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REVIEW

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Structures, Synthesis, and Human Nod1 Stimulation of Immunostimulatory Bacterial Peptidoglycan Fragments in the Environment

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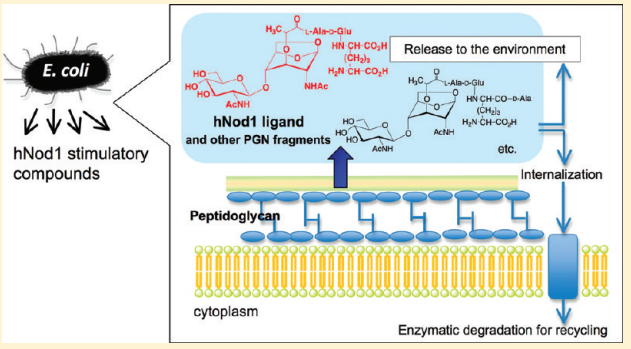
ABSTRACT: Bacteria release immunostimulatory compounds to the environment, and one of the stimulants is the ligand of nucleotide-binding oligomerization domain protein 1 (Nod1), an intracellular protein involved in the recognition of the bacterial component peptidoglycans having a diaminopimelic acid (DAP) structure. The polymorphisms of Nod1 have been linked to several inflammatory diseases and allergies that are strongly affected by environmental factors. The present paper summarizes recent results on the isolation and structural elucidation of natural human Nod1 (hNod1) ligands from the *Escherichia coli* (*E. coli*) K-12 culture supernatant, the first chemical synthesis of these natural ligands and related PGN fragments structures, and the hNod1 stimulatory activities of the chemically synthesized DAP-type PGN fragments. For structural characterization studies, the 7-(diethylamino)-coumarin-3-carbonyl (DEAC) labeling method was also used to enhance the sensitivity in mass spectrometry studies, in order to observe PGN fragments in a comprehensive manner. The results suggest that DAP-containing bacteria release certain hNod1 ligands to the environment and that these ligands accumulate in the environment and regulate the immune system through Nod1.



INTRODUCTION

The innate immune system exists in a wide range of multicellular organisms including plants, insects, and other animals and is activated by microbial components for the recognition of various pattern-recognition receptors (PRRs). In vertebrates, the innate immune system is the first line of defense, before activating the acquired immune system. Various PRRs have been recently identified in mammals, including toll-like receptors (TLRs),[1−3] nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs),[4−6] and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs).[7,8] Bacteria have immunostimulatory components in the cell-surface structure, such as cell wall peptidoglycans (PGN) and lipoproteins from Gram-positive and Gram-negative bacteria, lipopolysaccharides (LPS) of the outer membrane in Gram-negative bacteria, and lipoteichoic acids (LTA) in Gram-positive bacteria (Figure 1a and b). It was shown in 2003 that peptidoglycans are recognized with the intracellular receptors, Nod1 and Nod2, the founding family members of the NLRs.[9−12] The receptor proteins are composed of three main domains: the leucine-rich repeat (LRR) domain (for ligand recognition, located at the carboxy-terminal site), the NOD domain (facilitating self-oligomerization and having ATPase activity, located at the central site), and the caspase-recruitment domain (CARD) (located at the amino-terminal site). The target molecule, PGN, is known as a potent immunopotentiator and an adjuvant for antibody production. PGN has a structure composed of polysaccharide chains having alternating (β1−4)-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues, and the polysaccharide chains are linked to a peptide network to form the rigid structure of the cell wall. The branched position of the peptide usually has diamino acids such as L-lysine (in many Gram-positive bacteria; Figure 1c) or *meso*-diaminopimelic acid (*meso*-DAP) (in most Gram-negative bacteria; Figure 1d, and some Gram-positive bacteria). For activation of the immune system, Nod1 recognizes the site of DAP-containing fragments such as γ-D-Glu-*meso*-diaminopimelic acid (iE-DAP), whereas Nod2 recognizes muramyl dipeptide (MDP) (Figure 1c and d). The structures recognized were determined with chemically synthesized PGN fractions.[9,13−18]

Several investigations have shown that genetic variations of Nod1 and Nod2 are associated with a susceptibility to inflammatory diseases, including asthma and Crohn's disease, respectively, and also with other allergic diseases.[19−25] Polymorphisms in Nod1 have been linked to the previously observed inverse association[26−29] between exposure to microbial products and asthma and allergies in childhood.[30] An important function of Nod1 and Nod2 for the autophagic response to invasive bacteria was also revealed recently with the recruitment of the autophagy protein ATG16L1 to the plasma membrane at the bacterial entry site.[31] Autophagy induction was also observed with iE-DAP-containing PGN fragments in insect systems.[32]

