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Synthesis of Some Thieno Gamma Lactam Monocarboxylic Acids with High Antibacterial Activity: A New Look at an Old Molecular System

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Abstract—Synthesis and antibacterial activity of some novel monocyclic thienyl gamma lactams are reported. The compounds have been synthesized by a two-step process consisting of, first, intermolecular Michael addition, followed by intramolecular amidification between suitable arylamino malonate and 3-(2′-thienyl) acryloyl chloride and then hydrolysis cum in situ decarboxylation of the diacid. The compounds showed moderate to high antibacterial activity against gram positive and gram negative bacteria.

Introduction

Due to a growing number of bacterial strains acquiring resistance to the current arsenal of chemotherapeutic drugs, the search for novel mechanism-based antibacterial agents continues to be a major area of research on many fronts. The effort to produce a molecule containing the beta lactam motif alone has produced a plethora of novel chiral entities, many of which are presently marketed as antibiotic agents. The pronounced biological activity exhibited by beta lactam antibiotics is found to be associated with the beta lactam ring which in turn was believed to be due to the inherent strain of this four-membered cyclic ring which reduces amide resonance (between 'N' p-electrons and C=O group). However, the successful effectiveness of β-lactam antibiotics depends on the ability of bacteria to produce β-lactamase enzymes, which hydrolyse the β-lactam moiety in these antibiotics, whereby the drug is rendered inactive. These provoked further the design and synthesis of novel gamma lactam antibacterial derivatives[1–3] as well as isolating naturally occurring gamma lactam containing antibiotics.[4–8] These findings clearly challenged the long held notion that presence of a beta lactam ring is a prerequisite for antibacterial activity. It is now believed that the bioactivity of the lactam compounds depends on their ability to acylate several proteins to inhibit cross linking of the bacterial cell wall[9] which again is dependent on a suitably substituted and activated lactam ring.[10] During the last decade various groups have directed their efforts to synthesize non beta lactam mimics of several beta lactam antibiotics[11,12] and this has enriched the gamma lactam chemistry with development of many gamma lactam compounds with and without significant bioactivities.[1–3,13–30] In order to prepare novel gamma lactam analogues as a biological surrogate of beta lactam ring, recently we have described the synthesis of some novel monocyclic 1-aryl-4-(aryl/2′-furyl)-pyrrolidin-2-one-5-carboxylic acid derivatives.[31–33] The 2-furyl derivatives were found to exhibit gram positive and gram negative antibacterial activities at higher concentration.[32] Here we report the synthesis of some hitherto unknown thieno gamma lactam carboxylic acid derivatives **4(a–d)** with improved gram positive and gram negative bacterial growth inhibitory property (Scheme 1).

The rationale to design such type of molecules is based on the structural resemblance to well known antibiotics with lactam ring, a carboxylic acid functionality and the

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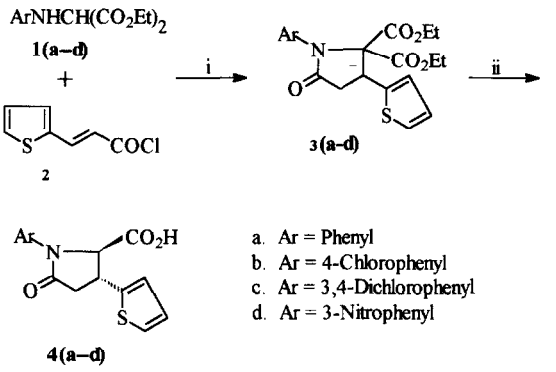
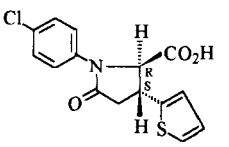


Scheme 1. Reagents and conditions: (i) Et_3N, benzene, reflux, 6 h; (ii) KOH (2.2 equiv), EtOH–H_2O, reflux, 3 h.

