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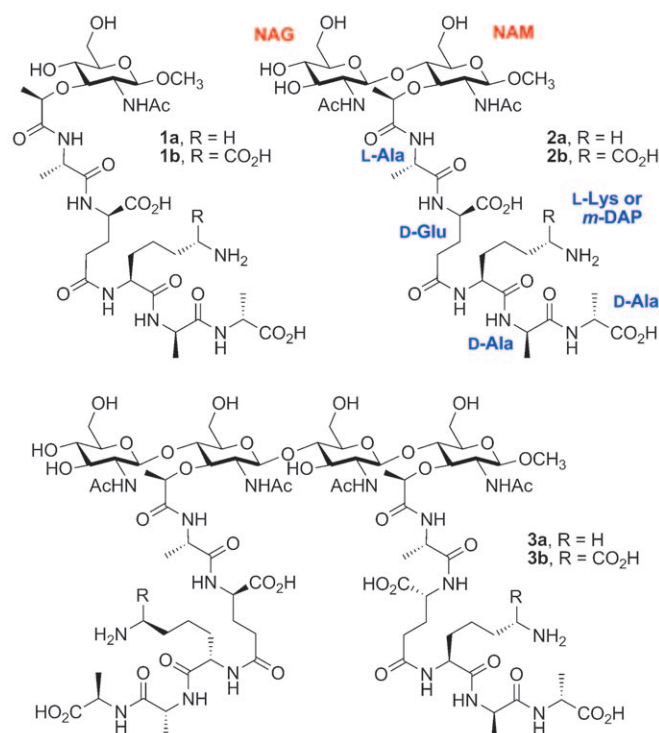
Synthetic Peptidoglycan Motifs for Germination of Bacterial Spores

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Certain Gram-positive bacteria—as exemplified by *Bacillus anthracis*, the causative agent of anthrax—can produce dormant and environmentally resistant spores under conditions of nutritional limitation. These spores exit from dormancy by the process of germination, which is triggered by exposure to specific molecules.^[1] While the precise chemical nature of these molecules, known as germinants, varies according to the organism, they are typically nutrients.^[2] Recently, we reported that supernatants from cultures of growing bacteria and constituents of the cell wall could serve as germinants of dormant *Bacillus subtilis* and *B. anthracis* spores.^[3] Since fragments of the cell wall are released in the course of bacterial growth, the presence of these molecules in the milieu as germination signals might be physiologically relevant.

The major constituent of the cell wall is the bacterial peptidoglycan. The peptidoglycan backbone is comprised of repeating units of *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM). A distinctive pentapeptide is appended to the NAM unit, which contains unusual amino acids, such as *D*-Glu, *D*-Ala and *meso*-diaminopimelate (DAP). We describe herein the study of three Lys- (**1a**, **2a**, and **3a**) and three DAP-containing peptidoglycan fragments (**1b**, **2b**, and **3b**) as spore germinants (Scheme 1). This study utilizes synthetic compounds with defined structures, with variations in the number of rings (1 to 4), different muramic acid moieties (muramic acid vs anhydromuramic acid), and different amino acids (Lys vs *meso*-DAP) at the third position of the stem pentapeptide, which is important for the recognition events. We document, for the first time, that the required minimal structural motif for germination of spores is the presence of one NAG–NAM-(peptide) unit. Compounds **2a/b** and **3a/b**, which possess this minimal structural motif, are potent spore germinants at the low nanomolar level.

The DAP-containing compounds **1b**, **2b**, and **3b** were prepared in multistep syntheses. For the synthesis of the pentapeptide, a preparation of *meso*-DAP was required. Whereas syntheses of DAP have been reported, only four publications have addressed the suitable functionalization of DAP for incorporation into peptidoglycan variants.^[4–7] Schmidt and co-worker^[7] used the Wittig–Horner reaction of *L*-glutamate aldehyde and a phosphoryl glycine derivative to produce the de-



Scheme 1. Structures of synthetic peptidoglycans with *L*-Lys- and *meso*-DAP-containing pentapeptides used for spore germination studies.