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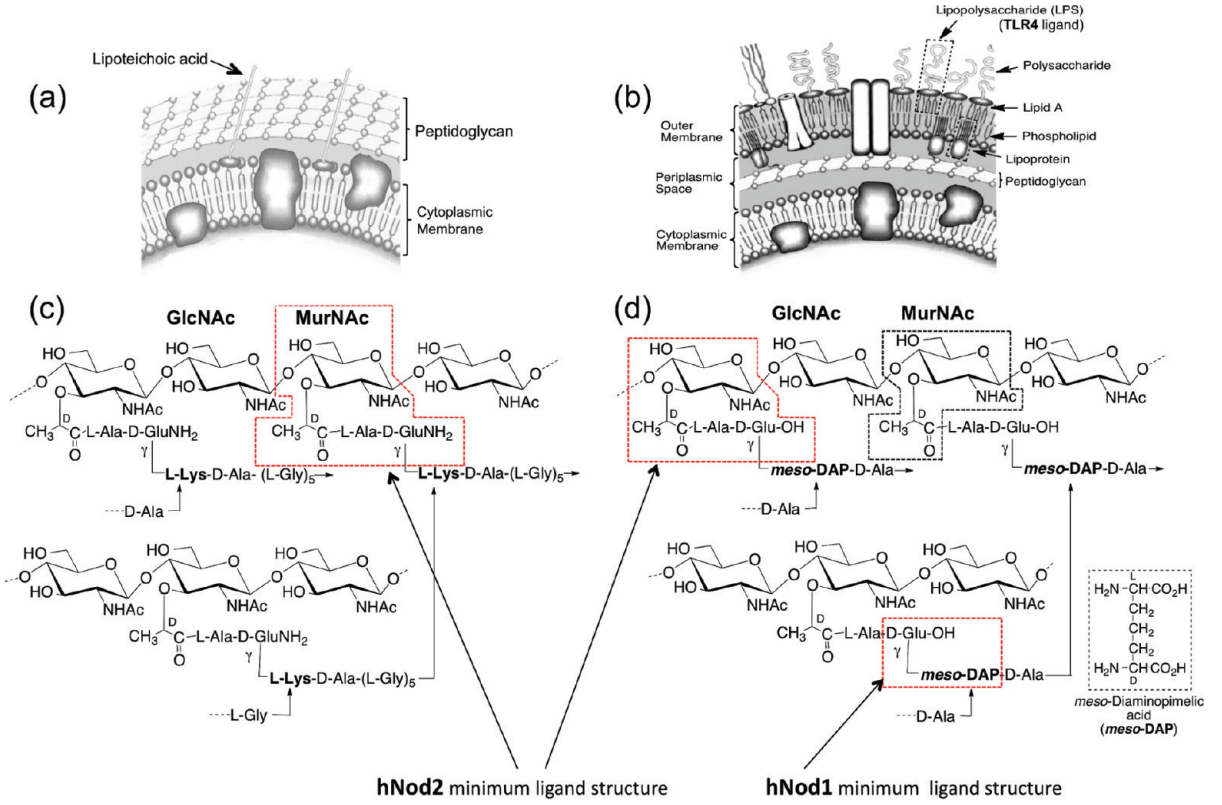

Figure 1. Bacterial surface structures and peptidoglycans. Cell surface of (a) Gram-positive and (b) -negative bacteria. The peptidoglycan structures are of (c) *Staphylococcus aureus* (as Gram-positive bacterium) and (d) *Escherichia coli* (as Gram-negative bacterium). hNod1: human nucleotide-binding oligomerization domain-containing protein 1; hNod2: human nucleotide-binding oligomerization domain-containing protein 2.

Recently, it was revealed that DAP-containing bacteria release Nod1 ligands to the environment, and it was suggested that these compounds modulate the development of the human immune system. Hence, in this review, recent results on the isolation and structure elucidation of natural human Nod1 (hNod1) ligands from the *Escherichia coli* culture supernatant are summarized, as representative immunomodulating compounds in the environment. Then, the first chemical syntheses of these natural ligands and related PGN fragment structures are also described. The syntheses include the first chemical synthesis of tracheal cytotoxin (TCT) and the repeating unit of the DAP-type PGN. The hNod1 stimulatory activities of the chemically synthesized DAP-type PGN fragments are also discussed.

■ STRUCTURE OF NOD1 STIMULATORY COMPOUND RELEASED FROM BACTERIA

DAP-containing bacteria release Nod1 ligands to the environment. It was found that both the bacterial bodies and the culture supernatants of various bacteria show human Nod1 stimulatory activities (Figure 2),[33] and the supernatants of some bacteria such as *E. coli* and *Bacillus* species exhibit more potent hNod1 stimulation than their bacterial bodies. In contrast, in the case of hNod2 and TLR4 (Figure 2), the ligands are not released to the same extent in the supernatants in comparison with the cell bodies. In these cases, in general, MDP in PGN is known as the ligand of hNod2, and LPS and the fragment structures including lipophilic terminal structures (e.g., lipid A) are known to stimulate TLR4. Nod1 ligands were also found to be more stable than the Nod2 or TLR4 ligands in stringent conditions such as higher temperatures or under acidic or basic conditions.[33] These results suggest a mechanism in which bacteria present in the environment stimulate the host immune system through Nod1, and this event may be related to the development of allergic diseases in some circumstances. Although synthetic studies have revealed that the recognition core of hNod1 stimulatory molecules is an iE-DAP-containing molecule,[9,15] the identification of the hNod1 ligands in the environment had not been elucidated until our own very recent results.[34]

During cell growth, previous investigators have shown that bacteria seem to recycle the cell wall components from about 60% of the parental cell wall; degradation occurs of the older cell wall, with transport of the fragments into the cell and reuse of the components for cell wall biosynthesis.[35−38] Lytic transglycosylases play an important role in the degradation of the bacterial cell wall in this recycling process, and this leads to *N*-acetyl-1,6-anhydromuramyl [(anh)MurNAc] moieties as the products.[39,40] The presence of the *N*-acetyl-1,6-anhydromuramyl [(anh)MurNAc] moiety in the cell wall was first reported in 1975,[41] and GlcNAc-(β1−4)-(anh)MurNAc-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala (tracheal cytotoxin; TCT), originally found in *Bordetela pertussis* (the causative agent of pertussis; whooping cough),[42] is thought to be a fragment released into the environment during the recycling of the cell wall.[43] However, TCT does not activate hNod1,[44] but only murine Nod1 (mNod1).[45] In addition, the sltY-deficient *E. coli* mutant MHD63 (*amiA*$^{-}$, *amiB*$^{-}$, *amiC*$^{-}$, *sltY*$^{-}$) culture supernatant, which is thought to produce scarcely *N*-acetyl-1,6-anhydromuramyl moieties, was found to induce hNod1 stimulation.[33]

