An antibiofilm vector based on a modified β-cyclodextrin: Complexation study and biological activity.

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**Materials and methods**

Solvents (HPLC quality) and reagents β-CD, ciprofloxacin and ciprofloxacin hydrochlorides were purchased from Sigma Aldrich (France, St. Louis, MO). Deuterated solvents were purchased from Euriso-top Thin layer chromatography (TLC) was performed on (silica gel 60 F254 aluminium-backed plates) and (silica gel 60 RP-18. F254s) purchased from Merck KGaA, Germany Spot detection was carried out with (ethanol/H2SO4) 92:8, nihydrine, UV light (λ=254 nm), Fluorescence light at 365 nm, Typical column chromatography on silica gel was used and chromatography on silica gel RP-18 using strata C18-E colon purchased from phenomenex. Electrospray mass spectrometry (ESI) spectra were recorded on an Esquire-LC ion-trap mass spectrometer (ITMS) equipped with an ESI source and the Esquire control 6.16 data system. The 1H NMR spectra were measured on a Bruker AC 300 spectrometer. Infrared analysis was done on spectrometer 100 FT-IR (Perkin-Elmer). Elementary analysis were realized by the laboratory of micro-analyses (IRCOF, University of Rouen, France). High–resolution mass spectra were performed an a LC-TOF Premier XE (Micromass, Manchester, UK) equipped with ESI source.

**Synthesis of β-CD derivatives**

β-Cyclodextrin was dried overnight at 100°C in vacuum prior to use. Pyridine and *N,N*-dimethylformamide (DMF) were freshly distilled from CaH2. All other reagents were of the highest available commercial quality and used without further purification.

**Heptakis (2,3-*O*-dimethyl)-β-cyclodextrin II** Compound 3 was prepared as described by [38].

**Heptakis (2,6-*O*-dimethyl)-β-cyclodextrin III** Compound 3 was prepared as described by [39].

***Permethyl mono-6-*amino*-β-cyclodextrin IV*** Compound **4** was prepared as described by [38].

***Adipic acid monomethyl Ester (a)***

Dimethyl adipate was obtained by reaction of adipic acid, (10 g, 43.45 mmol) in MeOH (34.09 ml) was added 0.75 mL of H2SO4 the mixture was refluxed over night. The solution was concentrated in vacuo. The resulting precipitate was poured on ice, and extracted with Et2O (3x40 ml) yield (11.2 g, 100%). KOH was added to the obtained compound in MeOH. Extensive washing with hexane removed the dodecanedioic acid monomethyl ester. Evaporation of the washing gave the title compound (**b**) as oil with 60% yield (4.1 g).

***Dodecanedioic acid Monomethyl Ester (b):*** The compound **b** was prepared from dodecanedioic acid following the same procedure described for compound ***a***.

***Permethyled β-cyclodextrin derivative CD1***  Compound **5** was prepared as described by [40].

***Permethyl β-cyclodextrin derivative CD2***

The compound **4** (0.33 g, 0.23 mmol) were dissolved in 40 mL of anhydrous DMF, and then reacted with DIC (519.7 µl, 3.30 mmol) and HOBt (0.5 g, 3.58 mmol) for 2 h at room temperature. A solution of adipic acid monomethyl ester (0.04 g, 0.27 mmoL) was added in 10 ml of dichloromethane with a few amount of triethylamine The mixture was stirred for 24 h at room temperature. The solvent was removed under vacuum. The residue was extracted in dichloromethane (20 mL) and washed with water (2 x 5 mL). The combined organic phase was dried in MgSO4 and evaporated under vacuum. The crude products were purified by chromatography on silica gel column eluting with DCM:MeOH, 7:3. The pure fractions were combined and then concentrated in vacuo to give the desired compound with 71% yield (0.26 mg)***.***

