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Laboratory note

Synthesis and antibacterial activity of dual-action agents of a β-lactam antibiotic with cytotoxic agent mitozolomide or temozolomide

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Abstract

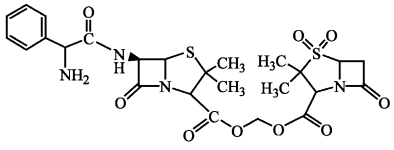
Dual-action agents **5a**–**f** and **12a**–**f**, a β-lactam antibiotic combined with a cytotoxic agent, mitozolomide (Meto) or temozolomide (Temo), were synthesised. The antibacterial activity (MICs) of the dual-action agents has been determined against a panel of bacteria including several β-lactamase producing strains. The tests showed **5a**–**f** active against the non-β-lactamase producing methicillin sensitive *Staphylococcus aureus* (MSSA) strains, however, little synergistic effect between the β-lactam and the cytotoxic agent was observed. **12a**–**f** demonstrated some synergistic effect against bacteria. **12a**, in particular, is active against ampicillin resistant (β-lactamase-producing) strains of *Serratia marcescens*. © 2002 Published by Éditions scientifiques et médicales Elsevier SAS.

Keywords: Dual-action; Antibacterial; β-lactam; Cytotoxic; Tetrazinone

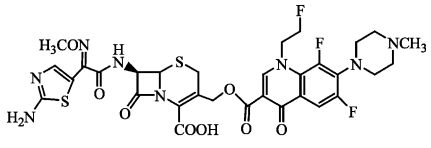
1. Introduction

β-Lactam antibiotics are unquestionably the most important group of antimicrobials and β-lactam nucleus is the most fruitful pharmacophore in producing therapeutic agents. However, because of increasing bacterial resistance, a number of novel approaches has been explored to improve activity of β-lactams [1]. Dual agent is one of the most successful developments, by employing a β-lactamase inhibitor (e.g. clavulanic acid, sulbactam or tazobactam) to protect a β-lactamase sensitive β-lactam (e.g. amoxicillin, ampicillin or piperacillin) in a 'cocktail' formula [2]. Another remarkable advance is to combine two effective drugs in a single molecule as a dual-action agent, e.g. sultamicillin [3] (Fig. 1) a conjugate of two drugs with expected combined bioactivities, and Ro 23-9424 [4,5] in which two antibiotics enhance each either through a mechanism: when a β-lactamase deactivates the cephalosporin by opening the β-lactam ring, the second antibacterial agent is released to exert its activity [6].

The dual-action agents designed in this study are a combination of a β-lactam antibiotic and a cytotoxic agent, mitozolomide (Mito) (**1a**) or temozolomide (Temo) (**1b**), small and readily available DNA cross-linking and alkylating agents [7]. In vivo, **1a** and **1b** undergo a hydrolysis to release the active species, chloroethyldiazonium [8] and methyldiazonium [9], responsible for cytotoxic activity. It also has been demon-



Sultamicillin



Ro 23-9424

Fig. 1. Structure of sultamicillin and Ro 23-9424.

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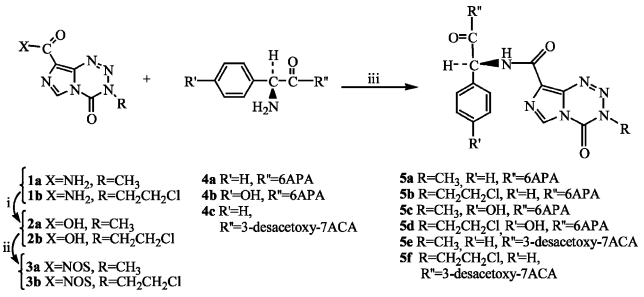

Fig. 2. (i) $NaNO_2$–H_2SO_4; (ii) NHS, DCC, DMF; (iii) TEA, DMF.

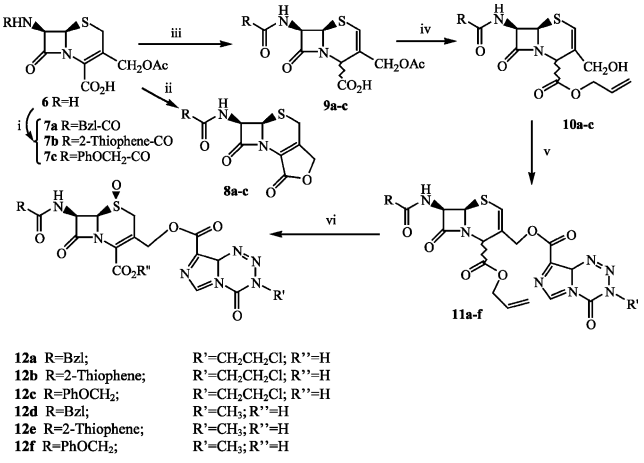

Fig. 3. (i) Ref. [17]; (ii) Ref. [18]; (iii) Py–$(Ac)_2O$–HCl; (iv) (a) NaOH–H_2O, (b) Allyl bromide–DMF; (v) **2a** or **2b**–PyBOP–DIPEA; (vi) (a) *m*-CPBA, DCM, (b) TPP, TKTPPP.

strated that Mito acid (**2a)** and Temo acid (**2b)** posses a similar cytotoxicity to their parent drugs [10,11]. In dual-action agents **5a**–**f**, the antibiotic and the cytotoxic agent were integrated through acylation α-amino group of α-aminoarylacetamido of ampicillin, amoxicillin and cefalexin to give a conjugate with two desired pharmacophores, antimicrobial and cytotoxic. Superior solution stability and β-lactamase affinity of sulfoxide cephalosporins [12] promoted us to synthesise **12a**–**f**, in which the acylations happened on hydroxy group of 3-hydroxymethyl of cephalosporins to furnish a molecule, which could undergo a cascade reaction to release a cytotoxic agent once the β-lactam has been attacked. The rationale was to combine the specificity of the β-lactam agent with the toxic properties of the cytotoxic agent to provide a selectively toxic antibacterial mode of action. It could be speculated that the agents would inhibit β-lactamase enzymes by the toxic activity of the cytotoxic agent, as the β-lactam agent was bound to the serine group of the β-lactamase. Even after the antibiotic is deactivated by the β-lactamases, the cytotoxic agents are lethal to bacteria because they are small molecules with an ability to penetrate cell membranes [13].

2. Results and discussion

2.1. Chemistry

A number of synthetic methods for acylation of α-amino group of α-aminobenzyl and α-amino(*p*-hydroxybenzyl) groups in ampicillin and amoxicillin was developed in 1970s when piperacillin and BL-P1908 were discovered [14]. *N*-hydroxy succinimide (NHS) active ester approach was adopted for syntheses of our target dual-action agents **5a**–**f** [15]. Conversion of 8-carboxamide in **1a** and **1b** to 8-carboxylic acid was accomplished with sodium nitrite and concentrated sulphuric acid in a quantitative yield [16]. Mitozolomide *N*-hydroxy succinimide active ester (Meto-NOS) (**3a**) and temozolomide *N*-hydroxy succinimide active ester (Temo-NOS) (**3b**) were prepared with DCC in dry DMF. Acylation of α-amino group of α-aminobenzyl and α-amino(*p*-hydroxybenzyl) groups in ampicillin, amoxicillin and cephalexin were carried out smoothly. First the mixture of equal mole of the β-lactam and Meto-NOS (**3a**) or Temo-NOS (**3b**) in dry DMF was cooled and exposed to triethylamine. The reaction normally was completed within 1 day at ambient temperature. After work-up with water and an organic solvent extraction to remove both water and organic soluble impurity, the dual-action agents **5a**–**f** were obtained (Fig. 2).

A reaction of 7β-aminocephalosporanic acid (7-ACA) (**6**) with phenylacetyl chloride, phenoxyacetyl chloride, and 2-thiopheneacetyl chloride in the presence of TEA in DMF at -40 °C gave high yields of **7a**–**c** as pure products after a participation of reaction mixture between an organic and an aqueous phase [17]. Hydrolysis of 3-acetoxymethyl of **7a**–**c** into 3-hydroxylmethyl was recorded in literature and foreseen as a conventional reaction [18]. However, when the reaction was performed accordingly, the only visible products were 2,3-lactone (**8a**–**c**) (Fig. 3) [19]. This outcome was attributed to a driving force of plane geometry of 3-hydroxylmethyl and 4-carboxylic acid in dilute HCl for the intramolecular esterification. It was predicated that 3,4-ene isomer **9a**–**c**, in which the plane geometry was destroyed, will stay in a free acid and alcohol form. The isomerization of the carbon=carbon double bond in **7a**–**c** was promoted by dry pyridine and acetic anhydride to furnish a pyridine salt first. After neutralisation with 2 N HCl, **9a**–**c** were obtained as a white powder. The hydrolysis of **9a**–**c** with 1 N NaOH followed by protection of the 3-carboxylic acid with ally bromide in DMF afforded cephem **10a**–**c**.

