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Discovery of RWJ-54428 (MC-02,479), a New Cephalosporin Active Against Resistant Gram-positive Bacteria

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The discovery of RWJ-54428 (MC-02,479), a new cephalosporin displaying promising activity against sensitive and resistant Gram-positive bacteria, is described. Progressive structural modification from the previously reported 3-phenylthiocephem MC-02,331 afforded an overall increase in potency against MRSA while retaining other key properties such as acceptable solubility and serum binding. Evaluation of the *in vitro* potency and *in vivo* efficacy of a series of closely related compounds resulted in selection of RWJ-54428 (MC-02,479) for further studies.

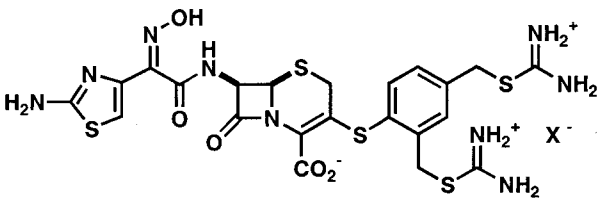
In response to the increasing incidence of infections due to multi-resistant Gram-positive pathogens in the nosocomial environment, a research program was initiated to find novel agents to combat methicillin-resistant staphylococci (MRS), vancomycin- and ampicillin-resistant enterococci, and penicillin-resistant pneumococci. The discovery of MC-02,331, a new cephalosporin antibiotic whose potent activity against MRS is attributable to its substantially higher affinity for PBP2a (the penicillin-binding protein responsible for methicillin resistance in staphylococci), has been previously described.[1] While MC-02,331 represented an important advance in the program, further improvement in potency against MRS was desired. This article describes a series of successive structural modifications, resulting in identification of RWJ-54428 (MC-02,479) as a compound worthy of further evaluation as a potential drug candidate.

Materials and Methods

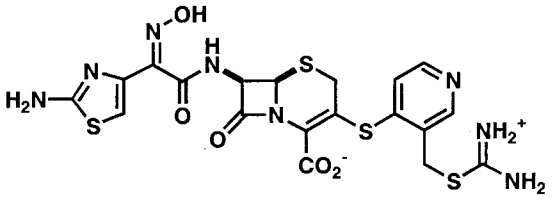
Synthesis

The methods described herein for preparation of RWJ-54428 (MC-02,479) are representative of those used to

Fig. 1. Replacement of (isothiouroniummethyl)phenyl by pyridyl.



MC-02,331



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synthesize the analogs reported.

(*Z*)-2-(2-Amino-5-chlorothiazol-4-yl)-2-(triphenylmethoxyimino)acetic Acid

To a solution of (*Z*)-2-(2-aminothiazol-4-yl)-2-(triphenylmethoxyimino)acetic acid (5.81 g, 13.47 mmol) in DMF (30 ml) at room temperature was added *N*-chlorosuccinimide (1.80 g, 13.47 mmol). After stirring overnight, the reaction mixture was poured into water (500 ml) and the resulting precipitate was filtered, washed with water and ethyl acetate, and dried under vacuum to afford 4.43 g (71%) of the title compound: ^{13}C NMR ($CDCl_3$) δ 108.5, 125.6, 126.2, 126.6, 127.3, 134.7, 141.8, 146.5, 162.1, 163.3.

(7*R*)-7-[(*Z*)-2-(2-Amino-5-chlorothiazol-4-yl)-2-(triphenylmethoxyimino)acetamido]-3-chloro-3-cephem-4-carboxylate Diphenylmethyl Ester

To a solution of 7-amino-3-chlorocephalosporanic acid diphenylmethyl ester toluenesulfonic acid salt (5.0 g, 8.72 mmol) in dry THF at room temperature (100 ml) was added pyridine (0.63 g, 10.0 mmol), followed by (*Z*)-2-(2-amino-5-chlorothiazol-4-yl)-2-(triphenylmethoxyimino)acetic acid (5.81 g, 13.47 mmol). The resulting slurry was cooled to −15°C and additional pyridine (1.42 g, 22.5 mmol) was added followed by dropwise addition of phosphorous oxychloride (1.64 g, 17.5 mmol), while maintaining reaction temperature below −10°C. After 30 minutes, ethyl acetate (200 ml) was added followed by water (150 ml). The aqueous layer was thoroughly extracted with ethyl acetate and the combined organic extracts were dried over sodium sulfate and concentrated under vacuum to yield the crude product, which was purified by flash column chromatography on silica gel (ethyl acetate/hexane 3 : 1) to afford the title compound (5.37 g, 65%): ^{1}H NMR ($CDCl_3/CD_3OD$) δ 3.35 (d, 1H, J=18), 3.68 (d, 1H, J=18), 5.07 (d, 1H, J=5), 5.80 (br s, 2H), 6.04 (dd, 1H, J=9, 5), 7.03 (s, 1H), 7.06 (d, 1H, J=9), 7.22~7.50 (m, 25H).

