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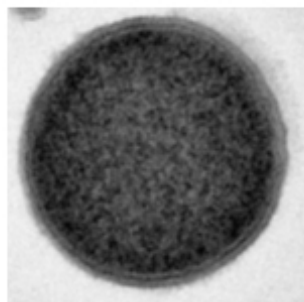
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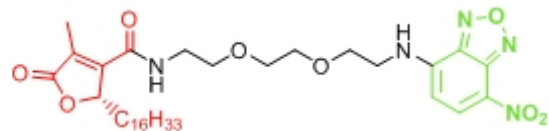
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S. gordonii

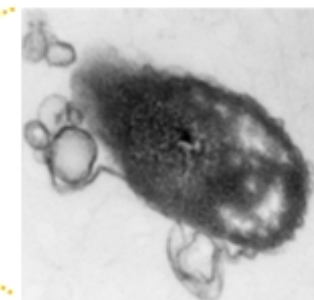
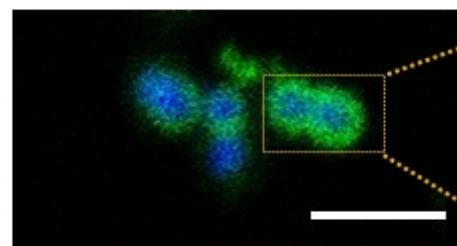
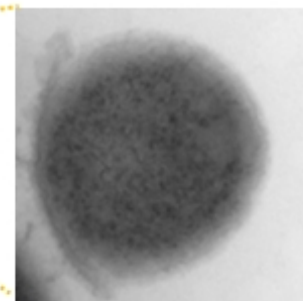
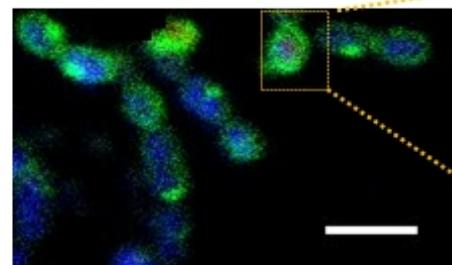


Butyrolactone derivatives



Membrane disruption

S. gordonii



P. gingivalis

P. gingivalis

Lichen butyrolactone derivatives disrupt oral bacterial membrane

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Abstract

We have previously demonstrated that out of the butyrolactones series synthesized based on the natural lichen metabolite lichesterinic acid, compound (**B-13**) was the most effective against oral bacteria. However, its antibacterial mechanism is still unknown. In this study, we have investigated its bacterial localization by synthesizing a fluorescently labeled **B-13** with NBD while maintaining its antibacterial activity. We showed that this compound binds to *Streptococcus gordonii* cell surface, as demonstrated by HPLC analysis. By adhering to cell surface, **B-13** induced cell wall disruption leading to the release of bacterial constituents and consequently, the death of *S. gordonii*, a Gram-positive bacterium. A Gram-negative counterpart, *Porphyromonas gingivalis*, showed also cracked and ruptured cells in the presence of **B-13**. Besides, we also demonstrated that the analog of **B-13**, **B-12**, has also induced disruption of *P. gingivalis* and *S. gordonii*. This study revealed that butyrolactones can be considered as potent antibacterial compounds against oral pathogens causing medical complications.

Keywords: Lichen; Butyrolactone; *Streptococcus gordonii*; *Porphyromonas gingivalis*; cell wall

1. INTRODUCTION

Treatment of infections with antibiotics reduces morbidity; however, the erroneous or unsuitable antibiotic can lead to the emergence of resistant pathogenic bacteria [1]. Facing this worldwide concern, alternative antimicrobial candidates against multidrug-resistant bacteria were developed.

The new drugs, which are of natural origin, are capable of surpassing the bacterial resistance mechanisms [2]. Among the natural sources is the association of fungus and alga and/or cyanobacterium forming a symbiotic organism named lichen which produces more than 1000 distinct secondary metabolites. They were shown to be effective against sensitive and several multi-drug resistant bacterial strains [3–6].

Among the bacteria sensitive to lichen secondary metabolites, we have previously described that the synthesized butyrolactone analogs, **B-13** and **B-12** (Fig. 1), can inhibit the growth of *Streptococcus gordonii* [7]. *S. gordonii* is a member of the viridans streptococci large category. In the oral cavity, *S. gordonii* adheres to the salivary pellicle which coats the teeth, proliferates and excretes an extracellular polysaccharide matrix protecting their developing microcolony on which late colonizers will adhere. The sites provided by *S. gordonii* are bound by *Porphyromonas gingivalis* and form a highly pathogenic complex microbial community [8], [9]. *S. gordonii*, as a pioneer initial colonizer, initiates the formation of dental plaques contributing in turn to the onset of dental caries and periodontal diseases as well as their progression [10]. Inhibiting *S. gordonii* might block the successive steps leading to acute oral diseases and introduce new antibiotics that might be able to prevent and treat the periodontal diseases.

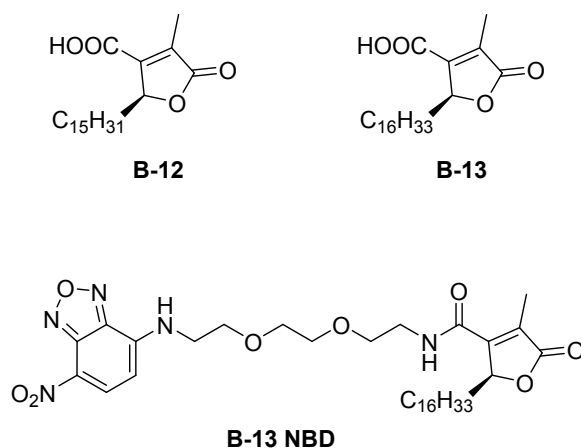


Fig. 1. Chemical structures of **B-12**, **B-13**, and **B-13 NBD**.

