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N-Thiolated β-lactams: A new family of anti-*Bacillus* agents

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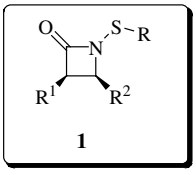
Abstract—This report describes the evaluation of *N*-thiolated β-lactam antibiotics as potential anti-*Bacillus* agents. *N*-Thiolated β-lactams are a new family of antibacterials that previously have been found to selectively inhibit the growth of *Staphylococcus* bacteria over many other genera of microbes. From the data presented herein, these lactams similarly inhibit a variety of *Bacillus* species, including *Bacillus anthracis*. The preliminary structure–activity studies suggest that there is a need to balance the lipophilic character of the C_3/C_4 groups in order to obtain optimal anti-*Bacillus* activity. Elongation or extensive branching of the organothio substitutent diminishes antibacterial effects, with the *sec*-butylthio derivative providing the strongest activity.

Bacillus anthracis is a rod-shaped Gram-positive bacterium that is the causative agent of anthrax infections.[1,2] If inhaled, spores of *B. anthracis* rapidly migrate to lymphonodes of the lungs, where they begin to germinate and release toxins that cripple the immune response, causing bacteremia, toxemia, and frequently, death.[3] Concerns about the possible use of *B. anthracis* as a biological weapon have led to widespread efforts to prevent or treat anthrax infections with vaccine or antibacterial drug development, and to detect the microbe.[4,5]

Our laboratory has recently identified a new family of anti-MRSA agents, *N*-thiolated β-lactams **1**, which have a mode of action distinct from that of all other β-lactam antibiotics.[6] Rather than interfering directly with cell wall biosynthesis through irreversible acylation of penicillin binding transpeptidases, these compounds seem to affect cellular processes through transfer of the *N*-organothio group to a bacterial thiol. We also note that these lactams exert anti-proliferative properties against only a narrow range of bacterial genera, most significantly, *Staphylococcus* (including MRSA), *Micrococcus*, and *Neisseria*. This selectivity seems to be related to the levels and types of cellular thiols present in each microbe that is sensitive to the lactams, not to whether the microbes are Gram-positive or Gram-negative classes. Given that *Staphylococcus* and *Bacillus* are both prominent members of the *Bacillales* taxonomic order of bacteria, we therefore turned to investigate whether these compounds could possess antibacterial properties against *Bacillus* spp.



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The lead compound in this study was *N*-methylthio-substituted lactam **1a**, which in previous studies was found to have one of the most potent antigrowth activities against *Staphylococcus* bacteria.[7] Prior studies on the overall structure–activity features of additional analogues of **1a** determined that substituents at the C_3 and C_4 centers of the lactam ring exerted rather subtle effects on anti-MRSA activity,[8] while relative and absolute stereochemistry at these locations was largely inconsequential. This led to the suggestion that the mode of action of the lactams requires passage of the lactam molecule through the bacterial membrane prior to interaction with a cytoplasmic thiol.

Keywords: *N*-Thiolated β-lactams; Anthrax; *Bacillus*; SAR; Antibiotics.
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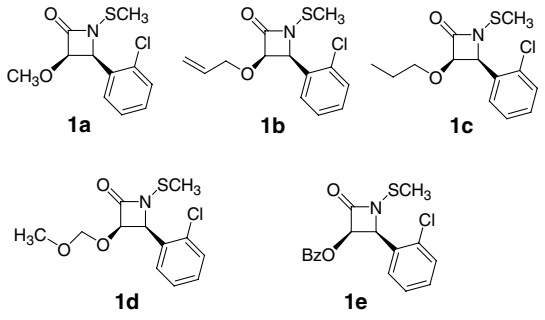
1. Microbiological testing of β-lactam analogues

