The Journal of Applied Pharmacology, 14, 220-225(2006)

β-Lactamase Inhibitory Activities of New 6-tricyclic Substituted Exomethylene Penam Sulfones

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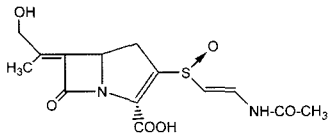
(Received October 11, 2006; Accepted November 29, 2006)

Abstract – Derivatives of penicillanic acid sulfones are known to be irreversible inhibitors of β-lactamase. Eight 6-tricyclic methylene penicillanic acid sulfones were prepared, and their β-lactamase inhibitory activities were evaluated against β-lactamase types I, II, III and IV. Among the tricycles attached to 6-exomethylenepenam sulfones, thiazolobenzimidazole (**12a-12b**), fluorene (**12c**), and carbazole (**12e**), showed inhibitory activity on type I, II and III β-lactamase. But phenanthrene (**12d**), and anthracene (**12f-12h**) derivatives showed little β-lactamase inhibitory activity. The synergic effects of the selected compound (12b) in 1:4 combination with piperacillin showed some protection to piperacillin for the resistant strains of *E. coli* DC2 and *P. aeruginosa* 1771.

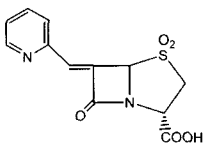
Keywords □ β-lactamase inhibitor, 6-exomethylenepenam, penam sulfones

INTRODUCTION

β-Lactamase inhibitors have little antibacterial activity, but these materials decrease the β-lactamase resistance and are used clinically to enhance the actions of those β-lactam antibiotics. Now clavulanic acid, sulbactam, and tazobactam are in market as β-lactamase inhibitors and several other compounds are under clinical study (Therrien and Levesque, 2000). They are potent, competitive and irreversible β-lactamase inhibitors and the mechanism of inhibition involved an irreversible acylation of serine hydroxyl group at the active site (Knowles, 1985). Recently 6-methylene penams and penems, their 1-oxides, 1,1-dioxides and esters have been reported as potent inhibitors of microbial β-lactamases (Venkatesan *et al* 2006; Weiss et al, 2004, Abe et al., 2004; Buynak et al, 1999; Bennett *et al.*, 1991). The first of these structures reported were naturally occuring asparenomycines (Tanaka *et al.*, 1981). Most of the compounds studied are hetercyclic mono- or bicycles such as pyridyl, furyl and thienyl, attached to 6-exomethylene like CP-65372 (Arisawa *et al,* 1982; Broom *et al*, 1990; Chen *et al*, 1986). However, little work has been done on tricyclic-substituted compounds. Therefore tricyclic-substituted methylene group attached to C-6 of the penam sulfone nucleus were synthesized and their β-lactamase inhibitory activities were examined in this study.



asparenomycines



CP-65372

MATERIALS AND METHODS

Materials

The ^{1}H NMR spectra were determined on a 400 MHz Varian FT-NMR spectrometer using tetramethylsilane as an internal standard. Samples were dissolved in $CDCl_3$ or DMSO-d_6. The chemical shifts are reported as parts per million. Most of the reagents were purchased from Sigma-Aldrich Chemical Company. β-Lactamase types I, II, III and IV were provided by Sigma company. Bacterial strains were from Hanmi Research Center's stock culture collection and were stored as suspensions in liquid nitrogen until use.

Synthesis of 6-tricyclic methylene penam sulfones

Synthesis of 6-tricyclic methylene penam sulfones is illus-

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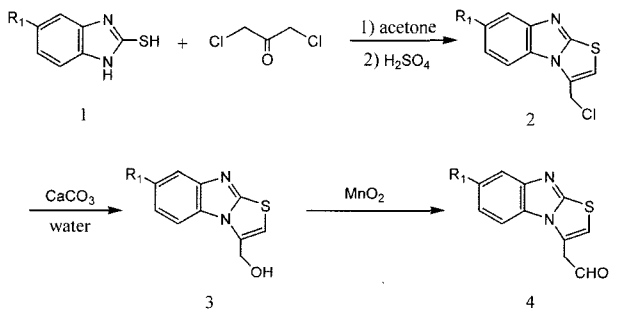
trated in Scheme 1 and 2. The procedure for the preparation of heterocyclic ring reported was modified to prepare 3-formyl thiazolo[3, 2, 4]benzimidazole (Oh *et al.*, 1995). The reaction of 2-mercaptobenzimidazole with 1,3-dichloroacetone gave 1-(benzimidazolyl-2-thio)-3-chloro-2-propanone hydrochloride and subsequent cyclization in sulfuric acid provided 3-(chloromethyl)-thiazolo[3, 2-a]benzimidazole **2**. Substitution of chloro group to hydroxy group in $CaCO_3$ / water and oxidation of resulting alcohol with MnO_2 gave 3-formyl thiazolo[3, 2, α] benzimidazole **4**. (Scheme 1)