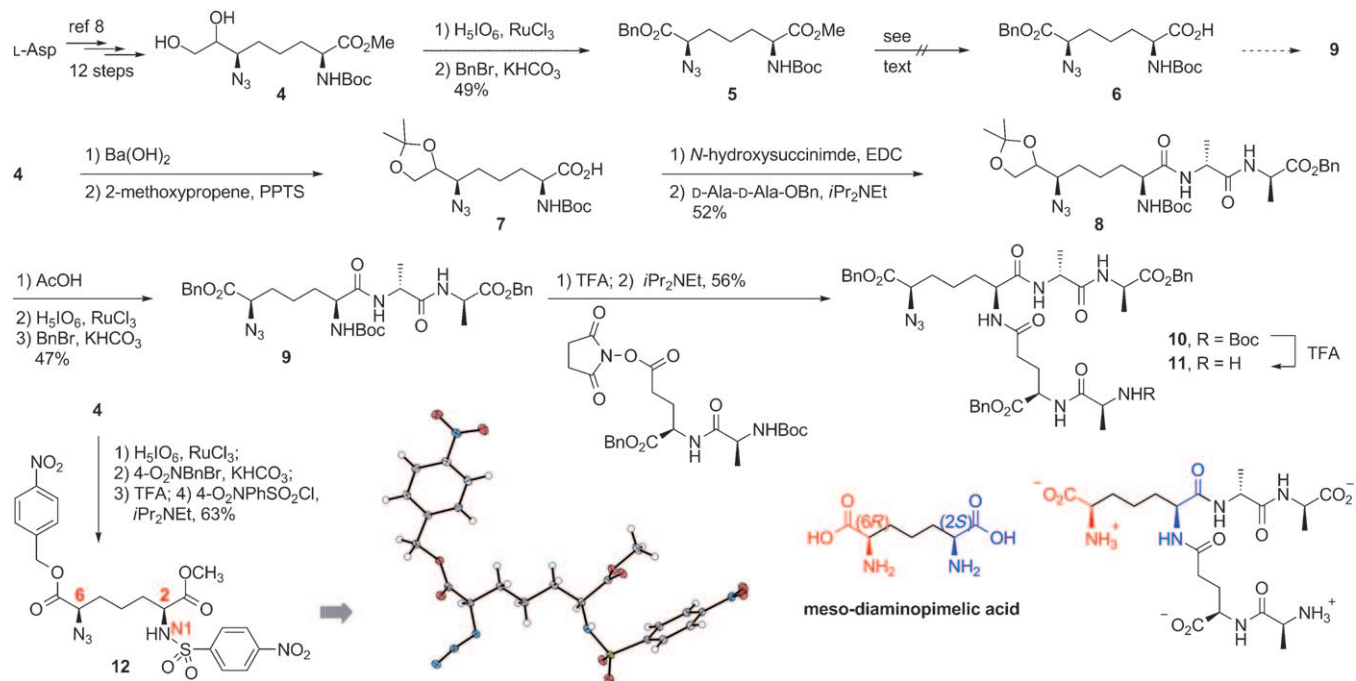
sired C-7 carboxylic acid derivative after C=C double bond reduction and chromatographic separation of diastereomers. Boons and co-workers^[5,6] synthesized *meso*-DAP using cross metathesis between allyl glycine and vinyl glycine derivatives, followed by reduction of the double bond of the resulting compound. Fukase et al.^[4] applied the Kocienski-modified Julia olefination, utilizing an aldehyde and a sulfone, both of which were derived from *D*-serine. The existing methods for DAP preparations proved not to be suitable in our hands for the large-scale reactions and orthogonal protection scheme that were needed. *Meso*-Diaminopimelic acid (*meso*-DAP) contains two stereogenic centers with configurations of 2*S* and 6*R*. Although it is a *meso* compound, the 2*S* and 6*R* carbons should be differentiated to be incorporated into the pentapeptide backbone. That is, the 2*S* carbon serves as the main-chain C_α and is connected to the *D*-Ala-*D*-Ala backbone, and the 6*R* carbon is part of the side-chain functionality. Hence, for the synthesis of *meso*-DAP, we needed to construct two stereogenic centers and the synthesis strategy had to allow for orthogonal protection of the two carboxylates and the two amino groups.