The most common way of antimicrobial killing is triggered by disruption of the cytoplasmic membrane. The bacterial cell membrane is responsible for many essential functions: transport, osmoregulation and respiration processes, biosynthesis and cross-linking of peptidoglycan, and synthesis of lipids. It is doubtless that for all these functions, membrane integrity is absolutely necessary, and its disturbance can directly or indirectly cause metabolic dysfunction and cell death. Alternative mechanisms of action include antimicrobial translocation into the cytoplasm where they interfere with metabolic processes, such as protein synthesis or DNA replication [11]. All of these modes of action have in common that the bacterial membrane will be severely damaged in the end leading to cell death. One of the most validated targets for antibacterial therapy is the enzymes of peptidoglycan biosynthesis. This biosynthesis is a complex process involving numerous steps in the cytoplasm and the inner and outer leaflets of the cytoplasmic membrane [12], [13].

Antimicrobials acting at the cell wall level are the most selective compared to other antibiotics. In contrast to all types of antibiotics, the cell wall-targeting ones as the butyrolactones may escape from a bacterial resistant strategy in treating the oral biofilms. Since the bacteria will grow significantly lower in its biofilm phase, thus, these metabolically reduced-activity bacteria regarding their biosynthesis of proteins, RNA, DNA, peptidoglycan, and folic acid, will be less inhibited by the antibiotics targeting these processes such as ribosome and DNA topoisomerases inhibitors [14]. For example, *Enterococcus faecalis* oral biofilms have been shown to require very high concentrations of antibiotics such as ampicillin and vancomycin (peptidoglycan synthesis inhibitor), and linezolid (protein synthesis inhibitor) [15,16]. However, targeting the cell wall can be by targeting the synthesis of its components as peptidoglycan or it can be by binding directly to the bacterial membrane bilayer thereby disrupting physically its integrity and its functions. Hurdle et al. has mentioned also that the antimicrobials can target either the bacterial membrane organization or the functions of membrane-associated respiratory enzymes [14].

In this study, we investigated the mechanisms of action of butyrolactone analog **B-13** on two oral bacteria implicated in periodontal diseases: *S. gordonii* and *P. gingivalis*. We have also compared its mechanism to another butyrolactone analog (**B-12**).

2. Material and Methods

2.1. General chemistry

All reagents of high quality were purchased from commercial suppliers and used without further purification. IR spectra were obtained with PerkinElmer UATR Two infrared spectrophotometer. ^1H (300 MHz) and ^{13}C (75 MHz) NMR spectra were performed on a Bruker GMX 300 spectrometer. Chemical shifts were referenced to the residual solvent signal (CDCl_3 : $\delta_{\text{H}} = 7.26$, $\delta_{\text{C}} = 77.16$). The δ values are given in parts per million (ppm), and the coupling constants (J values) are given in Hertz (Hz). The multiplicity of the signals is reported as s (singlet), d (doublet), t (triplet), q (quadruplet), m (multiplet). ESI-HRMS were carried out on a Bruker MicroTof QII spectrometer for electrospray ionization at the CRMPO (Centre Régional de Mesures Physiques de l'Ouest), University of Rennes 1. Elemental analyses were performed on a microanalyzer Flash EA1112 CHNS/O Thermo Electron at the CRMPO. Reactions were monitored by TLC on Merk 60 F254 (0.25 mm) plates which were visualized by UV detection or sprayed with KMnO_4 solutions, then heated. Log P values of the compounds **B-12**, **B-13** and the probe **B-13 NBD** have been calculated using ALOGPS 2.1 software.

(S)-4-methyl-5-oxo-2-pentadecyl-2,5-dihydrofuran-3-carboxylic acid B-12 and (S)-2-hexadecyl-4-methyl-5-oxo-2,5-dihydrofuran-3-carboxylic acid B-13:

B-13 and **B-12** derivatives described previously were used in this study [7]. Calculated log P = 7.29 (**B-12**) and 7.67 (**B-13**)

(2-{2-[2-(7-Nitro-benzo[1,2,5]oxadiazol-4-ylamino)-ethoxy]-ethoxy}-ethyl)-carbamic acid tert-butyl ester 2:

This compound was prepared as published [17]. Yield: 70%; red viscous oil. ^1H NMR (CDCl_3 , 300 MHz) δ 1.42 (s, 9H), 3.35 (q, $J = 6,1$ Hz, 2H), 3.59 (t, $J = 6$ Hz, 2H), 3.68 (m, 4H), 3.85 (m, 4H), 4.98 (s, 1H), 6.19 (d, $J = 8$ Hz, 1H), 6.87 (s, 1H), 8.49 (d, $J = 8,1$ Hz, 1H) ppm. ^{13}C NMR (CDCl_3 , 75 MHz) δ 28.6, 41.1, 44.4, 69.5, 70.8, 71.2, 71.3, 78.8, 100.2, 137.9, 145.2, 145.6, 146.1, 156.1.

N-(2-(2-(2-aminoethoxy)ethoxy)ethyl)-7-nitrobenzo[*c*][1,2,5]oxadiazol-4-amine **3**:

This compound was prepared as published [17] and used for the next step without any further purification.

