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Synergistic effect of CMP/KDO synthase inhibitors with antimicrobial agents on inhibition of production and release of vero toxin by enterohaemorrhagic *Escherichia coli* O157:H7

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Abstract—Synergistic effect of CMP/KDO synthase inhibitors in LPS biosynthesis of Gram-negative bacteria with kanamycin (KM) and fosfomycin (FOM) on the production and release of Vero toxins (VTs) by *Escherichia coli* O157 was evaluated in vitro. While CMP/KDO synthase inhibitors, KM and FOM showed no inhibitory effect on the production/release of VTs by themselves alone, both KM and FOM showed the remarkable inhibition of VT2 release through synergistic collaboration with CMP:KDO synthase inhibitor.

The rapid emergence of drug-resistant bacteria necessitates new and innovative antibiotics that act via inhibition of novel antibacterial targets. Of these targets, the biosynthesis of lipopolysaccharide (LPS) is attractive because LPS is characteristic of Gram-negative bacteria and believed to require for growth, virulence and drug resistance.[1] However, no inhibitor of any enzyme in the LPS pathway has yet been developed into a clinically useful drug. LPS consists of three covalently linked regions, that is lipid A, core oligosaccharide, and *O*-antigen. LPS formation requires 3-deoxy-D-*manno*-2-octulopyranosonic acid (KDO, **1**) which links lipid A with core oligosaccharide.[2] KDO (**1**) is activated by CMP/KDO synthase which reacts **1** with CTP to yield CMP/KDO (**2**), and the resulting **2** is the substrate for a series of CMP/KDO transferases able to incorporate **1** into LPS[3] (Fig. 1). Nucleotide-sugar CMP/KDO formation is the rate-determining step in LPS biosynthesis.[4] Two pioneering works on a new family of antibacterial agents acting on LPS biosynthesis by inhibition against CMP/KDO synthase were presented in 1987.[5] Dipeptide derivatives of α-*C*-(1,5-anhydro-7-amino-2,7-dideoxy-D-*manno*-heptopyranosyl)carboxylate (**3**), a specific inhibitor of CMP/KDO synthase ($K_i = 4\,\mu M$) are rationally designed for antibacterial action required an intact oligopeptide permease system of the cytoplasmic membrane and specific intracellular aminopeptidase activity to release an inhibitor from the peptide prodrug. Of these dipeptide prodrugs, alanyl-alanyl, norvalylalanyl and arginylnorvalyl derivatives (**4**, **5** and **6**) exhibit good antibacterial activity in vitro against the *Salmonella* and *Escherichia coli* strains.[5,6] It is also revealed that virulent strains of *Salmonella typhimurium* become avirulent by inhibition of CMP/KDO synthase which stops LPS synthesis, accumulates lipid A precursor in the outer membrane and causes structural perturbations.[7] On the other hand, vero toxins (VTs) produced by enterohaemorrhagic *E. coli* (EHEC) O157:H7 are responsible for the life-threatening complications of haemorrhagic colitis or haemolytic uremic syndrome (HUS) which makes EHEC infection a public health problem of serious concern.[8] There are evidences that treatment with antimicrobial agents is a risk factor for HUS during an infection of EHEC.[9,10] It could be because *E. coli* O157:H7 is killed, or VTs are released, or because the agent increase the production of VTs. Some antibiotics appear to be contraindicated in the treatment of EHEC infection. Under these investigations, we are interested in studying if inhibition of LPS biosynthesis influences secretion of pathogenic substances like exotoxins from Gram-negative bacterial cell-wall. We examined the known dipeptide prodrugs (**4** and **5**) and some derivatives as an initial probe of the profile of structure–inhibitory activity in pathogen secretion of this class of CMP/KDO synthase inhibitor. We here communicate the synergistic effect of these

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dipeptide prodrugs with antibiotics (fosfomycin and kanamycin) on the secretory inhibition of Vero toxins (VT1 and VT2)[11] of EHEC O157:H7.

The dipeptide prodrugs, alanylalanyl, norvalylalanyl and unknown arginylalanyl derivatives (**4**, **5** and **7**) were obtained by coupling of ethyl 8-amino-2,6-anhydro-3,8-dideoxy-D-*glycero*-D-*talo*-octonate (**8**) easily available from KDO with the corresponding Cbz-protected L,L-dipeptide succinimido ester (THF/MeOH) followed by removal of the protecting groups (LiOH, THF/H_2O; Pd/C, H_2, H_2O) according to the procedure of Claesson et al. (Fig. 2).[6] Peptide drugs are known to show often nofevorable effect in vivo, due to rapid degradation by action of mammalian peptidases. Therefore, those dipeptide prodrugs were further modified as an attempt to improve stability by the known N-terminal sarcosylation.[12] Thus, N-terminal sarcosyl analogues (**9**, **10** and **11**) were prepared by coupling of **8** with the corresponding Cbz-protected sarcosyl-L,L-dipeptide succinimido ester (THF/MeOH) and subsequent deprotections (LiOH, THF/H_2O; Pd/C, H_2, H_2O) by the method of Atherton et al. (Fig. 2).[12]