The convergent syntheses utilized the suitably protected DAP-based peptide and the corresponding saccharide units. We incorporated orthogonal protection scheme of *meso*-diami-

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Scheme 2. Synthesis of *meso*-DAP-containing pentapeptide 11.

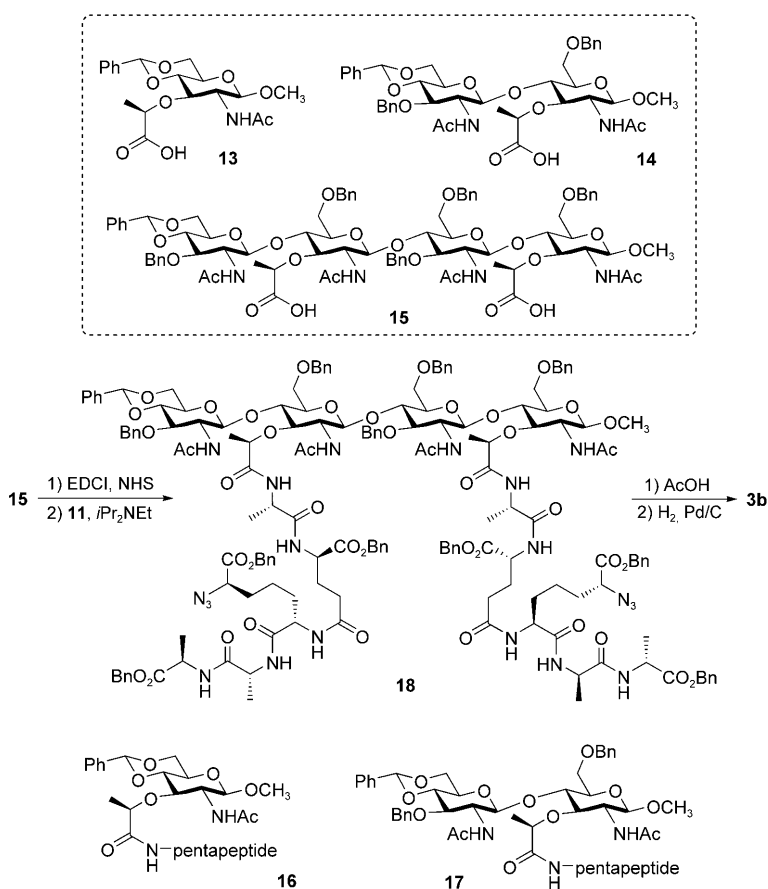
nopimelic acid to suit the need of our global deprotection strategy at the end of the synthesis into the method of Hernández and Martín,^[8] for construction of the template leading to the DAP structure. This approach uses L-aspartic acid in two Wittig reactions, followed by Sharpless epoxidation of allyl alcohol by using $\text{Ti}(\text{O}-i\text{Pr})_4$ and ethyl L-tartrate and its selective epoxide ring opening with azide. This twelve-step route for the preparation of known compound 4^[8] was amenable to the multigram scale. The transformations of 4 to the desired pentapeptide 11 are depicted in Scheme 2. We converted compound 4 to the benzyl monoester 5 by periodic acid oxidation of the diol in the presence of a catalytic amount of ruthenium trichloride, followed by treatment with benzyl bromide and potassium bicarbonate. However, selective hydrolysis of the methyl ester in 5 prior to attachment of D-Ala-D-Ala, proved challenging. Many conditions,^[9] including potassium trimethylsilylanolate, barium hydroxide, LiBr in the presence of triethylamine, resulted in the undesirable hydrolysis of the benzyl ester and the formation of the diacid. Directed hydrolysis of the methyl ester was tried; this involves deprotection of the Boc group under acidic condition, conversion of the free amine to the trifluoroacetamide ($(\text{CF}_3\text{CO})_2\text{O}$, THF), intramolecular base-catalyzed ring closure (NaH, THF) to the corresponding unstable oxazolidinone, and hydrolytic aqueous work-up to result in the corresponding carboxylic acid. Although this clever approach for selective intramolecular hydrolysis of the methyl ester has proven to be successful for a similar compound in the literature,^[10] it did not work for 5 in our hands. Instead of looking for different ester group for C-1, we decided to attach D-Ala-D-Ala to C-1 prior to chemistry at C-7 (oxidation and ester formation).