To a solution of obtained compound (0.2 g, 0.2 mmol) in 0.4 mL of MeOH, was added 0.1 ml of aqueous solution of KOH (1 M) and then the reaction mixture was stirred at room temperature for 1 h. The TLC showed the completely dispearation of the reagent. The reaction was stopped by removing of MeOH under vacuum. Then the residue was dissolved in 20 mL of water. The solution was then acidified by adding few ml of hydrochloric acid (1 M) at pH =2 to obtain the acidic product form. The product was extracted with (3 x 40 mL) dichloromethane; the combined organic phase was evaporated under vacuum The residue was taken up in ethanol (5 mL), filtered and the filtrate was then evaporated under vacuum and dried in vaccum at 80°C for 24 h. The desired product ***CD2*** was obtained as white solid (0.2 g, over yield 71%).

**TLC: *Rf***0.052 (DCM/MeOH:95/5); **mp** (**°C**): 138; **RMN 1H** (**300 MHz, CDCl3):** δ (ppm) 1.60-1.61 (m, 4H, (-CH2)2-CH2-COOH), 2.15-2.25 (m, 4H, -CH2-COOH), 3.13 (m, 1H, -CH- CH), 3.31 (s, 3H, OCH3), 3.34 (m, 1H, CH-CD), 3.43 (s, 3H, OCH3), 3.54 (m, 1H, CH-CD), 3.56 (s, 3H, OCH3), 3.73-3.75 (m, 2H, CH2-OCH3); **RMN 13C** (**75 MHz, CDCl3**): δ (ppm) 24.5-25.2 (-CH2-(CH2)2-COOH), 33.3 (-CH2-COOH), 36.1 (-NH-CO-CH2-COOH), 39.9 (-CH2-NH-CO-), 58.5 (-OCH3), 58.9 (-CH2-OCH3), 61.8 (-CH-OCH3), 70.8 (-CH-O-CH), 71.3 (-CH2-OCH3), 80.3 (-CH- CH-OCH3), 81.5 (-CH- CH-OCH3), 81.7 (-CH-CH-OCH3), 98.5-98.9 (-CH-O-); **ESI-MS**: calcd for C68H119NO37 1540,75. Found 1540.73 [M-H]-; **HR-MS:** TOF-MS-ES- m/z= 1540.73 [M-H]-, M=1541.75 g/mol-1.; **IR** ν(cm-1): ring vibration 946 cm-1, (C-C/C-O) 1032-1079,(C-H/O-H) ν=1297-1449,(CO-NH) 1731,(CO-OH) 1774, (CH2) 2931, (N-H) 3405.

***Permethyl β-cyclodextrin derivative CD3***

This compound was obtained as the same procedure described above. The compound **4** (1.2 g, 0.8 mmol) was added to the compound **b** (0.05 g, 51.5 mmol). The desired product ***CD3*** was obtained as white solid (0.05 g, over yield 84%).

**TLC**: ***Rf*** = 0.064 DCM/MeOH (95:5); **RMN 1H** (**300 MHz, CDCl3**): δ (ppm) 1.05-1.09 (m, 4H, -(CH2)3-(CH2)3-COOH), 1.21-2.25 (m, 4H, -(CH2)2-(CH2)2-COOH), 1.55 (m, 2H, -CH2-CH2-COOH), 2.12-2.23 (m, 4H, -CH2-COOH), 3.13 (m, 1H, -CH-OCH3), 3.32 (s, 3H, -CH-OCH3), 3.36 (m, 1H, -CH-OCH3), 3.43-3.44 (3H, s, -CH2-OCH3), 3.46 (m, 1H, -CH-OCH3), 3.54 (m, 1H, -CH-OCH3), 3.57-3.58 (s, 3H, -OCH3), 3.70-3.75 (s, 3H, -CH2-OCH3), 5.04-5.10 (m, 1H, -CH-O-CH-); **RMN 13C** (**75 MHz, CDCl3**): δ (ppm) 23.2-25.8 (-(CH2)3-(CH2)3-COOH), 24.8 -(CH2-CH2-COOH), 29.1-29.5 (-(CH2)2-(CH2)2-COOH), 34.2 (-CH2-COOH), 36.7 -(CH2-CO-NH), 39.9 (-CONH- CH2-OCH3), 58.3-58.6 (-OCH3), 58.8-59.9 (-OCH3), 61.2-61.5 (-OCH3), 71.0 (-CH- OCH3), 71.2-71.3 (-CH2-OCH3), 79.9-80.6 (-CH-OCH3), 81.3-82.2 (-CH-CH-OCH3), 98.8-99.0 (-CH-O-CH-), 173.5 (-CONH-), 177.4 (-COOH); **ESI-MS** calcd m/z = 1625, 84 [M-H]-, mass found m/z = 1625.0 [M-H]-; IR ν(cm-1): ring vibration 946 cm-1, (C-C/C-O) 1032-1079, (C-H/O-H) 1243-1436, (CO-NH) 1574, (-CO-OH) 1628; (CH2) 2849-2928, (N-H) 3329; **HR-MS** :**TOF-MS-ES**- m/z= 1625.83 [M-H]-, M=1626.83 g/mol-1.