Antimicrobial activities of these compounds (**4**, **5**, **7**, **9**, **10** and **11**) against non-pathogenic *E. coli* ATCC11303 ($VT1^-$, $VT2^-$) and EHEC O157:H7 ($VT1^+$, $VT2^+$)[13] were evaluated in peptide-free defined medium, glucose minimum medium.[14] The bacteria were grown in a medium deficient in peptides since the previous reports showed interference with the uptake of peptide antimicrobials.[6,12,15] The effect of the synthesized compounds (**4** and **5**) fosfomycin (FOM) and kanamycin (KM) on extracellular release of VTs was investigated using strain EHEC O157:H7 (10^8 cells/mL) which was grown in 1 mL of glucose minimum medium containing various concentrations of the compounds for 4 h. Number of viable cells were counted and the amounts of VT1 and VT2 released in the supernatants were measured with reverse passive latex agglutination (RPLA) using serially diluted sterilized culture supernatants.[16] In case of evaluation of the synergistic effect of **4** with FOM and KM on extracellular release of VTs, we examined first the dose of cytostatic stationary phase of **4** and the influence on extracellular release of VTs at this dose (Fig. 7). As shown in Figure. 7, **4** has no influence on extracellular release of VTs at the dose (1 mg/mL) of cytostatic stationary phase. Therefore, the synergistic effect of **4** with FOM and KM on extracellular release of VTs was studied in glucose minimum medium containing of **4** (1 mg/mL) and various concentrations of FOM and KM.[17] Of the synthesized compounds, alanylalanyl and norvalylalanyl derivatives (**4** and **5**) showed antibacterial activities, which are more potent against ATCC11303 than against EHEC O157:H7, shown in Figure 3. However, arginylalanyl derivative **7** and their N-terminal sarcosyl analogues (**9**, **10** and **11**) did not affect the bacterial growth (data not shown). These results suggest that the peptide linkages used in **7**, **9**, **10** and **11** could not produce a compound resembling a natural substrate for the bacterial peptide permeases[18] and/or the bacterial intracellular aminopeptidases.[18a,19] In the evaluation of the extracellular release of VT1 and VT2 by EHEC O157:H7, compounds **4** and **5** showed no inhibitory effect shown in Figure 4. In the case of KM, KM killed the bacteria efficiently, and release of VT1 and VT2 were also observed in the supernatant shown in Figure 5. FOM caused a marked increase of release and production of VT1 and VT2,[10] as revealed by an immunological toxin assay (RPLA) shown in Figure 6. However, as shown in Figures 8 and 9, it is noteworthy that both FOM and KM showed the remarkable inhibition of VT2 release through synergistic collaboration with compound **4**. Antibacterial activity of FOM and KM was also shown to be synergistically improved in combination with compound **4**. At the present stage, the reason for difference in production and release between VT1 and VT2

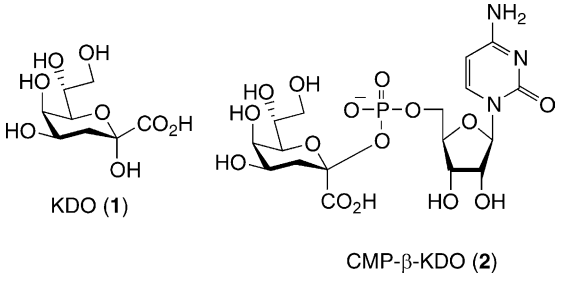
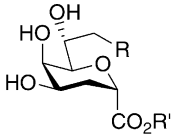


Figure 1. Structures of KDO and CMP-β-KDO.



3: R=NH_2, R'=H

4: R= -NH-L-Ala-L-Ala, R'=H

5: R= -NH-L-Nva-L-Ala, R'=H

6: R= -NH-L-Nva-L-Arg, R'=H

7: R= -NH-L-Arg-L-Ala, R'=H

8: R=NH_2, R'=CH_2CH_3

9: R= -NH-L-Ala-L-Ala-Sar, R'=H

10: R= -NH-L-Nva-L-Ala-Sar, R'=H

11: R= -NH-L-Arg-L-Ala-Sar, R'=H

Figure 2. Structures of CMP/KDO synthase inhibitors.