For the analysis of the natural hNod1 ligands, the *E. coli* K-12 strain was used, because the bacteria release the hNod1 ligands

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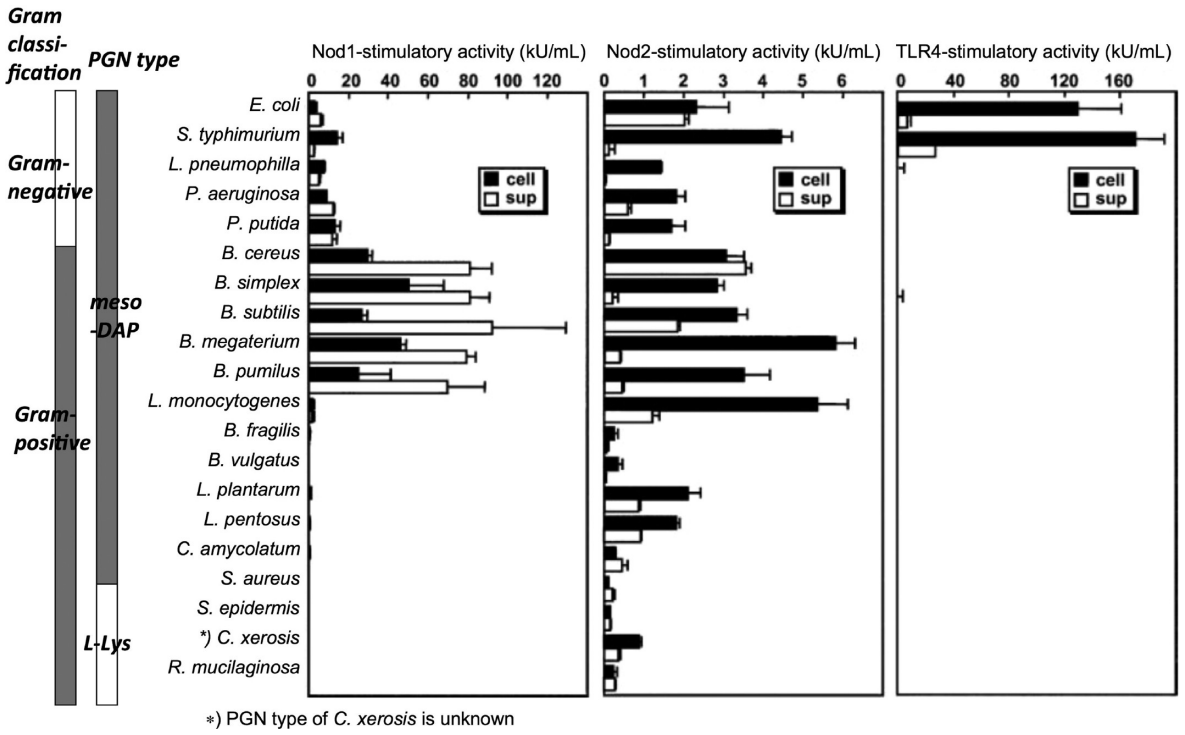

Figure 2. Human Nod1 and Nod2 (receptors of peptidoglycan (PGN)) and TLR4 (receptor of lipopolysaccharide (LPS)/lipid A) stimulatory activity in various bacterial cells and culture media.[33] Bacterial cells (cell) and culture supernatants (sup) were prepared from overnight cultures of bacteria. The Nod1, Nod2, and TLR4/MD2 stimulatory activities were determined by the HEK293T bioassay given in kilounits/mL. The activity of the bacterial cell extract is given in kilounits/mL of the original culture volume. One unit (U) of the Nod1, Nod2, and TLR4 stimulatory activity is equivalent to those of 1 ng of synthetic iE-DAP, MDP, and purified *E. coli* O55:B5 LPS, respectively. The strains used in the experiments were as follows: *Escherichia coli* K12, *Salmonella typhimurium* SL133, *Legionella pneumophila* O2, *Pseudomonas aeruginosa* PA01, *Bacteroides fragilis* NCTC10581, *Bacteroides vulgatus* ATCC8482, *Pseudomonas putida* NI395*a*, *Bacillus subtilis natto* NI146*a*, *Bacillus cereus* NI464*a*, *Bacillus megaterium* NI409*a*, *Bacillus pumilus* NI408*a*, *Listeria monocytogenes* EGD, *Lactobacillus plantarum* ATCC8041, *Lactobacillus pentosus* ATCC11580, *Corynebacterium amycolatum* NI355*a*, *Staphylococcus aureus* ATCC25923, *Staphylococcus epidermis* NI379*a*, *Rhodotorula mucilaginosa* NI343*a*, and *Corynebacterium xerosis* NI355*a*. This figure was originally published in the *Journal of Biological Chemistry* and has been partly modified. Hasegawa, M.; Yang, K.; Hashimoto, M.; Park, J. H.; Kim, Y. G.; Fujimoto, Y.; Nunez, G.; Fukase, K.; Inohara, N. Differential release and distribution of Nod1 and Nod2 immunostimulatory molecules among bacterial species and environments. *J. Biol. Chem.* **2006**, *281*, 29054−29063. ©The American Society for Biochemistry and Molecular Biology.