**Synthesis Cirpofloxacin-adamantyl derivative**

***N*-Boc protection of ciprofloxacin 2**

The mixture containing ciprofloxacin (3.61 g, 8.42 mmol), Boc2O (2.03g, 9.3 mmol) and 16.9 mL du NaOH aqueous solution in THF was stirred at room temperature overnight. The product precipitated from the reaction mixture and was collected by filtration as white solid, 100% yield (3.5 g).

**TLC:** **Rf** 0.55, DCM/MeH 90:10; **1H NMR (300 MHz, CDCl3):** δ 1.19-1.20 (m, 2H), 1.36-1.43 (m, 2H), 1.49 (s, 9H), 3.27-3.30 (t, *J* = 7.13, 13.14 Hz, 4H), 3.65-3.68 (t, *J* = 4.8, 10.2 Hz, 4H), 7.34-7.36 (d, *J* = 7.11 Hz, 1H), 7.97-8.20 (d, *J* = 13.4 Hz, 1H), 8.74 (s, 1H), 14.94 (s, 1H). **13C NMR (75 MHz, CDCl3):** δ 8.2 (CH2-cyclopropyl), 28.4 (C-Boc), 35.3 (CH-cyclopropyl), 49.9 (CH2-piperazin), 104.7 (CH-Ar), 107.9 (C-Ar), 111.7 (CH-Ar), 119.9 (C-Ar), 138.8 (C-Ar), 146.4 ( C-Ar), 152.1 (C-Ar), 155.2 (C-F), 167.2 (-COOH), 177.3 (Ar-C=O), 177.3 (Ar-C=O), 177.3 (-N-C=O); **ESI-MS:** calcd for (C28H33FN4O4+Na)+: 431,19. Found: 454.0 [M+Na]+.

**7-(4-((1*S*, 3*R*, 5*S*))-adamantane-1-carbonyl) piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4 dihydroquinoline-3-carboxylic (*tert*-butyl carbonic) anhydride 2**

To a solution of the compound **2** (1.4 g, 3.3 mmol) in DMF (16.7 mL), HBTU (2.5 g, 6.6 mmol) was added followed by HOBt (0.4 g, 3.3 mmol), DIPEA (0.6 g, 5 mmol), and 1-amino adamantane (0.604 g, 4 mmol). The mixture was stirred at room temperature under N2 for 30 min, and further DIPEA (0.85 mL, 5 mmol) was added. The reaction mixture was stirred at room temperature for 1 h, poured into water, and extracted with CH2Cl2. The organic layer was washed with brine, dried over anhydrous Na2SO4, and evaporated. The crude residue was purified by column chromatography on silica gel using DCM:MeOH 5:95 as eluant to provide the compound 3 (90% yield) as yellow solid.

