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Neutral β-Lactams Inactivate High Molecular Mass Penicillin-Binding Proteins of Class B1, Including PBP2a of MRSA

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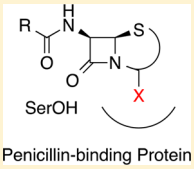
Supporting Information

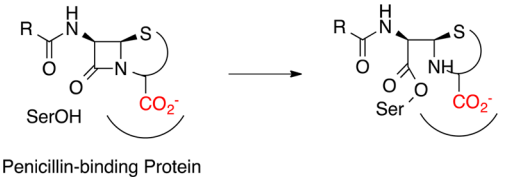
ABSTRACT: The targets of β-lactam antibiotics are bacterial DD-peptidases (penicillin-binding proteins). β-Lactam SAR studies over many years have demonstrated the importance of a specifically placed negative charge, usually carboxylate, on these molecules. We show here that neutral analogues of classical β-lactam antibiotics are of comparable activity to the originals against β-lactam-resistant high molecular mass DD-peptidases of the B1 class, a group that includes PBP2a of methicillin-resistant *Staphylococcus aureus*. These neutral β-lactams may direct new development of antibiotics against certain penicillin-resistant bacteria. These molecules do have antibiotic activity against Gram-positive bacteria.



KEYWORDS: *Penicillin-binding proteins, neutral β-lactams, inhibition, antibiotics*

Pathogenic bacteria continue to seriously impact human welfare.[1] They have been controlled to a considerable extent over the last seventy years by antibiotics, but this control has become seriously challenged by drug-resistant bacteria.[2] One of the few well-validated targets for antibiotics remains the DD-peptidases (penicillin-binding proteins, PBPs), which are, in principle, susceptible to β-lactams (Scheme 1).[3] In recent years, however, β-lactam-resistant DD-peptidases have evolved and spread though natural bacterial populations.[4,5] Our research on DD-peptidase substrate specificity[6] has now produced a new lead toward inhibitor design for one important class of β-lactam-resistant DD-peptidases, the one that includes PBP2a of *Staphylococcus aureus*, the cause of MRSA.[7] We describe here the results of modification of the carboxylate group of traditional β-lactam antibiotics on their inhibition of these enzymes.

Scheme 1



We recently showed that *Bacillus subtilis* PBP4a has enhanced reactivity toward the peptide substrate **1a**, and thus toward the penicillin amide **2a**,[8] with respect to the carboxylate analogues **1b** and **2b**, respectively, and we interpreted these results in terms of peptidoglycan structure; much of *B. subtilis* peptidoglycan is amidated on the free diaminopimelic acid carboxyl.[8] We then proceeded to test **2a** against the other *B. subtilis* PBPs, looking for a similar effect. The PBPs of *Escherichia coli* served as controls. Second-order rate constants for the reactions between β-lactams and the various PBPs were generally determined from competition experiments with the fluorescent β-lactam Bocillin FL[9,10] (representative raw data is available in Supporting Information, Figures S1 and S2) and listed in Table 1. Details of all experimental procedures are found in the Supporting Information.

The important result from Table 1 is that although **2b** is considerably more reactive than **2a** (see Chart 1 for structures), with most *B. subtilis* PBPs (although the difference is generally

Table 1. Rate Constants of Reaction of Penicillin V and Its Amide with *E. coli* and *B. subtilis* PBPs

		k_i (M^{-1} s^{-1})		
E. coli PBP	PBP class	**2a**	**2b**	k_i^{2a}/k_i^{2b}
1a/**1b**	HMMA	0.74 ± 0.10	184 ± 18	0.0040
2	HMMB	1.5 ± 0.2	128 ± 10	0.012
3	HMMB	18 ± 2	420 ± 80	0.044
4	LMMC	30 ± 4	3200 ± 450	0.0092
5/**6**	LMMA	0.60 ± 0.06	96 ± 8	0.0064
B. subtilis PBP				
1	HMMA	340 ± 40	(1.1 ± 0.1) × 10^5	0.0031
2[a]	HMMA/B	440 ± 20	(1.9 ± 0.2) × 10^4	0.024
3	HMMB	5.3 ± 0.6	28 ± 4	0.19
4[b]	HMMA	190 ± 20	(1.9 ± 0.2) × 10^4	0.010
5	LMMA	17 ± 2	620 ± 40	0.027

[a]"PBP2" of *B. subtilis* is actually a usually unresolved, spatially and temporally, mixture of PBP2a, **2b**, and **2c**, none of which is a HMMB1 enzyme. [b]PBP4a of *B. subtilis* is a LMMC enzyme, although it is not expressed at sufficiently high levels in the vegetative stage to be observed on gels.[4]