(7*R*)-7-[(*Z*)-2-(2-Amino-5-chlorothiazol-4-yl)-2-(triphenylmethoxyimino)acetamido]-3-mercapto-3-cephem-4-carboxylate Diphenylmethyl Ester

To a solution of (7*R*)-7-[(*Z*)-2-(2-amino-5-chlorothiazol-4-yl)-2-(triphenylmethoxyimino)acetamido]-3-chloro-3-cephem-4-carboxylate diphenylmethyl ester (4.0 g, 4.72 mmol) in DMF (30 ml) at −20°C was added in one portion powdered sodium hydrogen sulfide hydrate (1.10 g, 19.6 mmol). After 15 minutes the reaction mixture was poured into 0.5 M monosodium phosphate (about 100 ml). The mixture was extracted with ethyl acetate, and the organic layer was washed thoroughly with water. After concentrating under vacuum, the crude title product was obtained as a yellow foam 3.8 g (95%): ^{1}H NMR ($CDCl_3/CD_3OD$) δ 3.38 (d, 1H, J=15), 4.43 (d, 1H, J=15), 5.03 (d, 1H, J=5), 5.80 (d, 1H, J=5), 5.99 (br s, 1H), 6.80 (s, 1H), 7.05~7.50 (m, 25H).

3-Chloromethyl-4-chloropyridine Hydrochloride

Thionyl chloride (0.714 ml, 9.78 mmol) was added at room temperature to dry DMF (7 ml). After 30 minutes, the solution was cannulated into a solution of 3-hydroxymethyl-4-chloropyridine (700 mg, 4.89 mmol) in DMF (3 ml). After 45 minutes, the product was precipitated by addition of dry ether (100 ml), washed with ether, and dried under vacuum to yield 813 mg (84%) of the title compound: ^{1}H NMR (CD_3OD) δ 5.00 (s, 2H), 8.31 (d, 1H, J=5), 8.99 (d, 1H, J=5), 9.18 (s, 1H).

3-(*N-tert*-Butoxycarbonylaminoethylthiomethyl)-4-chloropyridine

To a solution of 3-chloromethyl-4-chloropyridine hydrochloride (513 mg, 2.59 mmol) in DMF (6 ml) at room temperature were added sodium iodide (386 mg, 2.59 mmol), diisopropylethylamine (1.12 ml, 6.47 mmol) and 2-(*N-tert*-butoxycarbonylamino)ethanethiol (458 mg, 2.59 mmol). After 2 hours, the reaction mixture was partitioned between dilute HCl and ethyl acetate. The organic layer was washed with water, dried over sodium sulfate, and concentrated to yield 750 mg of the oily product (96%), which was used in the next step without further purification. ^{1}H NMR ($CDCl_3$) δ 1.43 (s, 9H), 2.61 (m, 2H), 3.35 (m, 2H), 3.81 (s, 2H), 4.90 (br s, 1H), 7.35 (d, 1H, J=4), 8.40 (d, 1H, J=4), 8.57 (s, 1H).

(7*R*)-7-[(*Z*)-2-(2-Amino-5-chlorothiazol-4-yl)-2-(triphenylmethoxyimino)acetamido]-3-[3-(*N-tert*-butoxycarbonylaminoethylthiomethyl)pyrid-4-ylthio]-3-cephem-4-carboxylate Diphenylmethyl Ester

To a solution of (7*R*)-7-[(*Z*)-2-(2-amino-5-chlorothiazol-4-yl)-2-(triphenylmethoxyimino)acetamido]-3-mercapto-3-cephem-4-carboxylate diphenylmethyl ester (650 mg, 0.777 mmol) in DMF (3 ml) was added 3-(*N-tert*-butoxycarbonylaminoethylthiomethyl)-4-chloropyridine (242 mg, 0.80 mmol) at room temperature. After stirring overnight, the reaction mixture was partitioned between water and ethyl acetate. The organic layer was thoroughly washed with water, dried over sodium sulfate, and concentrated to yield the crude product which was purified by radial chromatography on silica gel (dichloromethane/methanol; v/v, 50/1) to afford 220 mg of the title compound

(26%). ^{1}H NMR ($CDCl_3/CD_3OD$) δ 1.23 (s, 9H), 2.32 (t, 2H, J=6), 2.98 (d, 1H, J=18), 3.06 (m, 2H), 3.40 (d, 1H, J=18), 3.46 (s, 2H), 5.03 (d, 1H, J=5), 5.52 (br s, 1H), 5.94 (d, 1H, J=5), 6.80 (s, 1H), 6.90 (d, 1H, J=6), 7.00~7.22 (m, 25H), 8.01 (d, 1H, J=6), 8.08 (s, 1H).