**TLC: *Rf*** 0.34 (DCM/MeOH 95:5); **mp (°C):** 232; **1H NMR** (**300 MHz, CDCl3**): δ (ppm) 1.14-1.16 (m, 2H), 1.28-1.31 (m, 2H), 1.48 (CH3, s, 9H), 1.65-1.71 (CH2-Ad, CH-Ad, m, 7.88), 2.09 (CH-Ad, 3H), 2.15 (CH2-Ad, m, 4.88 H), 3.20-3.24 (CH2-piperazin, m, 2H), 3.40-3.46 (CH-pentyl, m, 1H), 3.63-3.66 (CH2-piperazin, m, 2H), 7.29-7.32 (H-Ar, d, J = 7.11 Hz, 1H), 8.01-8.05 (H-Ar, d, J = 13.23 Hz, 1H), 8.80 (H-Ar, s, 1H), 9.86 (NH, s, 1H) ; **13C NMR** (**75 MHz, CDCl3**): δ (ppm) 8.0 (CH2-cyclopropyl), 28.4 ((CH3)3-C-BOC), 29.5 (Ad-CH), 34.6 (cyclopropyl-CH), 36.5 (Ad-CH2), 41.7 (Ad-CH2), 42.5 (CH2-piperazin), 49.9 (CH2-piperazin), 104.8 (CH-Ar), 112.3 (CH-Ar), 112.6 (CH-Ar), 146.4 (C-Ar). 155.2 (C-F), 154 (Boc-C=O), 163.6 (NH-C=O), 175.7 (Ar-C=O); **ESI:** calcd calcd for C32H41FN4O4 m/z = 564.31 found: 587 [M+Na]+ **; 19F NMR (282 MHZ, CDCl3):** δ (ppm) -123.33 (F-Ar), -113.36 (F-Ar); ***IR*** ν (cm-1)***:*** (C=C), 1656, (C=O) 1698, (CH2) 2231, 2906, (CH2), (N-H) 3439; **Anal. Calcd/Found**: calcd for (C27H33FN4O2): C, 69.80; H, 7.16; N, 12.06; found 62.80, 6.65, 9.77;

***HR-MS****:*TOF-MS-ES- m/z= 565.31 [M+H]+, M=¶565.31 g/mol-1.

**7-(4-((1*S*, 3*R*, 5*S*)-adamantane-1-carbonyl) piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4 dihydroquinoline-3-carboxylic acid 4**.

Cleavage of Boc group was achieved by treatment of conjugate **3** (4.3 g, 0.7 mmol) with 10% TFA solution in dichloromethane (19.7 mL). Complete reaction was confirmed by TLC. The compound was extracted with 25 mL of AcOEt and organic phase was then collected and evaporated under vacuum. The resulting residue was washed three times with 200 mL of hexane, dissolved in minimum volume of methanol, precipitate by addition of cold diethyl ether and kept at -20°C overnight. The solid precipitate was obtained by decantation after solvent removing by high vacuum. The residue was recovered in 200 mL of water and the pH was then adjusted to 9 by dropwise adding of aqueous 1 M NaOH solution. The desired product was precipitated and extracted by DCM to eliminate the trifluoroacetic acid sodium salt. The organic layer was washed with brine, dried over anhydrous Na2SO4 and evaporated to dryness.

***TLC***: ***Rf*** 0.27 (DCM/MeOH 95:5); ***mp (°C):*** 232; ***1H NMR*** (***300 MHz, CDCl3***): δ 1.1-1.14 (Bd, 2H), 1.24-1.28 (Bd, 2H), 1.67 (CH2, s, 6H), 2.06 (CH, s, 3H), 2.11 (CH2, s, 6H), 3.06-3.09 (CH2, m, 4H), 3.22-3.26 (CH2, m, 4H), 3.40-3.45 (CH, m, 1H), 7.26-7.28 (CH, d, *J* = 7.5 Hz, 1H), 7.92-7.96 (CH, d, *J* = 13.15 Hz, 1H), 8.74 (CH, s, 1H), 9.86 (NH, s, 1H); ***13C NMR*** (***300 MHz, CDCl3***): δ 8.0 (CH2), 28.5 (Ad-CH), 33.7 (cyclopropyl-CH), 35.5 (Ad-CH2), 40.7 (Ad-CH2), 44.7.