Probe B-13 NBD:

To a solution of **B-13** butyrolactone (44.6 mg, 0.122 mmol) and 1.28 equiv of TBTU (50.1 mg, 0.156 mmol) in anhydrous DMF (10 mL) under argon atmosphere, was added the NBD spacer **3** (40 mg, 0.122 mmol, 1.0 equiv) and anhydrous DIEA (42.5 μ L, 0.244 mmol, 2.0 equiv). After stirring overnight at room temperature, the reaction was quenched by adding H₂O, this solution was then extracted with diethyl ether (3 x 50 mL) and once with ethyl acetate. The combined organic layers were washed with brine, dried with MgSO₄ and concentrated under vacuum. The residue was purified over preparative TLC (CH₂Cl₂/EtOH 95:5) to yield the desired compound. Yield = 63%; dark red solid. R_f (SiO₂, CH₂Cl₂/EtOH (95:5)) = 0.6. IR (ATR): $\nu_{\text{C-O}}$ (ester) = 1133 cm⁻¹; $\nu_{\text{C-O}}$ (lactone) = 1213 cm⁻¹; $\nu_{\text{C=C}}$ (ar) = 1586 cm⁻¹; $\nu_{\text{C=C}}$ = 1630 cm⁻¹; $\nu_{\text{C=O}}$ (ester) = 1698 cm⁻¹; $\nu_{\text{C=O}}$ (lactone) = 1734 cm⁻¹. ¹H NMR (CDCl₃, 300 MHz) δ 0.87 (t, *J* = 6,7 Hz, 3H), 1.24 (m, 28H), 1.38 (m, 2H), 2.05 (s, 3H), 3.71 (m, 10H), 3.86 (t, *J* = 5,4 Hz, 2H), 5.15 (t, *J* = 5,8 Hz, 1H), 6.18 (d, *J* = 8,7 Hz, 1H), 6.42 (s, 1H), 6.82 (s, 1H), 8.46 (d, *J* = 8,7 Hz, 1H) ppm. ¹³C NMR (CDCl₃, 75 MHz) δ 10.8, 14.3, 21.2, 22.8, 25.1, 29.2, 29.3, 29.4, 29.5, 29.6, 29.7, 29.8, 29.8, 29.8, 30.2, 32.1, 32.9, 39.4, 43.6, 68.2, 69.7, 70.4, 70.6, 81.8, 99.1, 124.3, 129.1, 136.5, 143.8, 144.1, 153.8, 162.5, 171.3, 173.4 ppm. HRMS (ESI) *m/z* [M+Na]⁺ calcd. for C₃₄H₅₃N₅O₈Na 682.37918, found 682.37920. Anal. Calcd. for C₃₄H₅₃N₅O₈: C, 61.89; H, 8.10; N, 10.61. Found: C, 62.40; H, 8.06; N, 9.70. Calculated log P = 7.10.

2.2. Bacterial strains

We used in this study the oral bacteria, *Streptococcus gordonii* DL1 and *Porphyromonas gingivalis* ATCC 33277. We have grown them in an anaerobic environment (N₂-H₂-CO₂ [80:10:10]) at 37°C utilizing brain-heart infusion (BHI) medium (DIFCO, France) and blood Columbia agar plates (AES Chemunex, France) supplemented with hemin (5 μ g/mL) and menadione (1 μ g/mL) (Sigma Aldrich, France) [7].

2.3. Determination of the minimum inhibitory concentration and minimum bactericidal concentration

Broth microdilution test was done as described formerly [7]. In a nutshell, 1:2 serial dilutions of the butyrolactone derivatives were done in BHI medium in 96-well plate (Sterile, Flat bottom, with lid, Greiner Bio-one, Germany). Doxycycline antibiotic was used as a positive control [18]. The wells were inoculated with 3×10^7 CFU.mL⁻¹ of *P. gingivalis*. After that, the plate is incubated anaerobically for 48 hours at 37°C. The clear well corresponding to the lowest concentration was defined as the minimal inhibitory concentration or MIC. Then, all the clear wells were spread on Columbia agar plates to be incubated for 5 days to uncover the minimal bactericidal concentration or MBC which is the lowest concentration plate with no colonial growth. The experiment was repeated three times.

2.4. Cellular localization of butyrolactone derivatives in the oral bacteria

2.4.1. Confocal microscopy (CLSM)

After inoculating 1 mL of BHI medium to have finally 3×10^7 CFU/mL of *S. gordonii* or *P. gingivalis*, they were incubated anaerobically for 18 or 48 hours, respectively. Then, the samples were centrifuged, the pellets were resuspended in PBS to be labeled by SYTO 40 and/or B-13-NBD for 15 minutes. After that, each condition was transferred into a microscopic glass slide and visualized under CLSM.

For CLSM images, Leica-SP8 scanning microscope (Leica Microsystems, Wetzlar, Germany) equipped with an inverted microscope (Fluorescence Microscopy Platform, IFR 140 GFAS, Université de Rennes 1) was used to visualize the slides *in situ*. The resonant option with 8000 Hertz was selected to capture the images utilizing the oil immersion objective lens (HC PL Apo 63X, 1.4 NA) where the numerical zoom was set at 8. The 405-nm excitation laser and 430-440 nm band-pass emission filter were used for SYTO 40 and the 488-nm excitation laser and 506-535 nm band-pass emission filter in case of **B-13 NBD**.

The confocal microscopy was also used to evaluate the bacterial viability after their exposure to compound **B-13**. This viability was investigated by using two nucleic acid-specific dyes: SYTO 9 which is membrane-permeable, stains all cells and can be detected by green fluorescence, and propidium iodide (PI) which is membrane impermeable, stains cells with damaged membranes and gives red fluorescence. Bacteria treated with **B-13** were visualized *in situ* utilizing a Leica TCS-SP8 confocal laser scanning microscope (Leica

Microsystems, Wetzlar, Germany) equipped with an inverted microscope (Fluorescence Microscopy Platform, IFR 140 GFAS, Université de Rennes I). For an entire bacterial detection with the dyes, specific excitation lasers and emission filters were used. The 488-nm excitation laser and 506-539 nm band-pass emission filter were utilized for SYTO 9 and the 561-nm excitation laser and 600-700 nm band-pass emission filter in case of PI.

Image acquisition and microscope piloting were done by the Leica software (LAS AF V.2.2.1), and ImageJ software V1.48m (National Institute of Health) was used to analyze the images.