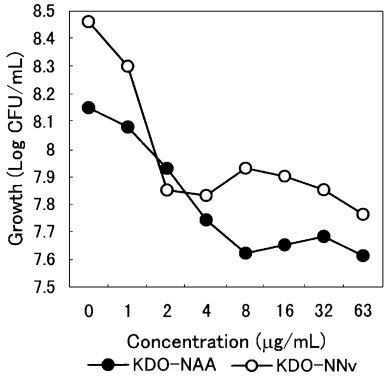


Figure 3. Antibacterial activity of KDO-NAA **4** and KDO-NNvA **5** against *E. coli* ATCC11303.

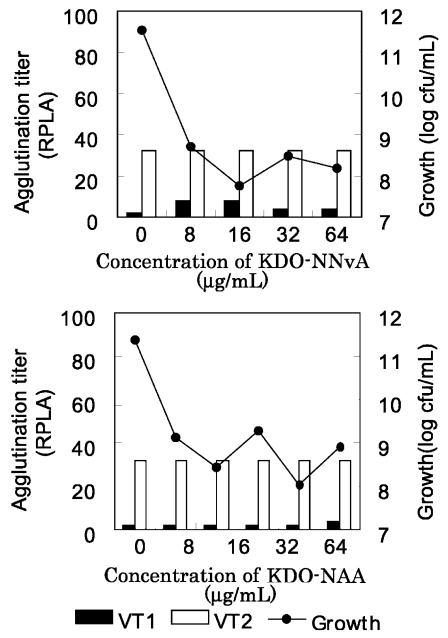


Figure 4. Effect of KDO-NAA **4** and KDO-NNvA **5** on extracellular VT1 and VT2. Closed bar: concentration of VT1 in supernatant, open bar; concentration of VT2 in supernatant; closed circle: decrease of CFU of *E. coli* O157:H7.

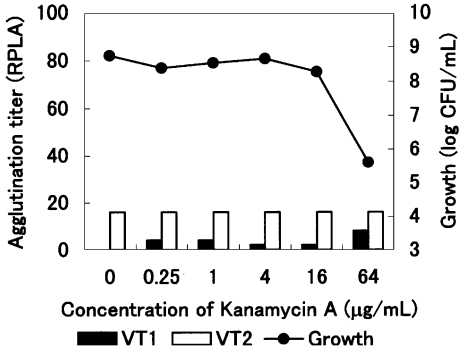


Figure 5. Effect of kanamycin (KM) on extracellelar VT1 and VT2. Closed bar: concentration of VT1 in supernatant; open bar: concentration of VT2 in supernatant; closed circle: decrease of CFU of *E. coli* O157:H7.

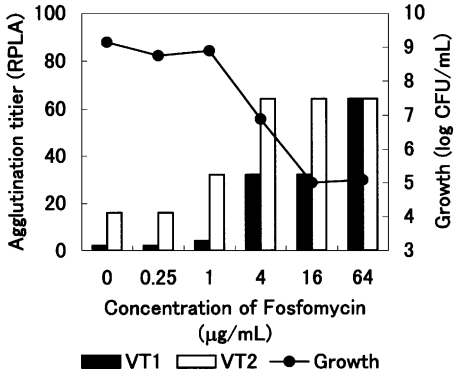


Figure 6. Effect of fosfomycin (FOM) on extracellelar VT1 and VT2. Closed bar: concentration of VT1 in supernatant; open bar: concentration of VT2 in supernatant; closed circle: decrease of CFU of *E. coli* O157:H7.

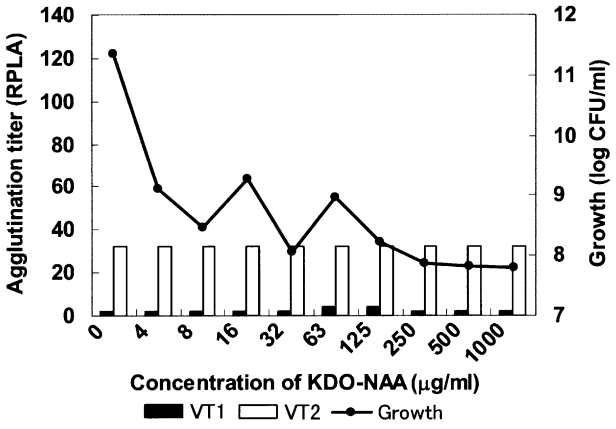


Figure 7. Effect of KDO-NAA **4** on extracellular VT1 and VT2 at high dose. Closed bar: concentration of VT1 in supernatant; open bar: concentration of VT2 in supernatant; closed circle: decrease of CFU of *E. coli* O157:H7.

