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Relationships Between Structure, Antibacterial Activity, Serum Stability, Pharmacokinetics and Efficacy in 3-(Heteroarylthio)cephems. Discovery of RWJ-333441 (MC-04,546)

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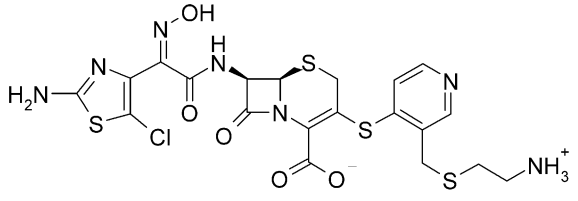
Abstract—SAR studies in a series of related 3-(heteroarylthio)cephems determined that a relatively high chemical reactivity of the β-lactam ring, modulated by electronic effects of substituents at C-3 and C-7, is necessary to achieve high in vitro activity against methicillin-resistant *Staphylococcus aureus* (MRSA). Such high reactivity results in lowered hydrolytic stability and concomitantly increases susceptibility to β-lactam ring opening mediated by serum enzymes. Therefore, optimization of anti-MRSA activity versus stability toward serum-mediated degradation required a fine balance of substituent effects. Serum stability studies (measured as percentage of parent drug degraded after 60 min incubation) revealed up to 80-fold difference in degradation rate in a series of closely related (3-heteroarylthio)cephems. Of the compounds evaluated, RWJ-333441 (MC-04,546) possessed the best balance of serum stability (6% degradation after 60 min incubation) and in vitro activity versus MRSA (*S. aureus* COL MIC = 1 μg/mL). Accordingly, RWJ-333441 displayed excellent in vivo efficacy versus methicillin-susceptible *Staphylococcus aureus* (MSSA, ED_{50} = 0.39 mg/kg in mouse sepsis model with *S. aureus* Smith) and good pharmacokinetic properties in the rat (Cl_{total} = 0.39 L/h/kg).

Introduction

The incidence of life-threatening nosocomial MRSA infections has been on the rise over the last three decades.[1] Since PBP2a-mediated MRSA resistance has rendered all clinically used β-lactams ineffective against such infections, the glycopeptide vancomycin, and to a lesser extent, new agents quinupristin/dalfopristin and linezolid,[2] represent the only available therapies. However, the recent occurrences of GISA (glycopeptide intermediate-resistant *Staphylococcus aureus*) infections[3] have prompted recommendation that vancomycin use be limited in order to control the emergence of vancomycin-resistant gram-positive pathogens.[4] The MRSA-active cephalosporin **RWJ-54428 (MC-02,479)**, presently under development by R. W. Johnson Pharmaceutical Research Institute and Essential Therapeutics (formerly Microcide), represents one of the recent examples of β-lactams in which the affinity toward PBP protein **2a** of *S. aureus* is restored.[5]



RWJ-54428 (MC-02,479)

We have reported previously on discovery of a wide variety of 3-heteroarylthio analogues with antimicrobial activities comparable to **RWJ-54428**.[6,7] The present publication describes our efforts aimed toward better understanding of the structural factors that govern pharmacokinetic properties within this class of compounds. In particular, the effects of structural modifications at positions C-3 and C-7 of 3-(heteroarylthio)cephalosporins on stability in serum, drug clearance and efficacy were studied in detail.

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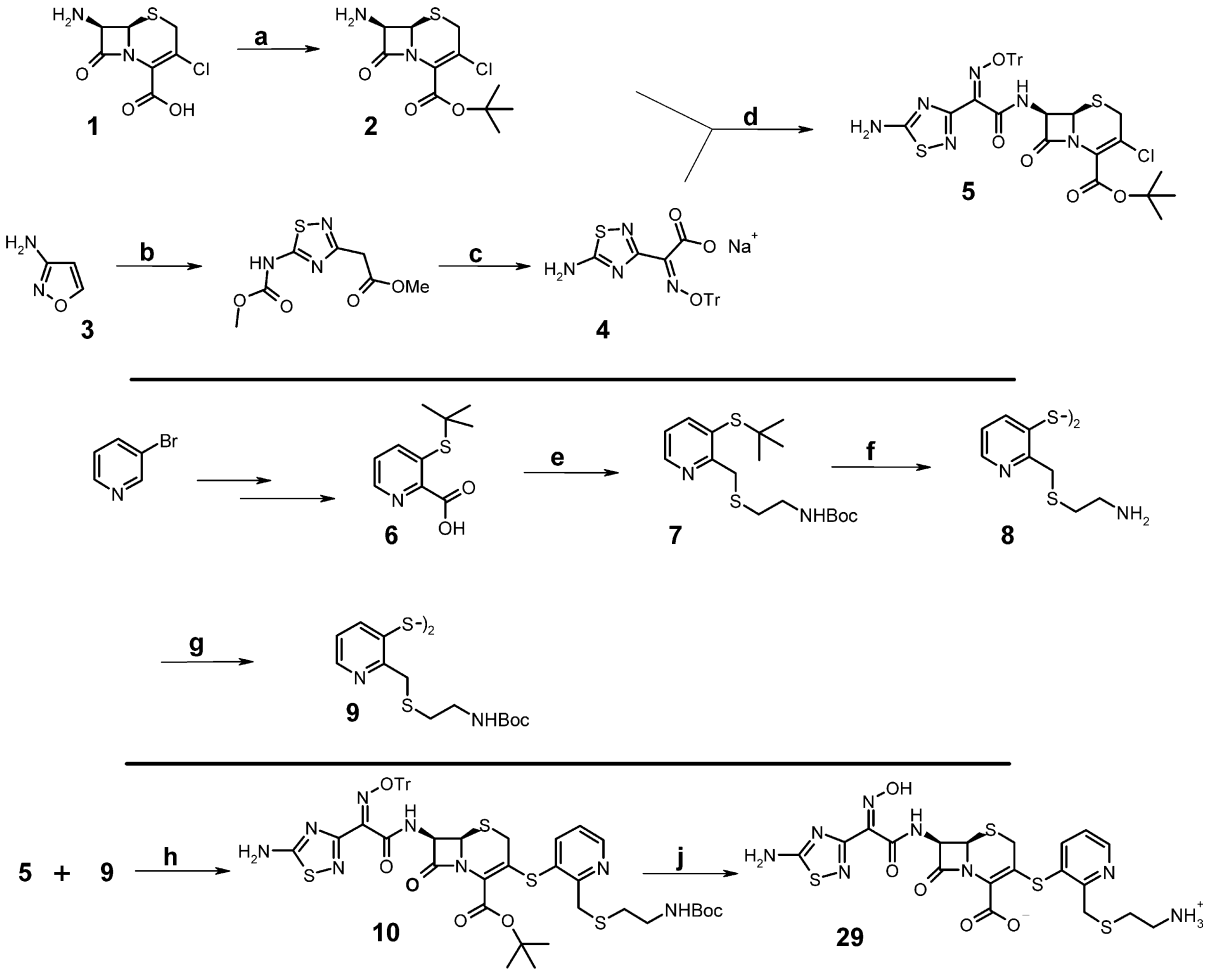
Chemistry