(piperazin-CH2), 49.8 (piperazin-CH2), 103.58-103.62 (CH-Ar), 112.2 (CH-Ar), 112.5 (CH-Ar), 121.8 (C-Ar), 138.2 (C-Ar), 145.1 (CH-Ar), 151.4 (C-Ar), 155.2 ( C-F), 163.6 (NH-C=O), 175.7 (Ar-C=O), ***19F (400MHZ, CDCL3***): δ 123.36 (s, 1F); ***ESI-MS***: calcd for (C27H33FN4O2+H)+: 465.30; found: 465.40 [M+H]+; ***HR-MS***: TOF-MS-ES- m/z= 464.26 [M+H]+, M=¶465.26 g/mol-1; ***IR*** ν (cm-1):(C=C), 1625, (C=O) 1297-1449, (CH2) 2349, 2844, 2908, (CH2) 2931, (N-H) 3446.; **Anal. Calcd/Found**: calcd for (C27H33FN4O2): C, 69.80; H, 7.16; N, 12.06; found 62.80, 6.65, 9.77.

***Isothermal Titration Calorimetry (ITC)***

Calorimetric studies were performed using the Nano ITC 2G Isothermal Titration Microcalorimeter from TA instruments (New Castle, DE, USA). This power compensation differential instrument has a reference cell and a sample cell of approximately 1.040 mL. Each experiment involved 25 injections of 10 µL of β-CD or derivatives into the thermostated sample cell containing solution of CIP-Ad. The reference cell was filled with degassed MilliQ-water. The time interval between two consecutive injections was fixed to 300 s and the agitation speed (via the paddle stirrer at the end of the syringe) at 250 rpm. Solutions of βCD or derivatives (4mM) and CIP-Ad (0.3mM) were prepared in chloroacetate buffer (pH = 3, 0.05 M) and filtered through 0.2 μm cellulose acetate membrane (Millipor®, France) before use. Experiments were realized at atmospheric pressure and at 310°K. For all experiments, corrections have be made to the heat data to account for heat effects associated with titrant dilution and any temperature difference between titrant and titrate solutions. These corrections were made by performing a blank titration experiment and subtracting the blank heat data from the experimental thermogram. The blank titration was carried out by introducing βCD or derivatives (4mM) in the sample cell containing buffer alone (without CIP-Ad), according the same experimental conditions (volume, agitation speed, temperature). All experiments were reproduced at least two times. The binding isotherms (without the first data point) were analyzed using an independent binding model of NanoAnalyse software (which models an interaction of “n” ligands with a macromolecule that has one binding site) to estimate the thermodynamic parameters of interaction such as the apparent binding stoichiometry (n), association constant (Ka) and enthalpy of binding (ΔH). Then, since temperature is held constant throughout, the free Gibbs energy (ΔG) of the binding reaction was determined by ΔG=-RTlnKa. Consequently, the entropy change (ΔS) was determined by ΔS= (ΔH-ΔG)/T.

**NMR Study**

***Preparation of samples for NMR analysis***

The CIP-Ad sample was prepared by dissolving an excess amount of CIP-Ad in D2O then the solution was sonicated using powerful ultrasonic bath for 2 h. The suspension was filtrated through a 0.2 µm sy-ringe filter (Millipor®, France) to eliminate the insol-uble supernatant. The filtrate was analyzed by NMR. A solution of 2,3-O-dimethyl-β-CD at 4 mM in D2O was prepared.