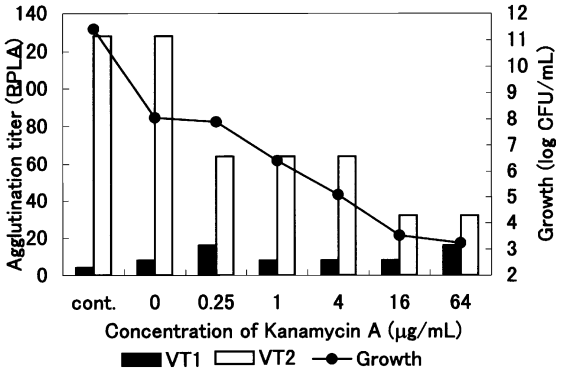


Figure 8. Synergistic effect of KDO-NAA **4** with kanamycin (KM) on extracellular VT1 and VT2. Closed bar: concentration of VT1 in supernatant; open bar: concentration of VT2 in supernatant; closed circle: decrease of CFU of *E. coli* O157:H7.

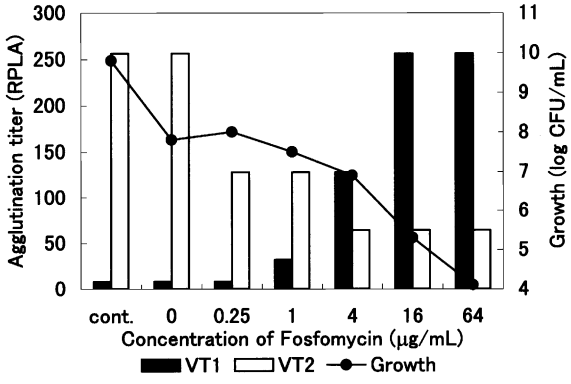


Figure 9. Synergistic effect of KDO-NAA **4** with fosfomycin (FOM) on extracellular VT1 and VT2. Closed bar: concentration of VT1 in supernatant; open bar: concentration of VT2 in supernatant; closed circle: decrease of CFU of *E. coli* O157:H7.

is unclear. As reported previously,[20] it has been proved that VT1 and VT2 are apparently different in both the secretion systems and the gene regulation systems. On the other hand, the biological activities of VT1 and VT2 have been reported to be different. VT2 was 1000-fold more active on human renal endotherial cells than VT1 was,[21] and VT2 also had a 400-fold higher 50% lethal dose for mice than compared to VT1.[22] These results and facts

seem to suggest that combination therapy of the CMP/KDO synthase inhibitor such as **4** in LPS biosynthesis with both the inhibitor of cell wall biosynthesis like as FOM and the inhibitor of protein synthesis like as KM could be one of the safe chemotherapy not causing the release of VT2 from the cells and thus preventing development of haemolytic uremic syndrome (HUS). That **4** shows potent synergistic effect on inhibition of production and/or release of Vero toxins by EHEC O157 with antimicrobial agents further supports the hypothesis of the design of the new CMP/KDO synthase inhibitor.

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13. Bacterial strains. A verotoxin producing strain *E. coli* O-157:H7 ($VT1^+$, $VT2^+$) was provided by Dr. K. Hotta, National Institute of Infectious Diseases Japan. *E. coli* B ATCC 11303 ($VT1^-$, $VT2^-$) ATCC 11303 was obtained from ATCC culture collection.
14. Effect of antibacterial agents for *E. coli* growth. Preculture broth was prepared from stock slant by inoculating one loopful of growth into 100 mL of glucose minimum medium, glucose; 0.5%, NH_4Cl; 0.2%, Na_2HPO_4; 0.066%, Na_2SO_4; 0.035%, KCl; 0.2%, $MgCl_2{\cdot}6H_2O$; 0.05% and 0.1 M Tris–HCl (pH 7.4), and then incubated at 37 °C overnight under static condition. The culture was then diluted with fresh medium to give an initial optical density at 500 nm (OD_{500}) of 0.37 (approximately 10^8 cells/mL). The culture (1 mL) was incubated with the antibacterial agent in various concentration at 37 °C for 4 h (*E. coli* O-157:H7) and 6 h (*E. coli* B ATCC 11303) under static condition. Viable cell numbers were estimated from spread plates of serial dilutions on standard method agar (Nissui) at 37 °C for 24 h.
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16. Determination of verotoxin (VT1 and VT2). After 4 h culture at 37 °C, the culture was centrifuged (10,000*g*, 15 min) to remove debris, and VT1 and VT2 agglutination titer were determined using a reversed passive latex agglutination (RPLA) kit (Denka Seiken Co., Tokyo) in accordance with the procedure described in the manufacturer's guidance.
17. Synergism test. KDO-NAA (1 mg/mL) was mixed with several concentration of kanamycin and fosfomycin, and the mixture was cultured with *E. coli* O-157:H7 at 37 °C for 4 h. Viable cell counts and agglutination titer of verotoxins were determined as described above.
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