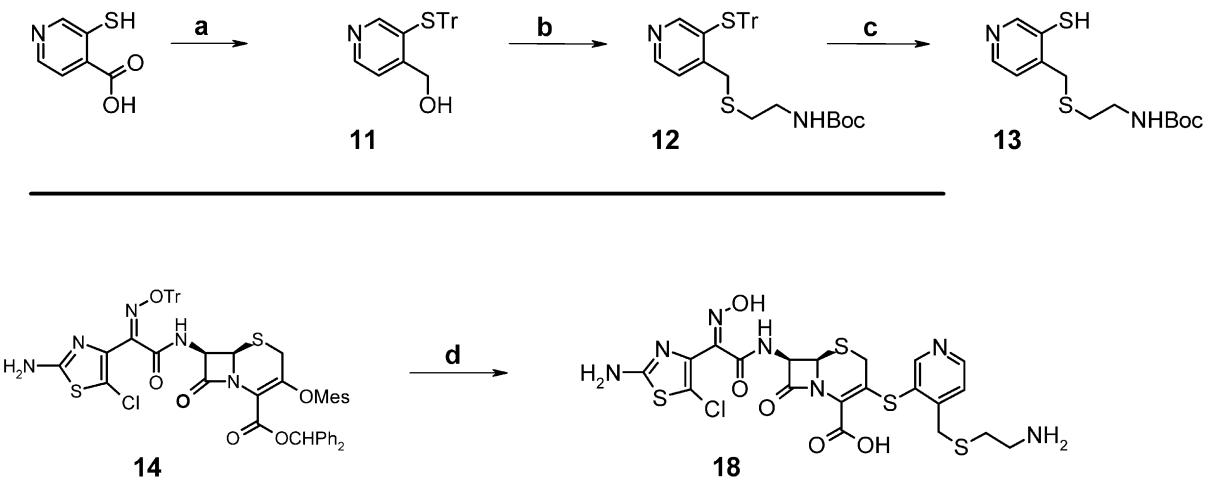
The syntheses of cephalosporin **29** (**RWJ-333441/MC-04,546**) and cephalosporin **18** exemplify the routes employed for the preparation of other cephalosporin analogues (Schemes 1 and 2). The 7-amino-3-chlorocephem carboxylic acid **1** (Otsuka Chemical Co.) was converted into the corresponding *t*-Bu ester **2** using a procedure described for other β-lactam intermediates.[8] Protected (5-amino-[1,2,4]thiadiazol-3-yl)-trityloxyimino-acetic acid (**4**) was synthesized by a modified Katayama route[9] starting from 3-aminoisoxazole (**3**). Acylation of **2** with the acid **4** provided the key 3-chlorocephem intermediate **5**. In the synthesis of the C-3 sidechain, 3-*tert*-butylthiopyridine-2-carboxylic acid (**6**), prepared from 3-bromopyridine,[10] was converted into the key intermediate **7**, which was then deprotected and oxidized to disulfide **8**. An aqueous solution of this hydrophilic intermediate was reacted in a biphasic system with Boc-anhydride in ethyl acetate solution to produce protected disulfide **9** in high yield. In situ reduction of **9** with triphenylphosphine provided the corresponding thiol, which was coupled with chlorocephem **5** to produce protected cephem **10**. Cephalosporin **29** (**RWJ-333441/MC-04,546**) was obtained by standard deprotection of **10** followed by desalting on an HP20 column and lyophilization. In order to synthesize the sidechain of cephalosporin **18**, 2-mercaptoisonicotinic acid was reduced to the corresponding alcohol and *S*-tritylated to produce intermediate **11**. Treatment with the Vilsmeyer reagent followed by reaction with *N*-Boc cysteamine gave the intermediate **12**, which upon further manipulation furnished the sidechain thiol **13** in suitably protected form. Coupling with the cephem 3-mesylate **14** followed by deprotection of acid-labile groups resulted in the target cephem. Preparations of other cephem analogues, which often required multi-step syntheses of C-3 sidechain thiols, generally followed similar synthetic schemes.

Results and Discussion

We have found that in order to obtain the desired level of anti-MRSA activity in the 3-(heteroarylthio)cephem series, it is necessary to append electron-withdrawing substituents at positions C-7 and especially C-3 of the cephem core, which increase the reactivity of the β-lactam ring. We observed that certain of these compounds displayed reduced stability in rat serum, and hypothesized

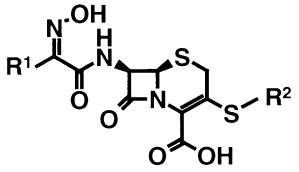


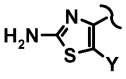
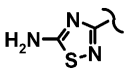
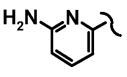
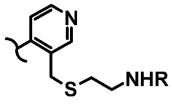
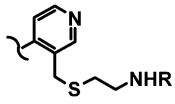
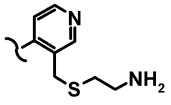
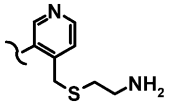
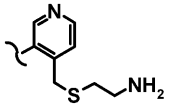
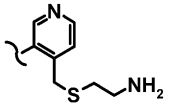
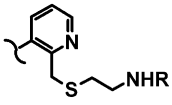
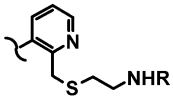
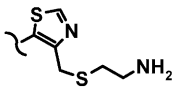
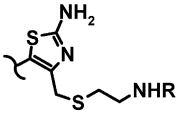
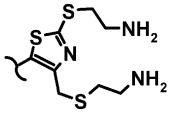
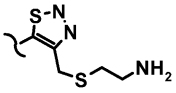
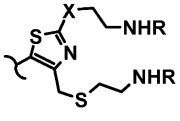
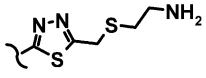
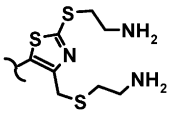
Scheme 1. (a) *t*-BuOAc, $BF_3{\cdot}Et_2O$; (b) (i) KSCN, MeOC(O)Cl; (ii) MeOH, 60°; (iii) peracetic acid; (iv) MeOH, $SOCl_2$; (c) (i) Br_2, MeOH; (ii) pyridine *N*-oxide, acetonitrile; (iii) NH_2OH, EtOH; (iv) trityl chloride, Et_3N, DMF; (v) NaOH, EtOH, water; (d) $(PhO)_2P(O)Cl$, 2,6-lutidine, THF; (e) (i) ClC(O)OEt, Et_3N; (ii) $NaBH_4$, THF; (iii) $SOCl_2$, DMF; (iv) *N*-Boc-cysteamine; (f) (i) 6M HCl; reflux; (ii) air, water; (g) Boc-anhydride, Et_3N, ethyl acetate, MeOH; (h) Ph_3P, $NaHCO_3$, DMF, water; (j) (i) trifluoroacetic acid, triethylsilane, CH_2Cl_2; (ii) HP20 resin, acetonitrile/water.



Scheme 2. (a) (i) B_2H_6, THF; (ii) TrCl, Et_3N, DMF; (b) (i) $SOCl_2$, DMF; (ii) $HSCH_2CH_2NHC(O)O$-*t*-Bu, DMF, K_2CO_3; (c) (i) trifluoroacetic acid, triethylsilane, CH_2Cl_2; (ii) di-*t*-butyldicarbonate, $(iPr)_2NEt$; (iii) sodium methoxide, MeOH; (d) (i) **13**, ethyl acetate; (ii) trifluoroacetic acid, triethylsilane, CH_2Cl_2.