2.4.2. HPLC-DAD analysis

In a 96-well plate (Sterile, Flat bottom, with lid, Biolite, Thermo Fisher Scientific, Korea) containing 100 μ L of BHI with or without **B-13**, 100 μ L of *S. gordonii* were added into each well to have a final concentration of 3×10^7 CFU/mL. A replicate for each condition was prepared. After incubation under anaerobic condition at 37°C for 18 hours, the contents of the MIC/2 wells were collected in an Eppendorf and extracted using an optimized protocol of that described by Leejae et al. 2013 [19]. Briefly, the samples were centrifuged at 5000 rpm for 10 minutes at 4 °C. The pellets obtained were washed 2 times with a buffer containing (10 mM Tris.HCl of pH = 8, 0.1 mM EDTA, 1 mM DTT, 5% ethanol, and 50 mM NaCl). Then, the pellets were lysed by sonication on ice for 5-10 min to be centrifuged at 5000 rpm for 10 min at 4°C. Supernatants comprising the cytoplasm and the pellets containing the cell wall and cell membrane were separated to be extracted by ethyl acetate. The organic upper layer was transferred into a new tube to be washed with distilled water. Again, the upper layer was transferred into another tube in which anhydrous sodium sulfate was added. The remaining liquid was finally transferred into a new tube and evaporated to dryness for the powder to be dissolved in THF. HPLC analysis were performed on a Prominence Shimadzu HPLC system (Marne La Vallée, France) equipped with a C18 hypersil Gold aQ column (250 x 4.6 mm, 5 μ m, Thermo Scientific) and consisting of a binary pump (LC-20ADSP), a surveyor autosampler (SIL-20AHT) and a diode array detector (SPD-M20A). The mobile phase consisted of (H₂O + 0.1% HCOOH) as solvent A and (ACN + 0.1% HCOOH) as solvent B with gradient: 0% of B during 5 min, 0%–100% of B during 5 min, 100% of B during 5 min, 100%–0% of B during 5 min, 0% of B during 10 min. The flow rate was 1 mL/min and 20 μ L of each sample were injected. DAD data were recorded at 228 and 254

nm and absorption spectra (210–400 nm) were recorded each second. The two samples, the pellet, and supernatant of each condition, had their own HPLC chromatograms allowing us to determine in which one (cell wall/cytoplasm) the butyrolactone was detected. Identification of **B-13** in the samples was done by comparison of its retention time and UV-spectra with the standard under the same chromatographic conditions.

2.4.3. Transmission electron microscopy (TEM):

Following overnight incubation, treated or untreated bacteria were collected, transferred to Eppendorf tubes and washed three times in Cacodilic buffer (0.15 M, pH 7.4). Fractions of bacterial suspension were fixed at 4°C for 60 min with 2% glutaraldehyde in 0.15 M Cacodilic buffer to be washed three times in the same buffer. Cells were post-fixed in 1% OsO₄ for 60 min at 4°C, rinsed in Cacodilic buffer and embedded in 2% Low melting agar (Sigma). After the dehydration series in acetone, the cells samples were embedded in conventional EPON (EMS 1420) and then polymerized at 60°C for 48h. Resins blocks were sectioned into 80 nm ultrathin sections using ultramicrotome LEICA UC7. These sections were mounted on copper grids and stained. Grids were observed using a TEM JEOL-JEM 1400 (Jeol Ltd, Tokyo, Japan) at an accelerating voltage of 120 kV and equipped with a Gatan Inc. Orius 1000 camera.

3. RESULTS AND DISCUSSION

3.1. Effect of butyrolactone analogs on the growth of *P. gingivalis* planktonic cells

The synthesized butyrolactones were shown to be potentially active against *P. gingivalis* where the COOH-containing compounds carrying an aliphatic chain with a minimal number of 13 carbon atoms were the best (Table 1). **B-7** was the least active with MIC = 75 µg ml⁻¹ and no MBC. It was followed by the unsaturated compounds (**B-3** and **B-4**) or saturated compounds substituted with a hydroxyl (**B-1** and **B-2**), or a vinyl group (**B-5** and **B-6**) which exhibited moderate activity. The activity increased to reach MICs = 0.037 and 0.293 µg ml⁻¹ and MBCs = 1.17 and 0.586 µg ml⁻¹ for **B-12** and **B-13**, respectively. These latter butyrolactones possess a conjugated carboxylic acid group and the longer aliphatic chains. They were even stronger than the reference antibiotic, doxycycline which presented an MIC = 0.13 µg ml⁻¹ and no MBC. It is worth noting that **B-12** was more efficient than doxycycline by about 3 times comparing their MICs and much more effective regarding their MBCs

277 Table 1. MIC and MBC of butyrolactones against *P. gingivalis* by broth microdilution

Compound	MIC (μg/mL)	MBC (μg/mL)
B-1	9.38	37.5
B-2	37.5	150
B-3	4.69	37.5
B-4	4.69	75
B-5	37.5	75
B-6	2.34	75
B-7	75	>i
B-8	9.38	150
B-9	37.5	150
B-10	0.073	9.38
B-11	0.586	4.69
B-12	0.037	1.17
B-13	0.293	0.586
Doxycycline	0.13	>i

278 >i: greater than the initial concentration

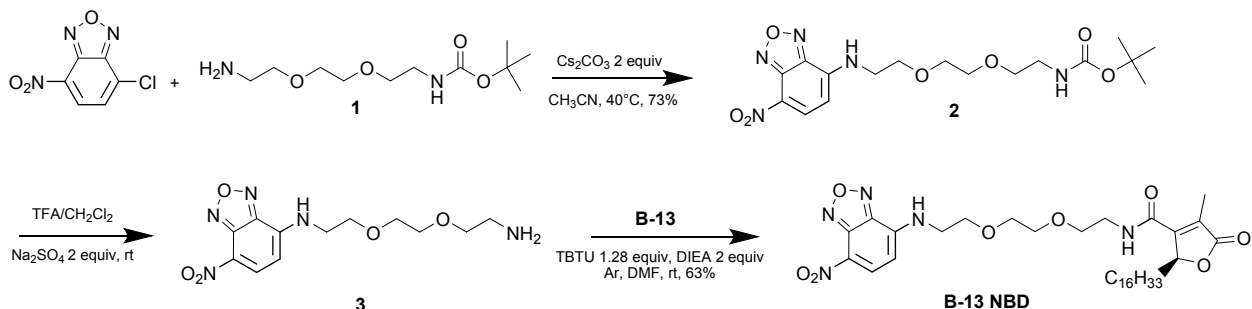
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280 3.2. Fluorescent B-13 butyrolactone adheres to *S. gordonii* membrane

281 Previously, we have demonstrated that lichen butyrolactone derivatives such as **B-13**
 282 inhibit two oral bacteria *S. gordonii* [7] and *P. gingivalis* (unpublished results).