In this study, we investigated the anti-*Bacillus* properties of a select number of differentially substituted β-lactams based on structure **1**. These analogues were prepared in racemic form according to our previous reports.[7–10] The β-lactams were individually tested for antibacterial activity against *B. anthracis* and six other species of *Bacillus* by the Kirby–Bauer method of well diffusion on agar plates. Previously, we have demonstrated that the growth inhibition zone sizes for *N*-methylthio β-lactams against *Staphylococcus* correlate well with their minimum inhibitory concentrations (MICs) obtained from broth dilution experiments, and thus represent a reliable way to gauge bioactivity within a closely related series of analogues.[7–10]

2. C_3-substituted lactams

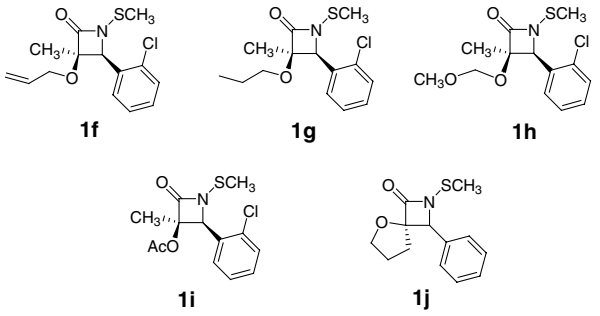
The first series of compounds examined in our study consists of C_3-oxygenated lactams **1b–e**. The selection of these five compounds was made based on their different structural features to evaluate the effects of lipophilicity and polarity on anti-*Bacillus* activity.



The growth inhibition zones observed for these compounds against the seven *Bacillus* microbes are given in Table 1. Lactams **1b** and **1c** showed enhanced activities compared to C_3-methoxy lactam **1a**, while the methoxymethyl ether and benzoyl ester derivatives **1d** and **1e** were consistently weaker. These results are somewhat different to the trend we observed for MRSA, where compound **1a** was the most active, suggesting that *Bacillus* may be more sensitive to lipophilicity within the C_3 alkoxy side chain.

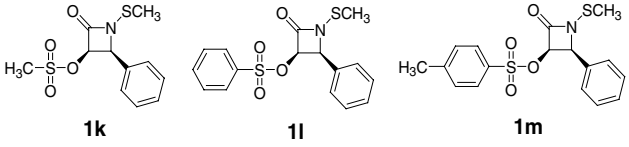
To evaluate the effects of steric crowding at the C_3 center, 3,3-disubstituted lactams **1f–i** were studied next.

The zone data in Table 2 for these sterically more crowded compounds indicate that the addition of lipophilicity at C_3 generally increases bioactivity, compared to the corresponding 3-monosubstituted lactam. Replacement of the C_3-alkoxy group for an acetoxy does not seem to alter bioactivity, as shown for lactam **1i**, whereas incorporation of the alkoxy- and alkyl residues into a spirocyclic ring (lactam **1j**)[11] significantly diminishes activity.



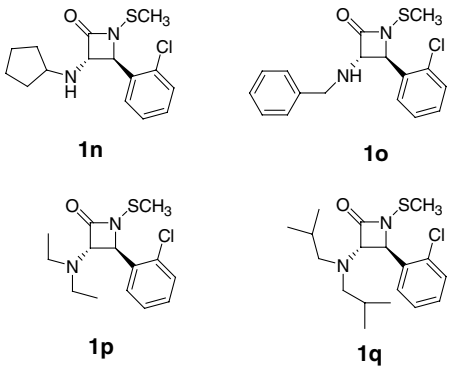
2.1. C_3-sulfonated lactams

Extending upon this survey of C_3-alkoxy- and acyloxy-substituted compounds, we tested three sulfonate-bearing derivatives **1k–m** (Table 3). Here, we found sizable differences in bioactivity which is dependent on the size (and lipophilicity) of the sulfonyl side chain. Whereas the methanesulfonyl compound **1k** is only weakly active against six of the seven *Bacillus* microbes, and totally inactive against *B. megaterium*, the phenylsulfonyl and toluenesulfonyl variants **1l** and **1m** are appreciably more active against *B. anthracis*.