presence of 'S' in thiophene with bicyclic frameworks and *trans* ring juncture. Accordingly,we present here a versatile synthetic route that permits easy preparation of novel thiopheno γ-lactams. Thus, arylamino malonate (**1**) on reaction with 3-(2′-thienyl) acryloyl chloride in the presence of triethylamine produced the gamma lactam diester derivative **3** in high yield. Reaction control is accomplished via sequential intermolecular Michael addition, followed by intramolecular amidification.[34] Compound **3** when subjected to saponification with about two equivalent of KOH underwent hydrolysis cum in situ decarboxylation to furnish exclusively the *trans* isomer of the thieno gamma lactam monocarboxylic acid **4** in 83–99% yield. The *trans* geometry of C-4 and C-5 substitution was assigned from the coupling constant value of C_4-H and C_5-H (~2.9–3.1 Hz) as well as by analogy.[31,32] All the compounds were characterized by usual spectroscopic data as well as by analysis (see Experimental). The thieno gamma lactams **3(a–d)** and **4(a–d)** synthesized by us were then taken for the detection of antibacterial properties against the growth of *Escherichia coli* and *Staphylococcus aureus* (ampicillin resistant) and *S. aureus* with lactamase producing strains. Interestingly the compound **4** shows moderate to high inhibition of the bacterial propagation against both the strains.

In a recent report[32] we disclosed that a phenyl ring at 4-position of *N*-aryl pyrrolidin-2-one-5-carboxylic acid system makes the gamma lactam inactive while replacement of the phenyl group by 2-furyl moiety made it moderately active at high concentration. We also observed that bacterial growth inhibitory property of the compound was dependent on the nature of *N*-aryl group and the maximum activity was observed with the substitution of *N*-*p*-chlorophenyl, C_4-furyl and C_5-CO_2H (the presence of which was a must to show the bacterial growth inhibition) functionality in the 2-pyrrolidinone system. In our current study we noticed that the racemic gamma lactam **4** [*N*-aryl-4-(2′-thienyl)-pyrrolidin-2-one-5-carboxylic acid] showed enhanced bacterial growth inhibitory property compared to its 2-furyl analogues. Once again, the presence of C_5-CO_2H as in compound **4** was essential for its bioactivity as evident from the fact that diester derivatives **3(a–d)** were completely inactive against both the strain under identical conditions. In the variation of the *N*-aryl substitution, we noticed that replacement of *N*-phenyl group of **4a** by an *N*-*p*-chlorophenyl group (**4b**) increased the activity significantly (about 1.5 times). We thought that it may be due to the presence of the halogen atom in the phenyl ring of **4b**. However, introduction of a second chlorine atom (as in **4c**) on *N*-aryl group caused a slight decrease in activity as compared to that of **4b**. Also, in general all the acids **4(a–d)** were found to be more active in inhibiting the bacterial growth against *E. coli* than *S. aureus* strains though in the latter strain the compounds were also active. The comparative results are listed in Table 1.

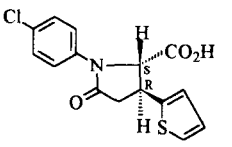
Insights from our previous experimental results have implied that the racemic acids **4** were quite active in both the strains. Now, in order to check selective activity (if any) of a particular enantiomer, we resolved the racemic acid **4b** into its enantiomers (−) 1-(4-chlorophenyl)-4-(2′-thienyl)-pyrrolidin-2-one-5-carboxylic acid and the corresponding (+) isomer.



(−) **4b** (4S,5R)

[α] = − 3.87 (at 33°C)

α = − 0.0104 at a conc. of 0.02683 mg/10 ml



(+) **4b** (4R,5S)

[α] = + 3.21 (at 34°C)

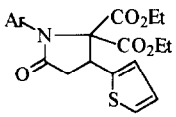
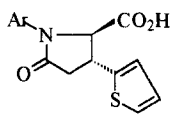
α = + 0.013 at a conc. of 0.04207 mg/10 ml

Specific rotation of the compounds were measured in ethyl acetate solution with a JASCO digital polarimeter (Model DIP-370) using Na-lamp (589 nm) as standard light source.

The absolute stereochemistry of (−) **4b** was determined by X-ray crystallographic study[35] and was found to have 4*S* and 5*R* configuration for the asymmetric center C_4 and C_5, respectively, while the stereochemistry of (+) **4b** was assigned by analogy.