(7*R*)-7-[(*Z*)-2-(2-Amino-5-chlorothiazol-4-yl)-2-(hydroxyimino)acetamido]-3-[3-(aminoethylthiomethyl)pyrid-4-ylthio]-3-cephem-4-carboxylate, Methanesulfonic Acid Salt

To a cold solution (0°C) of (7*R*)-7-[(*Z*)-2-(2-amino-5-chlorothiazol-4-yl)-2-(triphenylmethoxyimino)acetamido]-3-[3-(*N-tert*-butoxycarbonylaminoethylthiomethyl)pyrid-4-ylthio]-3-cephem-4-carboxylate diphenylmethyl ester (1.00 g, 0.907 mmol) in dichloromethane (10 ml) and anisole (1.0 ml) was added trifluoroacetic acid (13 ml). After 1.5 hours, the reaction mixture was concentrated under vacuum at room temperature, and the oily residue was dissolved in 98% formic acid (20 ml). After 4 hours at room temperature, formic acid was removed under vacuum, and the residue was dissolved in water (25 ml). The insoluble material was removed by centrifugation. The supernatant was purified on an HP20 column by elution with water, then 0.1 M aqueous ammonium acetate, then finally eluting the product with 1 : 4 acetonitrile/water. The eluate was concentrated to about 1/10 original volume, and the resulting precipitate was filtered, washed with water and dried in vacuum to yield zwitterionic product (260 mg). The methanesulfonate salt was prepared by suspending the above material in water (15 ml) followed by addition of methanesulfonic acid (1.0 M in water, 0.98 eq) and acetonitrile (5 ml). After evaporation of the resulting solution to dryness, the residue was dissolved in water (30 ml) and centrifuged to remove insoluble material, and the supernatant was lyophilized to produce the title compound (274 mg, 44%). ^{1}H NMR (D_2O) δ 3.11 (s, 3H), 3.19 (m, 2H), 3.52 (m, 2H), 3.67 (d, 1H, J=17), 4.22 (d, 1H, J=17), 4.33 (s, 2H), 5.76 (d, 1H, J=4), 6.29 (d, 1H, J=4), 7.93 (d, 1H, J=4), 8.78 (d, 1H, J=4), 8.87 (s, 1H).

Antibiotic Susceptibility Testing

Antibiotics: Cephalosporins synthesized in this program were prepared at a concentration of 2 mg/ml of active material in a 1 : 1 solution of DMSO : sterile water. Compounds were dissolved immediately prior to the susceptibility test. Vancomycin (Sigma Chemical Co.) and imipenem/cilastatin (Primaxin®, Merck & Co., Inc.) were used as comparator agents. These antibiotics were prepared at a concentration of 10 mg/ml in sterile water. Stock solutions were aliquoted and kept frozen at −80°C. Each aliquot was rapidly thawed and was used only once.

Bacterial Strains: Three characterized strains of *Staphylococcus aureus* and 2 strains of enterococci were used to follow structure-activity relationships. *S. aureus* ATCC13709 (Smith strain) and two methicillin-resistant strains of *S. aureus* (MRSA) including strain COL, a β-lactamase negative homogeneously resistant strain,[2] and MRSA 76, a β-lactamase positive strain,[3] were part of the study. *E. faecalis* ATCC29212 and *E. faecium* ATCC35667 were also included in the primary panel of evaluation. Both strains are susceptible to ampicillin.

MIC Determination: Susceptibility tests were performed using a broth microdilution assay according to NCCLS reference methods[4] with minor modifications. Assays were performed in a final volume of 100 μl using Mueller-Hinton broth (Difco). The bacterial inocula were adjusted to yield a density of 5×10^5 CFU/ml. Antibiotics were prepared at a concentration equivalent to 2-fold the highest desired final concentration in culture medium. Antibiotics were then diluted directly in the 96-well microtiter plates by serial 2-fold dilution using a multichannel pipette. Microtiter plates were incubated during 24 hours at 35°C and were read using a microtiterplate reader (Molecular Devices) at 650 nm as well as by visual observation using a microtiterplate reading mirror. The MIC is defined as the lowest concentration of antibiotic at which the visible growth of the organism is completely inhibited.

Systemic Infection Model

Antibiotics: All antibiotics were prepared in sterile water for injection and were administered in 0.1 ml volumes subcutaneously at 0 and 2 hours post infection. Vancomycin (Sigma Chemical Co.) and imipenem/cilastatin (Primaxin®, Merck & Co.) were used as comparator agents.