The aldol condensation route reported by Chen *et al* was modified to prepare 6-exomethylene penam sulfones as shown in Scheme 2(Chen *et al.*, 1991). 6-Aminopenicillanic acid was reacted with bromine and $NaNO_2$ to give 6,6-dibromopenicillanic acid **6**. Oxidation of **6** with $KMnO_4$, followed by protection of carbonyl group with PMB (p-methoxybenzyl) or DPM (diphenylmethyl) gave 6,6-dibromopenam sulfone ester **8**. Treatment of 6,6-dibromopenam sulfone ester **8** with methylmagnesium bromide, followed by quenching with various tricyclic aldehydes afforded 6-(substituted)hydroxy methyl penam sulfone esters **9**. The hydroxymethyl compound **9** was acetylated with acetic anhydride in the presence of 1 *eq* of pyridine. Elimination of **10** was accomplished by acetic acid and zinc to give an olefine **11**. Most of compounds gave olefine sulfone **11** as a mixture of *Z* and *E* isomers, but thiazolo[3, 2, α] benzimidazole attached compounds (**11a, 11b**) gave the *Z* isomer, while carbazole attached compound (**11d**) gave the *E* isomer only. The yield for *E/Z* isomer was determined by NMR spectra. Final deprotection of **11** with *m*-cresol gave the corresponding free carboxylic acid compounds (**12a-12h**).

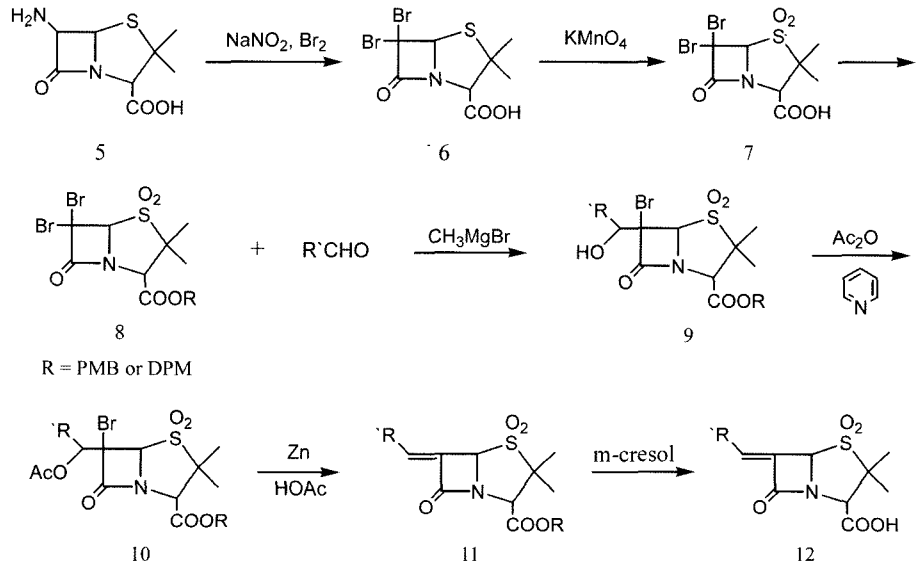


Scheme 1. Synthesis of 3-formylthiazolo[3, 2, a]benzimidazole

Assay for β-lactamase inhibition

The β-lactamase inhibitory activity of the prepared compounds was determined by microiodometry assay using β-lactamase types I, II, III and IV provided by Sigma company. The β-lactamases were incubated with various concentrations of synthesized compounds in 5% DMSO/pH 7.0 phosphate buffer and incubated 24°C for 20 minutes. To this partially inhibited enzyme the substrate nitrocefin was added and incubated 24°C for 1 h. The ultraviolet absorption at 292 nm was observed spectrophotometrically.