2.2. C_3-amino β-lactams

The trends observed thus far for lactams **1a–m** strongly suggest that polar side chains at C_3 have a detrimental influence on anti-*anthracis* activity. This pattern is also observed for C_3-amino-substituted analogues **1n–q**, of which only *N*-benzylamino compound **1o** possesses any bioactivity. This parallels what we found previously for MRSA (Table 4).



2.3. C_3-halogenated β-lactams

Our previous investigations have found that replacement of the C_3 methoxy substituent of lactam **1a** for a chloro group (**1r**) slightly increases antibacterial activity against MRSA, while replacement of methoxy for

Table 1. Bioactivities of C_3-alkoxy- or acyloxy-substituted lactams **1a–E** against *Bacillus* bacteria determined by the Kirby–Bauer method of well diffusion on agar plates

Compound	*B. anthracis*	*B. globigii*	*B. thuringensis*	*B. megaterium*	*B. coagulans*	*B. subtilis*	*B. cereus*
1a	25	18	19	16	20	18	21
1b	27	20	20	22	21	19	23
1c	27	24	19	20	19	18	22
1d	20	15	15	15	15	13	19
1e	20	18	11	12	18	13	19

Twenty micrograms of test compound in DMSO solution was used in each case. The values indicate the average diameters in mm (of three trials) for the zone of growth inhibition obtained for each compound after 24 h of incubation at 37 °C, with a margin of error of ±1 mm.

Table 2. Bioactivities of C_3-disubstituted lactams **1f–j** against *Bacillus* bacteria determined by the Kirby–Bauer method of well diffusion on agar plates

Compound	*B. anthracis*	*B. globigii*	*B. thuringensis*	*B. megaterium*	*B. coagulans*	*B subtilis*	*B. cereus*
1f	29	25	20	22	20	21	22
1g	30	nd	nd	nd	nd	nd	nd
1h	20	15	16	16	15	13	18
1i	29	20	nd	21	20	19	15
1j	14	10	9	9	13	0	17

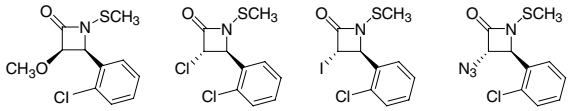
Twenty micrograms of test compound in DMSO solution was used in each case. The values indicate the average diameters in mm (of three trials) for the zone of growth inhibition obtained for each compound after 24 h of incubation at 37 °C, with a margin of error of ±1 mm (nd, not determined).

Table 3. Bioactivities of C_3-sulfonated lactams **1k–m** against *Bacillus* bacteria determined by the Kirby–Bauer method of well diffusion on agar plates

Compound	*B. anthracis*	*B. globigii*	*B. thuringensis*	*B. megaterium*	*B. coagulans*	*B. subtilis*	*B. cereus*
1k	10	8	10	0	10	10	14
1l	18	15	13	15	nd	nd	nd
1m	19	12	11	11	13	12	13

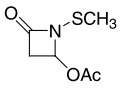
Twenty micrograms of test compound in DMSO solution was used in each case. The values indicate the average diameters in mm (of three trials) for the zone of growth inhibition obtained for each compound after 24 h of incubation at 37 °C, with a margin of error of ±1 mm (nd, not determined).

an iodo (**1s**) or azido (**1t**) group decreases activity. This is also observed for all seven of the *Bacillus* species (Table 5).[12]



1a **1r** **1s** **1t**

Finally, we note that the C_3 unsubstituted lactam **1u**, a compound with only weak anti-MRSA properties, has no in vitro activity against any of the *Bacillus* species tested. This may be due to a need for lipophilic groups at both the C_3 and C_4 centers, a feature that we now want to address.