Bacterial Strains: *Staphylococcus aureus* 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 hours at 37°C. The cells were harvested by centrifugation, washed twice with PBS, and adjusted to the desired O.D. at 600 nm (previously calibrated using CFU measurement). The cell suspension was mixed with an equal volume of sterile 14% hog-gastric mucin. Inoculum was kept in an ice bath until used (<1 hour).

Experimental Model of Peritonitis/Sepsis: A model of peritonitis/sepsis approved by the Institutional Animal Care and Use Committee was used.[5] Male Swiss-Webster mice (18~20 g) were challenged intraperitoneally with 0.5 ml of bacterial suspension. Animals were observed for 72 hours. The total dose resulting in survival of 50% of animals

(ED_{50}) was calculated by the probit method.[6]

Results and Discussion

The original design of MC-02,331 was based on the recognition that two positive charges would be required within the molecule in order for both protein binding and solubility to be within an acceptable range.[1] Further, these charges would have to reside within the C(3)-substituent in order to allow use of the widely accepted neutral aminothiazolyl(oximino)acetyl-type substituent at the 7-position. Earlier work by our group[1] and by others[7~9] had established the importance of a lipophilic group at the 3-position for high affinity to PBP2a. Based on a hypothesis that the existence of two charged groups at the 3-position of MC-02,331 was detrimental to its activity, a means was sought to accomplish the seemingly contradictory task of maintaining the solubilizing effect of the second charge while diminishing its impact on lipophilicity. One attractive approach to accomplishing this was to replace the 4-(isothiouroniummethyl)phenyl moiety by a 4-pyridyl group, which would be protonated at acidic pH to afford solubility for administration, but would be uncharged at physiological pH. This analog (**1**) was prepared, and its methanesulfonate salt demonstrated aqueous solubility of >20 mg/ml. However, its antibacterial potency was somewhat disappointing (Table 1).

Encouraged by the ability to form a soluble salt of compound **1**, a program of modification of the positively-charged moiety appended to the pyridylthio group at C(3) was initiated. Selected analogs are shown in Figure 2. The biological results on this series of compounds (Table 1) show clearly that an unsubstituted aminoethylthiomethyl

Table 1. *In vitro* and *in vivo* activity of pyridylthio analogs of MC-02,331.

(See Fig. 2 for structures)

Compound	MIC (μg/ml)					ED_{50} in mouse sepsis model, MSSA, mg/kg (95% C.I.)
	S. aureus Smith (MSSA)	*S. aureus* COL (Bla^-) (MRSA)	*S. aureus* 76 (Bla^+) (MRSA)	*E. faecalis* ATCC 29212 (Amp^S)	*E. faecium* ATCC 35667 (Amp^S)	
Imipenem	≤0.25	32	32	≤0.25	4	0.15 (0.06-0.25)
Vancomycin	0.5	1	0.5	1	0.25	2.1 (1.3-2.9)
MC-02,331	0.125	4	4	0.25	0.5	0.28 (0.11-0.35)
1	0.5	8	8	0.5	2	0.42 (0.21-0.63)
2	0.5	2	2	0.5	2	1.3 (0.78-1.8)
3	0.25	4	4	0.25	1	1.1 (0.73-1.5)
4	0.5	4	4	2	2	4.6 (0.63-9.9)
5	0.5	8	8	0.5	4	>2.5 (N.A.)
6	0.25	2	4	0.125	1	3.6 (1.4-8.6)
7	1	4	8	0.25	2	4.4 (1.6-7.3)
8	0.5	8	8	1	1	N.T.
9	0.5	2	4	1	1	>5 (N.A.)
10	1	8	8	0.5	4	N.T.
11	1	8	8	0.25	2	N.T.

Fig. 2. Variation of the appendage on the pyridyl substituent.