***Preparation of Complex between 2,3-di-O-methyl-β-CD and (CIP–Ad)***

A 2 equivalents of 2,3-*O*-dimethyl-β-CD (10.64 mg, 8 mM) were added to 1 equivalent of CIP–Ad (1.85 mg, 3.98 mmol) in 1 mL of D2O. The cloudy solution obtained was stirred and then filtrated through a 0.2 µm syringe filter (Millipor®, France) to eliminate the insoluble supernatant. The filtrate was analyzed by NMR. The reactants, 2,3-*O*-dimethyl-β-CD (in D2O) and CIP-Ad (in D2O and CDCl3), were analyzed with a Bruker AVIII300 NMR spectrometer. Experiments were carried out at 300 MHz for 1H and at 75 MHz for 13C. The complete assignment of all H and C atoms of 2,3-*O*-dimethyl-β-CD and CIP-Ad was achieved by conventional 2D experiments: HMQC (1H-13C) and HMBC (1H-13C). For 2D spectra, a total of 1024 (HMQC) or 2048 (HMBC) points in F2 and 128 experiments in F1 were recorded. The complex 2,3-*O*-dimethyl-β-CD:CIP–Ad (in D2O) was analyzed with a Bruker AVIII600 NMR spectrometer equipped with a 10 A gradient amplifier and a 5 mm CPTXI (1H, 13C, 15N) including shielded z-gradients. 1D 1H and 2D 1H-1H NMR were recorded at 298 K. All chemical shifts were measured relative to external TMS. 2D NOESY (1H-1H): was used to evidence the interaction between 2,3-O-dimethyl-β-CD and CIP–Ad. For 2D NOESY (1H-1H) spectrum, a total of 2048 points in F2 and 256 experiments in F1 were recorded. A mixing time of 300ms was used. The formation of the complex was confirmed using the diffusion coefficients measured by 1H DOSY experiments. The diffusion 1H NMR experiments were carried out by using the AVIII600 spectrometer, for 2,3-O-dimethyl-β-CD, CIP-Ad and complex. A pulsed-gradient stimulated echo (LED-PFGSTE) sequence, using bipolar gradient was used. Sequence delays were: diffusion delay (Δ) 150 ms, recovery delay after gradient (τ) 0.2 ms, and LED recovery delay 5 ms. For each data set, 66560 complex points were collected for each 32 experiments in which the gradient strength was linearly incremented from 0.681 to 32.35 G.cm*-1.* The gradient duration δ/2 was adjusted to observe a near complete signal loss at 32.35 G.cm-1. The δ/2 delay was fixed at 2 ms. A 2s recycle delay was used between scans for all data shown. The number of scans was fixed to 32 (for the free molecules) and 64 scans (for the complex).

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**Test of CIP-Ad solubility**

Triplicate saturated solutions of CIP-Ad in water were prepared by adding excess amount of compound in distilled water. Flasks with concentrated solutions were capped with plastic stoppers and mechanically shaken for 24 h at 25°C and 250 rpm. The solutions were then taken up into syringe filter, and filtered through 0.2 µm microcellulose filter (Merck Millipore, France). UV measurements (optical density, 280 nm) were performed to quantify the CIP-Ad amount in solutions (Perkin Elmer Carry 100 BIO UV-vis spectrophotometer) by using a calibration curve (R2 = 0.99).

**Phase solubility test:** The solubility tests were performed with CIP-Ad in presence of three methylated β-CD derivatives at various concentrations (0; 3; 12; 40; 75; 110; 155; 250 mM) using 5 mL glass flasks. 13.96-14 mg of CIP Ad was added to the aqueous CDs solution for a concentration of 14 mg/L. The tubes were recapped and mechanically shaken for 48 h at 37°C and 250 rpm. After 48 h, the solution was extracted using a 1 mL syringe and filtered with a 0.2 μm microcellulose filter. 0.5 ML of sample was diluted with 0.5 mL of ethanol in order to dissociate the μm microcellulose filter. 0.5 ML of sample was diluted with 0.5 mL of ethanol in order to dissociate the complex. Dilution (1/500) of each sample with 50% aqueous ethanol was necessary to performed UV absorption spectrophotometry and quantified the CIP-Ad in solution. A 50% aqueous ethanol solution was used as a blank. For solubility test with β-CD, the same procedure was followed. β-CD was added to four tubes at various concentrations in a range of 2; 4; 6; 8; 12; 16 mM. The experiment was performed in triplicate (n=3).