1u

3. C_4-substituted lactams

The role of the C_4 aryl group on anti-*Bacillus* activity of these *N*-methylthio lactams was studied by varying the *ortho*-chlorophenyl group for other substituents. First, we varied the halogen, and then its location on the aryl ring. For the *ortho*-substituted series of lactams **2a–e**, there is very little if any difference in bioactivity versus that of the chlorophenyl lead compound **1a**, indicating

Table 4. Bioactivities of C_3-amino-substituted lactams **1n–q** against *Bacillus* bacteria determined by the Kirby–Bauer method of well diffusion on agar plates

Compound	*B. anthracis*	*B. globigii*	*B. thuringensis*	*B. megaterium*	*B. coagulans*	*B. subtilis*	*B. cereus*
1n	0	0	0	0	0	0	0
1o	10	9	9	7	0	0	11
1p	0	0	0	0	0	0	0
1q	0	0	0	0	0	0	0

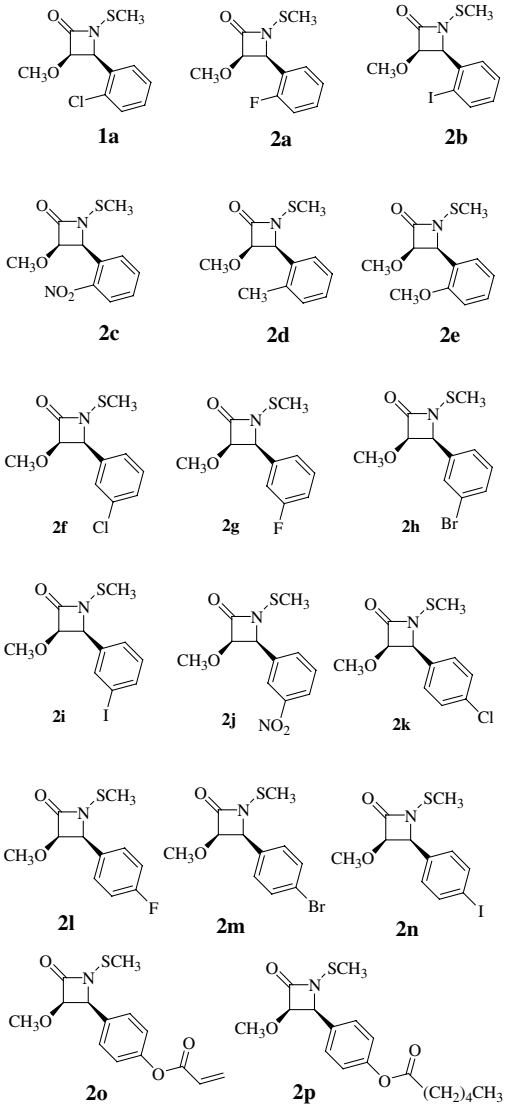
Twenty micrograms of test compound in DMSO solution was used in each case. The values indicate the average diameters in mm (of three trials) for the zone of growth inhibition obtained for each compound after 24 h of incubation at 37 °C, with a margin of error of ±1 mm.

Table 5. Bioactivities of C_3-halogen-substituted lactams **1r–t** against *Bacillus* bacteria determined by the Kirby–Bauer method of well diffusion on agar plates

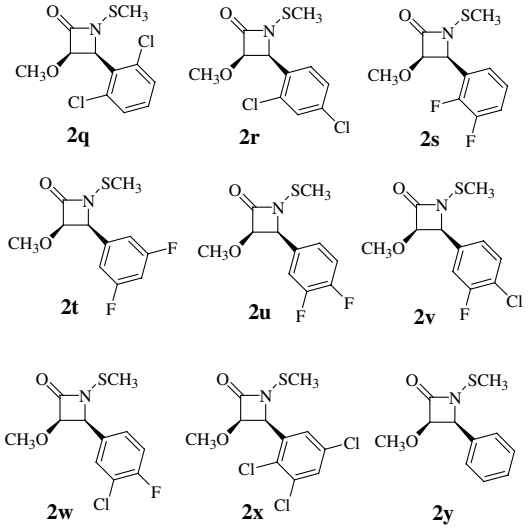
Compound	*B. anthracis*	*B. globigii*	*B. thuringensis*	*B. megaterium*	*B. coagulans*	*B. subtilis*	*B. cereus*
1a	25	18	19	16	20	18	21
1r	22	22	18	20	15	18	21
1s	22	24	15	13	11	13	17
1t	20	14	14	11	15	14	17

Twenty micrograms of test compound in DMSO solution was used in each case. The values indicate the average diameters in mm (of three trials) for the zone of growth inhibition obtained for each compound after 24 h of incubation at 37 °C, with a margin of error of ±1 mm.

that effects of electronegativity or lipophilicity of the substituent at this location of the ring are insignificant (Table 6).