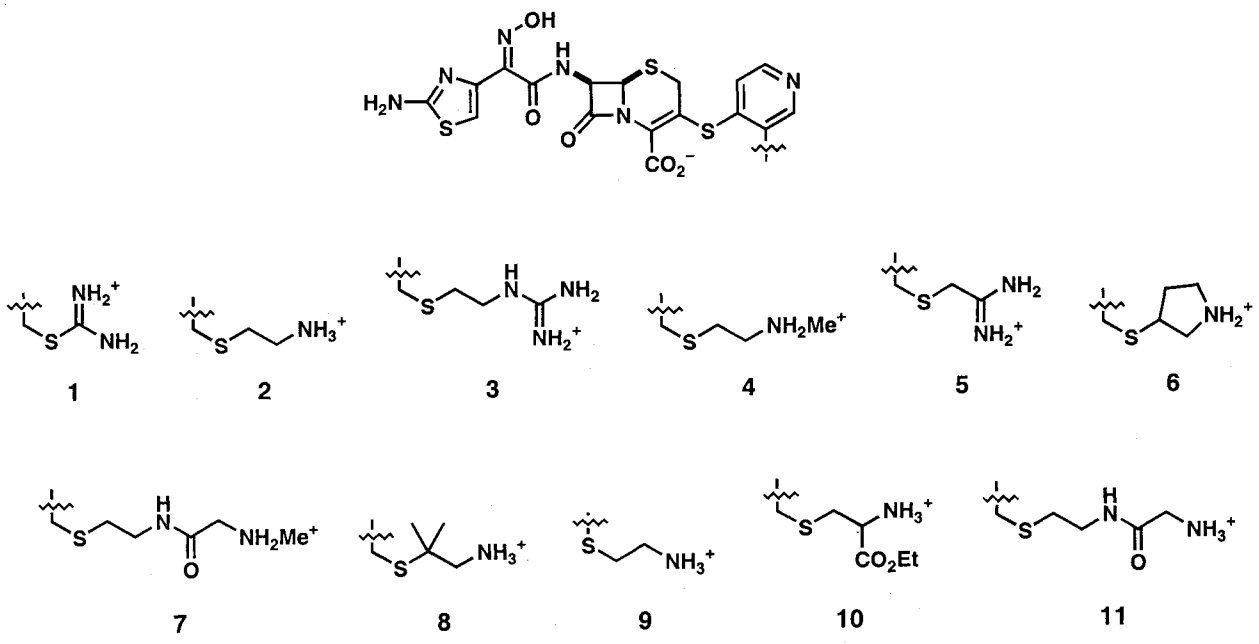
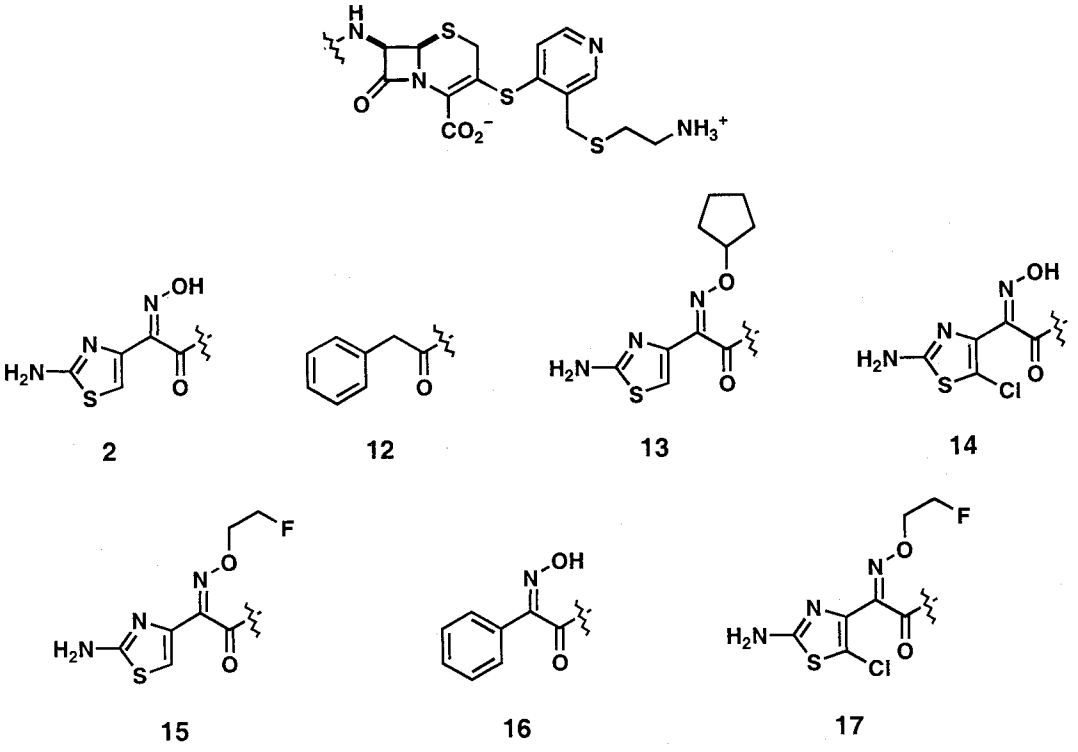


Table 2. *In vitro* and *in vivo* activity of 7-acyl analogs of compound **2**.

