Supporting information

Appendix S1

The inhibitory-effect of dMβCD on the anti-*H. pylori* action of progesterones

Materials and Methods

Progesterone (PS) and its synthetic derivative, 17α-hydroxyprogesterone caproate (17αPSCE), were dissolved in dimethyl sulfoxide (DMSO), and added into PPLO broth (3 ml) at concentrations of 50 and 10 μM, respectively. The 0.1% concentration of DMSO used in the experiments did not affect the viability of *H. pylori*. The *H. pylori* ($10^{5.3}$ CFU/ml) was cultured for 24 h in the presence or absence of dMβCD at concentrations of 0.03 and 0.3 mM using the PPLO broth containing either PS (50 μM) or 17αPSCE (10 μM), with continuous shaking under microaerobic conditions in the dark. Upon completion of the cultures, the CFUs were determined by the conventional method (Hosoda et al., 2011). The results are indicated as the mean CFU ± SD obtained from three independent experiments.

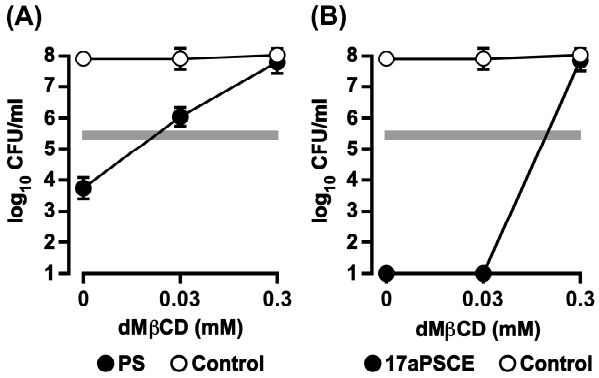

Fig. S1. Inhibitory effect of dMβCD on the anti-*H. pylori* actions of PS and 17αPSCE

The gray belts in the graphs indicate the baseline CFUs before the cultures.

Results

The anti-*H. pylori* action of PS (50 μM) was inhibited by dMβCD: the CFUs were higher than the pre-culture baseline CFU ($10^{5.3}$ CFU/ml) when *H. pylori* was cultured in the presence of 0.03 mM dMβCD and lower than the baseline CFU when *H. pylori* was cultured in the absence of dMβCD (Fig. S1A). In addition, the anti-*H. pylori* actions of PS (50 μM) and 17αPSCE (10 μM) were completely inhibited in the *H. pylori* cultured in the presence of 0.3 mM dMβCD: the CFUs at that time were comparable to the control CFU (10^{8} CFU/ml) of the *H. pylori* cultured without PS or 17αPSCE (Fig. S1A and B). These results indicate that dMβCD somehow prevents *H. pylori* cells from interacting with PS or 17αPSCE.

Appendix S2

Determination of dMβCD concentrations necessary to dissociate cholesterols and 3β-OH steroids from paper disks

Materials and Methods

The dMβCD concentration used in this study was determined by the following experiment. FC (100 nmol) dissolved in chloroform (20 μl) was dotted onto paper disks (8 mm diameter, 1 mm thickness) from Tokyo Roshi Kaisha Ltd. (Tokyo, Japan). After vaporization of the chloroform solvent, each FC-dotted paper disk was soaked in a 50 mM Tris (pH 7.5) buffer (2 ml) in a single well of a 12-well cell culture plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and incubated with dMβCD at 25˚C for

4 h, with shaking (100 rpm/min). The FC-dotted paper disks incubated with dMβCD at concentrations from 1 to 5 mM were then washed 6 times with distilled water (100 rpm/min, 10 min), dried with a centrifugal concentrator (Tomy Seiko Co., Ltd., Tokyo, Japan), and soaked in acetic acid (600 µl) to elute the FC remaining in the paper disk. A ferrous chloride-sulfuric acid reagent [phosphoric acid-sulfuric acid (2:25) solution containing 0.2% $FeCl_2$-$6H_2O$] (400 µl) was added into the acetic acid solution and vigorously stirred, and the reaction solution was incubated for 15 min at room temperature. After color reaction and cooling, the absorbance of acetic acid solution (200 µl) with ferrous chloride-sulfuric acid reagent was measured using a Versa max microplate reader (Molecular Devices Co, CA) at a wavelength of $A_{550\,nm}$. The FC remaining in the paper disk was quantified based on a regression line (y axis, $A_{550\,nm}$; x axis, FC amount) calculated using FC standard solutions. Results are indicated as the mean FC (nmol) ± SD obtained from three independent experiments. The experiments similar to that with FC were conducted on paper disks dotted with a 50 nmol of cholesterol ester (cholesterol hexanoate: CE), and with a 100 nmol of pregnenolone (PN) or dehydroepiandrosterone (dEA). After the measurement of $A_{550\,nm}$, the CE, PN, and dEA remaining in the paper disks were quantified based on the respective regression lines calculated using the CE, PN, and dEA standard solutions. The results are indicated as the mean CE (nmol) ± SD, the mean PN (nmol) ± SD, and the mean dEA (nmol) ± SD, obtained from three independent experiments.