Table 1. Structures of RWJ-333441 (MC-04,546; **29**) and related compounds



R^1 = 	R^1 = 	R^1 = 
R^2	R^2	R^2
15: Y=H; R=H 16: Y=Cl; R=H 17: Y=Cl; R=C(NH)NH_2 	26: R=H 27: R=C(NH)NH_2 	32: 
18: Y=Cl 	28: 	33: 
19: Y=Cl: R=H 20: Y=Cl; R=C(NH)NH_2 	29: R=H 30: R=C(NH)NH_2 	34: 
21: Y=Cl; R=H 22: Y=Cl; R=C(NH)NH_2 	31: 	35: 
23: X=S; Y=Cl; R=H 24: X=NH; Y=Cl; R=H 25: X=NH; Y=Cl; R=C(NH)NH_2 		36: 
		37: 

that this feature might influence their pharmacokinetic profiles (Table 1).

In our earlier SAR studies leading to the discovery of MC-02,331, basic functionality attached to the C-3 heteroarylthio substituent was found to be beneficial for the anti-MRSA activity of cephalosporins. Therefore while optimizing the pharmacokinetic properties of the C-3 heteroarylthio cephalosporins we maintained the presence of either amino or guanidino functionality in the structure. Several guanidine-substituted cephalosporins (**17**, **20**, **22**, **25**) displayed improved gram-positive

Table 2. Antimicrobial activity of cephalosporins **15**–**37** against gram-positive bacteria

	S.a.1	S.a.2	S.a.3	S.a.4	S.a.5	S.h.	E.fs.	E.fm.1	E.fm.2
15	0.25	0.13	1	1	1	2	0.13	1	2
16	0.25	0.13	0.5	1	1	2	≤0.06	0.5	0.25
17	0.13	≤0.06	0.5	0.5	0.5	1	≤0.06	0.25	0.5
18	0.13	0.13	1	1	1	2	0.13	1	2
19	0.25	0.13	0.5	1	1	1	0.06	0.5	1
20	0.13	0.06	0.5	0.5	0.5	1	0.06	0.25	0.5
21	0.25	0.13	1	1	2	1	0.13	1	8
22	0.06	0.06	0.25	0.5	0.5	0.5	0.06	0.5	1
23	≤0.06	0.13	0.5	0.5	0.5	0.5	≤0.06	0.5	4
24	0.13	0.13	1	1	1	1	≤0.06	0.5	4
25	≤0.06	≤0.06	0.5	0.5	0.5	0.5	≤0.06	0.25	1
26	0.13	0.13	1	1	1	2	0.13	0.5	1
27	0.25	0.13	1	1	1	2	0.13	0.5	1
28	0.5	0.13	8	8	8	16	1	4	8
29	0.25	0.25	1	1	1	2	1	1	2
30	0.13	0.13	1	1	1	2	1	0.5	1
31	0.5	0.13	1	1	1	2	0.5	2	8
32	0.5	0.25	1	2	2	4	0.25	1	4
33	0.13	≤0.06	8	8	4	8	0.5	4	4
34	0.13	≤0.06	2	2	1	2	0.25	1	2
35	0.13	≤0.06	1	1	1	2	0.13	1	1
36	0.25	≤0.06	8	16	8	32	0.25	1	2
37	0.13	≤0.06	1	1	1	1	0.25	1	2
IMI	≤0.008	≤0.008	32	32	32	64	≤0.25	4	4

S.a.1, *S. aureus* ATCC 29213 (methicillin-susceptible); S.a. 2, *S. aureus* ATCC 13709 Smith (methicillin-susceptible); S.a.3, *S. aureus* Col (methicillin-resistant, beta-lactamase negative); S.a. 4, *S. aureus* 76 (methicillin-resistant, beta-lactamase positive); S.a. 5, *S. aureus* ATCC 33593 (methicillin-resistant); S.h., *S. haemolyticus* 05 (methicillin-resistant); E.fs., *E. faecalis* ATCC 29212; E.fm.1, *E. faecium* ATCC 35667; E.fm.2, *E. faecium* (vancomycin resistant).

Table 3. Pharmacokinetic properties and serum stability of cephalosporins **15**–**37**

	ED_{50} (mg/kg) (95% CL)[a]	Total drug clearance in rat (L/h/kg) (cassette dosing)[a]	Human serum binding (%) (HSMHB/MHB MIC ratio)[a]	Aqueous solution decomposition (%/h[a])	Rat serum decomposition (%/h) (human serum decomposition)[a]
15	1.3 (0.8–1.8)	1.25	68 (–)	4.7	26
16	1.0 (0.7–1.6)	5.26 (2.41)	84 (3)	2.4	55 (22)
17	—	1.35 (1.67)	70 (–)	2.6	82
18	0.8 (0.5–1.0)	0.73	90 (2)	1.4	3
19	<0.31	0.79	90 (2)	2	9
20	—	—	83 (–)	1.2	38 (4)
21	0.2 (0.17–0.5)	0.95 (0.72)	88 (2)	1.7	3
22	—	—	– (2)	—	40 (1)
23	—	(14.7)	83 (–)	5.1	47 (15)
24	—	—	—	—	16 (6)
25	—	—	—	—	63 (8)
26	0.46 (0.3–0.6)	2.17 (1.59)	66 (2)	—	19
27	<0.31	1.12 (0.50)	47 (1)	—	45
28	—	—	– (4)	—	1
29	0.39 (0.3–0.5)	0.51 (0.39)	66 (2)	2	6 (1)
30	<0.31	—	60 (2)	—	10 (<1)
31	—	—	—	—	31 (13)
32	0.8 (0.6–1.0)	2.85	87 (–)	2.9	66
33	—	—	– (4)	—	1
34	0.4 (0.3–0.5)	0.81 (0.95)	94 (4)	3.2	15
35	2.0(1.3–2.7)	—	– (4)	—	70
36	—	—	—	—	63
37	—	5.39	91 (–)	4.8	42 (11)
IMI	0.07	—	– (2)	—	—

[a]For details see Experimental.

activity (including MRSA) in comparison to their amino analogues. The chlorine substituent present in the C-7 aminothiazole also proved to be beneficial to the anti-MRSA activity as evidenced by comparison with other C-7 substituents, of which the aminopyridine contributed the least to the anti-MRSA activity (e.g., **16** vs **15**, **26** and **32**). Interestingly, the 4-(2-aminoethylsulfanylmethyl)pyridin-3-yl substituent at C-3 position (e.g., **33**) consistently provided less anti-MRSA potency than the other two isomeric pyridines. In general, with the exception of a few analogues, the whole series of C-3 heteroarylthio cephalosporins displayed excellent gram-positive activity including β-lactam resistant strains of staphylococci and enterococci (Table 2).

Assessment of cephalosporin stability in rat serum was performed in fresh rat serum, and the results are reported in Table 3 as the percentage of compound decomposition over a period of 60 min (human serum stability was tested for only a limited number of analogues). With the exception of compound **16** (**RWJ-54428/MC-02,479**), where essentially all drug loss was accounted for in the form of β-lactam ring-opened product (data not shown), no attempt was made to rigorously identify the decomposition products. In several other cases however, the LC/MS/MS profiling of degradation mixtures suggested that the hydrolysis of the β-lactam ring represented the major decomposition pathway. The study revealed an 80-fold range in rat serum stability of (3-heteroarylthio)cephems (1% decomposition for pyridyl-3-thio compounds **28** and **33** versus 81% decomposition for pyridyl-4-thio compound **17**). A plausible explanation of this observation is that the effect of the more electronegative C-3 substituent extends over the cephem framework to the β-lactam ring making it more susceptible to the ring opening by serum enzymes. A considerable change in stability resulting from minor structural change in the **23**/**24** pair of analogues (2-(2-aminoethyl)thiothiazol-5-yl-thio versus 2-(2-aminoethyl)-aminothiazol-5-yl-thio substituent) points to a delicate balance between structure and serum stability. The lower stability of guanidine analogues revealed by comparison of pairs of analogues which differ only in a guanidine group replacing primary amine at the C-3 sidechain (e.g., **27** vs **26**) shows that the character of the C-3 heterocycle is not the only factor determining rat serum stability. Human serum stability data obtained for selected compounds showed less hydrolysis by human serum as compared to rat serum. Analogues tested in pH 7.9 buffer alone showed low levels of baseline aqueous hydrolysis, which supports the hypothesis that the decomposition observed in serum is enzyme-assisted (Table 3).