(See Fig. 3 for structures)

Compound	MIC (μg/ml)					ED_{50} in mouse sepsis model, MSSA, mg/kg (95% C.I.)
	S. aureus Smith (MSSA)	*S. aureus* COL (Bla^-) (MRSA)	*S. aureus* 76 (Bla^+) (MRSA)	*E. faecalis* ATCC 29212 (Amp^S)	*E. faecium* ATCC 35667 (Amp^S)	
Imipenem	≤0.25	32	32	≤0.25	4	0.15 (0.06-0.25)
Vancomycin	0.5	1	0.5	1	0.25	2.1 (1.3-2.9)
2	0.5	2	2	0.5	2	1.3 (0.78-1.8)
12	≤0.06	1	4	1	1	N.T.
13	2	4	8	1	4	N.T.
14	0.125	0.5	1	≤0.06	0.25	1.0 (0.7-1.6)
15	1	2	4	1	1	N.T.
16	≤0.06	0.5	1	≤0.06	0.25	1.1 (0.4-1.6)
17	0.125	1	1	0.125	0.5	1.2 (0.7-1.7)

Fig. 3. Variation of the 7-acyl substituent.



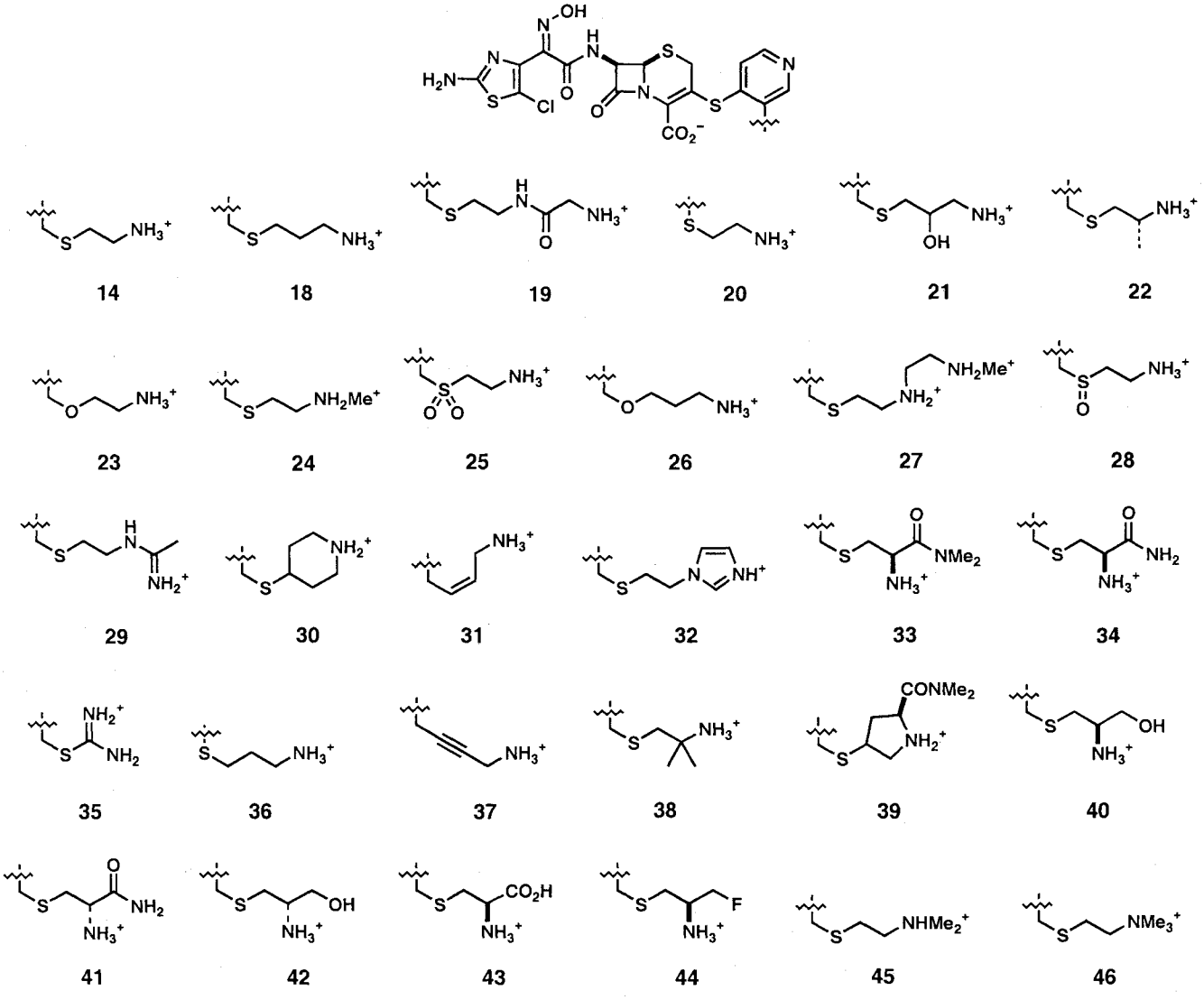
group (compound **2**) affords the optimum balance between *in vitro* potency against MRSA and enterococci, and *in vivo* efficacy in a murine septicemia model. In general, MICs against MRSA are more sensitive to structural changes than those either against MSSA or enterococci. Activity against MRSA was reduced by alkyl substitution on the amine or on the chain linking the heterocycle to the amine (compounds **4**, **6** and **8**), by incorporation of other polar groups (compounds **7**, **10** and **11**), and by replacing the amine with other groups affording positive charge (compounds **3** and **5**).