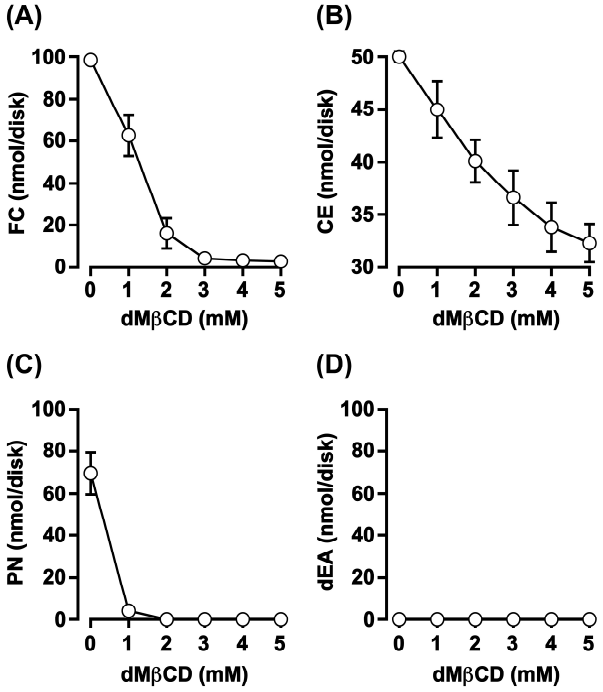

Fig. S2. Effect of dMβCD in eluting FC, CE, PN, and dEA from the paper disks

Results

FC was hardly eluted from the paper disk incubated without dMβCD: the FC amount (approximately 98 nmol) remaining in the paper disk after incubation was comparable to the FC amount (100 nmol) dotted onto the paper disk before the incubation. FC was efficiently eluted from the paper disks incubated in the presence of dMβCD, especially at the higher dMβCD concentrations: the FC amount remaining in the paper disk incubated in the presence of 5 mM dMβCD concentration was below the detection limits. This indicates that FC dissociates from the paper disk by forming an inclusion complex with dMβCD, and that the 5 mM dMβCD concentration is necessary to elute FC from the paper disk completely (Fig. S2A). In contrast, CE (50 nmol) was eluted from the paper disk incompletely: about 32 nmol of CE was retained in the paper disk even in the presence of the 5 mM

dMβCD (Fig. S2B). Approximately 30 nmol of PN was spontaneously dissociated from the paper disk even in the absence of dMβCD, and the complete elution of PN (100 nmol) from the paper disk was observed in the presence of dMβCD at the concentration of 2 mM (Fig. S2C). Surprisingly, dEA (100 nmol) was completely dissociated from the paper disk even in the absence of dMβCD (Fig. S2D). Though the elution of CE from the paper disk was incomplete in the presence of the 5 mM dMβCD, approximately 20 nmol CE was barely eluted from the paper disk dotted with a 50 nmol CE in the presence of the 5 mM dMβCD, and this dMβCD concentration was sufficient to elute the FC (100 nmol) and the 3β-OH steroids, namely PN (100 nmol) and dEA (100 nmol), from the paper disk. On this basis, we decided that the 5 mM dMβCD concentration was appropriate for use in this study.