Among the compounds showing high serum stability, compound **29**, the potent anti-MRSA cephem **RWJ-333441 (MC-04,546)** with low serum binding and good aqueous solubility (4.4 mg/mL at pH 7.2) was selected for further evaluation. **RWJ-333441 (MC-04,546)** also possessed the lowest rat clearance and a low ED_{50} in a mouse septicemia model against MSSA infection (MSSA Smith strain).

Conclusions

Considerable differences in stability of 3-(heteroarylthio)cephems toward enzyme-mediated decomposition in rat serum were observed. These differences appear to be related to the electron-withdrawing effects of both C-7 and C-3 substituents on the cephalosporin core, which influence the reactivity of the β-lactam ring. Accordingly, analogues with increased stability in rat serum displayed low clearance and improved efficacy in an animal model of infection. Based on these attributes, cephalosporin **RWJ-333441 (MC-04,546)**, which displayed an excellent antimicrobial profile (MRSA $MIC_{90} = 2$ μg/mL), high stability in rat serum (6% decomposition over 60 min) and improved pharmacokinetics in rat (total clearance 0.39 L/h/kg) was selected for further evaluation. Evaluation of pharmacokinetic properties and antimicrobial in vivo efficacy of **RWJ-333441 (MC-04,546)** will be described elsewhere.[11]

Experimental

Chemistry

Syntheses of cephalosporin analogues **29** and **18** represent the general routes employed in the preparation of the 3-(heteroarylthio)cephems.

(7R,6R)-7-Amino-3-chloro-8-oxo-5-thia-1-aza-bicyclo[4.2.0]-oct-2-ene-2-carboxylic acid *tert*-butyl ester (2). To a suspension of (7*R*,6*R*)-7-amino-3-chloro-8-oxo-5-thia-1-aza-bicyclo[4.2.0]oct-2-ene-2-carboxylic acid (**1**) (23.7 g, 101.2 mmol) in *t*-butyl acetate (510 mL), under nitrogen, was added boron trifluoride–ethyl ether complex (80 mL) and the mixture was stirred vigorously at room temperature until complete dissolution. The reaction mixture was poured into stirred ice water (1000 mL) and the organic layer was discarded. Aqueous layer was washed with 1:1 ethyl acetate/hexane (200 mL) and separated. To this aqueous solution with ice cooling and stirring was added ethyl acetate (500 mL) followed by portion-wise addition of sodium carbonate (216 g) until pH of 8–8.5 is reached. Organic layer was separated and the aqueous layer was extracted with ethyl acetate (100 mL). Combined aqueous extracts were dried over anhydrous sodium sulfate and concentrated under reduced pressure (not exceeding 30 °C) to oily residue (28 g). To this residue was added toluene (100 mL) followed by hexane (100 mL). After 10 min stirring crystalline title product (**2**) was filtered, washed with hexane and dried under reduced pressure (15.4 g, 52%). ^{1}H NMR ($CDCl_3/CD_3OD$) δ 1.40 (s, 9H), 3.30 (d, $J = 17$ Hz, 1H), 3.63 (d, $J = 17$ Hz, 1H), 4.59 (d, $J = 6$ Hz, 1H), 4.86 (d, $J = 6$, 1H).

***N*-(Isoxazol-3-yl)-*N*′-(carbomethoxy)thiourea.** A suspension of potassium thiocyanate (225 g, 2.3 mol) in fresh acetonitrile (1.65 L) was mechanically stirred at room temperature for 15 min and was then treated, dropwise, with methyl chloroformate (202 g, 2.1 mol). The suspension was warmed to 60 °C where it was stirred for 30 min becoming a thick, yellow mixture. The mixture was

cooled to 0 °C and treated dropwise with isoxazol-3-ylamine (**3**) (150 g, 1.8 mol). After additional 30 min at 0 °C the mixture was allowed to reach room temperature. The mixture was poured into rapidly stirred ice water (5.0 L) and was then stirred for 15 min. The precipitate was allowed to settle. After decanting the aqueous phase the yellow solid was washed with ice water (4.0 L). The aqueous phase was again decanted from the yellow solid. This was repeated twice more, the solid becoming more powdery with each wash. The solid was dried on the vacuum filter overnight yielding title product as an orange powder, 185 g (52%). ^{1}H NMR (DMSO-d_6) δ 3.95 (s, 3H), 7.52 (s, 1H), 9.14 (s, 1H).

[3-(2-Hydroxy-2-methoxy-ethyl)-[1,2,4]thiadiazol-5-yl]-carbamic acid methyl ester. A mechanically stirred solution of *N*-(isoxazol-3-yl)-*N*′-(carbomethoxy)thiourea (185 g, 0.9 mol) in methanol (2 L) was warmed to 60 °C and was stirred for 1 h. Concentration under reduced pressure gave a pale, yellow, moist solid. Residual water was removed azeotropically with toluene and the precipitate was filtered off producing title product as a cream colored solid (180 g, 86%). ^{1}H NMR (DMSO-d_6) δ 2.85–3.00 (m, 2H), 3.18 (s, 3H), 3.80 (s, 3H), 4.92 (br.s, 1H), 6.20 (br.s, 1H).

(5-Methoxycarbonylamino-[1,2,4]thiadiazol-3-yl)-acetic acid. A solution of [3-(2-hydroxy-2-methoxy-ethyl)-[1,2,4]thiadiazol-5-yl]-carbamic acid methyl ester (178 g, 0.76 mol) in glacial acetic acid (1500 mL) was stirred for 2 h then filtered, removing small amount of insoluble material. The filtrate was treated dropwise over 20 min with peracetic acid (32 wt.%, 195 mL). The reaction was stirred at room temperature for 16 h. The reaction mixture was then filtered and the resulting solid was washed repeatedly with ether, air-dried, then dried in vacuum yielding title product (110 g) as a white powder. ^{1}H NMR (DMSO-d_6) δ 3.75 (s, 2H), 3.80 (s, 3H).

(5-Methoxycarbonylamino-[1,2,4]thiadiazol-3-yl)-acetic acid methyl ester. A mechanically stirred suspension of (5-methoxycarbonylamino-[1,2,4]thiadiazol-3-yl)-acetic acid (108 g, 0.5 mol) in methanol, cooled to 0 °C (3 L), was treated dropwise with thionyl chloride (297 g, 2.5 mol). After stirring for 16 h at room temperature the mixture was filtered. The resulting white crystals were washed with ether, air-dried, then dried in vacuum yielding title product (60 g). Concentration of the mother liquors produced second crop of the product (53.3 g). Combined yield, 113 g (98%). ^{1}H NMR ($CDCl_3$) δ 3.75 (s, 3H), 3.92 (s, 3H), 4.00 (s, 2H).