The standard solution (0.19nM) of nitrocefin was prepared in 1% DMSO. A standard 8 μg/ml solution of β-lactamase (type I, II, III and IV) in pH 7.0 phosphate buffer was prepared.



Scheme 2. Synthesis of 1,1-dioxo-6-(substituted) methylene penicillanic acids.

A standard 1M solution of each inhibitor in 5% DMSO in pH 7.0 buffer was prepared, then it was diluted 10 times. To determine the rate of enzyme hydrolysis of nitrocefin in the absence of inhibitor, 200 μL of nitrocefin standard solution and 20 μL of enzyme standard solution were incubated 24°C for 1h. To evaluate inhibitors, standard enzyme solution 20 μL and inhibitor solution 20 μL were mixed and incubated 24°C for 20 min. This partially inhibited enzyme was then added to a 200 μL of nitorcefin standard solution and incubated 24°C for 1h. The ultraviolet absorption at 292 nm was observed spectrophotometrically. Decay of the absorption was immediately observed for 2 min. and the % inhibition calculated by comparing the rate with the rate observed for the hydrolysis without any inhibitor.

Assay for synergy

The minimum inhibitory concentrations (MIC) of piperacillin in the 1:4 combination with the prepared compound **12b** was determined against a series of β-lactamase producing bacteria. MICs were determined by the serial 2 folds agar dilution Mueller method. The bacteria cultivated in Mueller and diluted to 10^7 cfu/mL were inoculated into the same medium containing piperacillin and compound **12b** in a specific concentration and incubated at 37°C for 16 h. The growth of the microorganisms was observed to determine MIC.

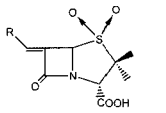
Bacterial strains were from Hanmi Research Center's stock culture collection and were stored as suspensions in liquid nitrogen until use.

RESULTS AND DISCUSSION

Structure of 1,1-dioxo-6-(substituted)methylene penicillanic esters synthesized and their nmr spectra are shown in Table I and II. Compounds in series **12a-12h** were tested against β-lactamase type I-IV provided by Sigma. The microiodometric assay result shown in Table III provides the comparison of β-lactamase inhibitory activity of sulbactam, tazobactam, prepared compounds and synthetic intermediates. The MICs of piperacillin in the presence of sulbactam, tazobactam and penam sulfone **12b** respectively were tested against various β-lactamase producing strains (Table IV).

All tricyclic substituted bromohydroxy **9a-h**, bromoacetoxy **10a-h**, and 6-exomethylene **12a-h** in our experiment were eval-

Table I. Structure of 1,1-dioxo-6-(substituted)methylene penicillanic esters



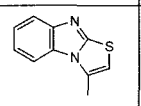
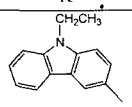
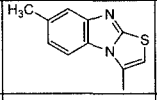
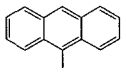
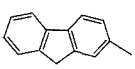
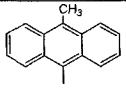
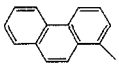
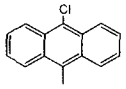
Compoun	R	E:Z	Compound	R	E:Z
12a		0:1	12e		1:0
12b		0:1	12f		2:7
12c		1:1	12g		3:7
12d		3:1	12h		7:3