***Preparation of Solid Inclusion Complex*:** The inclusion complex was prepared by freeze-drying method water by adding CIP-Ad into CD solution prepared using Milli-Q water. The concentrations of each component were calculated at the highest solubility given by the solubility curves. The aqueous CIP-Ad mixture was shaken for 1 night at -4°C at 250 rpm (Innova 4230, New Brunswick Scientific, Edison, NJ). The solution was filtered and placed into wide shallow dishes covered with plastic wrap and frozen to -80°C. Samples were freeze-dried over four days (Sentry Freezemobile 12SL, Virtis, Gardiner, NY). The solid sample was stored in sealed bottles at -4°C until analysis.

***Preparation of Dispersin B–CD conjugates:*** EDAC (30 mg) and 16 mg of *N*-hydroxysuccinimide were added to the reaction mixtures containing 0.5 mg of Dispersin B dissolved in 0.5 ml of 80 mM borate buffer, pH 8.0, and 76 mg of each CD derivative, i.e, CD1, CD2, and CD3. The solutions were stirred for 1 h at room temperature and then for 16 h at 4°C. Ultrafiltration of enzymatic solution was performed using centrifugal filter (Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-10 membrane, Millipore Merck, France) for concentrating and

desalting the enzyme and elimination of the entire reagent using a cut off 10KDa. The modified Dsp B was purified by coprecipitation using a 2-D Clean-Up Kit (Biorad, france).

***Minimal Inhibitory Concentration (MIC) determination:*** Antimicrobial activities of compounds were evaluated by MIC measurements through the micro dilution method based on ISO standard 20776-1[41].

Briefly, microplate wells containing TSB were enriched by a solution of CIP hydrochloride at various concentrations between 1024 and 0.25 µg/mL in 0.15 M, pH=7.4 phosphate buffer (PBS). Each well was inoculated by a bacterial suspension (final concentration, 106 colony forming units CFU/mL). After 24 h of incubation at 37°C, the bacterial growth was visualized by the broth turbidity. MIC was defined as the lowest concentration of CIP-Ad that inhibited the visible bacterial growth. Negative (without bacteria) and positive controls (without antibiotic) were included in each assay. The same procedure was used for CIP-Ad and each CDs complexes, i.e., CIP-Ad:2,3-*O*-dimethyl-β-CDs and CIP-Ad:permethyl β-CDs. In these cases CIP-Ad was solubilized in steril dimethylsulfoxide DMSO and CDs complexes in PBS.

***Biofilm formation assay:***Strain 5 was used. Biofilms were formed in 96-well microplates containing TSB inoculated from a preculture (initial cell concentration, 107 CFU/mL) as previously described [42]. After 24 h of incubation, the medium was removed and cells washed three times with PBS. Biofilm cells were then recovered by scrapping, re-suspended in sterile water and enumerated by plating out on Tryptic Soy agar medium.

***Enzymatic biofilm dispersion assays***:

Tests were adapted from the method described by O’Toole and Kolter [43]. Biofilms were formed as described above. After 24 h of incubation, unattached cells were removed by rinsing the microdishes thoroughly three times with PBS. Two hundred µl of solutions of Dsp B (final concentration, 5 µg/mL) or DspB-CD in PBS were added. After 2 h of incubation at 37°C, biofilms were washed again and attached cells were subsequently stained by incubation with 0.1% Crystal violet (CV, Sigma-Aldrich) for 30 min. The CV was then solubilized by adding 200 µL of ethanol/aceton (80/20, v/v) and the OD measured at 590 nm (Victor3 microplate reader, PerkinElmer Sciex). The OD590 values obtained at times zero (before DspB exposure) and after treatment were compared. For each strain the experiment was performed 3 times.