283 However, further studies are needed to determine the putative mechanisms for their
 284 antimicrobial activity. We were therefore interested in preparing a fluorescent tool to try to
 285 elucidate their mechanism. We envisioned that this could be accomplished by using NBD, a
 286 commonly used fluorescent probe as fluorophore and a pegylated diamine as a linker. So, we
 287 synthesized the NBD-tagged butyrolactone **B-13** (Scheme 1). Based on a reported
 288 methodology [17], the NBD-based probe **3** was obtained by anchoring *N*-Boc monoprotected
 289 Pegylated diamine linker **1** to chloro-NBD in the presence of cesium carbonate in acetonitrile
 290 followed by TFA mediated amine deprotection. Finally, this fluorescent probe was coupled
 291 to the active substrate. For this last step, we selected a peptidic coupling reaction between the

probe and **B-13** (step 3). This was carried out in the presence of TBTU and DIEA in anhydrous DMF. The final **B-13 NBD** compound was thus obtained with a yield of 63%.



Scheme 1. Synthesis of **B-13 NBD** fluorescent probe

In such an approach using a fluorescent tool, one critical point is the good positioning of the probe in the active compound so that the biological activity is maintained. So, **B-13 NBD** was evaluated ($\text{MIC} = 37.5 \mu\text{g ml}^{-1}$) and compared to **B-13** for its antimicrobial activity. Notably, the conjugation of NBD to **B-13** did not eliminate their biological activity as their antimicrobial activity against *S. gordonii* was seen. Zhao et al. 2016 [20] also have shown that NBD is a highly tolerated fluorescence label and Matijašić et al. in 2012 [21] have demonstrated that 9a-NBD-azithromycin has antimicrobial properties comparable to azithromycin. In order to compare the polarity of the probe and the active **B-12** and **B-13** compounds, their log P values have been calculated using ALOGPS 2.1 software giving a prediction of their relative lipophilicity. Regarding their Log P values (**B-13 NBD**: log P = 7.10; **B-12**: log P = 7.29 and **B-13**: log P = 7.67), the three compounds appear to be highly lipophilic and display the same polarity. So the probe could be used as model to support the direct binding of **B-12** and **B-13** to the bacterial membrane.

After that, we have used **B-13 NBD**, NBD (7-nitrobenz-2-oxa-1,3-diazole) is a small size fluorescent probe originally introduced to label proteins through binding to their hydrophobic region, to investigate the cellular localization of **B-13** in *S. gordonii* by confocal laser scanning microscopy (CLSM). Confocal microscopic images of *S. gordonii* following the treatment of **B-13 NBD** showed green fluorescence (Fig. 2) which indicated considerable binding of this compound onto the bacteria, as seen by the presence of green fluorescence on cell membranes. The green fluorescence has surrounded the bacterial DNA stained in blue with SYTO 40, suggesting a strong localization in the cell wall. Tripathi et al. 2015 used this

NBD fluorescent probe to label IsCT, antimicrobial peptide, and its analogs [22]. They showed that some IsCT-NBD labelled versions were accumulated onto the bacterial membrane, others were translocated through *E. coli* membrane and were localized in the cytoplasm. This study demonstrated that NBD did not prevent localization of molecules in cytoplasm

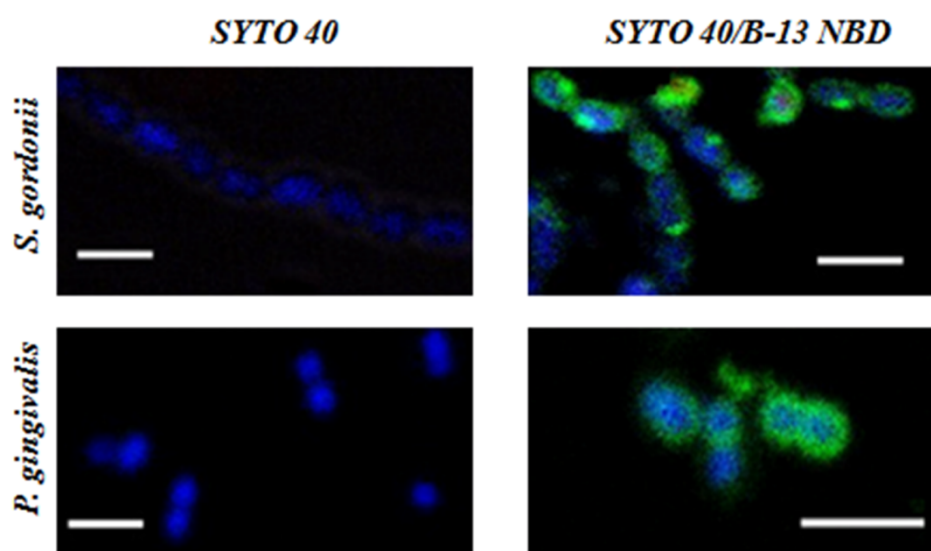


Fig. 2. Localization of fluorescently labeled **B-13 NBD** in *S. gordonii* and *P. gingivalis* by confocal microscopy. The bacterial DNA is stained by SYTO 40 in blue and **B-13 NBD** adhered to the bacterial surface and appears in green. Bars represent 2µm.