Next, we varied the location of the aryl substituent and found that the *meta*-substituted lactams (**2f–j**) and *para*-substituted lactams (**2k–n**) possessed nearly identical bioactivities against the seven *Bacillus* species as that displayed by the initial *ortho*-substituted derivatives (Tables 7 and 8). On the other hand, the *meta*-nitrophenyl and *para*-acyloxyphenyl lactams (**2j**, **2o**, and **2p**, respectively) were the only ones having significantly different, and sporadic, activities among the seven *Bacillus* species. This overall invariance in activities of the halogen-substituted lactams was also observed for lactams (**2q–x**) bearing multiple halogen substituents at different positions of the aryl ring, as well as for the unsubstituted phenyl analogue **2y** (Table 9).



Finally, we looked at analogues **3a–c** which have the aryl moiety linked to the β-lactam ring through either an alkenyl, alkynyl, or a more flexible alkyl tether. Once again, each of these derivatives shows about the same anti-*Bacillus* bioactivity as any of the other C_4-arylated compounds (Table 10).

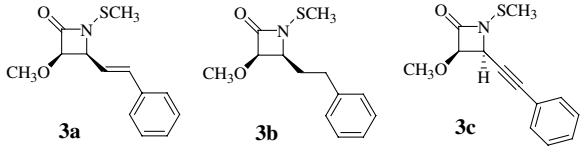


Table 6. Bioactivities of *ortho*-aryl-substituted lactams **1a** versus **2a–e** against *Bacillus* bacteria determined by the Kirby–Bauer method of well diffusion on agar plates

Compound	*B. anthracis*	*B. globigii*	*B. thuringensis*	*B. megaterium*	*B. coagulans*	*B. subtilis*	*B. cereus*
1a	25	18	19	16	20	18	21
2a	25	18	19	17	15	12	20
2b	25	17	17	15	13	14	19
2c	22	16	17	14	17	17	18
2d	21	14	15	12	15	10	18
2e	nd	14	13	10	13	0	nd

Twenty micrograms of test compound in DMSO solution was used in each case. The values indicate the average diameters in mm (of three trials) for the zone of growth inhibition obtained for each compound after 24 h of incubation at 37 °C, with a margin of error of ±1 mm (nd, not determined).

Table 7. Bioactivities of *meta*-aryl-substituted lactams **2f–j** against *Bacillus* bacteria determined by the Kirby–Bauer method of well diffusion on agar plates

Compound	*B. anthracis*	*B. globigii*	*B. thuringensis*	*B. megaterium*	*B. coagulans*	*B. subtilis*	*B. cereus*
2f	25	18	19	16	20	15	20
2g	25	19	18	17	18	12	20
2h	27	20	19	16	20	16	21
2i	22	19	15	14	13	14	19
2j	10	10	0	0	0	0	17

Twenty micrograms of test compound in DMSO solution was used in each case. The values indicate the average diameters in mm (of three trials) for the zone of growth inhibition obtained for each compound after 24 h of incubation at 37 °C, with a margin of error of ±1 mm.

Table 8. Bioactivities of *para*-aryl-substituted lactams **2k–p** against *Bacillus* bacteria determined by the Kirby–Bauer method of well diffusion on agar plates

Compound	*B. anthracis*	*B. globigii*	*B. thuringensis*	*B. megaterium*	*B. coagulans*	*B. subtilis*	*B. cereus*
2k	20	19	20	18	15	10	0
2l	20	17	17	15	16	10	18
2m	25	20	20	16	18	19	18
2n	22	20	19	17	22	18	23
2o	20	19	16	15	10	19	15
2p	14	0	10	10	0	0	13

Twenty micrograms of test compound in DMSO solution was used in each case. The values indicate the average diameters in mm (of three trials) for the zone of growth inhibition obtained for each compound after 24 h of incubation at 37 °C, with a margin of error of ±1 mm.