The reactions between **10a**–**c** and **2a** and **2b** were proved to need a strong catalyst. First we tried the mild reagents DCC and TBTU, no reaction was observed. Finally the reactions were completed by applying PyBOP and diisopropylethylamine (DIPEA) in

dichloromethane at 0 °C to afford **11a**–**f** in 23.6–77.1% yields after flash column purification. Since 3,4-cephem is not an active form of β-lactams, an isomerization was carried out to shift the double bond to 2,3-position. We found that from **11a**–**f** to the final target **12a**–**f** has to go isomerization first followed by deprotection. If deprotection first, the isomerisation will give a messy mixture. The oxidation-isomerization was achieved with *m*-CPBA in dichloromethane and the deprotection was accomplished with triphenylphosphine (TPP) and tetrakis(triphenylphosphine)palladium (TKTPPP) to give the targets **12a**–**f**.

Attempts to convert the dual-action agents **5a**–**f** and **12a**–**f** into their correspondent water soluble sodium salts with both organic and inorganic reagents resulted in decomposition, indicating that no decrease of sensitivity of the carbonyl group in the tetrazinone, the trigger for release of the cross-linking and alkylating agents, even to a weak base.

2.2. Antibacterial activity

Minimal inhibitory concentrations (MICs) of the dual-action agents **5a**–**f** and **12a**–**b**, **12d**–**f** for various bacteria were determined. As shown in Table 1, the dual-action agents **5a**–**f** were active against the non-β-lactamase producing methicillin sensitive *S. aureus* (MSSA) strains. While **5a** and **5e** proved the most active against MSSA NCTC 10788 (MIC of < 0.03 gg mL^{-1}), and against MSSA NCTC 6571 with MIC values 0.98 ng mL^{-1}. None of **5a**–**f** showed a good activity against methicillin-resistant *S. aureus* (MRSA) strains, either β-lactamase positive (MRSA 96-7778 and MRSA Innsbruck) or β-lactamase negative (MRSA 967992 and MRSA 96-5665), except **5b** displayed activity against the β-lactamase producing MSSA strain HW. The dual-action agents **5a**–**f** were active against the permeability mutant *E. coli* DC2, with **5a** proving the most active with a MIC of 0.491 μg mL^{-1}. However, None of **5a**–**f** displayed activity against *E. coli* DC0 and the extended spectrum β-lactamase producing *E. coli* strain (the *E. cloacae* strain producing a Group 1 β-lactamase or the *K. pneumoniae* candidate which also produces a β-lactamase enzyme).

In comparison of MIC values of **5a**–**f** with those of their components, the β-lactams and the cytotoxic agents, determined against the same panel of organisms Table 1, **5a**–**f** were virtually not more active than the β-lactam agents. Only in the case of **5a**, small degree of synergistic activity against the non-β-lactamase producing MSSA strains was demonstrated. It seems the cytotoxic agent in **5a**–**f** does not protect the β-lactam from β-lactamase hydrolysis, nor does it augment significant the activity of spectrum of the β-lactam agents. Antibacterial activity of **5a**–**f** appears to be due to the presence of the β-lactam, rather that due to a synergistic and selectively toxic mechanism of the dual-action agents.

Table 2 showed dual-action agents **12a**–**b** and **12d**–**f** were active in inhibiting the growth of some of strains, but not as effective as ampicillin, particularly, *S. aureus* NCTC6571. Exceptions to this were *Serratia marcescens* (7) that was resistant to ampicillin but was susceptible to **12a**–**b** and **12d**–**f** and *S. marcescens* (8) which was also ampicillin resistant but attacked by **12a**. These results do show that **12a** have potentially good antimicrobial activity at levels low enough for it to be

Table 1
Minimum inhibitory concentrations (MICs μg mL^{-1}) of **5a**–**f** and their components

Organism	**5a**	**5b**	**5c**	**5d**	**5e**	**5f**	Amp [a]	Amo [b]	Cep [c]	Temo	Mito
MRSA 96-7778*	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5
MRSA Innsbruck*	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5
MRSA 96-7992	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	62.5	>62.5	>62.5
MRSA 96-5665	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	31.25	>62.5	>62.5
MSSA NCTC 6571	0.98	0.98	3.9	3.9	7.81	7.81	1.95	0.49	3.9	>62.5	>62.5
MSSA NCTC 10788	<0.03	<0.03	<0.03	0.06	0.06	0.06	<0.03	<0.03	<0.03	>62.5	>62.5
MSS HW*	>62.5	>62.5	>62.5	>62.5	15.62	>62.5	>62.5	>62.5	1.95	>62.5	>62.5
E. coli DC0	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	3.9	15.62	62.5	>62.5	>62.5
E. coli DC2	0.49	15.62	3.9	1.95	15.62	7.81	0.24	0.98	3.9	>62.5	>62.5
E. coli ESI3L+*	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5
E. cloacae 1051E*	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5
K. pneumoniae 1082E*	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5

* Strains producing a β-lactamase enzyme. MSSA, Methicillin-sensitive *Staphylococcus aureus*; MRSA, Methicillin-resistant *Staphylococcus aureus*; *E. coli*, DC2-permeability mutant of parent strain *E. coli* DC0.

[a] Amp, ampicillin.

[b] Amo, amoxicillin.

[c] Cep, cephalexin.

Table 2
Minimum inhibitory concentrations (MICs μg ml^{-1}) of **12a–b** and **12d–f**

Organism	Amp	**12a**	**12b**	**12d**	**12e**	**12f**
Escherichia coli	>64	>64	>64	>64	>64	>64
Serratia marcescens	64	1	1	8	16	8
Serratia marcescens	64	1	64	64	>64	>64
Serratia marcescens	1	1	1	16	4	1
Serratia marcescens	1	1	1	8	>64	1
Pseudomonas aeruginosa	1	1	32	8	64	1
Stenotrophomonas maltophilia	1	4	64	4	64	64
Klebsiella pneumoniae	8	>64	32	8	1	>64
Staphylococcus haemolyticus	32	>64	>64	>64	>64	>64
Bacillus subtilis	1	8	64	8	32	>64
Enterococcus faecalis	1	>64	64	>64	>64	>64
Enterococcus faecalis	1	>64	>64	>64	>64	>64
Enterococcus faecium	1	>64	64	16	>64	>64
Staphylococcus epidermidis	1	8	8	8	16	2
Staphylococcus epidermidis	2	8	16	16	32	
Staphylococcus epidermidis	32	>64	>64	64	>64	>64
Staphylococcus epidermidis	1	64	1	4	32	64
Staphylococcus aureus MRSA	1	>64	64	16	8	>64
Staphylococcus aureus MRSA	1	32	32	16	16	>64
Staphylococcus aureus MRSA	8	64	>64	32	64	>64
MRSA 424	64	64	32	32	16	32
B. cereus 5	32	16	32	32	4	8

considered for further investigation and that they infer activity against ampicillin resistant strains does exist.

In conclusion, the dual-action agents **5a–f** and **12a–f** are a unique class of compounds, combining a β-lactam antibiotic with a cytotoxic agent, with the aim of enhancing the spectrum of activity of the parent β-lactam agent. Unfortunately the results of the MIC tests against a range of Gram-positive and Gram-negative organisms were not very promising. The agents failed to display activity against MRSA strains, and other bacterial strains which produce β-lactamase enzymes. Strains which produce a β-lactamase enzyme appear to destroy the β-lactam, while the cytotoxic moiety lacks antibacterial activity. Antibacterial activity of the agents appears to be due mostly to the presence of the β-lactam moiety, with little synergistic or selective toxicity.