into the supernatant,[33] and the strain is culturable in a minimum salt medium, which simplifies the purification process of Nod1 ligands. The Nod1 stimulatory fractions were separated and purified as outlined in Figure 3. Thus, after the fermentation of *E. coli* K-12 in M9 minimum medium, the culture supernatant was first desalted by filtration through an ODS open, short column and then further purified by RP-HPLC (Figure 3a).[34] The fractions obtained from the RP-HPLC separation were evaluated for hNod1 and murine Nod1 stimulatory activities. The most potent hNod1 stimulatory fraction (0.15 mg from 22 L of supernatant) was analyzed by ESIQTOFMS, MS/MS (Figure 3b), and NMR spectroscopy. The structure was determined to be DS(anh)-3P(DAP) [GlcNAc-(β1−4)-(anhydro) MurNAc-L-Ala-γ-D-Glu-*meso*-DAP] by ESIMS/MS and NMR spectroscopy and found to be identical with the previously synthesized compound[44] (Figure 3c). This compound, DS-(anh)-3P(DAP), activated hNod1 but not mNod1, and the result was consistent with a previous report.[45] For the comprehensive analysis of PGN fragments in the *E. coli* supernatant, a fluorescent tag, a 7-(diethylamino)coumarin-3-carbonyl (DEAC) group, was used for the enhancement of sensitivity in MS analysis.[46] The DEAC tags were coupled to amino groups in the PGN fragments through *N*-hydroxysuccinimidyl ester intermediates to generate amides, and the DEAC-modified fragments DS-4P$_{(DAP)}$, DS(anh)-3P$_{(DAP)}$, MS-5P$_{(DAP)}$, MS(anh)-4P$_{(DAP)}$, and MS-3P$_{(DAP)}$ were observed by ESIMS/MS analysis (Figure 4). DS(anh)-4P$_{(DAP)}$ (tracheal cytotoxin) was also observed by ESIMS/MS in the *E. coli* supernatant without any labeling. Tripeptide-containing fragments such as MS-3P$_{(DAP)}$ and DS(anh)-3P$_{(DAP)}$ proved to have potent hNod1 stimulatory activities using synthetic hNod1 ligands, as described in the following section.[44] Both 1,6-anhydro- and non-anhydro-GlcNAc moieties were found in the active fraction, and the fact that MurNAc-containing (non-anhydro-type) PGN fragments were released might explain the results of hNod1 activation with the *sltY*-deficient *E. coli* culture supernatant.

An incidence difference in allergic diseases has been shown in terms of rural (especially "farming") and urban environments. Recent studies have shown that exposure to bacterial components at an early age decreases the risk of allergic sensitization later in life ("hygiene hypothesis"). The genetic variations of Nod1 are associated with a susceptibility to inflammatory diseases including athsma and other allergic diseases, and Nod1 ligands in the environment might control the development of an innate immune system of humans at an early age.[26−30] Considering other proposed molecules such as LPS and microbial CpG motifs[28,47−49] and also recently shown allergy-protective components such as LPS from *Acinetobacter lwoffi*,[50−52]