It was found that both the (+) and (−) isomer of **4b** were potent to inhibit bacterial growth. However (+) **4b** (optical purity ~83%) was slightly less active than (−) **4b** against both the strains. MIC value for the

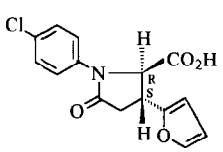
Table 1.

Compd	Ar	Inhibition	
		E. coli	*S. aureus* (ampicillin res.)
 3(a–d)	**3a**. phenyl	Nil	Nil
	3b. 4-chlorophenyl	Nil	Nil
	3c. 3,4-dichlorophenyl	Nil	Nil
	3d. 3-nitrophenyl	Nil	Nil
 4(a–d)	**4a**. phenyl	35–40%	25–30%
	4b. 4-chlorophenyl	62–68%	45–48%
	4c. 3,4-dichlorophenyl	59–65%	42–45%
	4d. 3-nitrophenyl	57–64%	37–42%

Concentration of gamma lactam derivatives used = 1.8 mg/0.02 mL of THF.
% refers to zone of inhibition with respect to ampicillin.

compound (+) **4b** and (−) **4b** was found to be 0.0285 mg/0.2 mL and 0.0280 mg/0.2 mL, respectively.

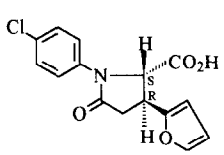
Identical results were also observed for the 2-furyl analogue[32] **5** where also the (−) isomer was slightly more active than the (+) isomer. The 2-furyl analogue **5** was resolved with (−) quinine in a similar way as used to resolve (±) **4b**. The stereochemistry was assigned in analogy with **4b**.



(−) **5** (4S,5R)

[α] = − 37.43 (at 28°C)

α = − 0.427 at a conc. of 0.1142 mg/10 ml



(+) **5** (4R,5S)

[α] = + 34.40 (at 30°C)

α = + 0.229 at a conc. of 0.0666 mg/10 ml

It is clear from these studies that the presence of thiophene moiety at C-4 position of the 2-pyrrolidinone ring system contributes to enhancing the bacterial growth inhibition property of gamma lactams.

Experimental

All melting points are uncorrected and were checked in a one-side open-glass capillary using concd H_2SO_4 bath. Unless otherwise mentioned ^1H NMR spectra were recorded on a 200 MHz (Brucker) machine while ^{13}C NMR spectra were recorded on 50 MHz (Brucker) spectrometer. IR spectra were recorded on a Perkin–Elmer 800 spectrometer. Mass spectral data were obtained from CDRI Lucknow, India, and RSIC, IIT Madras, India. Elemental analyses were obtained from CDRI Lucknow.

General procedure for 1-aryl-4-(2-thienyl)-5,5-dicarbethoxypyrrolidin-2-one 3(a–d)

To a stirred soln of arylaminomalonate **1** (19.0 mmol) and triethylamine (46.0 mmol) in dry benzene (40 mL), the acid chloride **2** (21.0 mmol) in 30 mL dry benzene was added dropwise. The reaction mixture was warmed up and a white precipitate separated out. It was then refluxed on a water bath for 6 h, protecting it from moisture. After cooling to rt, the reaction mixture was successively washed with 5% ice cold HCl soln, 5% ice cold sodium bicarbonate soln and finally with water several times. The organic layer was collected, dried (Na_2SO_4) and solvent removed to furnish a highly viscous brownish yellow oil which solidified on standing. It was further purified by column chromatography and/or recrystallization from suitable solvent.

3a. Colourless sol (purified by column chromatography over silica gel using petroleum ether (60–80 °C) + ethylacetate (7:3) mixture as eluent), mp 92–93 °C (isopropyl alcohol), yield 68.8%. IR (KBr) ν_{max} 1720 cm^{-1} (br). ^1H NMR ($CDCl_3$) (90 MHz) δ: 0.90 (t, 3H, J~7.5 Hz), 0.95 (t, 3H, J~7.5 Hz), 3.05 (d, 2H, J~10.5 Hz), 3.75–4.25 (m, 4H), 4.85 (t, 1H, J~10.5 Hz), 6.95–7.15 (m, 2H), 7.20–7.50 (m, 6H) ppm. ^{13}C NMR ($CDCl_3$) δ: 13.31, 13.45, 35.91, 41.13, 62.26, 125.34, 126.53, 126.77, 128.26, 128.41, 128.91, 136.80, 138.62, 166.34, 173.53 ppm. MS (*m/z*): 387 (M^+), 314 (B^+) (M−CO_2Et), 249, 241, 212, 199, 176, 167, 137, 122, 110, 109, 104, 97, 96, 93, 77. Anal. calcd for $C_{20}H_{21}NO_5S$: C, 62.02; H, 5.43; N, 3.62; found: C, 61.84; H, 5.21; N, 3.49%.