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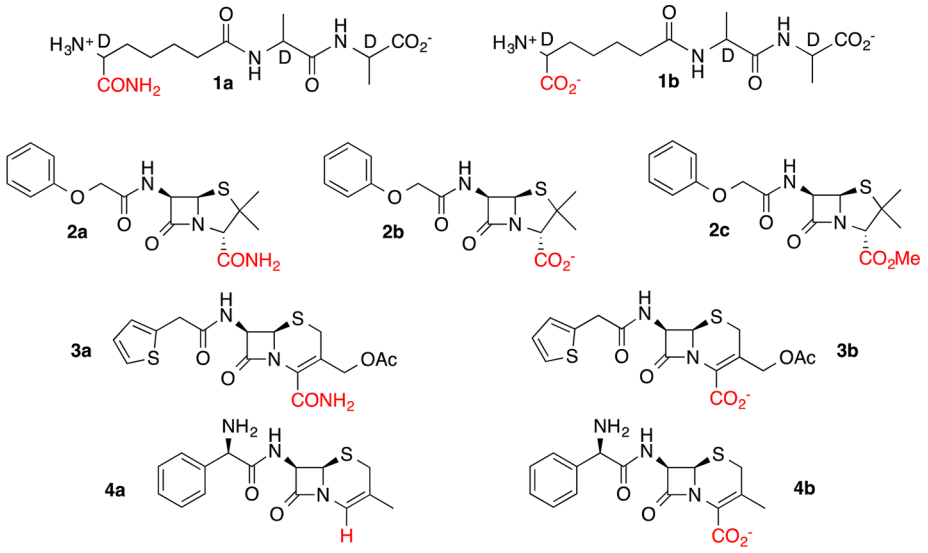
Chart 1



less than that for the *E. coli* enzymes, presumably because of the difference in peptidoglycan structure between these species[8]), they show comparable reactivity with *B. subtilis* PBP3. This result is very striking, since the presence of a carboxylate group on β-lactam antibiotics has long been a given.[11] It appears that the free carboxylate of the penicillin is not essential to its activity with PBP3. This is not true, however, for the other *B. subtilis* PBPs, which behave classically, reflecting a positive effect of the penicillin carboxylate. β-Lactamases also strongly prefer the charged carboxylate.[12]

B. subtilis PBP3 is a high molecular mass (HMM) class B1 PBP.[13] This class of DD-peptidase is found in Gram-positive bacteria, is known to be intrinsically β-lactam-resistant, and includes, in particular, PBP2a of MRSA. Other examples are found in enterococci, e.g. PBP5fm of *Enterococcus faecium*.[14] Many bacilli, other than *B. subtilis*, carry one, including pathogens such as *B. anthracis* (PBP3).[15] These enzymes are thought to be able, aided by a transglycosylase, to maintain cell wall synthesis when all other DD-transpeptidases have been β-lactam-inactivated.[16] Crystal structures are available of *S. aureus* PBP2a[17] and *E. faecium* PBP5fm.[18]

We have found that **2a** is also comparably reactive to **2b** with *S. aureus* PBP2a (Figure 1, Table 2). We suggest that these

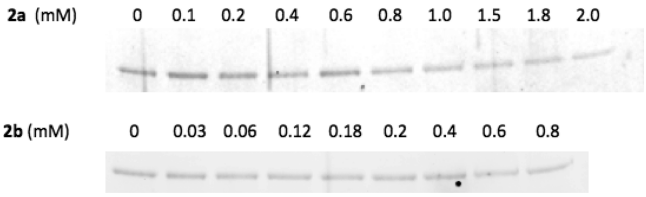


Figure 1. Extent of fluorescent labeling of *S. aureus* PBP2a (0.2 μM) by Bocillin FL (20 μM) in the presence of increasing concentrations of **2a** (upper panel) and **2b** (lower panel).

results may indicate a general property of HMMB1 DD-peptidases, a reactivity with neutral β-lactams comparable to that with the original β-lactams themselves.

To further support this hypothesis, we found that the neutral cephalosporins cephalothin amide **3a** and descarboxycephalexin **4a** are also comparably reactive with *B. subtilis* PBP3 and *S. aureus* PBP2a as the reference carboxylates **3b** and **4b**, respectively (Tables 2 and 3). Both **3a** and **4a** are only weakly

Table 2. Rate Constant of Reactions between β-Lactams and *S. aureus* PBP2a

	k_i (M^{-1} s^{-1})	
2a	2.0 ± 0.9	
2b	5.0 ± 0.6	k_i^{2a}/k_i^{2b} = 0.40
2c	1.5 ± 0.2	k_i^{2c}/k_i^{2b} = 0.30
3a	1.3 ± 0.1[a]	
3b	0.50 ± 0.01[a]	k_i^{3a}/k_i^{3b} = 2.6
4a	0.34 ± 0.04	
4b	0.23 ± 0.06	k_i^{4a}/k_i^{4b} = 1.7

[a]These rate constants were determined directly by monitoring changes in protein fluorescence (Supporting Information, Figure S3).