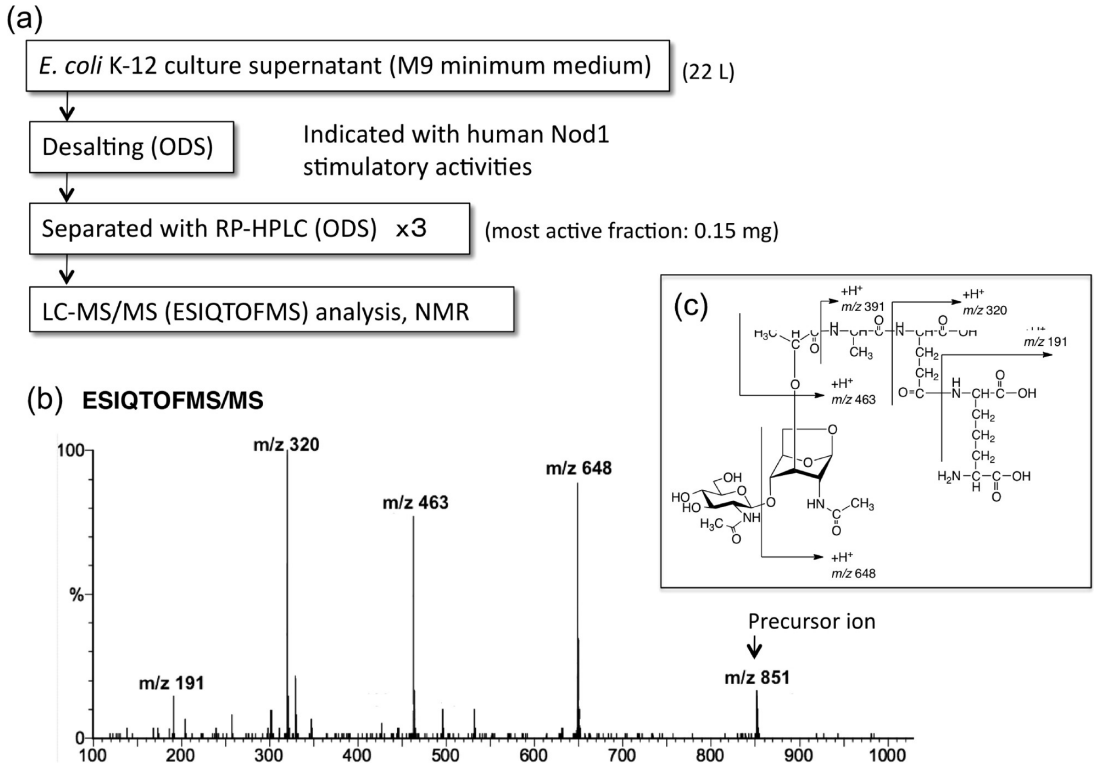

Figure 3. (a) Isolation of fractions with hNod1 stimulatory activity from *Escherichia coli* K-12 culture supernatant (M9 minimum medium). (b) ESIQTOFMS/MS of the most active fraction from the *E. coli* culture supernatant. (c) MS/MS fragment ion analysis of the most active fraction for human Nod1 stimulatory activity, DS(anh)-3P(DAP). This figure was originally published in the *Journal of Biological Chemistry* and has been partly modified. Pradipta A. R.; Fujimoto, Y.; Hasegawa, M.; Inohara, N.; Fukase, K. Characterization of natural human nucleotide-binding oligomerization domain protein 1 (Nod1) ligands from bacterial culture supernatant for elucidation of immune modulators in the environment. *J. Biol. Chem.* **2010**, *285*, 23607−23613. ©The American Society for Biochemistry and Molecular Biology.

arabinogalactan from *Alopecurus pratensis*,[53] and spores from *Bacillus licheniformis*,[54] it is fundamental that the molecular structures of hNod1 ligands in the environment be understood for investigations on their effect on the human immune system.

SYNTHESIS OF *MESO*-DIAMINOPIMELIC ACID (*MESO*-DAP) CONTAINING PEPTIDOGLYCAN (PGN) FRAGMENTS

In general, it is usually difficult to obtain highly pure bacterial glycoconjugates including PGN fragments from natural sources, because of their heterogeneous characteristics. In addition, the possibility of contamination by other immunostimulatory compounds should not be ruled out even after extensive purification. We have thus synthesized a series of PGN fragments, including DAP-containing fragments[44] and also Lys-containing fragments,[16−18] to find out the key molecules in immune system activation and to investigate the functions of ligands and receptors.[13,14] The differences of Nod1 stimulatory activities depending on the configuration of DAP structure have been examined.[44] The investigated isomers include three DAP isomers, namely, *meso*-, (2*S*,6*S*)-, and (2*R*,6*R*)-,[55] and four iE-DAP isomers, (2*R*,6*R*)-, (2*R*,6*S*)-, (2*S*,6*R*)-, and (2*S*,6*S*)-DAP (Figure 5).[56] The iE-DAP with a natural (2*S*,6*R*)-*meso*-DAP structure showed the most potent Nod1 stimulatory activity among the isomers. The structure of (2*S*,6*S*)-DAP, which is also natural but a minor component, showed weaker but definite activity, and other (2*R*,6*R*)- and (2*R*,6*S*)-DAP analogues showed much weaker activities. No antagonistic activities were observed for these isomers.

After several synthetic approaches to relatively small DAP-containing fragments, including the synthesis of monosaccharide DAP-type fragments[57,58] and DAP-containing peptides of PGN,[59] the repeating unit of DAP-type PGN fragments and tracheal cytotoxin, GlcNAc-(β1−4)-(anh)MurNAc-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala) (**5**), were synthesized chemically for the first time (Figure 6).[44] The fragment structures of DS-4P(DAP) and TCT (**2**−**4** and **6**−**8**) were also prepared with the same synthetic strategy. The strategy for the synthesis of DS-4P-(DAP) (**1**), TCT (**5**), and other fragment structures is shown in Figure 6.

For the preparation of the glycan part, the appropriately protected disaccharides, **14** (GlcNTroc(β1−4)MurNTroc) and **15** (GlcNTroc(β1−4)(anh)MurNTroc), were prepared by β-selective glycosylation between the MurNTroc acceptor **9** and the GlcNTroc donor **11** (for **14**), and the (anh)MurNTroc acceptor **10** and the GlcNTroc donor **11** (for **15**), with activation by the Lewis acid trimethylsilyl trifluoromethane sulfonate (TMSOTf) at 0 °C. The Troc protections of the amino group at the 2-position of the glycosyl donors **9** and **10** were effective for the reactivities and also for the β-selective glycosylation by neighboring effects. The glycans obtained were used for further condensation with peptides. The monosaccharides **12** and **13** were also used for the synthesis of the monosaccharide fragment structures **3**, **4**, **7**, and **8** via condensation with the peptide moieties.