3. Experimental

3.1. Chemistry

3.1.1. General

Melting points (m.p.) were determined on Gallenkamp apparatus and are uncorrected. NMR spectra were recorded on a Bruker AC250 Spectrometer at ^{1}H (250.1 MHz) and ^{13}C (62.9MHz). Chemical shifts are downfield of tetramethylsilane. Mass spectroscopic analysis was carried out on a Hewlett–Packard 5989B MS engine with an HP 5998A API Electrospray LC | MS interface; the LC being an HP1100 system with autosampler. High resolution mass spectra (HRMS) were measured on Finnigan MAT 900 XLT high resolution double focussing mass spectrometer using electrospray method. Infrared spectra were recorded on a Mattson 3000 FTIR Spectrometer; solid samples were prepared as KBr discs and liquids as thin films between sodium chloride plates. Flash column chromatography was performed using Sorbsil C60 silica gel. TLC was carried out using aluminium backed Merck Silica Cel 60 F_{254} plates and visualised under UV (254 nm). Potassium permanganate was used where appropriate to develop TLC plates. Elemental analyses (C, H, N) were performed on Leeman 440 analyzer.

3.1.2. General procedure for syntheses of *N*-hydroxysuccinimide active esters ***3a*** *and* ***3b***

A dry dimethylformamide (14 mL) solution of **2a** or **2b** (2.87 mmol), *N*-hydroxysuccinimide (2.87 mmol) and DCC (752 mg, 3.64 mmol) was stirred at room temperature (r.t.) for 8 h. After dicyclohexylurea was removed by filtration and the solution was concentrated under high vacuum to give the title compound as white solid. Without further purification, **3a** and **3b** were used in the subsequent reactions.

3.1.3. *3-Methyl-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxylic acid, NHS ester* ***3a***

In 60% yield, m.p.: 219–221 °C; IR (KBr): 3505, 3324, 3112, 2925 and 1742 cm^{-1}; ^{1}H NMR (DMSO): δ 2.88 (d; 4H, CH_2–CH_2), 3.92(s; 3H, CH_3) and 9.03(s; 1H, *H*-6); MS: m/z: 292 [M^+].

3.1.4. 3-(2-Chloroethyl)-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxylic acid, NHS ester ***3b***

In 50% yield, m.p.: 216–219 °C; IR (KBr): 3324, 2925, 2847, 2362, 2338, 1623 and1577 cm^{-1}; ^1H NMR (DMSO): δ 2.90 (m; 4H, CH_2–CH_2), 4.02 (t; 2H, CH_2), 4.70 (t; 2H, CH_2), 9.07 (s; 1H, 6-*H*); MS: *m*/*z*: 340 $[M^+]$.

3.1.5. General procedure for syntheses of ***5a–f***

To a stirred solution of **3a** or **3b** (0.30 mmol) and ampicillin (or amoxicillin or cephalexin) (0.30 mmol) in dry DMF (3 mL) in an ice water bath was added dropwise triethylamine (0.05 mL, 0.33 mmol). The mixture was warmed up to 30 °C and then stirred for 4 h while temperature gradually came down to ambient. To the reaction solution was added 10 mL of ice water and was washed with ethyl acetate (3 × 20 mL). The aqueous layer was adjusted to the pH 1–2 with 1 M HCl and ice, then extracted with ethyl acetate (3 × 30 mL). The combined organic layer was washed with brine (5 × 20 mL) and concentrated under high vacuum to give a white solid product.

3.1.6. α-[(3-Methyl-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxyl)amino benzyl] penicillin ***(5a)***

In 37% yield, m.p.: 179–188 °C; IR (KBr): 3343, 3127, 3058, 3031, 2928, 2856, 2360, 1751 and 1670 cm^{-1}; ^1H NMR (DMSO): δ 1.39 (s; 3H, CH_3), 1.53 (s; 3H, CH_3), 3.87 (s; 3H, CH_3), 4.20 (s; 1H, 2-*H*), 5.41 (d; $J = 4.0$ Hz, 1H, 5-*H*), 5.59 (dd; $J = 4.0$, 7.6 Hz, 1H, 6-*H*), 5.95 (d; $J = 8.7$ Hz, 1H, Ph-C*H*), 7.32, 7.49 (2 × m; 5H, Ph-*H*), 8.60 (d; $J = 7.6$ Hz, 1H, *H*N), 8.89 (s; 1H, 6′-*H*), 9.38 (d; $J = 8.7$ Hz, 1H, *H*N); ^{13}C NMR (DMSO): δ 26.6 (CH_3), 30.3 (CH_3), 36.4 (CH_3), 55.0 (*C*-5), 58.24 (*C*-6), 63.8 (*C*-3), 67.1 (Ph-*C*H), 70.3 (*C*-2), 126.8, 127.9, 128.5 and 128.9 (*C*H-aromatic) and 129.2 (*C*-6′), 134.9 (*C*-aromatic), 138.2 (*C*-9′), 139.1 (*C*-8′), 158.5 (*C*O), 168.9 (2 × *C*O), 169.7 (*C*O), 173.1 (*C*O); MS: *m*/*z*: 527 $[M + H]^+$. Anal. for $C_{22}H_{22}N_8O_6S$ $3H_2O$: C, 45.5; H, 4.8; N, 19.3. Found: C, 45.9; H, 4.7; N, 19.7%.

3.1.7. α-[(3-(2-Chloroethyl)-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxyl) aminobenzyl] penicillin ***(5b)***

In yield 61%; m.p.: 145–153 °C; IR (KBr): 3365, 2928, 2362, 1752 and 1661 cm^{-1}; ^1H NMR (DMSO): δ 1.39 (s; 3H, CH_3), 1.53 (s; 3H, CH_3), 4.01 (t; $J = 6.0$ Hz, 2H, CH_2CH_2Cl), 4.21 (s; 1H, 2-*H*), 4.66 (t; $J = 6.0$ Hz, 2H, CH_2CH_2Cl), 5.42 (d; $J = 4.0$ Hz, 1H, 5-*H*), 5.58 (dd; $J = 4.0$, 7.6 Hz, 1H, 6-*H*), 5.95 (d; $J = 8.7$ Hz, 1H, Ph-C*H*), 7.36 7.49 (2 × m; 5H, Ph-*H*), 8.64 (d; $J = 7.6$ Hz, 1H, N*H*), 8.94 (s; 1H, 6′-*H*), 9.41 (d; $J = 8.7$ Hz, 1H, N*H*); ^{13}C NMR (MSO) δ 26.6 (CH_3), 30.3 (CH_3), 41.5 (CH_2), 50.2 (CH_2), 55.1 (*C*-5), 58.2 (*C*-6), 63.8 (*C*-3), 67.1 (Ph-*C*H), 70.3 (*C*-2), 126.8, 127.98 and 128.5 (5 × *C*H-aromatic) and 129.6 (*C*-6′), 134.3 (aromatic-*C*), 138.2 (*C*-9′), 1389.0 (*C*-8′), 158.4 (*C*O), 168.9 (2 × *C*O), 169.6 (*C*O), 173.1 (*C*O); MS: *m*/*z*: 575 $[M + H]^+$. Anal. for $C_{23}H_{23}ClN_8O_6S{\cdot}3H_2O$: C, 43.9; H, 4.6; N, 17.8. Found: C, 43.5; H, 4.4; N, 17.4%.

3.1.8. α-[(3-Methyl-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxyl)amino-p-hydroxy benzyl] penicillin ***(5c)***

In yield 69%, m.p.: 196–235 °C; IR (KBr): 3359, 3127, 3029, 2928, 2849, 2358, 2338, 1744 and 1662 cm^{-1}; ^1H NMR (DMSO): δ 1.42 (s; 3H, CH_3), 1.56 (s; 3H, CH_3), 3.87 (s; 3H, CH_3), 4.19 (s; 1H, 2-*H*), 5.41 (d; $J = 4.1$ Hz, 1H, 5-*H*), 5.57 (dd; $J = 4.1$, 7.8 Hz, 1H, 6-*H*), 5.83 (d; $J = 8.2$ Hz, 1H, Ph-C*H*), 6.73 (d; $J = 8.6$ Hz, 2H, aromatic-*H*), 7.27 (d; $J = 8.6$ Hz, 2H, aromatic-*H*), 8.47 (d; $J = 8.2$ Hz, 1H, *H*N), 8.89 (s; 1H, 6′-*H*), 9.30 (d; $J = 7.8$ Hz, 1H, *H*N), 9.42 (s; 1H, *H*O); ^{13}C NMR (DMSO): δ 26.6 (CH_3), 30.1 (CH_3), 36.3 (CH_3), 54.5 (*C*-5), 58.1 (*C*-6), 63.8 (*C*-3), 67.0 (Ph-*C*H), 70.4 (*C*-2), 115.2 (2 × *C*H-aromatic), 128.1 (2 × *C*H-aromatic), 128.8 (*C*-aromatic), 129.9 (*C*-6′), 134.8 (*C*-9′), 139.1 (*C*-8′), 157.1 (aromatic-*C*-OH), 158.4 (*C*O), 169.0 (2 × *C*O), 170.1 (*C*O), 173.3 (*C*O); MS: *m*/*z*: 543 $[M + H]^+$. Anal. for $C_{22}H_{22}N_8O_7S$ $3H_2O$: C, 44.3; H, 4.7; N, 18.8. Found: C, 44.0; H, 4.8; N, 18.4%.