With the C(3)-substituent optimized, attention was next turned to modification at the 7-position. The poor beta-lactamase stability of 7-phenylacetyl analog **12** is shown (Table 2) by the 4-fold difference in MICs against *S. aureus* COL (beta-lactamase negative) and *S. aureus* 76 (beta-lactamase positive). Among the (oximino)acetyl analogs, good anti-MRSA and anti-enterococcal activity was observed with chloro-substituted analog **14**, its fluoroethyloxime analog **17**, and phenyl analog **16**. Due in large part to its synthetic accessibility, the chloroaminothiazolyl group found in compound **14** was selected as the 7-substituent of choice for further SAR work.

The substituents attached to the C(3)-pyridylthio group shown in Figure 4 explore a variety of spacer lengths, linking groups, substituent effects, and positively-charged groups. While a few compounds display potency equivalent to that of **14**, the majority are less potent either *in vitro* or *in vivo* (Table 3). The changes in activity in response to alkyl substitution and incorporation of polar groups are similar to those described above for the compounds in Table 1. As with compound **1**, the methanesulfonate salt of **14** demonstrated aqueous solubility of >20 mg/ml. As a result of these observations, larger quantites of **14** (MC-02,479, RWJ-54428) were prepared in order to permit further microbiological and pharmacological evaluation. The synthesis of RWJ-54428 (MC-02,479) is shown in Figure 5.

In summary, replacement of the 2,4-bis(isothiouroniummethyl)phenylthio moiety of MC-02,331 with a 3-(aminoethylthiomethyl)-4-pyridylthio moiety, and concomitant chlorine substitution on the aminothiazole ring at the 7-position, resulted in a significant improvement in *in*

Fig. 4. Optimization of the pyridyl substituent.



vitro potency against Gram-positive bacteria, while retaining the ability to form a soluble salt. Due to its high activity against the organisms of interest both *in vitro* and *in vivo*, RWJ-54428 (MC-02,479) was advanced to further microbiological and pharmacological characterization. Results of these studies will be forthcoming.

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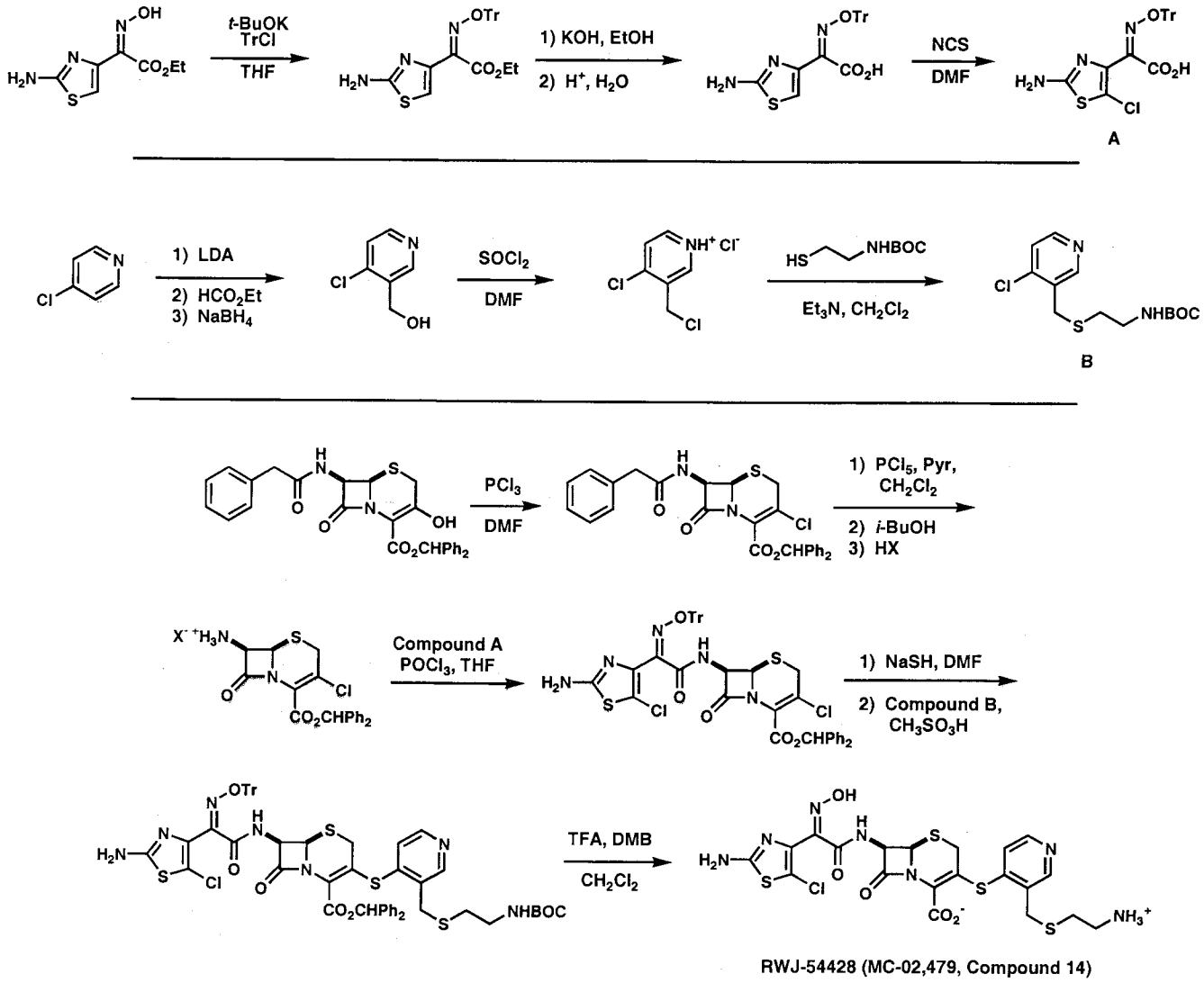
Table 3. *In vitro* and *in vivo* activity of substituted-pyridylthio analogs of compound **14**.