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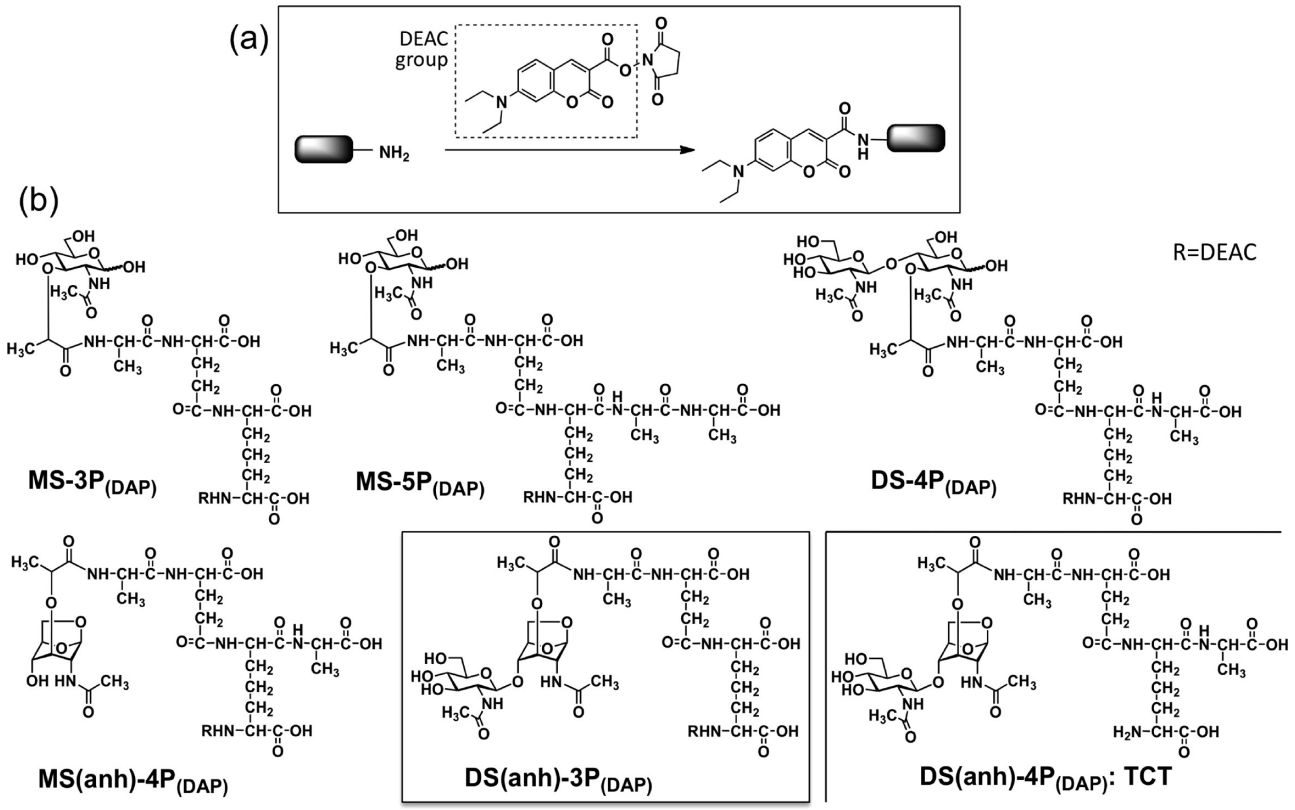

Figure 4. (a) DEAC labeling reaction of amino groups in the Nod1 stimulatory fraction of the *E. coli* supernatant. (b) Observed peptidoglycan fraction structures from the *E. coli* supernatant with DEAC labeling. DS(anh)-4P(DAP) (TCT) was also observed without labeling.

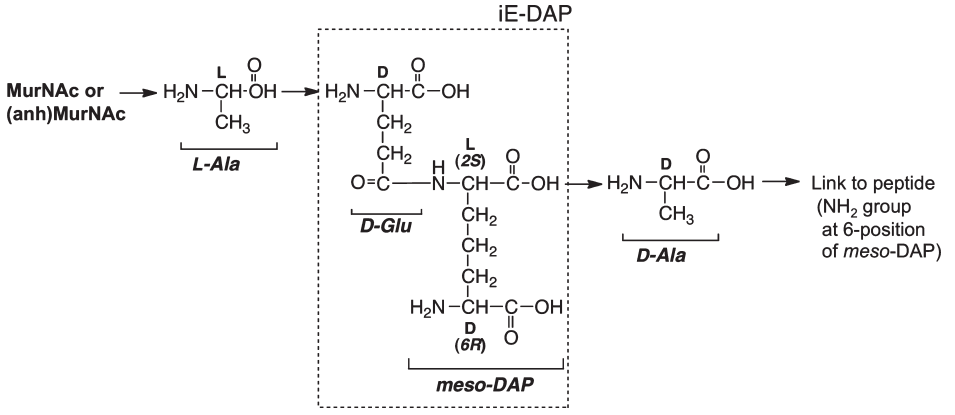

Figure 5. Peptide moiety of DAP-type peptidoglycan (*Escherichia coli*) and the iE-DAP structure.

As for the preparation of orthogonally protected *meso*-diaminopimelic acid (*meso*-DAP), several syntheses of orthogonally protected *meso*-DAP analogues have been reported,[57,58,60−72] as reviewed by Dzierzbicka.[73] In the strategy for TCT (Figure 5), two α-amino acid derivatives (the aldehyde, **16**,[74] and the sulfone, **17**,[75] derived from D-serine) were coupled by utilizing the Julia−Kochenski olefination reaction[76] to afford the appropriately protected *meso*-DAP precursor, **18**, while maintaining both chiral centers. Compound **18** was converted to the orthogonally protected *meso*-diaminopimelic acid **19**, which led to the tripeptide (L-Ala-γ-D-Glu-*meso*-DAP) and the tetrapeptide (L-Ala-γ-D-Glu-*meso*-DAP-D-Ala). The condensation of the carboxy group in the muramic acid unit of the glycan parts and the amine group of the peptide moieties and then subsequent a deprotection reaction gave the target compounds **1**−**8** successfully.