Table 9. Bioactivities of C_4-aryl-disubstituted lactams **2q–2y** against *Bacillus* bacteria determined by the Kirby–Bauer method of well diffusion on agar plates

Compound	*B. anthracis*	*B. globigii*	*B. thuringensis*	*B. megaterium*	*B. coagulans*	*B. subtilis*	*B. cereus*
2q	24	16	17	19	14	12	19
2r	27	22	20	21	17	21	21
2s	22	20	18	20	20	15	20
2t	20	14	13	15	15	10	18
2u	25	20	17	16	18	13	20
2v	27	21	17	19	20	19	22
2w	26	20	19	22	18	17	21
2x	20	20	20	20	22	18	20
2y	21	14	15	11	14	12	18

Twenty micrograms of test compound in DMSO solution was used in each case. The values indicate the average diameters in mm (of three trials) for the zone of growth inhibition obtained for each compound after 24 h of incubation at 37 °C, with a margin of error of ±1 mm.

Table 10. Bioactivities of C_4-organoaryl-substituted lactams **3a–c** against *Bacillus* bacteria determined by the Kirby–Bauer method of well diffusion on agar plates

Compound	*B. anthracis*	*B. globigii*	*B. thuringensis*	*B. megaterium*	*B. coagulans*	*B. subtilis*	*B. cereus*
3a	23	18	16	17	16	14	23
3b	20	15	14	12	17	11	20
3c	23	16	16	18	18	16	20

Twenty micrograms of test compound in DMSO solution was used in each case. The values indicate the average diameters in mm (of three trials) for the zone of growth inhibition obtained for each compound after 24 h of incubation at 37 °C, with a margin of error of ±1 mm.

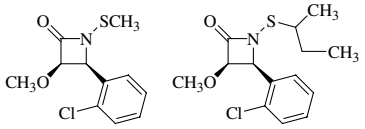
Table 11. Bioactivities of N-organothio-substituted lactams **1a** versus **4** and 5–7 against *Bacillus* bacteria determined by the Kirby–Bauer method of well diffusion on agar plates

Compound	*B. anthracis*	*B. globigii*	*B. thuringensis*	*B. megaterium*	*B. coagulans*	*B. subtilis*	*B. cereus*
1a	25	18	19	16	20	18	21
4	40	39	30	36	37	39	30
5	10	10	0	0	0	0	0
6	10	0	9	10	0	nd	nd
7	0	0	0	0	0	0	0
Cip	39	33	40	41	42	41	33

Twenty micrograms of test compound in DMSO solution was used in each case. The values indicate the average diameters in mm (of three trials) for the zone of growth inhibition obtained for each compound after 24 h of incubation at 37 °C, with a margin of error of ±1 mm. Ciprofloxacin (Cip) is included as a reference (nd, not determined).

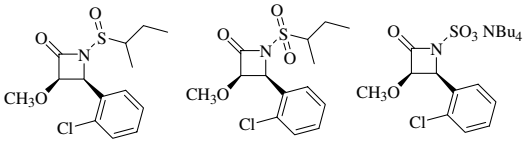
4. Effect of the *N*-organothio substituent on anti-*Bacillus* bioactivity

Previously, we reported that the antibacterial properties of *N*-thiolated β-lactams against MRSA are highly dependent upon the organothio substituent, in terms of both the nature of the organo chain as well as the oxidation state of the sulfur center. The most active derivative from this study was found to be the branched *N*-*sec*-butylthio lactam **4** (Table 1). Examination of **4** against the seven *Bacillus* bacteria likewise determined that this compound was significantly more active than *N*-methylthio lactam **1a**, with zone sizes being on average more than two times larger than those produced by **1a** (Fig. 1).