Bromo-(5-methoxycarbonylamino-[1,2,4]thiadiazol-3-yl)-acetic acid methyl ester. A stirred solution of (5-methoxycarbonylamino-[1,2,4]thiadiazol-3-yl)-acetic acid methyl ester (58 g, 0.25 mol) in 1:1 methanol/dichloromethane mixture (0.6 L) was cooled to 0 °C and treated dropwise with bromine (40.1 g, 0.25 mol). The red-brown solution was allowed to rise slowly to room temperature and stirred for 16 h. The resulting pale yellow solution was partitioned between ethyl acetate (0.75 L) and water (0.5 L). The organic layer was washed further with water (0.5 L) then saturated sodium bicarbonate (2×0.5 L), then brine. The organic layer was dried with sodium sulfate and concentrated to produce a white solid (66.5 g). NMR revealed presence of 80% (molar) of title product contaminated with about 10% of both non-brominated starting material and bis-brominated by-product. ^{1}H NMR ($CDCl_3$) δ 3.80 (s, 3H), 3.95 (s, 3H), 5.73 (s, 1H).

(5-Methoxycarbonylamino-[1,2,4]thiadiazol-3-yl)-oxo-acetic acid methyl ester. A stirred solution of bromo-(5-methoxycarbonylamino-[1,2,4]thiadiazol-3-yl)-acetic acid methyl ester (50 g, 0.13 mol, about 80% pure) in acetonitrile (0.3 L) was treated with pyridine-*N*-oxide (38.3 g, 0.40 mol). After stirring at reflux for 1 h the reaction was concentrated. The residue was partitioned between dichloromethane (0.5 L) and brine (0.15 L). The brine was washed with dichloromethane. The organic layers were combined, dried on sodium sulfate, and concentrated to produce crude title product as a brown oil (45.5 g). This material was taken into next step without purification. ^{1}H NMR ($CDCl_3$) δ 3.92 (s, 3H), 3.96 (s, 3H).

(*Z*)-Hydroxyimino-(5-methoxycarbonylamino-[1,2,4]thiadiazol-3-yl)-acetic acid methyl ester. A solution of (5-methoxycarbonylamino-[1,2,4]thiadiazol-3-yl)-oxo-acetic acid methyl ester (45.5 g, 0.18 mol), pyridine (19.6 g, 0.25 mol), and hydroxylamine hydrochloride (16.8 g, 0.24 mol) in absolute ethanol (0.4 L) was stirred at room temperature for 16 h. Concentration gave a yellow syrup which was partitioned between ethyl acetate (0.5 L) and water (0.15 L). The organic was again washed with water (0.15 L) then one molar hydrochloric acid (0.2 L) then brine. The organic was dried on sodium sulfate and concentrated to 0.1 L. The solution was allowed to crystallize. The product was collected, washed with ether and dried yielding pure title product as white crystals (12.3 g, 26%). Concentration of mother liquors gave an orange glass (15.1 g), which by NMR was estimated to contain 60–70% of the desired material. ^{1}H NMR ($CDCl_3$) δ 3.85 (s, 3H), 3.93 (s, 3H).

(5-Methoxycarbonylamino-[1,2,4]thiadiazol-3-yl)-(*Z*)-trityloxyimino-acetic acid methyl ester. A solution of (*Z*)-hydroxyimino-(5-methoxycarbonylamino-[1,2,4]thiadiazol-3-yl)-acetic acid methyl ester (10.0 g, 38.5 mmol) in dichloromethane (0.15 L) was cooled to 0 °C. The cold mixture was treated with triethylamine (4.0 g, 39.5 mmol) then portion-wise with triphenylmethyl chloride (10.7 g, 38.5 mmol). The resulting yellow solution was stirred at 0 °C until TLC showed the reaction complete (approximately 2 h). The reaction solution was washed with brine, dried on sodium sulfate then concentrated to 100 mL. The slightly opaque mixture was placed in the freezer. The resulting thick mixture was filtered. The white crystals were washed with ether and dried under reduced pressure yielding title product as colorless crystals (12.0 g, 62%, pure *syn* isomer as confirmed by NMR). ^{1}H NMR ($CDCl_3$) δ 3.53 (s, 3H), 4.05 (s, 3H), 7.20–7.35 (m, 15H).

(5-Amino-[1,2,4]thiadiazol-3-yl)-(*Z*)-trityloxyimino-acetic acid sodium salt (4). A suspension of (5-methoxycarbonylamino-[1,2,4]thiadiazol-3-yl)-(*Z*)-trityloxyimino-acetic acid methyl ester (9.5 g, 18.9 mmol) in 2:1 mixture

of 2.5 M aqueous NaOH and ethanol (75 mL) was heated to gentle reflux for 8 h and stirring was continued for 16 h at room temperature (initial suspension becomes clear solution on heating). Crystalline product was collected by filtration, washed with water, air dried and then dried in high vacuum for 72 h producing white crystalline title product (6.0 g, 70%).

(6*R*,7*R*)-7-[2-(5-Amino-[1,2,4]thiadiazol-3-yl)-2-(*Z*)-trityloxyimino-acetylamino]-3-chloro-8-oxo-5-thia-1-aza-bicyclo[4.2.0]oct-2-ene-2-carboxylic acid *tert*-butyl ester (5). To a stirred suspension of (5-amino-[1,2,4]thiadiazol-3-yl)-(*Z*)-trityloxyimino-acetic acid sodium salt (**4**) (14.38 g, 31.7 mmol, containing 0.33 equiv of water according to Karl Fisher method determination) in dry THF (170 g) was added diphenylchlorophosphonate (9.9 mL, 47.5 mmol). After a few minutes of stirring most of starting material dissolved. Stirring was continued for 1 h at ambient temperature and solid (6*R*,7*R*)-7-amino-3-chloro-8-oxo-5-thia-1-aza-bicyclo[4.2.0]oct-2-ene-2-carboxylic acid *tert*-butyl ester (**2**) (9.19 g, 31.7 mmol) was added followed by addition of 2,6-lutidine (3.7 mL, 31.7 mmol). After 3 h at room temperature reaction mixture was partitioned between ethyl acetate (200 mL) and 0.5 M hydrochloric acid (100 mL). Organic layer was washed with 0.5 M hydrochloric acid (100 mL) and then washed twice with 0.5 M aqueous sodium bicarbonate (100 mL). Organic solution was dried over anhydrous sodium sulfate and concentrated to about 60 mL volume. To this solution 1:2 mixture of ethyl acetate/hexane (80 mL) was added and the product was allowed to crystallize overnight. Filtration and drying under reduced pressure yielded 14.1 g of crystalline product. ^{1}H NMR ($CDCl_3$/CD_3OD) δ 1.40 (s, 9H), 3.20 (d, J = 17, 1H), 3.60 (d, J = 17, 1H), 4.97 (d, J = 6, 1H), 5.80 (d, J = 6, 1H), 7.10–7.30 (m, 15H).

(3-*tert*-Butylsulfanyl-pyridin-2-yl)-methanol. To a suspension of 3-*tert*-butylsulfanyl-pyridine-2-carboxylic acid (**6**) (10.0 g, 47.4 mmol) in tetrahydrofuran (200 mL) cooled to −5 °C was added triethylamine (8.25 mL, 47.4 mmol) followed by addition of ethyl chloroformate (4.38 g, 47.4 mmol) and reaction was stirred for 30 min at 0 °C. Lithium borohydride (2.58 g, 118 mmol) was added in portions, maintaining the temperature below −5 °C. After the addition was complete the reaction was allowed to warm to room temperature and stirred for 1 h. Temperature was lowered to −5 °C and methanol (10 mL) was added followed by addition of aqueous sodium hydroxide (10 mL, 10%). After the addition of ethyl acetate (50 mL) and water (40 mL) dilute hydrochloric acid was added to obtain pH = 5.0. Precipitated inorganic salt was filtered off and organic layer of the filtrate was separated. After washing aqueous layer thoroughly with ethyl acetate the combined organic extracts were dried over sodium sulfate and concentrated to produce yellow oil (7.21 g) of title product. ^{1}H NMR ($CDCl_3$) δ 1.40 (s, 9H), 4.50 (br.s, 1H), 4.90 (s, 2H), 7.20 (m, 1H), 7.80 (d, J = 7, 1H), 8.55 (d, J = 5, 1H).