Cellular dysfunctions can result from the interaction of an antimicrobial with the microbial cell membrane [23]. The antimicrobial can only attach to the cell membrane and alter its structure, permeability, and/or transport activity. On the other hand, the antimicrobial can follow another pathway. After binding the cell membrane, it may penetrate inside the cells and affect vital cellular functions. To determine the antibacterial mechanisms of **B-13** in *S. gordonii*, localization of **B-13** in the bacteria was performed after incubating the latter with the compound for 1 and 18h. Lysed bacteria were centrifuged and their cell wall and membrane pellet (Fig. 3a) and cytoplasmic supernatant (Fig. 3b) fractions were analyzed using HPLC. Chromatograms in Figure 3 indicated that the compound was clearly visualized

only in the cell wall and membrane fraction of the treated cells in the same manner as the control **B-13** after 1h of incubation. After 18h of treatment, a low level of **B-13** was detected in the cell membrane. This signal decrease of the compound may be caused by the degradation or the release by bacteria of this butyrolactone. In contrast, **B-13** was not detected in the cytoplasm fraction. This kinetic study suggests that **B-13** binds the membrane and does not cross to the cytoplasm.

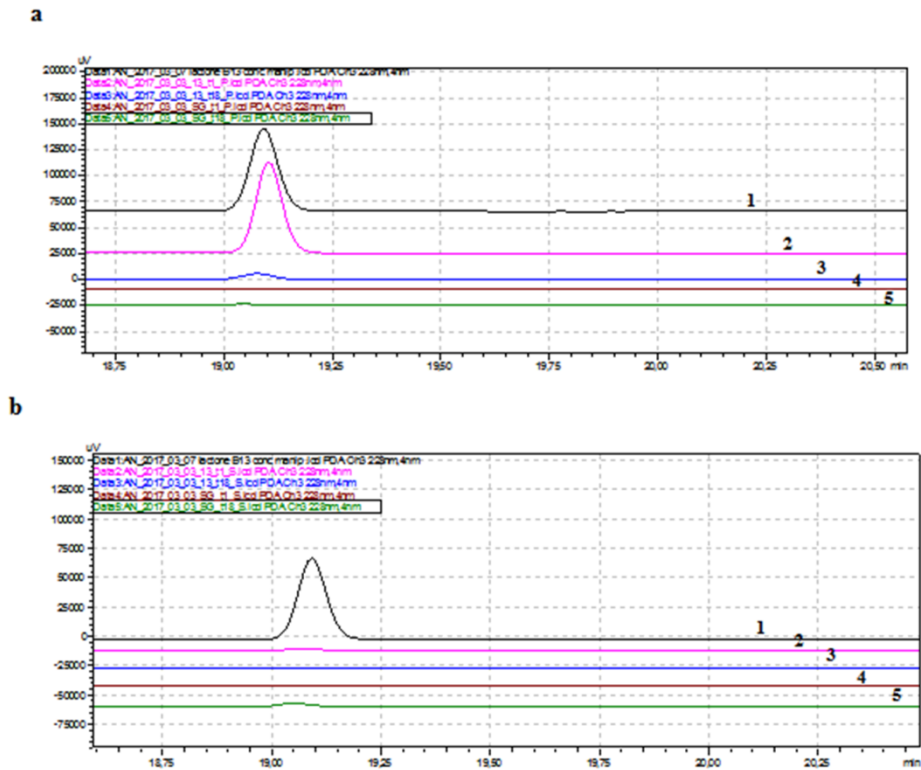


Fig. 3. Localization of **B-13** in *S. gordonii* using HPLC method. After incubation with **B-13**, *S. gordonii* was lysed, and the cell wall and cell membrane were separated from the cytoplasm by centrifugation. (a) HPLC analysis of cell wall and membrane; (b) HPLC analysis of cytoplasm. **B-13** was detected in the cell wall and membrane fraction of lysed *S. gordonii*. **B-13** as a reference (1), *S. gordonii* incubated with **B-13** for 1h (2) or 18h (3), *S. gordonii* incubated alone for 1h (4) or 18h (5).

3.3 B-13 Butyrolactone induced cell wall disruption of *S. gordonii*

To obtain a deeper insight into the mode of action of **B-13**, the morphology of *S. gordonii* was visualized by transmission electron microscopy (TEM) after treatment with this

compound (Fig. 4). Untreated bacteria appeared intact with a typically delimited cell wall and well-defined membrane, and a cytoplasmic content with few electron-dense areas. Treatment with doxycycline, the reference antibiotic [18], caused cytoplasmic alterations as suggested by the presence of round bodies in the cytoplasm with a similar electron density like clear vesicles. However, in presence of **B-13**, the bacteria appeared lysed with broken walls and membranes, and heterogeneous electron density zones in the cytoplasm (Fig. 4). The cells showed aberrant morphology; they were cracked and ruptured leading to their death. In some cells, the externalization of cytoplasmic material could be seen by the way of vesicles and rupture of the cell. Altogether, the results suggested that **B-13** inflicted considerable damage to *S. gordonii* membrane and this membrane disruption property could be the basis of the antibacterial activity of this compound.