3b. Colourless sol [purified by column chromatography over silica gel using petroleum ether (60–80 °C) + ethyl acetate, 7:3), mp 114–115 °C (isopropyl alcohol), yield 73.7%. IR (KBr) ν_{max} 1730 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 0.93 (t, 3H, *J*~7.2 Hz), 1.00 (t, 3H, *J*~7.2 Hz), 3.04 (d, 2H, *J*~10.0 Hz), 3.68–4.20 (m, 4H), 4.82 (t, 1H, *J*~10.0 Hz), 6.98–7.02 (m, 2H), 7.19 (d, 2H, *J*~8.9 Hz), 7.25–7.27 (m, 1H), 7.35 (d, 1H, *J*~8.9 Hz) ppm. MS (*m/z*): 423 (M+2), 421 (M^+), 350, 348 (M−CO_2Et), 278, 277, 276, 175 (M−2×CO_2Et), 250, 215, 213, 144, 116. Anal. calcd for $C_{20}H_{20}NO_5SCl$: C, 56.94; H, 4.74; N, 3.32; found: C, 56.73; H, 4.61; N, 3.16%.

3c. Colourless sol, mp 124–125 °C (isopropyl alcohol), yield 64.8%. IR (KBr) ν_{max} 1720, 1756 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 0.93 (t, 3H, *J*~7.1 Hz), 1.06 (t, 3H, *J*~7.1 Hz), 3.00 (d, 2H, *J*~9.6 Hz), 3.65–4.22 (m, 4H), 4.77 (t, 1H, *J*~9.6 Hz), 6.94–7.01 (m, 2H), 7.11 (dd, 1H, *J*~2.1 Hz), 7.22 (d, 1H, *J*~1.2 Hz), 7.34 (d, 1H, *J*~2.1 Hz), 7.44 (d, 1H, *J*~8.5 Hz) ppm. ^{13}C NMR ($CDCl_3$) δ: 13.54, 13.65, 36.01, 41.14, 62.43, 62.56, 78.63, 125.47, 126.74, 126.86, 128.20, 130.38, 130.75, 132.49, 132.64, 136.34, 138.58, 166.13, 166.73, 173.15 ppm. Anal. calcd for $C_{20}H_{19}NO_5SCl_2$: C, 52.63; H, 4.17; N, 3.07; found: C, 52.55; H, 4.05; N, 2.99%.

3d. Colourless sol, mp 154–156 °C (isopropyl alcohol), yield 75.0%. IR (KBr) ν_{max} 612, 1331, 1558, 1722, 1740 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 0.93 (t, 3H, *J*~7.2 Hz), 1.01 (t, 3H, *J*~7.1 Hz), 3.04 (d, 2H, *J*~9.5 Hz), 3.70–4.29 (m, 4H), 4.79 (t, 1H, *J*~9.5 Hz), 6.93–7.25 (m, 4H), 7.49–7.65 (m, 2H), 8.09–8.18 (m, 2H) ppm. Anal. calcd for $C_{20}H_{20}N_2O_7S$: C, 55.56; H, 4.63; N, 6.48; found: C, 55.32; H, 4.44; N, 6.27%.

General procedure for 1-aryl-4-(2-thienyl)-pyrrolidin-2-one-5-carboxylic acid 4(a–d)

To a soln of the gamma lactam diester derivative **3** (8.0 mmol) in 50 mL ethanol, a soln of KOH (17.0 mmol) in 20–25 mL water was added and refluxed for 4 h. Excess of ethanol was removed by distillation and the residue was diluted further with water (20–25 mL). It was then cooled in ice and acidified with ice cold concd HCl. The precipitated sol was filtered, redissolved in $NaHCO_3$ soln, and extracted with ethylacetate to remove neutral matter (if any). The aqueous layer was then cooled in ice-bath and carefully acidified with concd HCl (ice cold). The white sol separated was filtered, washed well with ice water and finally dried. An analytical sample was prepared by further recrystallization of the acid from ethyl acetate benzene or benzene–chloroform mixture.