[2-(3-*tert*-Butylsulfanyl-pyridin-2-ylmethylsulfanyl)-ethyl]-carbamic acid *tert*-butyl ester (7). A solution of Vilsmeier reagent was prepared by addition of thionyl chloride (1.09 g, 9.17 mmol) to dry dimethylformamide (10 mL) at room temperature. After 30 min the above solution was transferred to a solution of (3-*tert*-butylsulfanyl-pyridin-2-yl)-methanol (1.20 g, 6.09 mmol) in dry dimethylformamide (5 mL). After stirring for 30 min at room temperature, powdered potassium carbonate (4.15 g, 30 mmol) was added followed by addition of (2-mercapto-ethyl)-carbamic acid *tert*-butyl ester (5.73 g, 30.0 mmol) and sodium iodide (0.15 g, 1.05 mmol) and vigorous stirring was continued for 16 h. Reaction mixture was partitioned between ethyl acetate and water. Organic extract was thoroughly washed with water, then dried over sodium sulfate and evaporated to produce oily residue, which was purified by flash chromatography on silica gel (ethyl acetate/ hexane 1:2) to afford oily title product (1.10 g). ^{1}H NMR ($CDCl_3$) δ 1.40 (s, 9H), 1.57 (s, 9H), 2.80 (t, J = 6, 2H), 3.43 (t, J = 6, 2H), 4.35 (s, 2H), 5.40 (br.s, 1H), 7.28 (dd, J = 6, J = 4, 1H), 7.95 (d, J = 6, 1H), 8.63 (d, J = 4, 1H).

2-(2-Amino-ethylsulfanylmethyl)-pyridine-3-thiol dihydrochloride. A solution of [2-(3-*tert*-butylsulfanyl-pyridin-2-ylmethylsulfanyl)-ethyl]-carbamic acid *tert*-butyl ester (**7**) (0.60 g) in hydrochloric acid (5 mL, 6.0 M) was refluxed for 72 h (until NMR of a sample in D_2O does not show any *t*-butyl signal) and the reaction mixture was evaporated to dryness to produce solid material of dihydrochloride of title product (0.40 g), which was used for next step without further purification. ^{1}H NMR (D_2O) δ 2.85 (t, J = 6, 2H), 3.15 (t, J = 6, 2H), 4.20 (s, 2H), 7.62 (m, 1H), 7.30 (d, J = 4, 1H), 7.43 (d, J = 6, 1H).

2-{3-[2-(2-Amino-ethylsulfanylmethyl)-pyridin-3-yldisulfanyl]-pyridin-2-ylmethylsulfanyl}-ethylamine (8). A solution of 2-(2-amino-ethylsulfanylmethyl)-pyridine-3-thiol dihydrochloride (0.40 g) in water (4 mL) basified with addition of concentrated ammonium hydroxide and a stream of air was bubbled through it for 16 h with occasional addition of more concentrated ammonium hydroxide. Reaction mixture was evaporated to dryness to produce solid residue of title product and ammonium chloride, which was used for next step without further purification. ^{1}H NMR (D_2O) δ 2.75 (t, J = 6, 2H), 3.15 (t, J = 6, 2H), 4.00 (s, 2H), 7.38 (dd, J = 4, J = 6, 1H), 8.21 (d, J = 6, 1H), 8.38 (d, J = 4, 1H).

(2-{3-[2-(2-*tert*-Butoxycarbonylamino-ethylsulfanylmethyl)-pyridin-3-yldisulfanyl]-pyridin-2-ylmethylsulfanyl}-ethyl)-carbamic acid *tert*-butyl ester (9). To a solution of crude 2-{3-[2-(2-amino-ethylsulfanylmethyl)-pyridin-3-yldisulfanyl]-pyridin-2-ylmethylsulfanyl}-ethylamine (**8**) in methanol (50 mL) were added di-*t*-butyldicarbonate (1.19 g, 5.46 mmol) and triethylamine (0.73 g, 7.20 mmol). After 45 min at room temperature reaction mixture was evaporated to dryness and re-dissolved in dichloromethane. The insoluble residue was filtered off and the filtrate was concentrated under reduced pressure to produce oily residue. Flash chromatography on silica gel (5% methanol in dichloromethane) yielded oily title product (0.28 g). ^{1}H NMR ($CDCl_3$) δ 1.32 (s, 9H), 2.60–2.70 (m, 2H), 3.20–3.30 (m, 2H), 4.00 (s, 2H), 5.10 (br.s, 2H), 4.00 (s, 2H), 7.17 (dd, J = 5, J = 8, 1H), 7.90 (d, J = 8, 1H), 8.38 (d, J = 5, 1H).

(7*R*,6*R*)-7-[2-(5-Amino-[1,2,4]thiadiazol-3-yl)-2-(*Z*)-trityloxyimino-acetylamino]-3-[2-(2-tert-butoxycarbonylamino-ethylsulfanylmethyl)-pyridin-3-ylsulfanyl]-8-oxo-5-thia-1-aza-bicyclo[4.2.0]oct-2-ene-2-carboxylic acid *tert*-butyl ester (10). To a solution of (6*R*,7*R*)-7-[2-(5-amino-[1,2,4]thiadiazol-3-yl)-2-(*Z*)-trityloxyimino-acetylamino]-3-chloro-8-oxo-5-thia-1-aza-bicyclo[4.2.0]oct-2-ene-2-carboxylic acid *tert*-butyl ester (**5**) (2.00 g, 2.84 mmol) in DMF (30 mL), under nitrogen, was added (2-{3-[2-(2-*tert*-butoxycarbonylamino-ethylsulfanylmethyl)-pyridin-3-yldisulfanyl]-pyridin-2-ylmethylsulfanyl}-ethyl)-carbamic acid *tert*-butyl ester (**9**) (852 mg, 1.42 mmol) followed by addition of triphenylphosphine (1.12 g, 4.27 mmol), water (0.41 mL) and 0.5 M aqueous sodium bicarbonate (5.68 mL, 2.84 mmol). After stirring at ambient temperature overnight the reaction mixture was diluted with ice cold water and thoroughly extracted with ethyl acetate/hexane mixture (3:1). The combined extracts were washed with water. The title product was isolated by chromatography on silica gel column to produce quantitative yield of final product (2.91 g) as yellow-orange foam. ^{1}H NMR ($CDCl_3/CD_3OD$) δ 1.35 (s, 9H), 1.49 (s, 9H), 2.62 (t, $J=7$, 9H), 3.02 (s, $J=17$, 9H), 3.10–3.20 (m, 3H), 3.96 (s, 2H), 5.07 (d, $J=6$, 1H), 6.00 (d, $J=6$, 1H), 7.14 (dd, $J=5$, $J=8$, 1H), 7.20–7.35 (m, 15H), 8.40 (br.s, 1H).