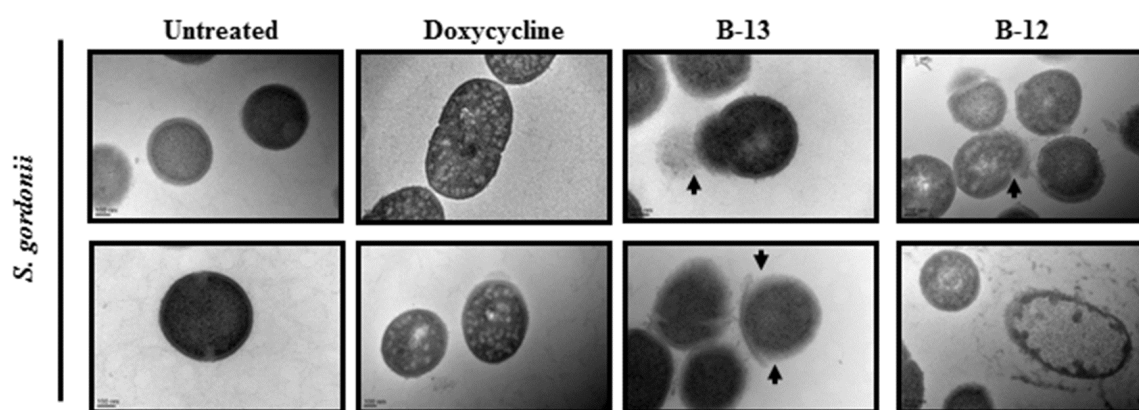


Fig. 4. Transmission electron micrographs of *S. gordonii*. **B-13**, as **B-12**, disrupted *S. gordonii* cell wall (as indicated by black arrows).

Furthermore, we have counted the damaged bacterial cells upon treatment with butyrolactones or doxycycline in three random regions from the electron micrographs of each condition and calculated their percentages (Table 2). *P. gingivalis* was shown to be more sensitive than *S. gordonii*, and this coincides with our results from the microdilution test. Butyrolactones had similar values which were higher than doxycycline by 1.5 to 2 times.

Table 2. Percentage of damaged bacterial cells upon treatment with butyrolactones or doxycycline (enumerated in the transmission electron micrographs).

	<i>S. gordonii</i>	<i>P. gingivalis</i>
B-12	58.5±1.3	92.5±2.8
B-13	55.7±1.6	86.1±3.1
Doxycycline	32.6±2.6	66.4±1.2

CLSM analysis was used to provide further confirmation of our hypothesis on this membrane disruption. In order to follow permeability modification due to **B-13** exposure, we monitored the fluorescence intensity of bacterial culture mixed with propidium iodide (PI) and SYTO 9 [24]. SYTO 9 can penetrate intact or damaged membranes giving green fluorescence, however, PI fluoresces in red and can only penetrate damaged membranes. As a result, the dead cells have appeared nearly in orange color due to the presence of the two labels. As shown in figure 5, untreated control *S. gordonii* cells appeared predominantly green (demonstrating living cells); whereas the **B-13** treated cells appeared substantially red, indicating the high permeability of PI dye and that most of the cells were dead. This result confirmed that **B-13** had a destructive effect on the cell membranes where the majority of the bacteria were dead.

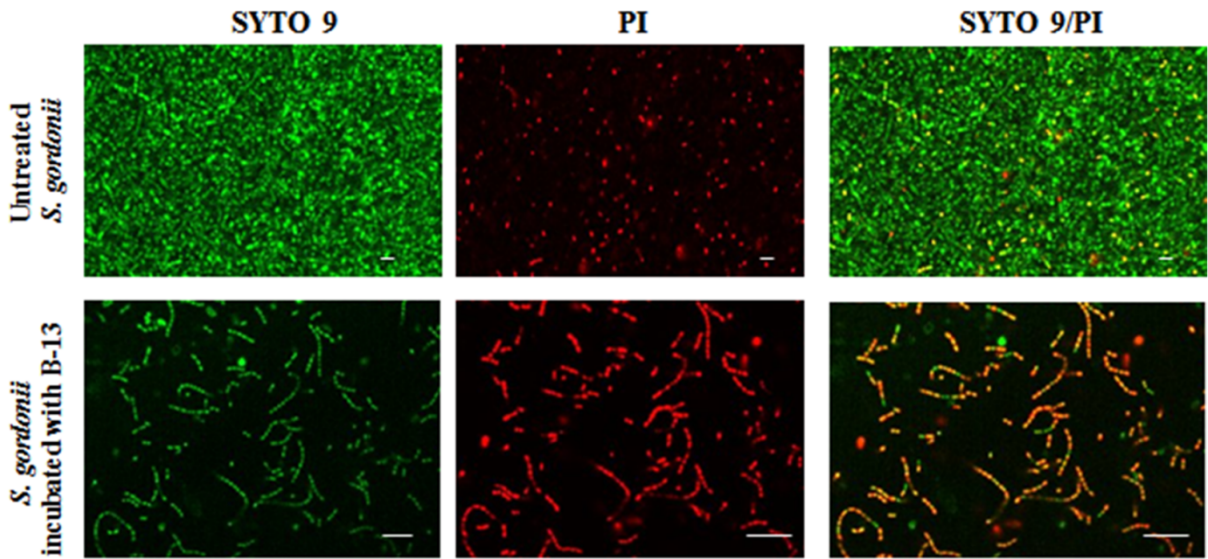


Fig. 5. *S. gordonii* cell suspension, untreated or treated with **B-13**, stained with SYTO 9 and PI, and analyzed by CLSM. Cells with intact membranes were stained with green fluorescence, whereas cells with ruptured membranes were stained with red fluorescence. The overlap of the two stains appears in orange. Bars represent 10µm.

3.4 B-13 Butyrolactone has also disrupted the Gram-negative bacterial membrane of *P. gingivalis*

In order to determine if its antimicrobial potential is influenced by the cell wall composition of the microorganisms, we have investigated the effect of **B-13** on *P. gingivalis*, a Gram-negative strain. When we used **B-13 NBD** on *P. gingivalis* (Fig. 2), we have observed a prominent green fluorescence on cell membranes. Figure 6 showed TEM micrograph of *P. gingivalis* control with a typical cell wall, outer and cytoplasmic membrane, periplasmic space and cytoplasmic content with few electron-dense areas. When *P. gingivalis* was treated with **B-13**, cells were damaged showing either localized or complete separation of the cell membrane from the cell wall. The cellular degradation was also accompanied by electron-translucent cytoplasm and cellular disruption in the damaged cells. These differences in structure, thickness, and composition of the cell can explain why Gram-positive *S. gordonii* were less inhibited and Gram-negative *P. gingivalis* showed substantial inhibition even at lower antibiotic concentration.