The synthesis of GlcNAc-(β1−4)-(anh)MurNAc-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala-D-Ala was also reported by the Mobashery group for the analysis of enzymatic function in the peptidoglycan recycling system of bacteria.[40]

■ HUMAN NOD1 STIMULATORY ACTIVITIES OF PGN FRAGMENTS (1−8)

The human Nod1 stimulating activity of each synthetic peptidoglycan fragment **1**−**8** (Figure 6) was evaluated by a HEK293T bioassay, using HEK293T cells transfected with expression plasmids of Nod1, as previously described (Figure 7).[9,44] First,

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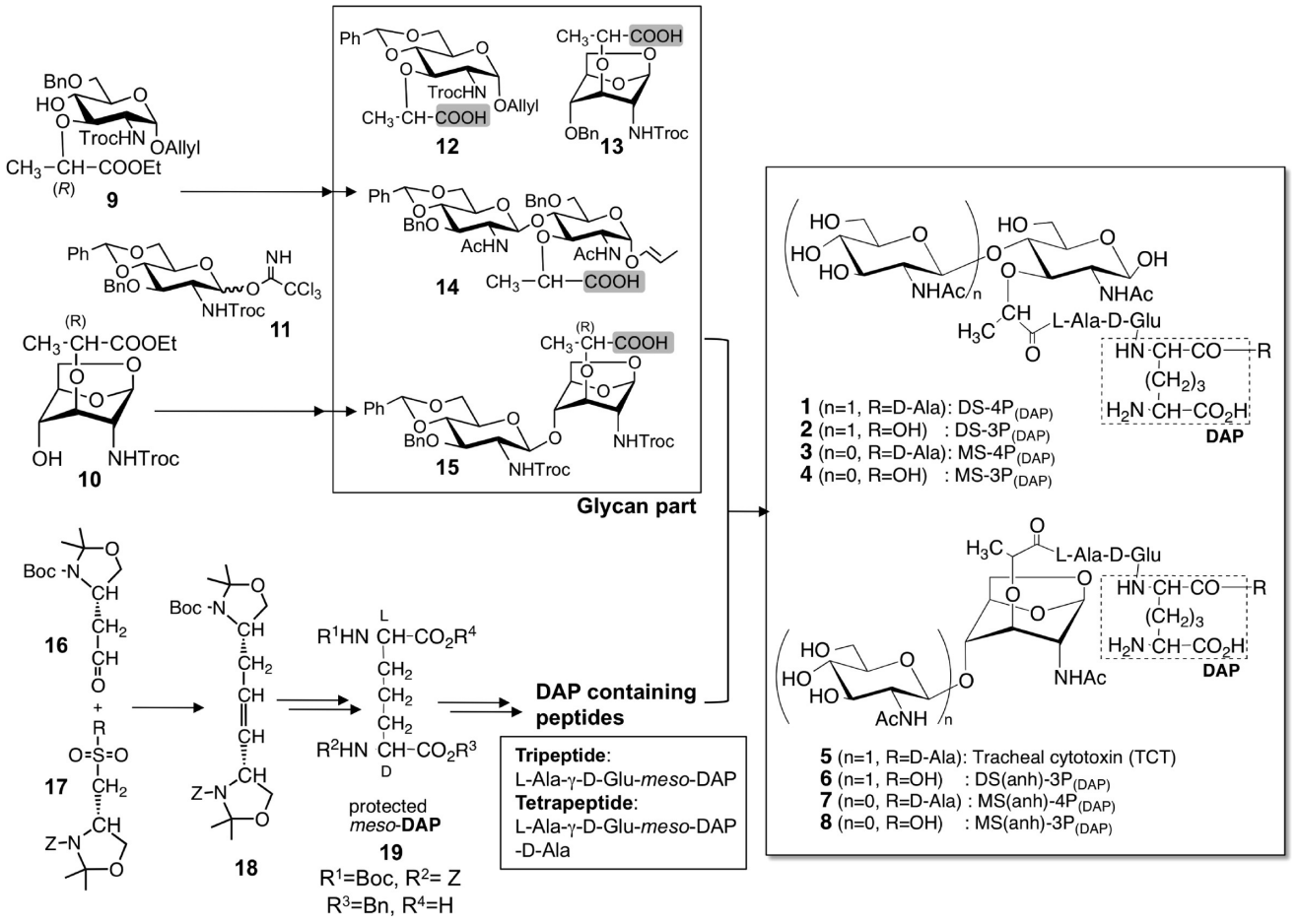

Figure 6. Synthesis of DAP-type peptidoglycan fragment compounds.[44].