3.1.9. α-[(3-(2-Chloroethyl)-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxyl) amino-p-hydroxy benzyl] penicillin ***(5d)***

In 65% yield; m.p: 138–142 °C; IR (KBr): 3742, 3370, 3121, 3023, 2928, 2851, 2358, 2344, 1748 and 1664 cm^{-1}; ^1H NMR (DMSO): δ 1.41 (s; 3H, CH_3), 1.55 (s; 3H, CH_3), 4.03 (t; $J = 5.9$ Hz, 2H, CH_2CH_2Cl), 4.21 (s; 1H, 2-*H*), 4.64 (t; $J = 5.9$ Hz, 2H, CH_2CH_2Cl), 5.42 (d; $J = 4.0$ Hz, 1H, 5-*H*), 5.58 (dd; $J = 4.1$, 7.7 Hz, 1H, 6-*H*), 5.83 (d; $J = 7.9$ Hz, 1H, Ph-C*H*), 6.74 (d; $J = 8.4$ Hz, 2H, aromatic-*H*), 7.29 (d; $J = 8.4$ Hz, 2H, aromatic-*H*), 8.52 (d; $J = 7.9$ Hz, 1H, N*H*), 8.94 (s; 1H, 6′-*H*), 9.28 (d; $J = 7.7$ Hz, 1H, N*H*), 9.48 (s; 1H, *H*O); ^{13}C NMR (MSO): 26.6 (CH_3), 30.2 (CH_3), 41.5 (CH_2), 50.2 (CH_2), 54.6 (*C*-5), 58.1 (*C*-6), 63.8 (*C*-3), 67.1 (Ph-*C*H), 70.4 (*C*-2), 115.2 (2 × *C*H-aromatic), 128.1 (2 × *C*H-aromatic), 128.5 (*C*-aromatic), 129.2 (*C*-6′), 134.8 (*C*-9′), 139.0 (*C*-8′), 157.1 (aromatic-*C*-OH), 158.3 (*C*O), 168.9 (2 × *C*O), 170.1 (*C*O), 173.3 (*C*O); MS: *m*/*z*: 591 $[M + H]^+$. Anal. for $C_{23}H_{23}ClN_8O_7S$ $3H_2O$: C, 42.9; H, 4.5; N, 17.4. Found: C, 43.1; H, 4.3; N, 17.0%.

3.1.10. 7-{D-2-(3-Methyl-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxyl) amino-2-phenyl acetamido)-3-methyl-8-oxo-5-thia-azabicyclo[4, 2, 0]oct-2-ene-2-carboxylic acid ***(5e)***

In 34% yield; m.p.: 250–256 °C; IR (KBr): 3324, 3276, 3125, 3058, 2925, 2847, 2362, 2338, 1752 and

1671 cm^{-1}; ^1H NMR (DMSO): δ 1.98 (s; 3H, CH_3), 3.23–343 (m; 2H, S-CH_2), 3.92 (s; 3H, CH_3), 5.00 (d; $J = 4.7$ Hz, 1H, 6-*H*), 5.68 (dd; $J = 4.7$, 8.2 Hz, 1H, 7-*H*), 5.90 (d; $J = 7.9$ Hz, 1H, Ph-C*H*), 7.35, 7.50 (2m; 5H, Ph-*H* d), 8.61 (d; $J = 7.9$ Hz, 1H, *H*N), 8.90 (s; 1H, 6′-*H*), 9.50 (d; $J = 8.2$ Hz 1H, *H*N); ^{13}C NMR (DMSO): δ 19.4 (CH_3), 30.7 (CH_2-4) 36.4 (CH_3), 55.2 (*C*-6), 57.0 (*C*-7), 58.5 (Ph-*C*H), 122.6 (*C*-2), 126.8 (*C*-3), 128.0, 128.5, 128.9 and 129.1 (5 × aromatic-*C*H), 129.8 (*C*-6′), 134.9 (aromatic-*C*), 138.2 (*C*-8′), 139 (C-9′), 158.5 (*C*O), 163.5 (2 × *C*O), 163.8 (*C*O), 170.3 (*C*O); MS: *m*/*z*: 525 $[M + H]^+$. Anal. for $C_{22}H_{20}N_8O_6S$ $3H_2O$: C, 45.7; H, 4.5; N, 19.4. Found: C, 45.4; H, 4.6; N, 19.1%.

3.1.11. 7-{D-2-[3-(2-Chloroethyl)-4-oxo-imidazo[5,1-d]-1,2,3,5-tetrazine-8-carboxyl]amino-2-phenylacetamido)-3-methyl-8-oxo-5-thia-azabicyclo [4, 2, 0]oct-2-ene-2-carboxylic acid (**5f**)

In 71%, m.p.: 182–187 °C; IR (KBr): 3372, 3127, 3062, 2927, 2851, 2358, 2344, 1750 and 1668 cm^{-1}; ^1H NMR (DMSO): δ 1.91 (s; 3H, CH_3), 3.23–3.43 (m; 2H, S-CH_2), 4.03 (t; $J = 5.9$ Hz, 2H, CH_2CH_2Cl), 4.65 (t; $J = 5.9$ Hz, 2H, CH_2CH_2Cl), 4.98 (d; $J = 4.7$ Hz, 1H, 6-*H*), 5.68 (dd; $J = 4.7$, 8.2 Hz, 1H, 7-*H*), 5.90 (d; $J = 7.9$ Hz, 1H, Ph-C*H*), 7.38, 7.50 (2m; 5H, Ph-*H*), 8.64 (d; $J = 7.9$ Hz, 1H, *H*N), 8.94 (s; 1H, 6′-*H*), 9.54 (d; $J = 8.2$ Hz, 1H, *H*N); ^{13}C NMR (DMSO): δ 19.4 (CH_3), 30.8 (CH_2-4), 41.5 (CH_2), 50.2 (CH_2), 55.3 (*C*-6), 57.0 (*C*-7), 58.5 (Ph-*C*H), 122.6 (*C*-2), 126.8 (*C*-3), 128.0, 128.5 and 129.6 (5 × aromatic-*C*H), 129.8 (*C*-6′), 134.3 (aromatic-*C*), 138.1 (8′-*C*), 139.0 (*C*-9′), 158.4 (*C*O), 162.4 (*C*O), 163.5 (*C*O), 163.7 (*C*O), 170.2 (*C*O); MS: *m*/*z*: 573 $[M + H]^+$. Anal. for $C_{23}H_{21}ClN_8O_6S{\cdot}3H_2O$: C, 44.1; H, 4.3; N, 17.9. Found: C, 43.8; H, 4.0; N, 17.7%.

3.1.12. General procedure for synthesis of allyl 3-hydroxymethyl-3-cephem-2-carboxylate (**10a–c**)

3-Hydroxymethyl-3-cephem-2-carboxylic acids **9a–c** were prepared following the literature method [20]. To a solution of 3-hydroxymethyl-3-cephem-2-carboxylic acid (1.69 mmol) in DMF (9 mL) and 1,4-dioxane (6 mL) was added sodium bicarbonate (3.38 mmol) followed by allyl bromide (2.54 mmol). The resulting solution was refluxed for 1 h, then cooled to r.t.. The reaction solution was partitioned between ethyl acetate (100 mL) and brine (100 mL). The organic phase was washed with water (2 × 20 mL) and brine (2 × 20 mL), dried over anhydrous magnesium sulfate. Evaporation and flash chromatography (ethyl acetate–hexane, 1:2) gave the title compound.