(7*R*,6*R*)-3-[2-(2-Amino-ethylsulfanylmethyl)-pyridin-3-yl-sulfanyl]-7-[2-(5-amino-[1,2,4]thiadiazol-3-yl)-2-(*Z*)-hydroxyimino-acetylamino]-8-oxo-5-thia-1-aza-bicyclo[4.2.0]oct-2-ene-2-carboxylic acid (29). To a solution of (7*R*,6*R*)-7-[2-(5-amino-[1,2,4]thiadiazol-3-yl)-2-(*Z*)-trityloxyimino-acetylamino]-3-[2-(2-*tert*-butoxycarbonylamino-ethylsulfanylmethyl)-pyridin-3-ylsulfanyl]-8-oxo-5-thia-1-aza-bicyclo[4.2.0]oct-2-ene-2-carboxylic acid *tert*-butyl ester (**10**) (2.91 g, 3.0 mmol) in dichloromethane (15 mL) was added triethylsilane (15 mL). To this mixture trifluoroacetic acid (58 mL) was added slowly with ice cooling. The reaction was stirred for 2 h at 10 °C and then for 3 h at ambient temperature. The bis-trifluoroacetic acid salt of the title product (2.13 g, 89%) was isolated by precipitation with slow addition of diisopropyl ether (120 mL) with ice cooling, filtration and thorough washing with diisopropyl ether. ^{1}H NMR (D_2O) δ 2.91 (t, $J=6$, 2H), 3.31 (t, $J=6$, 2H), 3.36 (d, $J=16$, 1H), 3.76 (d, $J=16$, 1H), 4.28 (s, 2H), 5.37 (d, $J=6$, 2H), 5.91 (d, $J=6$, 1H), 7.92 (dd, $J=7$, $J=6$, 1H), 8.43 (d, $J=7$, 1H), 8.60 (d, $J=6$, 1H).

A 1 inch diameter column was packed with 30 g of HP20 resin and the solution of the bis-trifluoroacetic acid salt of the title product (1.20 g) in water (20 mL) was loaded. Distilled water was run through the column until the pH of the effluent reached 5.5. The solvent was changed immediately to 4:1 water/acetonitrile and the zwitterionic product was eluted in approximately 100 mL of effluent. The acetonitrile was removed in vacuum at <40 °C and the remaining water was lyophilized to give the title compound (0.79 g, 92%).

(3-Mercapto-pyridin-4-yl)-methanol. To a suspension of 3-mercaptoisonicotinic acid (1.80 g, 11.6 mmol) in dry THF (70 mL) was added slowly borane in THF (52 mL, 1.0 M, 52 mmol) and the reaction mixture was stirred for 30 min. Solvent was evaporated under reduced pressure, methanol (40 mL) was added and after gas evolution stopped concentrated hydrochloric acid (3.6 mL) was added. The solution was filtered and the filtrate was evaporated to dryness. The residue was redissolved in small volume of water, concentrated aqueous ammonia (3.6 mL) was added and the reaction mixture was evaporated to dryness. After overnight drying under vacuum quantitative yield of the title product was obtained, which was used for next step without purification. ^{1}H NMR (CD_3OD) δ 4.786 (s, 2H), 8.13 (d, $J=6$, 2H), 8.60 (d, $J=6$, 2H), 8.70 (s, 1H).

(3-Tritylsulfanyl-pyridin-4-yl)-methanol (11). (3-Mercapto-pyridin-4-yl)-methanol (100 mg, 0.71 mmol) was dissolved in DMF (5 mL) and diisipropylethylamine (0.12 mL, 0.71 mmol) was added followed by trityl chloride (197 mg, 0.71 mmol). After 30 min the reaction mixture was partitioned between water and ethyl acetate, the organic layer was thoroughly washed with water and dried with anhydrous sodium sulfate. Purification by radial chromatography on silica gel produced pure title material (67 mg, 25% yield). ^{1}H NMR ($CDCl_3$) δ 4.17 (s, 2H), 7.20–7.40 (m, 15H), 7.42 (d, $J=6$, 1H), 8.22 (s, 1H), 8.40 (d, $J=6$, 1H).

[2-(3-Tritylsulfanyl-pyridin-4-ylmethylsulfanyl)-ethyl]-carbamic acid *tert*-butyl ester (12). A solution of thionyl chloride (126 mg, 1.05 mmol) in dry DMF (2 mL) was stirred for 30 min at room temperature. Such solution was then cannulated into a solution of (3-tritylsulfanyl-pyridin-4-yl)-methanol (**11**) (270 mg, 0.70 mmol) in DMF (2 mL) at room temperature and the reaction was stirred for 30 min. (2-Mercapto-ethyl)-carbamic acid *tert*-butyl ester (187 mg, 1.05 mmol) and powdered potassium carbonate (486 mg, 3.52 mmol) were added to the reaction mixture and stirring was continued for additional 30 min. The reaction was partitioned between water and ethyl acetate and the organic layer was thoroughly washed with water and dried. After removing the solvent under reduced pressure the residue was purified by radial chromatography on silica gel (hexane/ethyl acetate 4:1) to yield title material as off-white solid (220 mg, 58%). ^{1}H NMR ($CDCl_3$) δ 1.46 (s, 9H), 2.42 (t, $J=6$, 2H), 3.18 (s, 2H), 3.23 (m, 2H), 4.80 (br.s, 1H), 7.10 (d, $J=6$, 1H), 7.20–7.45 (m, 15H), 8.28 (m, 2H).

Thiocarbonic acid *S*-[4-(2-*tert*-butoxycarbonylamino-ethylsulfanylmethyl)-pyridin-3-yl] ester *O*-*tert*-butyl ester. To [2-(3-tritylsulfanyl-pyridin-4-ylmethylsulfanyl)-ethyl]-carbamic acid *tert*-butyl ester (**12**) (790 mg, 1.45 mmol) and triethylsilane (2 mL, 12.5 mmol) dissolved in dichloromethane (15 mL) was added trifluoroacetic acid (15 mL). After 1 h stirring at room temperature reaction mixture was evaporated to dryness under reduced pressure and the residue was dissolved in dry tetrahydrofuran (10 mL). To this solution diisopropylethylamine (1.40 mL, 7.84 mmol) was added, followed by di-*t*-butyldicarbonate (1.28 g, 5.88 mmol) and after 3 h reaction at room temperature solvent was removed at reduced pressure. Purification on silica gel

by radial chromatography (dichloromethane/methanol 50:1) produced pure title product as yellow foam (460 mg, 79%). ^{1}H NMR ($CDCl_3$) δ 1.42 (s, 9H), 1.50 (s, 9H), 2.58 (t, $J=6$, 2H), 3.23 (t, $J=6$, 2H), 3.84 (s, 2H), 4.95 (br.s, 1H), 7.42 (d, $J=6$, 1H), 8.58 (d, $J=6$, 1H), 8.70 (s, 1H).

[2-(3-Mercapto-pyridin-4-ylmethylsulfanyl)-ethyl]-carbamic acid *tert*-butyl ester (13). To a solution of thiocarbonic acid *S*-[4-(2-*tert*-butoxycarbonylamino-ethylsulfanylmethyl)-pyridin-3-yl] ester *O*-*tert*-butyl ester (415 mg, 1.04 mmol) in methanol (2 mL) was added under nitrogen methanolic solution of sodium methoxide (1.04 mL, 1.0 M) and the reaction mixture was heated to 50 °C for 30 min. After evaporating the solvent in vacuum the residue was partitioned between water and ethyl acetate with addition of acetic acid (125 mg, 2.08 mmol). Evaporation of the solvent under reduced pressure yielded title material (298 mg, 96% yield), which was used in the next step without further purification. ^{1}H NMR (CD_3OD) δ 1.42 (s, 9H), 2.60 (m, 2H), 3.20 (m, 2H), 4.0 (s, 2H), 4.95 (br.s, 1H), 7.52 (d, $J=6$, 1H), 8.00 (d, $J=6$, 1H), 8.40 (s, 1H).