1a **4**

Inspection of the actual plate from the Kirby–Bauer assay (Fig. 2) shows this clear distinction in activities between lactams **1a** and **4** versus ciprofloxacin (Cip). This well diffusion experiment indicates that **4** is as potent a growth inhibitor of *Bacillus* spp. as the current clinical benchmark, ciprofloxacin. The minimum inhibition concentration (MIC) values for lactams **1a** and **4** against both the avirulent Sterne and virulent Ames strains of *B. anthracis* were determined by broth microdilution to be 4 and 0.5 μg/ml, respectively.[13]



5 **6** **7**

Another interesting finding is that the more highly oxidized *N*-sulfinyl analogue **5**,[14] *N*-sulfonyl lactam **6**,[14] and *N*-sulfonate compound **7** are all significantly weaker in activity than the *N*-methylsulfenyl lactam **1a**.

In this study, we have discovered a new family of antibacterial agents for *B. anthracis* and other *Bacillus* species. The structure–activity profiles of these N-thio-

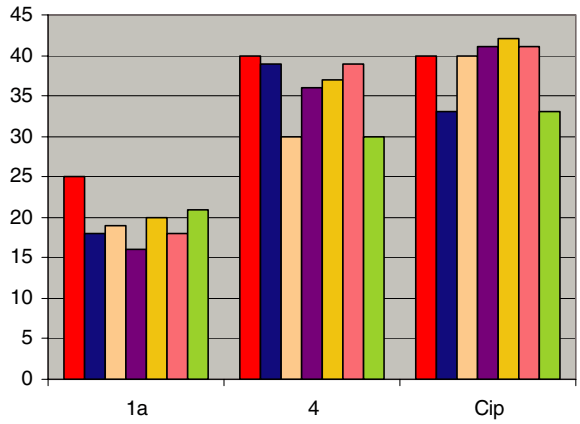


Figure 1. Comparison of anti-*Bacillus* activities of lactams **1a** and **4** to that of ciprofloxacin. Each colored bar represents, in sequential order, the seven *Bacillus* microbes listed in Table 11

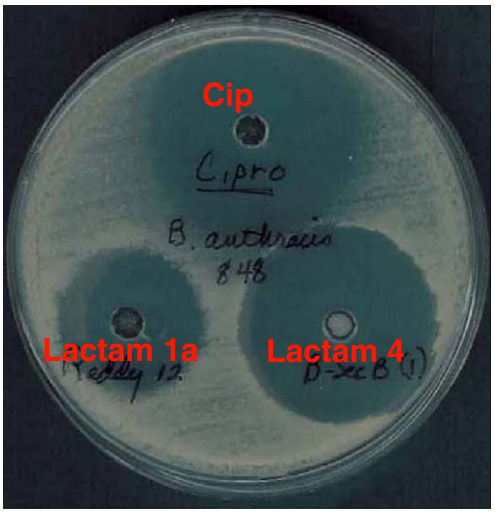


Figure 2. Kirby–Bauer disk diffusion assay comparing the relative effectiveness of lactams **1a**, **4**, and ciprofloxacin (Cip). Twenty micrograms of each compound in DMSO solution was used.

lated β-lactams mirror to a large extent those observed previously for MRSA, with some notable exceptions. In general, lipophilic acyloxy or alkoxy groups at C_3 of the lactam ring lead to the strongest growth inhibition properties against each of the seven *Bacillus* microbes examined. The C_3 allyloxy and propoxy compounds were on average slightly more active than the parent C_3 methoxy derivative. Spirocyclic ethers at C_3 gave lower activity than the open chain variants. At the C_4 center, both aryl and strain chain organoaryl moieties were found to be about equally potent, regardless of the presence of unsaturation or aryl ring substituents. The most important determinant of anti-*Bacillus* activity, as in the case for MRSA, was found to be the *N*-organothio moiety, with the *sec*-butylthio compound **4** having the best overall bioactivity. The mode of action of these lactams in *Bacillus* most likely parallels that in *Staphylococcus*, with transfer of the *N*-organothio substituent from the lactam to a cellular thiol occurring within the cytoplasm of the bacterium. A more complete study on the mechanism of action of these compounds is underway to identify the target and reasons for bacterial species selectivity.

Acknowledgments

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Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.01.070.

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