3.1.13. Allyl 3-hydroxymethyl-7-phenylacetamido-3-cephem-2-carboxylate (**10a**)

In 25.6% yield as a white solid; m.p.: 106–108 °C. IR (KBr): 3417, 3309 (N–H, O–H), 3081, 2968 (C–H), 1784 (β-lactam C=O), 1751, 1353 (O–C), 1660, 1531 (N–C), 1409, 1178 and 695. ^1H NMR ($CDCl_3$): δ 3.61 (s; 2 H, PhCH_2), 4.10, 4.20 (AB_q; $J = 13.4$ Hz, 2H, CH_2OH), 4.66 (d; $J = 5.8$ Hz, 2H, $CH_2CH{=}CH_2$), 5.08 (s; 1H, *H*-2), 5.23 (d; $J = 3.9$ Hz, 1H, *H*-6), 5.28–5.38 (m; 2 H, $CH_2CH{=}CH_2$), 5.59 (dd; $J = 3.9$, 8.5 Hz, 1H, 7-*H*), 5.83–5.98 (m; 1 H, $CH_2CH{=}CH_2$), 6.27 (s; 1 H, *H*-4), 7.07 (d; $J = 8.5$ Hz, *H*N), 7.26–7.33 (m; 5H, aromatic-*H*); ^{13}C NMR ($CDCl_3$): δ 42.8 (PhCH_2), 49.7 (*C*-2), 53.5 (*C*-6), 60.3 (*C*-7), 64.2 (CH_2OH), 66.7 ($OCH_2CH{=}CH_2$), 117.37 ($CH_2CH{=}CH_2$), 119.6 ($CH_2CH{=}CH_2$), 123.8 (*C*-4), 127.2, 128.7 and 129.3 (aromatic-*C*H), 130.8 (*C*-3), 134.1 (aromatic-*C*), 164.6 (*C*O), 167.1 (*C*O), 171.8 (*C*O). HRMS (ES^+): *m*/*z* 406.1436 ($M + NH_4^+$, $C_{19}H_{24}N_3O_5S$ requires 406.1431).

3.1.14. Allyl 3-hydroxymethyl-7-(2-thienacetamido)-3-cephem-2-carboxylate (**10b**)

In 47.8% yield as a yellow foam and its m.p. and spectroscopic properties are identical to those reported in literature [21].

3.1.15. Allyl 3-hydroxymethyl-7-phenoxyacetamido-3-cephem-2-carboxylate (**10c**)

In 47.8% yield as a yellow foam; m.p.; 102–103 °C; IR (KBr) 3417, 3309 (NH, OH), 3081, 2968 (CH), 1784 (β-lactam C=O), 1751, 1353 (O–C), 1660, 1531 (N-C), 1409, 1178 and 695 cm^{-1}. ^1H NMR ($CDCl_3$): δ 4.22–4.31 (AB_q; $J = 13.2$ Hz, 2H, CH_2OH), 4.59 (s; 2H, PhOCH_2), 4.71 (m; 2H, $CH_2CH{=}CH_2$), 5.18 (s; 1H, 2-*H*), 5.34 (d; $J = 4.1$ Hz, 6-*H*), 5.33–5.43 (m; 2H, $CH_2CH{=}CH_2$), 5.78 (dd; $J = 4.0$, 9.1 Hz, 1H, 7-*H*), 5.87–6.00 (m; 1H, $CH_2CH{=}CH_2$), 6.36 (s; 1H, 4-*H*), 6.94–7.10, 7.19–7.48 (2 × m; 5H, aromatic-*H*). ^{13}C NMR ($CDCl_3$): δ 49.8 (*C*-2), 53.2 (*C*-6), 59.4 (*C*-7), 66.7 (2C, CH_2OH and $CH_2CH{=}CH_2$), 67.0 (PhOCH_2), 114.7 (2 × aromatic-*C*H) 117.6 ($CH_2CH{=}CH_2$), 119.7 ($CH_2CH{=}CH_2$), 122.2 (*C*-4), 124.0 and 129.7 (2 × aromatic-*C*H), 130.8 (*C*-3), 162.1 (aromatic-*C*-O), 167.1 (*C*O), 168.6(2 × *C*O). HRMS (ES^+): *m*/*z* 422.1483 ($M + NH_4^+$, $C_{19}H_{24}N_3O_6S$ requires 422.1380).

3.1.16. General procedure for syntheses of **11a–f**

10 (0.257 mmol) and **2a** or **2b** (0.257 mmol) dissolved in DCM (8 mL) with *N*,*N*-diisopropylethylamine (0.514 mmol) at r.t. PyBOP (0.257 mmol) was added in one portion at 0 °C. After stirring for a period of 1–3 h, the mixture was diluted with DCM (8 mL), washed with brine and dried over anhydrous magnesium sulfate. Evaporation and flash chromatography (ethyl acetate–hexane 2:1) yielded the title compounds as yellowish powder.

*3.1.17. 2-(Allyloxy)carbonyl-7-phenylacetamido-3-cephem-3-methyl, 3-(2-chloroethyl)-4-oxo-imidazo [5,1-d][1,2,3,5]tetrazin-8-carboxylate (**11a**)*

In 41.4% yield, m.p.: 85–88 °C; IR (KBr): 3450, 3411 (N–H), 3128, 3027, 2956 (C–H), 1774 (β-lactam C=O), 1741 (C=O), 1240 (O–C), 1674, 1527 (N–H), 1458, 1168, and 734 (C–Cl) cm^{-1}; ^1H NMR ($CDCl_3$): δ 3.65 (s; 2H, PhCH_2), 4.01 (t; $J = 6.0$ Hz, 2H, CH_2CH$_2$Cl), 4.68 (d; $J = 6.0$ Hz, 2H, CH_2CH=CH$_2$), 4.78 (t; $J = 6.0$ Hz, 2H, CH$_2$CH_2Cl), 5.05 (AB_q; $J =$ 12.6 Hz, 2H, CH_2O), 5.23–5.52 (m; 4H, 2-*H*, 6-*H*, CH=CH_2), 5.66 (dd; $J = 4.0$, 8.6 Hz, 1H, 7-*H*), 5.83–5.96 (m; 1H, CH$_2$C*H*=CH$_2$), 6.53 (d; $J = 8.6$ Hz, 1H, *H*N), 6.61 (s; 1H, 4-H), 7.26–7.36 (m, 5H, Ph-*H*), 8.48 (s, 1H, 6′-*H*); ^{13}C NMR ($CDCl_3$): δ 40.6 (CH$_2$*C*H$_2$Cl), 43.1 (*C*H$_2$Ph), 49.8 (*C*-2), 50.4 (*C*H$_2$CH$_2$Cl), 53.4 (*C*-6), 60.4 (*C*-7), 66.4 (O*C*H$_2$CH=CH$_2$), 66.9 (*C*H$_2$O), 118.4 (CH$_2$CH=*C*H$_2$), 119.8 (CH$_2$*C*H=CH$_2$), 123.1(*C*-4), 127.5 128.9 and 129.2 (5 × aromatic-*C*H), 129.3 (*C*-6′), 130.7 (*C*-3), 133.8 (aromatic-*C*), 135.5 (*C*-9′), 138.3 (*C*-8′), 159.6 (*C*O), 164.1 (*C*O), 166.6 (2 × *C*O), 171.2 (*C*O). HRMS (ES^+): m/z 614.1218 ($M + H^+$, $C_{26}H_{25}ClN_7O_7S$ requires 614.1220).

*3.1.18. 2-(Allyloxy)carbonyl-7-(2-thien-2-yl)acetamido-3-cephem-3-methyl 3-(2-chloroethyl)-4-oxoimi dazo-[5,1-d][1,2,3,5]tetrazin-8-carboxylate (**11b**)*

In 53% yield, m.p.: 92 °C (dec.); IR (KBr): 3450, 3411 (N–H), 3116, 3039, 2960 (C–H), 1774 (β-lactam C=O), 1745, 1240 (O–C), 1682, 1527 (N–C), 1458, 1169 and 740 (C–Cl) cm^{-1}; ^1H NMR ($CDCl_3$): δ 3.88 (s; 2H, CH_2CO), 4.02 (t; $J = 6.0$ Hz, 2H, CH_2CH$_2$Cl), 4.69 (d; 2H, $J = 5.9$ Hz, CH_2CH=CH$_2$), 4.80 (t; $J = 6.0$ Hz, 2H, CH$_2$CH_2Cl), 5.07 (AB_q; $J = 12.7$ Hz, 2H, CH_2O), 5.25–5.53 (m; 4H, H-2, H-6,CH=CH_2), 5.69 (dd; $J = 4.0$, 8.8 Hz, 1H, 7-*H*), 5.83–5.96 (M, 1H, CH$_2$C*H*=CH$_2$), 6.55 (d; $J = 8.8$ Hz, 1H, *H*N), 6.63 (s; 1H, 4-*H*), 7.00, 7.29 (2 × m, 3H, H-thiophene), 8.50 (s; 1H, 6′-*H*); ^{13}C NMR ($CDCl_3$): δ 37.0 (*C*H$_2$CO), 40.6 (*C*H$_2$*C*H$_2$Cl), 49.8 (*C*-2), 50.3 (*C*H$_2$*C*H$_2$Cl), 53.4 (*C*-6), 60.3 (*C*-7), 66.4 (O*C*H$_2$CH=CH$_2$) 66.9 (*C*H$_2$O), 118.5 (CH$_2$CH=*C*H$_2$), 119.9 (CH$_2$*C*H=CH$_2$), 123.0 (*C*-4), 126.1, 127.4 and 127.8 (3 × thiophene-*C*H), 129.1 (*C*-6′), 130.7 (*C*-3), 134.6 (thiophene-*C*), 135.5 (*C*-9′), 138.3 (*C*-8′), 159.6 (CO), 163.9 (CO), 166.6(2 × CO), 169.8 (CO). HRMS (ES^+): m/z 620.0784 ($M + H^+$, $C_{24}H_{23}ClN_7O_7S_2$ requires 620.0785).