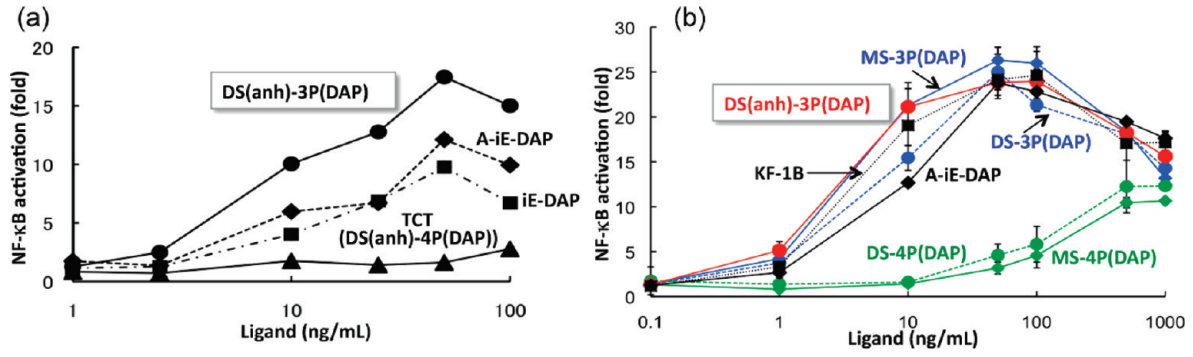

Figure 7. Stimulation of hNod1 by synthesized PGN fragments.[44] (a) iE-DAP (γ-D-glutamyldiaminopimelic acid), A-iE-DAP (L-alanyl-γ-D-glutamyldiaminopimelic acid), **5** (TCT), and **6** (DS(anh)-3P$_{DAP}$). (b) **1**−**5**. HEK293T cells were transfected with human-Nod1, the indicated amount of each compound was added to the cells, and the ability of each compound to activate NF-κB was determined by a luciferase reporter assay.[33]

as shown in Figure 7a, the anhydromuramic acid [(anh)-MurNAc]-containing compounds, **5** (TCT) and **6** (DS(anh)-3P$_{(DAP)}$), were examined in comparison with Nod1 ligands having shorter peptide chains, A-iE-DAP (L-alanyl-γ-D-glutaminyldiaminopimelic acid)[15] and iE-DAP (γ-D-glutaminyldiaminopimelic acid)[9] (Figure 5). In these compounds, **5** (TCT) showed only very weak human-Nod1 stimulatory activity. The results are consistent with a report using TCT from a natural source.[45] On the other hand, **6** (DS(anh)-3P$_{(DAP)}$) showed much higher activity than the other compounds including A-iE-DAP, iE-DAP, and TCT. The results confirmed the definite, strong hNod1 stimulatory activity of **6** (DS(anh)-3P$_{(DAP)}$), which is obtained from the active fraction of the *E. coli* supernatant. These results also demonstrated that a D-alanine residue adjacent to DAP strongly affected recognition by human Nod1. Having a free carboxyl group at the C-2 position of DAP is therefore favorable for the recognition with hNod1.

Nod1 stimulatory activities of the PGN fragments, **1** (DS-4P$_{(DAP)}$), **2** (DS-3P$_{(DAP)}$), **3** (MS-4P$_{(DAP)}$), and **4** (MS-3P$_{(DAP)}$), were also observed (Figure 7b),[44] in comparison with the anhydro-MurNAc-containing **6** (DS(anh)-3P$_{(DAP)}$), A-iE-DAP, and a known potent ligand, C14-iE-DAP (KF1B;

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N-myristoyl-iE-DAP).[15] These results also showed similar tendencies of the activity determined by a peptide structure. The tripeptide compounds (**2** and **4**) showed more potent human Nod1 stimulation than the tetrapeptide compounds (**1** and **3**). These results suggest that the substitution of the N-terminus of iE-DAP is necessary for stronger Nod1 recognition, but the structure of the substituent seems not to be strictly recognized. The importance of the carboxyl group at the C-2 position of DAP for hNod1 stimulation is also shown by these results. In previously reported results, **3** (MS-4P$_{(DAP)}$) and **4** (MS-3P$_{(DAP)}$) showed similar activities for human Nod1 stimulation at a higher concentration (5 mM),[77] and in Figure 7b, at a high concentration up to 1000 ng/mL (1.4−1.6 mM), **3** (MS-4P$_{(DAP)}$) and **4** (MS-3P$_{(DAP)}$) showed similar stimulatory activities. However, detailed hNod1 stimulatory activities were observed at relatively lower doses from 0.1 to 100 ng/mL, and at these concentrations, among the PGN fragments, **4** (MS-3P$_{(DAP)}$) showed the most potent activity and much stronger stimulation than **3** (MS-4P$_{(DAP)}$). The hNod1 activation of **4** (MS-3P$_{(DAP)}$) was similarly potent to those of **6** (DS(anh)-3P$_{(DAP)}$) and C14-iE-DAP (KF1B).

The hNod1 stimulatory activities of the chemically synthesized DAP-type PGN fragments clearly showed characteristic biological effects depending on compound structure. The results are also consistent with the activities of the isolated compounds from natural bacterial supernatants.

CONCLUSION

Bacteria of the DAP-type PGN release Nod1 ligands into the environment, with the structure of hNod1 ligands in the *E. coli* K-12 culture supernatant elucidated as DS(anh)-3P(DAP). At the same time, other PGN fragments also have been observed. Their structures were determined using the DEAC-labeling method, which enhances the sensitivity of mass spectrometry analysis. DAP-type fragment structures were also synthesized chemically, including the first total synthesis of repeating units of *E. coli* PGN and TCT. Synthesis was achieved based on a new method for the preparation of orthogonally protected *meso*-DAP and also as a result of an efficient β-selective glycosylation. The synthesized compounds were compared to the naturally obtained structures for structure elucidation. It was also demonstrated that the DS(anh)-3P(DAP) structure is one of the most hNod1 stimulatory compounds among the PGN fragments and as potent as other glycopeptides having a tripeptide (L-Ala-γ-D-Glu-*meso*-DAP) fragment, as shown by the results of hNod1 stimulatory activities of the structurally defined chemically synthesized compounds. These findings of stable hNod1 ligand structures in the environment will lead to a better understanding of the key molecules to affect and modulate the human immune system.

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DEDICATION

Dedicated to Dr. Koji Nakanishi of Columbia University for his pioneering work on bioactive natural products.

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