After hydrolysis of the methyl ester in 4 with barium hydroxide, the diol was converted to the acetone 7. The carboxylate was subsequently activated with N-hydroxysuccinimide and the fragment was coupled to D-Ala-D-Ala-OBn to give 8. The treatment of 8 with aqueous acetic acid gave the desired diol without effecting the Boc group. The resulting diol was subsequently transformed to the benzyl ester 9 by a two-step treatment (oxidative cleavage of the resultant diol, and benzyl protection of the carboxylate). The Boc group in compound 9 was subsequently removed by treatment with trifluoroacetic acid (TFA) and the resultant deprotected amine was coupled with Boc-L-Ala- γ -D-Glu(OHNS)-OBn to give the pentapeptide 10. The Boc group in 10 was removed by TFA treatment, before the coupling reaction with the suitable saccharide derivatives (vide infra).

Since the stereocenter at C-6 in the *meso*-DAP derivatives was newly formed, its absolute stereochemistry needed to be established. The diol in 4 was oxidized to the carboxylate. Several ester and amine protective groups for C-7 carboxylate and N-1, respectively, were tried in order to attempt crystallization, and ultimately, structure determination by X-ray crystallography. The effort was successful with compound 12, the X-ray structure of which confirmed that nitrogen atoms at C-2 and C-6 exist as *syn* to each other and their respective configurations were 2S and 6R, as desired.^[11]

Completion of the syntheses of compounds 1, 2, and 3 started with the orthogonally protected derivatives 13, 14, and 15, which were individually synthesized from D-glucosamine in 6, 21, and 31 steps, respectively, by methodology developed in our laboratory.^[12,13] In each case, the lactyl moiety was activated by the formation of succinimide esters, which were treated with 11 to give the corresponding peptidoglycan derivatives

(**16**, **17**, and **18**; Scheme 3). The protective groups in sugar and peptide moieties were designed for global removal in one-pot by treatment with acetic acid, followed by hydrogenolysis. Compounds **1b**, **2b**, and **3b** were prepared for the



Scheme 3. Synthesis of *meso*-DAP-containing fragments of the peptidoglycans **1b**, **2b**, and **3b**.

first time for this study in convergent syntheses involving 29, 44, and 63 synthesis steps, respectively. When L-Lys was used in place of *meso*-DAP in the protected pentapeptide during the coupling reaction with derivatives **13**, **14**, and **15**, compounds **1a**, **2a**, and **3a** were produced.^[12,13]

Germination of *B. subtilis* spores by the synthetic samples was assessed. This response requires PrkC, a well-conserved bacterial Ser/Thr membrane kinase, which contains an extracellular domain capable of binding peptidoglycan.^[3] The *B. subtilis* peptidoglycan is DAP-based, and its spores responded only to *meso*-DAP-containing peptidoglycan fragments. However, *B. subtilis* spores lacking PrkC but expressing the PrkC homologue from *Staphylococcus aureus* (PrkC_{Sa}), responded to both L-Lys- and *meso*-DAP-containing peptidoglycan fragments. We, therefore, addressed whether the identity of the residue at the third position (Lys vs *meso*-DAP) was responsible for this specificity by assaying the ability of synthetic muropeptides of both types to germinate spores expressing only one of the kinase homologues. The *B. subtilis* system recognized only