4a. Colourless sol, mp 194–195 °C, yield 83.0%. IR (KBr) ν_{max} 1652, 1732 cm^{-1}. ^1H NMR ($CDCl_3$) of the methyl ester derivative prepared by diazomethane, δ: 2.73 (dd, 1H, *J*~3.9 Hz and 17.0 Hz), 3.19 (dd, 1H, *J*~8.6 Hz and 17.0 Hz), 3.77 (s, 3H), 3.87–3.95 (m, 1H), 4.69 (d, 1H, *J*~3.0 Hz), 6.93–6.98 (m, 2H), 7.16–7.22 (m, 2H), 7.31–7.46 (m, 4H) ppm. MS (*m/z*): 287 (M^+), 286 (M−1), 242, 214, 212, 200, 167, 158, 149, 138, 125, 111, 107, 100. Anal. calcd for $C_{15}H_{13}NO_3S$: C, 62.72; H, 4.53; N, 4.88; found: C, 62.60; H, 4.41; N, 4.73%.

4b. Colourless sol, mp 216–217 °C (benzene–ethylacetate), yield 99.0%. IR (KBr) ν_{max} 1654, 1742, 3466 cm^{-1}. ^1H NMR ($CDCl_3$) of the methyl ester derivative prepared by action of diazomethane, δ: 2.72 (dd, 1H, *J*~4.0 Hz and 17.0 Hz), 3.18 (dd, 1H, *J*~8.7 Hz and 17.0 Hz), 3.77 (s, 3H), 3.87–3.95 (m, 1H), 4.65 (d, 1H, *J*~3.1 Hz), 6.95 (d, 2H, *J*~3.5 Hz), 7.20–7.22 (m, 1H), 7.31 (dd, 1H, *J*~2.4 and 6.7 Hz), 7.40 (dd, 1H, *J*~2.4 and 6.7 Hz) ppm. ^{13}C NMR ($CDCl_3$) of the methyl ester derivatives δ: 37.48, 39.36, 52.94, 69.47, 123.44, 124.48, 124.62, 127.14, 129.22, 131.39, 136.32, 144.36, 170.74, 172.40 ppm. MS (*m/z*): 323 (M+2), 321 (M+), 278, 276 (M−CO_2H), 236, 234, 212, 199, 140, 138, 123, 112, 111, 110, 109, 97. Anal. calcd for $C_{15}H_{12}NO_3ClS$: C, 55.99; H, 3.73; N, 4.35; found: C, 55.88; H, 3.62; N, 4.24%.

4c. Colourless sol, mp 163–164 °C (benzene–chloroform), yield 84.0%. IR (KBr) ν_{max} 1658, 1738 cm^{-1}. ^1H NMR ($CDCl_3$) of the methyl ester derivative prepared by action of diazomethane, δ: 2.73 (dd, 1H, *J*~3.8 Hz and 17.1 Hz), 3.18 (dd, 1H, *J*~8.6 Hz and 17.1 Hz), 3.81 (s, 3H), 3.88–3.96 (m, 1H), 4.65 (d, 1H, *J*~2.9 Hz), 6.96 (d, 2H, *J*~3.5 Hz), 7.19–7.23 (m, 1H), 7.32 (dd, 1H, *J*~2.4 and 8.8 Hz), 7.37 (d, 1H, *J*~8.8 Hz), 7.64 (d, 1H, *J*~2.4 Hz) ppm. Anal. calcd for $C_{15}H_{11}NO_3SCl_2$: C, 50.56; H, 3.10; N, 3.93; found: C, 50.45; H, 2.93; N, 3.81%.