Table 3. Ratio of Rate Constants for Reactions between Cephalosporin Derivatives and *B. subtilis* PBPs

B. subtilis PBP	k_i^{3a}/k_i^{3b}	k_i^{4a}/k_i^{4b}
1	0.033	0.080
2	0.018	0.0030
3	1.0	0.11
4	0.049	0.0014
5	0.80	0.25

active with respect to **3b** and **4b** against other *B. subtilis* PBPs (except, curiously, against the nonessential PBP5, although the rates here are very small, <5 M^{-1} s^{-1}).

It seems possible, therefore, that HMMB1 enzymes in general may be as susceptible to neutral bicyclic β-lactams as they are to their classical negatively charged analogues. Not all such compounds and enzymes react, however, since we have found that penicillin V methyl ester (**2c**) does not have this activity against *B. subtilis* PBP3 (or any other *B. subtilis* PBP). The methyl ester **2c** did, however, inhibit *S. aureus* PBP2a (Table 2). Amides may be better able than methyl esters to take advantage of carboxylate binding sites.[6] There have also been some previous indications of the effectiveness of other neutral compounds against these enzymes.[19] These compounds, bis-2-oxazetidinyl macrocycles, are not, however, close analogues of

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classical β-lactams. Certain derivatives of naturally occurring descarboxy bicyclic β-lactams, the clavams, are also reported to have modest activity against a DD-peptidase.[20] The low reactivity of classical β-lactams against HMMB1 enzymes must reflect the inability of the carboxylate to facilitate catalysis at these active sites, perhaps because of their unusual narrow active site cleft[17,18] and the probable need for allosteric activation of the site.[21] In crystal structures of the (inert) acyl-enzymes generated, however, the carboxylates of classical β-lactams do appear to interact with the active site in the usual fashion.[17,18]

It was also striking that antibiotic susceptibility tests showed that **2a** was more effective than **2b** against *B. subtilis* (Table 4).

Table 4. MIC Values of Penicillin V (2b), Its Amide (2a), and Its Methyl Ester (2c)

bacterial species	MIC		
	2b	**2a**	**2c**
E. coli	0.75 mM (0.25 g/L)	10 mM (3.4 g/L)	ND[a]
B. subtilis	1.4 mM (0.45 g/L)	2.3 μM (0.76 mg/L)	25 μM (8 mg/L)
S. epidermis	0.37 mM (0.12 g/L)	95 μM (31 mg/L)	ND
B. licheniformis	1.5 mM (0.50 g/L)	1.5 μM (0.5 mg/L)	ND

[a]ND = not determined.

We also saw this difference against other Gram-positive bacteria, *Staphylococcus epidermis* and *Bacillus licheniformis*, but not against the Gram-negative *E. coli*, where **2b** was much more effective (Table 4). This phenomenon has previously been observed with *S. aureus*,[22] although whether PBPs were the target of **2a** was not established. Our preliminary experiments to address this showed that exposure of growing *B. subtilis* cells to **2a** at concentrations around the MIC led to its reaction with all PBPs (data not shown).

Compounds **2a**, **3a**, and **4a**, β-lactams lacking the usual carboxylate, may be useful leads to new antibiotics targeted specifically at the normally β-lactam-resistant HMMB1 DD-peptidases that are often found in pathogenic Gram-positive bacteria such as MRSA. Structure–activity relationships of neutral β-lactams may be different than those of the parent carboxylates. Such compounds could be administered in combination with a regular β-lactam, the latter to inactivate the other HMM PBPs. Much research has focused on the discovery of anti-MRSA cephalosporins.[23] Perhaps some of these may be readily carboxyl modified to yield more effective inhibitors, and ones less susceptible to β-lactamases.[12] Such compounds may also have useful activity against other PBP classes.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1, showing typical SDS PAGE gels for determination of rate constants. Figure S2, showing typical fits to the gel data for rate constant determination. Figure S3, showing fluorescence data for direct determination of rates of reaction of solubilized PBP2a with **3a** and **3b**. Detailed experimental procedures: bacteria, enzyme, and membrane preparation, and determination of rate constants. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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