DAP-containing variants. In contrast, the system with the *S. aureus* protein recognized both DAP- and Lys-containing samples. The synthetic *meso*-DAP-containing peptidoglycan analogues **2b** and **3b** germinated spores expressing either native PrkC or *S. aureus* PrkC (Figure 1 A and B) at an EC₅₀ of approximately 10 nM. In contrast, L-Lys-containing synthetic molecules (**2a** and **3a**) only stimulated germination of spores expressing the PrkC_{Sa} homologue (Figure 1 B). Thus, both classes of synthetic compounds are biologically active for the respective target proteins.

Compounds **1a** and **1b** lacking the NAG residue failed to stimulate germination entirely, indicating that the presence of the NAG–NAM disaccharide is a strict minimal structural motif for germination. Hence, the third amino acid (Lys or *meso*-DAP) appears to be crucial for the recognition event by the given target protein, and the binding requires the presence of one NAG–NAM unit. Interestingly, the critical role of this third amino acid residue is also observed with the eukaryotic proteins that recognize peptidoglycan.^[14] For example, Nod1 is an intracellular pattern recognition molecule activated during bacterial infection and is stimulated by *meso*-DAP-containing peptidoglycan, but not Lys-containing peptidoglycan.^[15–17] A mechanistic explanation of such discrimination is found in the binding of two peptidoglycan recognition proteins (PGRPs) from *Drosophila* to a *meso*-DAP-containing muropeptide, in which the side-chain carboxylate of *meso*-DAP forms a bidentate salt bridge with a conserved arginine residue in the PGRP.^[18,19]

Although there is no sequence similarity between the PGRPs and the extracellular domain of the bacterial kinase that binds peptidoglycan, future structural studies with different kinases and their respective ligands should resolve whether the kinases use a similar strategy for ligand discrimination. As much as 50% of the bacterial cell wall is turned over in the course of normal growth through the action of a muralytic family of lytic transglycosylases. These enzymes facilitate the formation of a transient oxocarbenium species at the C-1 of the NAM unit, which entraps the C-6 hydroxyl group, resulting in what is referred to as NAG-1,6-anhydromuramyl-peptide (**19a** and **19b**).^[20–23] This process releases two sugars at a time from the polymeric cell wall. Both **19a** and **19b** have been prepared in our laboratories,^[20] hence we explored if they could serve as spore germinants. The ability of **19a** and **19b** to stimulate germination was significantly less than that of **2** and **3** (Figure 1). This result indicates that the products of lytic transglycosylases are not involved in stimulating the kinase activity, and further suggest that peptidoglycan fragments produced by other muralytic enzymes are responsible for the germination event.

Secreted murein hydrolases analogous to the muralytic re-suscitation promoting factors, which play an important role in *Mycobacterium tuberculosis* pathogenesis, stimulate the activity of these kinases,^[24] although their reaction products have not