4d. Colourless sol, mp 179–181 °C (ethylacetate–benzene), yield 90.2%. IR (KBr) ν_{max} 616, 1331, 1648, 1740 cm^{-1}. ^1H NMR (DMSO-d_6 + $CDCl_3$) δ: 2.65 (dd, 1H, *J*~3.4 Hz and 17.1 Hz), 3.15 (dd, 1H, *J*~8.6 Hz and 17.1 Hz), 3.97–4.01 (m, 1H), 4.69 (brs, 1H), 6.86–6.93 (m, 2H), 7.16 (d, 1H, *J*~4.9 Hz), 7.52 (d, 1H, *J*~8.2 Hz), 7.77–7.82 (m, 1H), 7.93 (dd, 1H, *J*~1.6 and 8.2 Hz), 8.41 (d, 1H, *J*~1.6 Hz) ppm. ^1H NMR ($CDCl_3$) of the methyl ester derivative prepared by action of diazomethane, δ: 2.79 (dd, 1H, *J*~3.9 Hz and 17.2 Hz), 3.23 (dd, 1H, *J*~8.6 Hz and 17.2 Hz), 3.82 (s, 3H), 3.96–4.00 (m, 1H), 4.77 (d, 1H, *J*~3.1 Hz), 6.95–6.98 (m, 2H), 7.21–7.25 (m, 1H), 7.55 (t, 1H, *J*~8.2 Hz), 8.01–8.07 (m, 2H) 8.20 (t, 1H, *J*~2.1 Hz) ppm. MS (*m/z*): 332 (M^+), 315 (M−OH), 302 (M−NO), 290, 288, 287, 271, 259, 257, 247, 245, 241, 240, 228, 213, 212, 203, 198, 197, 195, 194, 171, 168, 166, 154, 148, 138, 137, 123, 121, 111, 110, 109, 103, 97. Anal. calcd for $C_{15}H_{12}N_2O_5S$: C, 54.22; H, 3.61; N, 8.43; found: C, 53.97; H, 3.39; N, 8.21%.

Resolution of the racemic acid 4b into its enantiomers (+) 4b and (−) 4b

600 mg (1.87 mmol) of the racemic acid **4b**, 590 mg (2.00 mmol) of (−) cinchonine in ethylacetate (8–10 mL) were warmed together on a water bath to dissolve. It was then filtered to remove insoluble matter (if any). The filtrate on removal of solvent afforded the salt which was repeatedly crystallized from dichloromethane–ether mixture until a solid with constant melting point (190–192 °C) was obtained. (Every time the mother liquor was reserved.) The sol thus obtained was redissolved in methanol and the free acid was regenerated by stirring with 1 N HCl. The precipitated acid was filtered, washed well with water and dried to furnish the (−) **4b** as a colourless sol (mp 216–217 °C, $[\alpha]\sim-3.87$ at 33 °C, $\alpha\sim-0.0104$ at a concn of 0.02683 mg/10 mL of $CHCl_3$).

The (+) isomer of **4b** was recovered from the mother liquor. Then the filtrate after separation of the (−) cinchonine salt of (−) **4b** on removal of solvent afforded the sol which was repeatedly crystallized as before using dichloromethane–ether mixture to produce the constant melting (mp 181–182 °C) salt of (−) cinchonine and (+) **4b**. This was decomposed with 1 N HCl, in methanol and after usual work up it furnished (+) **4b** as a colourless sol, mp 215–216 °C, $[\alpha]\sim+3.21$ at 34 °C ($\alpha\sim0.013$ at a concn of 0.04207 mg/10 mL of $CHCl_3$).

General procedure for bioassay of gamma lactams

Escherichia coli C 600 K-12 sensitive to ampicillin was collected from NCL, Pune, India, in addition to that the strain was mutated to ampicillin resistant, concn up to 1000–2000 mg/mL. *Staphylococcus aureus* with lactamase producing activity was also used in this investigation. Mutations were followed according to the method (measuring of antibiotic activity by Oxford cylinder plate method) described by Sale[36] with some modification. The strains were grown on standard media containing nutrient broth purchased from HIMEDIA, India.

Nutrient (15 gm/L) and agar–agar (15 gm/L) were applied when sol agar Petri dishes were used. In these cases bacteria were grown at least 12 h previously in an incubator at 35–36 °C with constant agitation. Then 5:20 ratios of 5 mL of bacterial culture 10^9 cells/mL of total cells were mixed with 20 mL of molten agar at about 50 °C and poured quickly into sterile and clean Petri dishes. After solidification, dishes were immediately preserved in a refrigerator at 5–7 °C to stop all further bacterial propagations.