Table II. NMR spectra of 6-tricyclicsubstituted-exomethylene penam sulfones

compounds	δ (ppm)
11a	7.8(m, 3H), 7.4(t, 1H), 7.3(m, 4H), 6.9(d, 2H), 5.2(m, 2H), 5.1(d, 1H), 4.5(s, 1H), 3.8(s, 3H), 1.5(s, 3H), 1.2(s, 3H)
11b	7.77(s, 0.4H), 7.76(s, 0.4H), 7.70(d, 0.6H), 7.65(m, 0.6H), 7.6(s, 0.6H), 7.58(s, 0.4H), 7.33(m, 2H), 7.29(s, 1H), 7.24(d, 0.4H), 7.13(d, 0.4H), 6.9(m, 2H), 5.28(m, 2H), 5.1(d, 1H), 4.5(m, 1H), 3.8(m, 3H), 2.5(m, 3H), 1.6(s, 3H), 1.35(s, 3H)
11c	7.85(m, 2H), 7.7(s, 1H), 7.5(dd, 2H), 7.45(s, 1H), 7.3~7.35(m, 4H), 6.9(d, 2H), 5.45(s, 1H), 5.3(d, 1H), 5.1(d, 1H), 4.5(s, 1H), 395(s, 2H), 3.8(s, 3H), 1.5(s, 3H), 1.3(s, 3H)
11d	8.6(dd, 2H), 8.1(s, 1H), 7.95(m, 3H), 7.6(m, 4H), 7.25(m, 2H), 6.8(m, 2H), 5.2(m, 2.75H), 5.0(s, 0.25H), 4.48(s, 1H), 3.75(s, 3H), 1.6(s, 3H), 1.2(s, 3H)
11e	8.27(s, 1H), 1.17(d, 1H), 7.6(m, 2H), 7.5(m, 1H), 7.2~7.3(m, 13H), 7.0(s, 1H), 5.4(s, 1H), 4.5(s, 1H), 4.38(q, 2H), 1.6(s, 3H), 1.45(t, 3H), 1.2(s, 3H)
11f	8.5(s, 1H), 8.3(s, 0.7H), 8.15(d, 1H), 8.0(d, 2H), 7.8(s, 0.2H), 7.5~7.6(m, 4H), 7.35 (m, 10H), 7.0(s, 1H), 5.45(s, 0.2H), 4.8(s, 0.7H), 4.6(s, 1H), 1.4(s, 3H), 0.9(s, 3H)
11g	8.35(m, 2H), 8.15(m, 2H), 7.6(m, 4H), 7.35(m, 11H), 7.0(d, 1H), 5.45(s, 0.3H), 4.85(s, 0.7H), 4.6(s, 1H), 3.15(s, 3H), 1.5(s, 3H), 0.9(s, 3H)
11h	8.65(d, 2H), 8.3(s, 0.3H), 8.21(d, 1.4H), 8.19(d, 0.6H), 7.7(s, 0.7H), 7.6(m, 4H), 7.35(m, 10H), 7.02(s, 0.3H), 6.97(s, 0.7H), 5.4(s, 0.7H), 4.78(s, 0.3H), 4.6(s, 0.3H), 4.5(s, 0.7H), 1.6(s, 2.1H), 1.4(s, 0.9H), 1.25(s, 2.1H), 0.9(s, 0.9H)

Table III. β-Lactamase inhibitory activity IC_{50} (μM/ml)

Compound \ Enzyme type	Type I	Type II	Type III	Type IV
sulbactam	N	4.29	8.57	N
tazobactam	N	0.36	1.08	0.11
clavulanic acid	N	5.03	N	N
9a*	N	N	N	N
9b	N	N	N	N
9c	N	N	0.01	0.001
9d	N	1.89	1.32	0.06
9e	N	N	0.56	0.02
10c	N	N	0.05	0.001
10d	N	N	N	N
10e	N	1.78	N	N
12a	N	N	N	0.23
12b	N	0.23	N	2.26
12c	N	0.71	1.19	0.71
12d	N	N	N	N
12e	N	2.29	N	N
12f	N	N	N	0.72
12g	N	N	N	N
12h	N	N	N	N

Penicillinase Type I, II: *Bacillus cereus*, Type III: *Enterobacter cloacae* (Chromatographically purified), TypeIV: *Enterobacter cloacae* (Lyophilized), N; no inhibition
*Compounds **9a-e**, **10c-e** were tested after hydrolysis of PMB or DPM group by *m*-cresol.

uated in their enzyme inhibitory activity after deprotection in *m*-cresol. For bromohydroxy **9a-h**, and bromoacetoxy **10a-h** compounds, fluorenyl substitution (**9c and 10C**) showed a high inhibition of enzyme type III and IV, the enzyme derived from *Enterobacter cloacae*. Phenanthrene substituted bromohydroxymethyl penam sulfone **9d** showed good activity for emzyme type I, II, and III, but no activity was observed for 6-phenanthrene substituted bromoacetoxy penam sulfone **10d**.

Among the tricycles attached to 6-exomethylenepenam sulfones, thiazolobenzimidazole (**12a-12b**), fluorene (**12c**), and carbazole (**12e**), showed moderate β-lactamase inhibitory activity, but phenanthrene (**12d**), and anthracene (**12f-12h**) derivatives showed little β-lactamase inhibitory activity. In general, the tricycles with heteroatom which are electron withdrawing because of electronegativity of heteroatom showed good activity. However, phenathrene and anthracene which do not have heteroatom, showed poor activity. Fluorene can accept electrons because negatively charged fluorene becomes aromatic, and showed good activity.