*3.1.19. 2-(Allyloxy)carbonyl-7-phenoxyacetamido-3-cephem-3-methyl 3-(2-chloroethyl)-4-oxoimidazo [5,1-d][1,2,3,5]tetrazin-8-carboxylate (**11c**)*

In 36.5%, m.p.: 85 °C (dec.); IR (KBr): 3450, 3411 (N–H), 3128, 3041, 2939 (C–H), 1774 (β-lactam C=O), 1747, 1238 (O–C), 1683, 1527 (N–C), 1490, 1172, 760 (C–Cl) cm^{-1}; ^1H NMR ($CDCl_3$): δ 4.03 (t; $J = 6.0$ Hz, 2H, CH_2CH$_2$Cl), 4.59 (s; 2H, CH_2CO), 4.72 (d; $J = 5.8$ Hz, 2H, CH_2CH=CH$_2$), 4.81 (t; $J = 6.0$ Hz, 2H, CH$_2$CH_2Cl), 5.12 (AB_q; $J = 12.6$ Hz, 2H, CH_2O), 5.30–5.41 (m, 4H, 2-*H*, 6-*H*, CH=CH_2), 5.88 (dd; $J = 4.1$, 8.1 Hz, 1H, 7-*H*), 5.83–5.96 (M; 1H, CH$_2$C*H*=CH$_2$), 6.68 (s; 1H, 4-*H*), 6.95–7.10, 7.32–7.39 (2 × m, 5H, aromatic-*H*), 8.52 (s; 1H, 6′-*H*); ^{13}C NMR ($CDCl_3$): δ 40.6 (CH$_2$*C*H$_2$Cl), 49.8 (*C*-2), 50.3 (*C*H$_2$CH$_2$Cl), 53.1 (*C*-6), 59.5 (*C*-7), 66.4 (O*C*H$_2$CH=CH$_2$) 66.8 (*C*H$_2$O), 66.9 (O*C*H$_2$CO), 114.6 (2 × aromatic-*C*H), 118.5 (CH$_2$CH=*C*H$_2$), 119.7 (CH$_2$*C*H=CH$_2$), 122.1 (*C*-4), 122.8, 128.6 and 129.2 (5 × aromatic-*C*H), 129.6 (*C*-6′), 130.7 (*C*-3) 135.5 (C-9′), 138.3 (C-8′), 156.8 (aromatic-*C*), 159.6 (CO), 163.6 (CO), 166.7 (2 × CO), 168.4 (CO). HRMS (ES^+): m/z 647.1431 ($M + NH_4^+$, $C_{26}H_{24}ClN_7O_8S$ requires 647.1434).

*3.1.20. (6R,7R)-2-(Allyloxy)carbonyl-7-phenyla-cetamido-3-cephem-3-methyl, 3-methyl-4-oxoimidazo [5,1-d] [1,2,3,5]tetrazin-8-carboxylate (**11d**)*

In 77.1% yield, m.p.: 123–125 °C (dec.); IR (KBr): 3288, 3122 (N–H), 3083, 3031, 2956 (C–H), 1774 (β-lactam C=O), 1737, 1248 (O–C), 1654, 1537 (N–C), 1457, 1326, 1172, 1052 and 950 cm^{-1}; ^1H NMR ($CDCl_3$): δ 3.67 (s; 2H, PhCH_2), 4.08 (s; 3H, CH_3), 4.68 (d; $J = 5.8$ Hz, 2H, CH_2CH=CH$_2$), 5.05 (AB_q; $J = 12.7$ Hz, 2H, CH_2O), 5.27–5.38 (m; 4H, 2-*H*, 6-*H*, CH=CH_2), 5.68 (dd; $J = 4.0$, 8.7 Hz, 1H, 7-*H*), 5.83–5.99 (m; 1H, CH$_2$C*H*=CH$_2$), 6.40 (d; $J = 8.7$ Hz, 1H, *H*N), 6.60 (s; 1H, 4-*H*), 7.27–7.40 (m; 5H, aromatic-*H*), 8.46 (s; 1H, 6′-H); ^{13}C NMR ($CDCl_3$): δ 36.7 (*C*H$_3$), 43.2 (*C*H$_2$Ph), 49.8 (*C*-2), 53.4 (*C*-6), 60.4 (*C*-7), 66.4 (O*C*H$_2$CH=CH$_2$), 66.9 (*C*H$_2$O), 118.5 (CH$_2$CH=*C*H$_2$), 119.8 (CH$_2$*C*H=CH$_2$), 122.9 (*C*-4), 127.6 128.3, 128.6 and 129.0 (5 × aromatic-*C*H), 129.3 (*C*-6′), 130.7 (*C*-3), 133.6 (aromatic-*C*), 138.3 (*C*-8′ and *C*-9′), 159.7 (*C*O), 164.0 (*C*O), 166.6 (2 × *C*O), 171.0 (*C*O). HRMS (ES^+): m/z 566.1463 ($M + H^+$, $C_{25}H_{24}N_7O_7S$ requires 566.1458).

*3.1.21. (6R,7R)-2-(Allyloxy)carbonyl-7-(2-thien-2-yl)-acetamido-3-cephem-3-methyl 3-methyl-4-oxoimi-dazo[5,1-d] [1,2,3,5]tetrazin-8-carboxylate (**11e**)*

In 55% yield; m.p.: 94–95 °C (dec.); IR (KBr): 3288, 3122 (N–H), 3041, 2956 (C–H), 1780 (β-lactam C=O), 1747, 1242 (O–C), 1679, 1542 (N–C), 1457, 1327, 1164 and 945 cm^{-1}; ^1H NMR ($CDCl_3$): δ 3.88 (s; 2H, CH_2CO), 4.09 (s; 3H, CH_3), 4.69 (d; $J = 6.0$ Hz, 2H, CH_2CH=CH$_2$), 5.07 (AB_q; $J = 12.7$ Hz, 2H, CH_2O), 5.25–5.39 (m; 4 H, 2-*H*, 6-*H*, CH=CH_2), 5.70 (dd; $J = 4.0$, 8.7 Hz, 1H, 7-*H*), 5.86–5.97 (m; 1H, CH$_2$C*H*=CH$_2$), 6.46 (d; $J = 8.7$ Hz, 2H, *H*N), 6.62 (s; 1H, 4-*H*), 7.01, 7.29 (2 × m; 3H, thiophene-*H*), 8.48 (s; 1H, *H*-6′); ^{13}C NMR ($CDCl_3$): δ 36.7 (*C*H$_3$), 37.0 (*C*H$_2$CO), 49.8 (*C*-2), 53.4 (*C*-6), 60.4 (*C*-7), 66.4, (O*C*H$_2$CH=CH$_2$), 66.9 (*C*H$_2$O), 118.6 (CH$_2$CH=*C*H$_2$),

119.9 ($CH_2CH{=}CH_2$), 122.9 (*C*-4), 125.9 127.4 and 127.7 (3 × thiophene-*C*H), 130.8 (*C*-6′), 131.1 (*C*-3), 134.7 (thiophene-*C*), 136.0 (*C*-8′), 138.4 (*C*-9′), 159.8 (*C*O), 164.0 (*C*O), 166.6 (2 × *C*O), 170.0 (*C*O). HRMS (ES^+): *m*/*z* 572.1022 ($M + H^+$, $C_{23}H_{22}N_7O_7S_2$ requires 572.1022).