Solidified Petri dishes with bacterial cells 10^{6-7}/mL were grooved with one cork borer at absolute sterility. Alcohol burning was applied to sterile needles, forceps and glasswares against local contamination. However, a major sterilization was mostly carried out in an autoclave. After making four individual grooves on each four inch diametric dishes (plates) they were placed carefully under a luminar hood. Dissolved gamma lactam (1.8 mg/0.02 mL) in acetonitrile or THF was added carefully, with all precautions, into each groove. After all solutions were diffused from the grooves the plates with gamma lactam and bacteria were incubated overnight/48 h at 35–36 °C, with respect to ampicillin. Clean plaques were observed to show the positive results.

Quantitative measurement of the bacterial growth inhibition by 4b against *E. coli* and *S. aureus*

Six, properly steriled and labeled (as 1, 2, ..., 6) test tubes (5 mL capacity) were taken, charged with 1 mL of nutrient broth and were plugged with cotton properly. To test tubes 1–3, 20 μL of a solution of **4b** (1.955 mg , 20 μL) in THF was added and plugged with cotton immediately. To test tubes numbered 4–6, 8 μL of an aq soln of ampicillin (2.0 mg/8 μL) was added taking every precaution during addition. To each of the test tubes 1 and 4, 10 μL of the bacterial culture of *E. coli* (containing $\approx 10^6$ cells/1000 μL) were added and plugged with cotton properly. Similarly to each of the test tubes 2 and 5, 10 μL of bacterial culture of *E. coli.* (ampicillin res.) (containing $\approx 10^7$ cells/1000 μL) and to test tubes 3 and 6, 10 μL of *S. aureus* culture (containing $\approx 10^6$ cells/1000 μL) was added, respectively, taking every sterility precaution during transfer. After plugging properly with cotton the test tubes were placed in an incubator (34–35 °C) overnight.

From each of these test tubes (1–6) 10 μL of the mixture was taken out and applied separately to six sterile agar Petri dishes. After incubation overnight at 34–35 °C the number of colonies developed on each of these Petri dishes was counted. It was found that no colony developed for the solutions as used from test tubes 2, 4 or 5, whereas 3, 4 and 13 colonies developed respectively for the solutions as used from the test tubes 1, 3 and 6. The results are tabulated in Table 2.

Determination of MIC values[37,38]

For MIC values we have applied the following procedure in which overnight grown bacterial counts 10^6 to 10^7 cells/mL were diluted up to 1000 cells/0.2 mL and were distributed on Petri dishes. The dishes were loaded, prior to application of viable bacteria of 1000 cells/0.2 mL, with different concentration of products (−) and (+) **4b** whereas concentration variables of 6.0, 2.5, 1.0 and 0.5 mg/100 μL in different plates (4×4). In all cases viable bacterial cells of 1000 cells/0.2 mL of *E. coli* were

Table 2.

Bacteria	Viability rate with	
	4b	Ampicillin
E. coli	~3%	0%
E. coli (ampicillin res.)	0%	0%
S. aureus	~4%	~13%

Concentration of **4b** and 1.9555 mg/10 μL of THF.
Concentration of ampicillin used 2.0 mg/8 μL of H_2O.

separated. After overnight incubation at 37 °C, cells were counted and their viability counts against the product concn were graphically extrapolated. From the extrapolation, it was observed that the viability of cells in thousand and the total deaths coincide in one point which showed the product concn of 5.3 and 5.35 mg, respectively. MIC value of the product could be estimated as 5.325 as mean value used for both (−) and (+) product **4b** which is to be required for direct inoculum with bacterial cells to kill 10% of cell growth as they are already being estimated in plaque formation. In Oxford plaque studies we used 1.8 mg/0.2 mL in 5 mL diameter. Similarly (5.3 mg/0.2 mL) concn in a groove was spread over 80 times larger surface area in the Petri dishes for (−) **4b** compound. So, it may be concluded that the bacterial minimum inhibition count must be considered 0.028 mg/0.2 mL in this case.

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