This result is correlated well with the report by Chen and coworkers. They reported a π-deficient 2-heteroaryl methylene group at the C-6 position possessed potent β-lactamase inhibitory activity, while π-excessive 2-heteroaryl analogs showed

Table IV. Antibacterial activity (MIC) of piperacillin in the presence of sulbactam, tazobactam and compound **12b,** respectively.

	Strains	Minimal Inhibitory Concentration(μg/ml)			
		Piperacillin	Piperacillin:sulbactam = 1 : 4	Piperacillin :tazobactam = 1 : 4	Piperacillin: 12b = 1: 4
1	*Streptococcus pyogenes* 308A	0.049	N.D	N.D	0.013
2	*Streptococcus pyogenes* 77A	0.049	N.D	N.D	0.013
3	*Streptococcus faecium* MC 8b	3.125	N.D	N.D	3.125
4	*Staphylococcus aureus* SG 511	0.781	0.391	0.391	0.391
5	*Staphylococcus aureus* 285	1.563	0.781	0.781	1.563
6	*Staphylococcus aureus* 503	1.563	0.781	0.391	1.563
7	*Escherichia coli* 055	0.781	0.781	0.781	0.781
8	*Escherichia coli* DC O	0.781	0.781	0.781	0.781
9	*Escherichia coli* DC 2	0.098	0.098	0.098	0.049
10	*Escherichia coli* TEM	>100	6.250	1.563	100
11	*Escherichia coli* 1507E	3.125	1.563	1.563	3.125
12	*Pseudomonas aeruginosa* 9027	3.125	3.125	3.125	3.125
13	*Pseudomonas aeruginosa* 1592E	3.125	3.125	3.125	3.125
14	*Pseudomonas aeruginosa* 1771	1.563	1.563	1.563	0.781
15	*Pseudomonas aeruginosa* 1771M	0.195	0.195	0.195	0.195
16	*Salmonella typhimurium*	0.391	0.391	0.391	0.391
17	*Klebsiella oxytoca* 1082E	>100	>100	12.500	>100
18	*Klebsiella aerogenes* 1522E	1.563	0.781	0.781	1.563
19	*Enterobacter cloacae* P99	>100	12.500	6.250	>100
20	*Enterobacter cloacae* 1321E	0.781	0.781	0.391	0.391

N.D : not determined.

weak activity (Chen *et al.*, 1987).

Generally penicillanic acid sulfones are known to be the mechanism based irreversible inhibitors of β-lactamase (Knowles, 1985). They form the acyl-enzyme intermediate and stabilization of this intermediate, restricted rotation necessary for the hydrolysis of the intermediate, or linear vinologous enamine formation by elimination of SO_2 would lead to inhibition of the enzyme. A conjugated acyl-enzyme ester formation was proposed for the mechanism of 6- (heterocyclyl)-methylene penam sulfones (Arisawa *et al.*, 1982), where the intermediate was stable and the acyl-enzyme ester was expected to resistant to hydrolysis to regain active enzyme.

The synergy study shown in Table III, compound **12b** was able to afford protection to piperacillin for the resistant strains of *E. coli* DC2 and *P. aeruginosa* 1771. However, the synergy effect was much less potent than tazobactam against *Escherichia coli* TEM, *Klebsiella oxytoca* 1082E, and *Enterobacter cloacae* P99. None of the examined compounds showed protection effect against *Pseudomonas aeruginosa* 9027 and *Pseudomonas aeruginosa* 1592E.

In conclusion, some 6-(tricycle substituted methylene) penam sulfones and related compounds showed β-lactamase inhibitory activity. Among the synthesized compounds, the 6-bromo-6-(2-fluorenyl)hydroxymethyl penam sulfone **9c** and 6-bromo-6-(2-fluorenyl)acetoxymethyl penam sulfone **10C** have been proven to be a highly effective inhibitor of enzyme type III and IV, the enzyme derived from *Enterobacter cloacae*. Also 6-(2-fluorenyl) exomethylenepenam sulfones **12C** showed inhibitory activity for Type II β-lactamase. Therefore it could be conceived that further modification of fluorenyl group could increase the activity.

ACKNOWLEDGEMENTS

This Research was supported by grant from the Korea Science and Engineering Foundation.

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