3.1.22. (6R,7R)-2-(Allyloxy)carbonyl-7-phenoxyacetamido-3-cephem-3-methyl 3-methyl-4-oxoimidazo[5,1-d][1,2,3,5]tetrazin-8-carboxylate (**11f**)

In 23.6% yield, m.p.: 75–76 °C (dec.); IR (KBr): 3288, 3122 (N–H), 3072, 3036, 2948 (C–H), 1776 (β-lactam C=O), 1741, 1246 (O–C), 1683, 1523 (N–C), 1490, 1462, 1172, 1049 and 949 cm^{-1}; ^1H NMR ($CDCl_3$): δ 4.07 (s; 3H, CH_3), 4.57 (s; 2H, CH_2CO), 4.70 (d; *J* = 5.8 Hz, 2H, $CH_2CH{=}CH_2$), 5.08 (AB_q; *J* = 12.7 Hz, 2H, CH_2O), 5.28–5.39 (m; 4H, 2-*H*, 6-*H*, CH=CH_2), 5.78 (dd: *J* = 4.0, 9.0 Hz, 1H, 7-*H*), 5.87–6.00 (m; 1H, $CH_2CH{=}CH_2$), 6.65 (s; 1H, 4-*H*), 6.90–7.07, 7.29–7.36 (2 × m; 5H, Ph-*H*), 7.52 (d; *J* = 9.0 Hz, 1H, *H*N), 8.47 (s; 1H, H-6′); ^{13}C NMR ($CDCl_3$): δ 36.7 (CH_3), 49.8 (*C*-2), 53.2 (*C*-6), 59.6 (*C*-7), 66.4 ($OCH_2CH{=}CH_2$), 66.9 (CH_2O), 67.0 (OCH_2CO), 114.5 (2 × aromatic-*C*H), 118.6 ($CH_2CH{=}CH_2$), 119.9 ($CH_2CH{=}CH_2$), 122.8 (*C*-4), 128.7 and 129.7 (3 × aromatic-*C*H), 130.7 (*C*-6′), 132.4 (*C*-3) 134.5 (*C*-9′), 138.3 (*C*-8′), 156.8 (aromatic-*C*), 159.7 (*C*O), 163.8 (*C*O), 166.7 (2 × *C*O), 168.4 (*C*O). HRMS (ES^+): *m*/*z* 599.1679 ($M + NH_4^+$, $C_{25}H_{27}N_7O_8S$ requires 599.1673).

3.1.23. General procedure fore synthesis of **12a–f**

To a solution of **11** (0.5 mmol) in dry DCM (2 mL) cooled at 5 °C was added dropwise a solution of *m*-CPBA (57–85%, 0.5 mmol) in DCM (1 mL). The reaction mixture was stirred overnight at r.t. and TLC (ethyl acetate) showed no starting material and one main product. The precipitate was collected by filtration to give a 7-substituted 2-(allyloxy)carbonyl-5-sulfoxide-2-cephem-3-methyl-4-oxoimidazo[5,1-*d*][1,2,3,5]-tetrazin-8-carboxylate as yellowish solid, which was dissolved in dry DMF (6 mL) with TPP (0.13 mmol) and tetrabis (triphenylphosphine)palladium (0.05 mmol) and stirred at 5 °C for 3 h. TLC (ethyl acetate: acetic acid 2:1) showed no starting material and one main product. The yellow solution was concentrated under reduced pressure and the crude product was purified by dry-column flash chromatography to give the title compound as a yellowish solid.

3.1.24. 2-Carboxyl-7-phenylacetamido-5-sulfoxide-2-cephem-3-methyl 3-(2-chloroethyl)-4-oxoimidazo[5,1-d][1,2,3,5]tetrazin-8-carboxylate (**12a**)

In 38.8% yield, m.p.: 194 °C. IR (KBr): 3450, 3297 (N–H), 3128, 3027, 2960 (C–H), 1781 (β-lactam C=O), 1749, 1243 (O–C), 1724, 1648, 1529 (N–C), 1458, 1164, 1047 (S–O), 734 (C–Cl) cm^{-1}; ^1H NMR ((DMSO-d_6): δ 3.53, 3.69 (AB_q; *J* = 13.9 Hz, 2H, $PhCH_2$), 3.66, 4.03 (AB_q; *J* = 13.9 Hz, 2H, 4-H-4), 4.02 (t; *J* = 5.9 Hz, 2H, CH_2CH_2Cl), 4.65 (t; *J* = 5.9 Hz, 2H, CH_2CH_2Cl), 4.92 (d; *J* = 4.9 Hz, 1H, 6-*H*), 4.96, 5.59 (AB_q; *J* = 13.2 Hz, 2H, CH_2O), 5.81 (dd; *J* = 4.9, 8.2 Hz, 1H, 7-*H*), 7.21–7.30 (m, 5H, aromatic-H), 8.45 (d; *J* = 8.2 Hz, 1H, *H*N), 8.93 (s; 1H, 6′-*H*); ^{13}C NMR (DMSO): δ 41.6 (2C, CH_2CH_2Cl and Ph-CH_2), 45.6 (CH_2-4), 50.5 (CH_2CH_2Cl), 58.4 (*C*-7), 64.4 (CH_2O), 66.5 (*C*-6), 118.9 (*C*-2), 126.8 (*C*-3), 126.7, 128.5 and 129.3 (5 × aromatic-*C*H), 130.4 (*C*-6′), 136.0 (aromatic-*C*), 136.5 (*C*-9′), 139.0 (*C*-8′), 160.1 (*C*O), 162.3 (*C*O), 164.4 (2 × *C*O), 171.2 (*C*O). Anal. for $C_{23}H_{20}ClN_7O_8S{\cdot}5H_2O$: C, 40.5; H 4.4; N, 14.4. Found: C, 40.2; H 4.5; N, 14.4%.

3.1.25. 2-Carboxyl-5-sulfoxide-7-(2-thien-2-yl)-acetamido-2-cephem-3-methyl 3-(2-chloroethyl)-4-oxoimidazo[5,1-d][1,2,3,5]tetrazin-8-carboxylate (**12b**)

In 71.5% yield, m.p.: > 200 °C (dec.); IR (KBr): 3450, 3297 (N-H), 3128, 3027, 2964 (C–H), 1781 (β-lactam C=O), 1761, 1242 (O–C), 1654, 1610, 1565 (N–C), 1454, 1172, 1035 (S–O) and 786 (C–Cl) cm^{-1}; ^1H NMR (DMSO): δ 3.48, 3.70 (AB_q; *J* = 18.1 Hz, 2H, 4-H), 3.78, 3.89 (AB_q; *J* = 15.1 Hz, 2H, CH_2CO), 4.01 (t; *J* = 5.9 Hz, 2H, CH_2CH_2Cl), 4.64 (t; *J* = 5.9 Hz, 2H, CH_2CH_2Cl), 4.82 (d; *J* = 4.9 Hz, 1H, 6-*H*), 4.97, 5.69 (AB_q; *J* = 13.1 Hz, 2H, CH_2O), 5.65 (dd; *J* = 4.9, 8.6 Hz, 1H, 7-*H*), 6.94 and 7.36 (2 × m, 3H, thiophene-*H*), 8.31 (d; *J* = 8.6 Hz, 1H, *H*N), 8.90 (s; 1H, 6′-H); ^{13}C NMR (DMSO): δ 41.6 (CH_2CH_2Cl), 45.4 (CH_2-4), 50.5 (CH_2CH_2Cl), 57.9 (*C*-7), 65.1 (CH_2O), 66.2 (*C*-6), 125.3 (*C*-2), 126.7 (*C*-3), 126.9 and 128.8 (3 × H*C*-thiophene), 135.0 (*C*-6′), 136.4 (*C*-9′), 137.1 (*C*-thiophene), 139.0 (*C*-8′), 160.5 (*C*O), 161.3 (*C*O), 163.3 (2 × *C*O), 170.2 (*C*O). Anal. for $C_{21}H_{18}ClN_7O_8S_2{\cdot}H_2O$: C, 41.4; H 3.5; N, 16.4. Found C, 41.1; H 3.3; N, 16.0%.