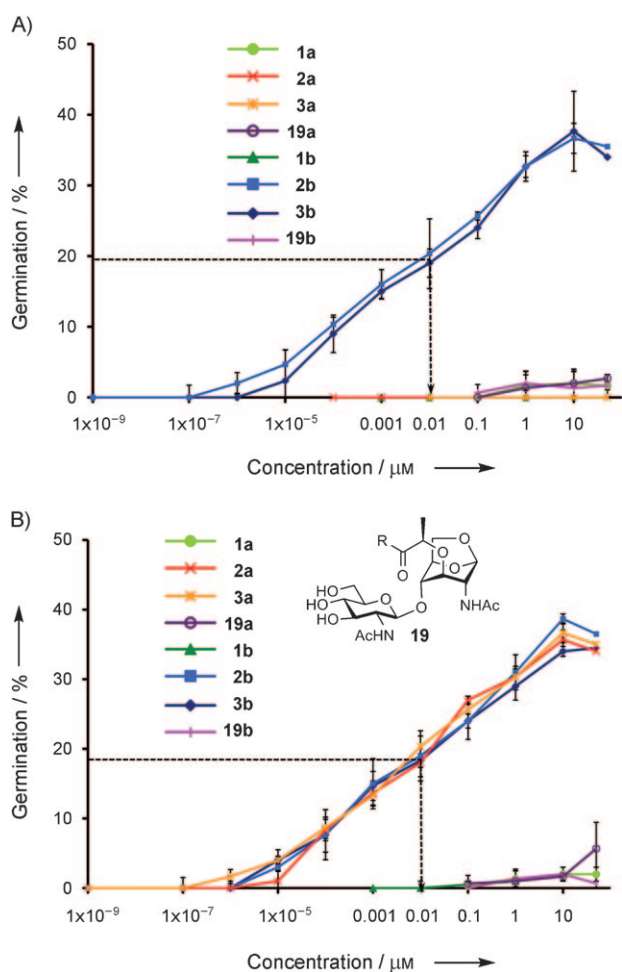


Figure 1. Dose-response curves for spore germination by using the synthetic samples. A) Wild-type *B. subtilis* spores expressing the native PrkC, and B) *B. subtilis* spores expressing the *S. aureus* PrkC protein were incubated with indicated concentrations of synthetic samples, and percentage germination was determined. The dashed gray line was used for EC₅₀ determination. Compound **19a**, R = Lys-pentapeptide = L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala; compound **19b**, R = DAP-pentapeptide = L-Ala-γ-D-Glu-m-DAP-D-Ala-D-Ala.

been characterized. The maximal observed germination with these synthetic compounds (~45%) is similar to that reported previously for purified samples from digested bacterial cell wall;^[3] this indicates that only a fraction of the spores might be able to respond. Nevertheless, it is clear that synthetic compounds **2** and **3** are potent stimulants of spore germination. These peptidoglycan derivatives reveal that two or more sugar moieties are required in the germinant and that the single-sugar variants and the anhydromuramyl derivatives, which are products of turnover of peptidoglycan, fail to stimulate germination. These reagents will be useful mechanistic tools for further exploration of the details of the complex processes involved in germination of bacterial spores. In addition, these molecules will be useful in elucidating the mechanisms underlying the essential function of PrkC homologues in pathogenesis and antibiotic resistance of a number of other important nonspore-forming pathogens, such as *S. aureus*^[25,26] and *Enterococcus faecalis*.^[27]

Experimental Section

Germination assay: Spore germination assays were performed with compounds **1a/b**–**3a/b** and **19a/b**, at concentrations ranging from 50 μM to 1 fM.^[3] Briefly, we incubated 10⁶ *B. subtilis* wild-type spores or those expressing PrkC_{sa} with a synthetic compound in germination buffer (50 μL; 10 mM Tris-HCl, pH 8.0, 1 mM glucose) for 60 min at 37 °C. Since spores that have initiated germination, but not dormant spores, are temperature sensitive, the response to the compounds was assessed by subjecting the samples to wet heat (80 °C, 20 min), followed by incubation at 37 °C, overnight, in LB agar plates to determine survival. Percentage germination was determined by calculating the ratio of the colony forming units (CFUs) obtained following incubation with germinant to that obtained after incubation with buffer only. EC₅₀ values were determined for active compounds by using the dose-response curves.

Syntheses of compounds 1b, 2b, and 3b: Detailed chemical syntheses of compounds **1b**, **2b**, and **3b** are given in the Supporting Information.

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