(See Fig. 4 for structures)

Compound	MIC (μg/ml)					ED_{50} in mouse sepsis model, MSSA, mg/kg (95% C.I.)
	S. aureus Smith (MSSA)	*S. aureus* COL (Bla^-) (MRSA)	*S. aureus* 76 (Bla^+) (MRSA)	*E. faecalis* ATCC 29212 (Amp^S)	*E. faecium* ATCC 35667 (Amp^S)	
Imipenem	≤0.25	32	32	≤0.25	4	0.15 (0.06-0.25)
Vancomycin	0.5	1	0.5	1	0.25	2.1 (1.3-2.9)
14	0.125	0.5	1	≤0.06	0.25	1.0 (0.7-1.6)
18	0.125	1	1	≤0.06	0.25	2.6 (1.6-3.6)
19	0.125	1	1	≤0.06	0.25	1.3 (0.6-2.0)
20	≤0.25	0.5	2	≤0.06	0.5	1.7 (0.9-2.5)
21	0.125	1	2	≤0.06	0.5	2.0 (1.1-2.9)
22	0.25	1	2	0.25	0.5	N.T.
23	0.25	2	2	0.125	1	1.4 (0.8-2.1)
24	0.125	1	1	≤0.06	0.25	>5 (N.A.)
25	2	4	4	0.125	2	N.T.
26	2	2	2	0.125	2	N.T.
27	0.5	4	4	0.125	1	1.1 (0.2-2.0)
28	0.25	4	4	0.125	1	N.T.
29	0.25	2	2	0.125	0.5	N.T.
30	0.5	2	2	≤0.06	≤0.06	N.T.
31	0.125	2	2	0.125	1	N.T.
32	0.125	4	4	0.5	2	>5 (N.A.)
33	0.5	4	4	0.5	1	4.1 (0.3-7.9)
34	0.125	2	2	0.5	1	>5 (N.A.)
35	0.125	2	1	0.5	1	2.0 (1.4-2.5)
36	0.5	2	4	0.125	0.125	N.T.
37	0.25	4	8	0.25	1	N.T.
38	0.5	4	4	0.125	1	N.T.
39	0.25	4	4	0.25	0.5	>5 (N.A.)
40	0.5	2	4	0.25	1	1.5 (0.9-2.2)
41	0.25	2	2	0.125	0.5	1.1 (0.6-1.6)
42	0.25	1	1	≤0.06	0.5	1.2 (0.6-1.8)
43	2	4	4	0.5	1	>5 (N.A.)
44	0.125	1	1	0.25	0.25	>5 (N.A.)
45	0.5	2	2	0.125	1	N.T.
46	0.5	2	2	0.125	1	1.7 (1.2-2.1)

Fig. 5. Synthesis of RWJ-54428 (MC-02,479, compound **14**).



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