3.1.26. 2-Carboxyl-7-phenoxyacetamido-5-sulfoxide-2-cephem-3-methyl 3-(2-chloroethyl)-4-oxoimidazo-[5,1-d][1,2,3,5]tetrazin-8-carboxylate (**12c**)

In 43.6% yield; m.p.: 150–151 °C (dec.). IR (KBr): 3420, 3297 (N–H), 3128, 3082, 2962 (C–H), 1783 (β-lactam C=O), 1758, 1250 (O–C), 1724, 1698, 1526 (N–C), 1492, 1159, 1033 (S–O) and 754 (C–Cl) cm^{-1}; ^1H NMR (DMSO): δ 3.54, 3.81 (AB_q; *J* = 17.7 Hz, 2H, 4-*H*), 4.02 (t; *J* = 5.6 Hz, 2H, CH_2CH_2Cl), 4.65 (t; *J* = 5.6 Hz, 2H, CH_2CH_2Cl), 4.67 (s; 2H, $PhOCH_2$), 4.93 (d; *J* = 4.6 Hz, 1H, 6-*H*), 5.00, 5.68 (AB_q; *J* = 13.0 Hz, 2H, CH_2O), 5.85 (dd; *J* = 4.6, 9.7 Hz, 1H, 7-*H*), 6.96, 7.30 (2 × m, 5H, aromatic-*H*), 8.14 (d; *J* = 9.7 Hz, 1H, *H*N), 8.93 (s; 1H, 6′-H); ^{13}C NMR (DMSO): δ 41.6 (CH_2CH_2Cl), 45.1 (CH_2-4), 50.5 (CH_2CH_2Cl), 57.5 (C-7), 65.7 (CH_2O), 65.8 (*C*-6), 70.2 (OCH_2CO), 114.9 (2 × aromatic-*C*H), 121.8 (*C*-2), 127.3 (*C*-3), 129.8 (2 × aromatic-*C*H), 130.3 (*C*-6 ×), 136.4 (*C*-9′), 139.0

(*C*-8′), 157.4 (aromatic-*C*), 160.4 (*C*O), 163.6 (*C*O), 168.3 (2 × *C*O), 172.5 (*C*O). Anal. for $C_{23}H_{20}ClN_7O_9S\cdot 2H_2O$: C, 43.0; H 3.9; N, 15.3. Found: C, 42.8; H, 3.8; N, 15.2%.

3.1.27. 2-Carboxyl-7-phenylacetamido-5-sulfoxide-2-cephem-3-methyl, 3-methyl-4-oxoimidazo[5,1-d] [1,2,3,5]tetrazin-8-carboxylate (**12d**)

In 40.9% yield; m.p.: 182–183 °C (dec.), IR (KBr): 3280, 3128 (N–H), 3031, 2954, 2929 (C–H), 1783 (β-lactam C=O), 1749, 1245 (O–C), 1720, 1646, 1533 (N–C), 1051 (S–O), 953 cm^{-1}; ^1H NMR (DMSO) δ 3.53, 3.68 (Abq; $J = 13.9$ Hz, 2H, PhCH_2), 3.64, 4.02 (Abq; $J = 18.5$ Hz, 2H, 4-*H*), 3.88 (s; 3H, CH_3), 4.92 (d; $J = 4.7$ Hz, 6-*H*), 4.96, 5.57 (Abq; $J = 13.2$ Hz, 2H, CH_2O), 5.81 (dd; $J = 4.7$, 8.2 Hz, 1H, 7-*H*), 7.21–7.30 (m; 5H, aromatic-*H*), 8.44 (d; $J = 8.2$ Hz, 1H, *H*N), 8.87 (s; 1H, 6′-*H*); ^{13}C NMR (DMSO) δ 36.7 (*C*H_3), 41.6 (*C*H_2Ph), 45.6 (*C*H_2-4), 58.4 (*C*-7), 64.3 (*C*H_2O), 66.5 (*C*-6), 126.2 (C-2), 126.8 (C-3), 128.5, 129.3 and 129.7 (4 × aromatic-C*H*), 129.8 (C-6′), 136.0 (aromatic-C), 137.2 (C-9′) 139.1 (C-8′), 160.2 (CO), 162.4 (CO), 164.4 (CO), 171.3 (2 × CO). Anal. for $C_{22}H_{19}N_7O_8S$ $4H_2O$: C, 43.0; H, 4.4; N, 16.0. Found: C, 42.7; H, 4.7; N, 15.7%.

3.1.28. 2-Carboxyl-5-sulfoxide-7-[2-thien-2-yl)-acetamido]-2-cephem-3-methyl 3-methyl-4-oxoimidazo[5,1-d] [1,2,3,5]tetrazin-8-carboxylate (**12e**)

In 32.2% yield; m.p.: 179–181 °C (dec.), IR (KBr): 3280, 3128 (N–H), 3031, 2954, 2929 (C–H), 1781 (β-lactam C=O), 1760, 1243 (O–C), 1720, 1634, 1533 (N–C), 1039 (S–O) and 808; ^1H NMR (DMSO) δ 3.68, 4.02 (Abq; $J = 17.4$ Hz, 2H, 4-*H*), 3.79, 3.89 (Abq; $J = 14.8$ Hz, 2H, CH_2CO), 3.93 (s; 3H, CH_3), 4.79 (d; $J = 4.8$ Hz, 1H, 6-*H*), 4.95, 5.67 (Abq; $J = 12.7$ Hz, 2H, CH_2O), 5.58 (dd; $J = 4.8$, 8.6 Hz, 1H, 7-*H*), 6.94, 7.36 (M; 3H, thiophene-*H*), 8.28 (d; $J = 8.6$ Hz, 1H, *H*N), 8.84 (s; 1H, 6′-*H*); ^{13}C NMR (DMSO) δ 35.8 (*C*H_2-thiophene), 36.4 (*C*H_3), 45.2 (*C*H_2-4), 57.7 (*C*-7), 66.0 (*C*H_2O), 66.2 (*C*-6), 125.1 (*C*-2), 126.4 (*C*-3), 126.7 and 129.4, (3 × *C*H-thiophene) 130.4 (*C*-6′), 134.7 (*C*H-thiophene), 136.9 (*C*-9′), 139.0 (*C*-8′), 160.4 (*C*O), 163.1 (*C*O), 170.0 (2 × *C*O), 175.8 (*C*O): Anal. for $C_{20}H_{17}N_7O_8S_2$ $6H_2O$: C, 36.6; H, 4.4; N, 14.9. Found: C, 36.4; H, 4.5; N, 14.8%.

3.1.29. 2-Carboxyl-7-phenoxyacetamide-5-sulfoxide-2-cephem-3-methyl 3-methyl-4-oxoimidazo[5,1-d] [1,2,3,5]tetrazin-8-carboxylate (**12f**)

In 60.1% yield; m.p.: 207–208 °C (dec.), IR (KBr): 3280, 3128 (N–H), 3031, 2954, 2921 (C–H), 1782 (β-lactam C=O), 1751, 1245 (O–C), 1720, 1616, 1564 (N–C), 1407, 1051 (S–O) and 956 cm^{-1}; ^1H NMR (DMSO) δ 3.53, 3.78 (Abq; $J = 19.1$ Hz, 2H, 4-*H*), 3.88 (s; 3H, CH_3), 4.67 (s; 2H, PhOCH_2), 4.91 (d; $J = 4.7$ Hz, 1H, 6-*H*), 4.97, 5.65 (Abq; $J = 12.4$ Hz, 2H, CH_2O), 5.84 (dd; $J = 4.7$, 9.7 Hz, 1H, 7-*H*), 7.21–7.30 (M, 5H, aromatic-*H*), 8.12 (d; $J = 9.7$ Hz, 1H, *H*N), 8.85 (s; 1H, 6′-*H*); ^{13}C NMR (DMSO) δ 36.6 (*C*H_3), 45.0 (*C*H_2-4), 57.4 (*C*-7), 65.7 (*C*-6), 66.7 (2C, OCH_2COI and *C*H_2O), 114.9 (2 × aromatic-*C*H), 121.8 (*C*-2), 126.2 (*C*-3), 128.9 and 129.7 (3 × aromatic-*C*H), 130.4 (C-6′), 138.1 (C-9′), 139.2 (C-8′), 157.4 (aromatic-*C*), 160.2 (*C*O), 163.5 (*C*O), 168.3 (2 × *C*O), 172.5 (*C*O). Anal. for $C_{22}H_{19}N_7O_8S\cdot 3H_2O$: C, 43.2; H, 4.1; N, 16.0. Found: C, 42.9; H, 4.2; N, 15.7%.

3.2. MIC (minimal inhibitory concentration) determination

Minimum inhibitory concentration (MIC) values were determined by a broth microdilution method carried out according to NCCLS guidelines. Test organisms were suspended in saline and the concentration adjusted to give an initial inoculum of 106 colony forming units per milliliter (CFU per mL) in Mueller–Hinton broth (MHB). Test conjugates were dissolved in a minimum of DMSO where necessary, and made up to volume in MHB. Test concentrations of the dual-action agents ranged from 0.03 to 62.5 μg mL^{-1} in the microtitre plate. Microtitre plates were incubated overnight at 37 °C. Growth of test organisms was observed as a button of cells at the base of the well.

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