***Antibiotic resistance assays*:** Biofilms were formed in 96-well microplates as described above. After 24 h of incubation at 37°C, the medium was removed and cells washed three times with PBS. Wells were then filled with 200 µl of TSB containing different concentrations of ciprofloxacin, i.e., 5 MIC,

**Biological tests**

***Bacterial strains:*** *S. epidermidis* clinical strains (1457, 9142, RP62A and 5) were kindly provided by Pr. J. Kaplan from the University of Medicine and Dentistry of New Jersey (USA). Strains were stored at -80°C in glycerol and used as required. For precultures, bacteria were grown for 24 h in Tryptic soy broth (TSB) (Difco, Beckton Dickinson, Franklin Lakes, NJ US) at 37°C with shaking. All antibiotics, CIP or CIP-Ad was conserved at-4°C.

***Bacterial growth monitoring:*** The growth curve was obtained by incubating the strain 5 in TSB for 26 h at 37°C and monitoring the OD595 (Figure 12). 10 MIC, 20 MIC, 50 MIC and 100 MIC. After two hours of incubation at 37°C, the culture medium was removed and the number of adherent cells was enumerated as described above. The number of adherent cells before and after treatment was compared. A control without antibiotic was performed. For each strain, the experiment was performed 3 times.

The same protocol was used for the complex CIP-Ad:2,3-*O*-dimethyl-β-CD by using concentrations corresponding to 5 MIC, 10 MIC, 20 MIC, 50 MIC. The solid CIP-Ad:2,3-*O*-dimethyl-β-CD complex was prepared as described above and solubilized in PBS.

***Combined effect of Dsp B and ciprofloxacin on established biofilms of S. epidermidis:***

Biofilms were performed as described above. After 24 h of incubation at 37°C, the supernatant was gently aspirated with a micropipette in each well. Wells were then washed three times with PBS under aseptic conditions to eliminate the unbound bacteria, without disturbing the adherent film.

Ciprofloxacin (10xMIC) and/or, Dsp B (5 µg/mL) were added in 200 µL PBS. Plates were incubated at 37°C under shaking. After 2 h of incubation, the growth medium was removed and wells washed three times with PBS. Biofilm cells were recovered by scrapping and enumerated as described above.

***Combined effect of DspB with (CIP-Ad:2,3-O-dimethyl-β-CD) complex, Dsp B-CD3 conjugate and Dsp B-CD3 :CIP-Ad conjugate on biofilms***

The same procedure was used to test the effect of the Dsp B CIP-Ad:2,3-*O*-dimethyl-β-CD complex (5 MIC) association. The complex was prepared as described above and then solubilized in PBS. The solution was sterilized by filtration on 0.2 µm filter and then diluted in PBS at 1000 mg/L.

Likewise, the Dsp B-CD3 and *Dsp B-CD3:CIP-Ad conjugate* conjugates was tested (final concentration 30 µg/mL), alone or associated with CIP-Ad. Finally, the antibiofilm activity of the Dsp B-CD3-:CIP-Ad conjugate was tested (final concentration, 30 µg/mL)*.*

[***Bradford protein assay***](http://www.google.fr/url?sa=t&rct=j&q=%20bradford%20proteine%20asssa&source=web&cd=2&cad=rja&ved=0CDgQFjAB&url=http%3A%2F%2Fen.wikipedia.org%2Fwiki%2FBradford_protein_assay&ei=nNwLUqqKG-nQ0QW664GwBw&usg=AFQjCNEjNK6_E-mvfXLBGjFxSVU9-x4YmA&bvm=bv.50723672,d.d2k)***:*** Twenty µL of protein sample was added to 1 mL of a Bradford solution (BioRad Protein Assay) diluted (1/5, v/v). After 30 min of reaction, the absorbance was read at 595 nm and the protein

concentration was determined using a calibration curve realized with a range concentration of Bovine Serum Albumin (BSA).