(7*R*,6*R*)-7-[2-(2-Amino-5-chloro-thiazol-4-yl)-2-(*Z*)-trityloxyimino-acetylamino]-3-[4-(2-tert-butoxycarbonylamino-ethylsulfanylmethyl)-pyridin-3-ylsulfanyl]-8-oxo-5-thia-1-aza-bicyclo[4.2.0]oct-2-ene-2-carboxylic acid benzhydryl ester. To a solution of [2-(3-mercapto-pyridin-4-ylmethylsulfanyl)-ethyl]-carbamic acid *tert*-butyl ester (**13**) (298 mg, 0.99 mmol) in ethyl acetate (5 mL) was added (7*R*,6*R*)-7-[2-(2-amino-5-chloro-thiazol-4-yl)-2-(*Z*)-trityloxyimino-acetylamino]-3-methanesulfonyloxy-8-oxo-5-thia-1-aza-bicyclo[4.2.0]oct-2-ene-2-carboxylic acid benzhydryl ester. After 1 h stirring at room temperature the reaction was partitioned between ethyl acetate and dilute sodium bicarbonate solution (30 mL, 1%). The organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was purified by radial chromatography on silica gel to yield pure title product (366 mg, 33%). ^{1}H NMR (CD_3OD) δ 1.40 (s, 9H), 2.50 (m, 2H), 3.20 (m, 2H), 3.12 (d, $J=16$, 1H), 3.80 (s, 2H), 3.38 (d, $J=16$, 1H), 5.25 (d, $J=6$, 1H), 6.00 (d, $J=6$, 1H), 7.00 (s, 1H), 7.20–7.40 (m, 25H), 7.42 (d, $J=6$, 1H), 8.40 (d, $J=6$, 1H), 8.50 (s, 1H).

(7*R*,6*R*)-3-[4-(2-Amino-ethylsulfanylmethyl)-pyridin-3-ylsulfanyl]-7-[2-(2-amino-5-chloro-thiazol-4-yl)-2-(*Z*)-hydroxyimino-acetylamino]-8-oxo-5-thia-1-aza-bicyclo[4.2.0]oct-2-ene-2-carboxylic acid bis-trifluoroacetic acid salt (18). To a suspension of protected cephem (342 mg, 0.31 mmol) precursor in dichloromethane (3.5 mL) was added triethylsilane (1.7 mL, 4.06 mmol) followed by addition of trifluoroacetic acid (4.5 mL). After stirring at room temperature for 1 h, reaction was cooled to 0 °C and isopropyl ether (30 mL) was added. Stirring was continued at 0 °C for 10 min and the resulting precipitate was filtered off and washed thoroughly with diisopropyl ether and dried in vacuum, yielding title deprotected cephem bis-trifluoroacetate (222 mg, 85%). ^{1}H NMR (D_2O) δ 2.90 (t, $J=6$, 2H), 3.31 (t, $J=6$, 2H), 3.43 (d, $J=17$, 1H), 3.85 (d, $J=17$, 1H), 4.18 (s, 2H), 5.43 (d, $J=4.5$, 1H), 5.97 (d, $J=4.5$, 1H), 8.10 (d, $J=6$, 1H), 8.66–8.68 (m, 2H).

Microbiology and pharmacology

Susceptibility testing. Compounds were evaluated for antimicrobial activity against a panel of bacterial strains using a broth microdilution assay performed using NCCLS reference methods.[12] The minimum inhibitory concentration (MIC) is defined as the lowest concentration of a compound that prevents the growth of the bacteria.

Effect of human serum on MIC; serum binding determination. The effect of human serum on antimicrobial activity against *S. aureus* ATCC 29213 was determined using a 1:1 mixture of human serum and growth medium (HS–MHB). The magnitude of change in MIC is expressed as a ratio of the MIC in serum-supplemented to unsupplemented media (Table 3 — 'MIC ratio HSMHB/MHB'). For selected compounds, the binding in pooled human serum was determined using ultrafiltration. Compounds were incubated in serum for 10 min at 37 °C in a shaking water bath. Serum ultrafiltrate was obtained by centrifugation of ultrafiltration units (Amicon Centrifree) for 20 min at 25 °C. Drug content in ultrafiltrate was quantified by HPLC using standards prepared in blank ultrafiltrate undergoing similar processing.

Mouse model of sepsis. Inoculum preparation: *Staphylococcus aureus* (strain Smith; ATCC 13709, penicillin-susceptible) was grown overnight at 37 °C in brain–heart infusion broth (BHIB). The following morning, it was subcultured to fresh BHIB and incubated for 4–5 h at 37 °C. The cells were harvested by centrifugation, washed twice with PBS, and adjusted to the desired inoculum. The cell suspension was mixed with an equal volume of sterile 14% hog-gastric mucin.[13] The inoculum was kept in an ice bath until used (<1 h).

Experimental infection. Male Swiss–Webster mice were challenged intraperitoneally with 0.5 mL of bacterial suspension (ca $10\times LD_{50}$). Test compounds were administered subcutaneously in 0.1 mL volumes immediately after inoculation and 2 h later. The total dose associated with 50% survival (ED_{50}) at 72 h was determined using the probit method.[14]

Pharmacokinetic studies in rats. Single dose pharmacokinetic (PK) studies were conducted in male Sprague–Dawley (CD) rats (250–300 g). Drugs were studied as single compounds (20 mg/kg) or in a cassette up to 4 compounds (1 mg/kg of each compound) with a control agent **RWJ-54428**. Doses were administered through a jugular venous catheter as a bolus injection (cassette) or over 20 min (single compounds). Blood (serum) samples were collected from tail vein or the venous catheter and assayed for drug content using HPLC. For selected compounds, PK parameters were determined in cassette experiments. Cassette dosing of a cocktail of up to 4 compounds (1 mg/kg of each compound) and a control of **RWJ-54428** was conducted in single catheterized (jugular vein) male CD rats. Serum samples were assayed using LC/MS/MS and the data were analyzed using WinNonlin program (Pharsight).

Serum and buffer stability. Rat serum was obtained from a euthanized rat immediately prior to the experiment. Solutions of β-lactam (50 μL, 1 mg/mL) were added to fresh rat serum (950 μL, preincubated at 37 °C for 10 min). Aliquots were removed at varying time points. The pH prior to and after the addition of compound was in the 7.5–8.0 range and at the end of the 1 h incubation was in the 8.5–9.0 range. Samples for HPLC were prepared by addition of 100 μL aliquots to trichloroacetic acid solution (200 μL, 4%), vortexing and centrifugation at 14,000 rpm in an Eppendorf microcentrifuge. A 25 μL sample of each supernatant was injected onto the HPLC. Human serum stabilities were determined in analogous fashion.

For buffer stability determinations, each compound was dissolved at a concentration of 500 μg/mL (or 250 μg/mL for less soluble compounds) in a 0.1 M MOPS buffer, pH 7.9. Solutions were placed in 37 °C bath. At 0, 1, 2, 4 and 6 h time an aliquot of solution was removed and diluted to concentration that fit into predetermined calibration curve. Quantification was done by reverse phase HPLC using a five-point curve.

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