with a purified P99 enzyme from *Enterobacter cloacae* (0.8 mg, 0.32 μM) using a 13 mM solution of **8**.

4.5. Susceptibility assays

For determination of minimum inhibitory concentration (MIC) of peptides, the broth microdilution method was used according to the guidelines of the Clinical Laboratory Standards Institute (CLSI) with modifications for testing of cationic peptides as described by Wu and Hancock.[26] Briefly bacterial strains from the American Type Culture Collection (ATCC) or the National Collection of Type Cultures (NCTC) or isogenic strains (this study) were grown overnight on Mueller Hinton (MH) agar at 37 °C and single colonies were resuspended in MH broth to the density of a 1 McFarland standard (5×10^7 cells approx) which was further diluted 1/100 in MH broth as the inoculum. Peptides were resuspended in a solution containing 0.2% (w/v) bovine serum albumin and 0.01% (v/v) acetic acid and serial doubling dilutions were prepared at ten times the required concentration in Eppendorf tubes (dilutions tested were from 160 to 0.3125 μg ml^{-1} final peptide concentration per well). Assays were prepared in 96 well polypropylene plates (Costar Corp., Cambridge MA) and contained 10 μl of each dilution of peptide and 90 μl of inoculum. Sterility controls for each peptide dilution and for MH medium were included. Plates were incubated overnight (18 h) and MIC was recorded as the lowest concentration of peptide at which no growth was observed with reference to wells containing no peptide.

4.6. Generation of isogenic strains ± $bla_{CTX\text{-}M\text{-}15}$

Plasmid pET-30a(+) (Novagen) was double-restricted with Eco R I/Hind III in a reaction containing 10 U of each restriction enzyme and 1 U of thermosensitive alkaline phosphatase (TSAP) (all Promega) for 3 h at 37 °C. Linearized pET30a plasmid was separated by agarose gel electrophoresis and purified using Qiagen's QIAquick® Gel Extraction Kit. The $bla_{CTX\text{-}M\text{-}15}$ insert (924 bp) was amplified from genomic DNA isolated from a CTX-M-producing clinical *E. coli* isolate using PCR primers; CTX-MEco-FW, 5′-GGAATTCGACTATTCATGTTGTTG-TTATT-3′[27] and CTX-M-R, 5′-CCCAAGCTTTTACAAAC-CGTCGGTGACGAT-3′.[28] The forward primer contained a 5′ EcoR I restriction site (underlined) and included a homologous promoter sequence 29-42 bp upstream of the start codon.[27] The reverse primer included a 3′ Hind III restriction site (underlined).[28] Insert DNA was purified, double-digested with Eco R I/Hind III restriction enzymes (Promega) and re-purified. A Clonables™ Ligation/Transformation Kit (Novagen) was used to ligate the $bla_{CTX\text{-}M\text{-}15}$ insert into the linearized pET-30a(+) vector. NovaBlue Singles™ Competent Cells were transformed with the ligation mixture by heat shock. Positive transformants were selected by growth on Luria-Bertani (LB) agar containing 30 μg ml^{-1} kanamycin (Kam) and identification of transformants containing the $bla_{CTX\text{-}M\text{-}15}$ insert was confirmed by colony PCR screening using T7 vector specific primers which annealed to the T7 promoter (T7F) and T7 terminator (T7R) regions on the pET-30a(+) vector amplifying a 1150 bp target sequence that incorporated the $bla_{CTX\text{-}M\text{-}15}$ insert. The recombinant vector pET-CTX-M-15 was purified from a 10 ml overnight LB Kam 30 μg ml^{-1} broth culture.

The expression host *E. coli* BL21(DE3) was transformed with recombinant plasmid pET-CTX-M-15 or the empty vector pET30a(+) and positive transformants were selected on Mueller Hinton (MH) Kam 30 μg mL^{-1} agar. Transformation screening was performed by colony PCR using the T7 primers as previously described. Cloning success was confirmed by sequence analysis of $bla_{CTX\text{-}M\text{-}15}$ amplified from pET-CTX-M-15 using CLC DNA Workbench 6 software (CLC bio, Denmark) by comparison to the $bla_{CTX\text{-}M\text{-}15}$ sequence from Genbank (Accession no: ACX54236.1). Functional CTX-M-15 enzyme was confirmed in the BL21 cells containing pET-CTX-M-15 by ESBL disk diffusion phenotypic confirmatory tests using MASTDISCS™ ID ceftazidime and cefotaxime ESBL ID Disc Sets (Mast Diagnostics, Merseyside, UK) and by ESBL broth microdilution phenotypic confirmatory tests. Both tests were performed and interpreted according to CLSI guidelines.[29] Lack of expression of CTX-M-15 in BL21 cells containing pET30a(+) was confirmed using the same methods.

Acknowledgements

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