Appendix S3

Distribution of dMβCD via the chloroform-methanol-water solvent system

Materials and Methods

After dMβCD (10 nmol) was dissolved in chloroform-methanol (2:1) solvent (800 µl), distilled water (160 µl) was added into the dMβCD solution and vigorously mixed. The water phase and chloroform phase separated via the above-described organic solvent distribution method were recovered as two fractions and dried with a centrifugal concentrator (Tomy Seiko Co., Ltd.). After replacing the solvent with 50 mM Tris (pH 7.5) buffer (100 µl), adding a 5% phenol solution (100 µl) into each fraction from the water phase and chloroform phase, and mixing vigorously, the color reaction in the phenol mixed-solution was induced by vigorous mixing with a sulfuric acid solution (500 µl). After 20 min, the absorbance ($A_{490\ nm}$) of the reaction solution (200 µl) was measured at a wavelength of 490 nm (Versa max microplate reader) to quantify the dMβCD in each fraction from the water phase and chloroform phase. The amounts of dMβCD were calculated based on a dMβCD standard curve, and indicated as the mean dMβCD (nmol) ± SD obtained from three independent experiments. Meanwhile, a thin-layer chromatography (TLC) analysis was performed on the dMβCD profiles in fractions of the water phase and chloroform phase. Chloroform-methanol (2:1) solution (40 µl) was added into each dried fraction from the water phase and chloroform phase, and dotted onto a silica gel G60 plate (Merck, Darmstadt, Germany). After TLC by the chloroform-methanol-water (70:30:5) solvent system, the silica gel G60 plate was sprayed with a 60% sulfuric acid solution and heated at 180˚C to visualize the dMβCD spotted on the plate surface.

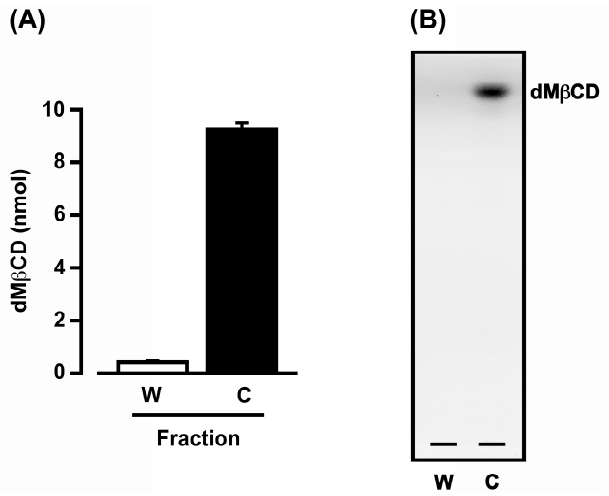

Fig. S3. Levels of dMβCD detected in the water phase and chloroform phase fractions

W, water phase fraction; C, chloroform phase fraction

Results

Almost all of the dMβCD (about 9.3 nmol) was distributed into the chloroform phase fraction when 10 nmol dMβCD was treated with the chloroform-methanol-water (10:5:3) solvent (Fig. S3A). In contrast, the water phase fraction contained only negligible amounts (about 0.4 nmol) of dMβCD. In addition, the TLC analysis detected the dMβCD spot (R_f: 0.94) at a high density in the chloroform phase fraction, but not in the water phase fraction (Fig. S3B). These results indicate that dMβCD is recovered at the chloroform phase separated via the organic solvent distribution method used in this study.

Appendix S4

Binding of the dMβCD molecule itself to *H. pylori* PE

Materials and Methods

PE (300 μg)-fixed paper disks were incubated with FC (100 nmol)-fixed paper disks or FC-free paper disks in the presence of dMβCD (5 mM) in a 50 mM Tris (pH 7.5) buffer (2 ml) for 4 h at 25˚C, with shaking (100 rpm/min). After the incubations, the dMβCD in the PE-fixed paper disks was quantified by the same method described in the section on the "Assay of binding of cholesterols, 3β-OH steroids, and dMβCD to PE and PG-CL" in the Materials and Methods. The dMβCD amounts were indicated as the mean dMβCD (nmol) ± SD obtained from three independent experiments.

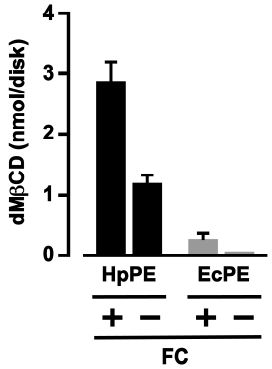

Fig. S4. Binding of the FC-dMβCD inclusion complex and dMβCD to *H. pylori* PE

Results

H. pylori PE (HpPE) clearly interacted with the dMβCD molecule even in the absence of FC, although less dMβCD bound to HpPE in the absence of FC than in the presence of FC (Fig. S4). In contrast, *E. coli* PE (EcPE) did not bind dMβCD in the absence of FC, and the binding of dMβCD to EcPE was negligible even in the presence of FC. These results indicate that *H. pylori* PE has sufficient potency to bind not only FC-dMβCD inclusion complex, but also the dMβCD molecule, without FC.

References

Hosoda K, Shimomura H, Hayashi S, Yokota K, Hirai Y. 2011. Steroid hormones as bactericidal agents to *Helicobacter pylori*. FEMS Microbiol. Lett. **318**: 68-75.