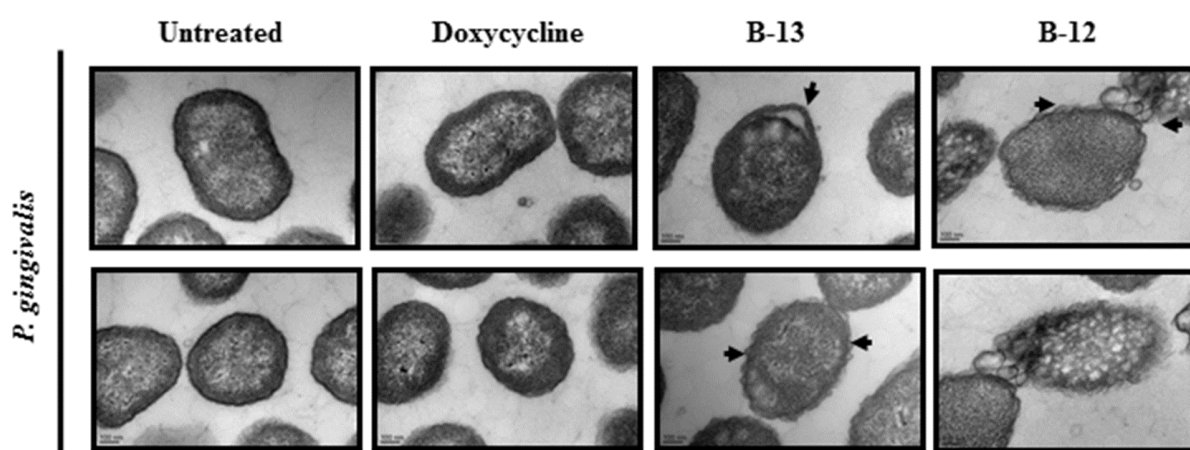


Fig. 6. Transmission electron micrographs of *P. gingivalis*. **B-13**, as **B-12**, disrupted *P. gingivalis* cell wall (as indicated by black arrows).

3.5 B-13 Butyrolactone analog, B-12, has also disrupted the membrane of oral bacteria

In order to determine if this membrane disruption is specific to the synthesized butyrolactone, we have used an analog of **B-13**, **B-12** with a shorter aliphatic chain (Fig. 1), which we have already described in Sweidan et al. 2016 [7]. Electron Micrographs of *S. gordonii* or *P. gingivalis* treated with **B-12** showed broken membrane and dead bacteria (Fig. 4 and Fig. 6). However, as for **B-13**, we found that the effect of **B-12** is also more active against Gram-negative as seen by the formation of unwinded structures in the cell wall, irregularly shaped cells, and scratched cell surface of *P. gingivalis* (Fig. 6).

The finding that **B-12** has the ability to break and damage the bacterial membrane leads to propose that the bactericidal effect of our synthesized butyrolactones is based on destroying the cell surface of bacteria.

4. Conclusion

S. gordonii and *P. gingivalis* are oral bacteria initiating the formation of dental plaques contributing in turn to the onset of dental caries and periodontal diseases as well as their progression [10]. We have previously demonstrated that these bacteria are sensitive to new drugs which are of natural origin as they are derived from lichen secondary metabolites [7,25]. However, its antibacterial mechanisms are still unknown. By using the compound (**B-13**) which is one of the most active butyrolactones series synthesized based on the natural compound, lichesterinic acid, we have shown that this compound has bound to the bacterial surface and induced membrane modification breaking the cell wall and releasing the cytoplasmic constituents leading to bacterial death. These results suggested that its antimicrobial potential is influenced by the composition of the cell wall of the microorganisms [26].

This study shows for the first time the mechanism of action of synthesized butyrolactones, analogs of lichesterinic acid. To our knowledge, there are only two studies on the mechanism of action of lichen-derived compounds [27], [3]. Gupta et al. (2012) demonstrated that usnic acid, a commonly occurring polyphenolic compound in many species of lichens, can destabilize the membrane integrity of Methicillin-Resistant *Staphylococcus aureus* (MRSA) [27]. Shrestha et al. 2016 demonstrated also that *L. vulpina*

extracts disrupted the integrity of MRSA membranes and targeted its cell division processes in MRSA [28]. These studies have only investigated Gram-positive strains whereas both Gram types were investigated in the present study.

Our study opens the door for future mechanistic research on lichen secondary metabolites which will give a better understanding of lichen as an association of fungus and alga and/or cyanobacterium forming a symbiotic organism and how to use its secondary metabolites as antibiotics. In addition, the structures of the butyrolactone analogs are different from those of all the antibiotics discovered to date including the cell wall-targeting ones. This fact supported by the way these compounds target the bacteria as described in our study, may introduce a new generation of antibiotics with a new mode of action. However, to better develop a new antibiotic, it is necessary to make further investigations on lichen secondary metabolites in general and **B-13** in particular.

Conflicts of interest

The authors declare no conflicts of interest.

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We would like to acknowledge C. Le Lann, (NUMECAN – Rennes I University, France), Marie Thérèse Lavault (Plateforme microscopie électronique MRic, Campus Santé, Rennes 1, France) and Stephanie Dutertre (Plateforme Microscopie, Biosit, Campus Santé, Rennes 1, France) for their technical help in CLSM.

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Figures captions

Scheme 1. Synthesis of **B-13 NBD** fluorescent probe.

Fig. 1. Chemical structures of **B-12**, **B-13**, and **B-13 NBD**.

Fig. 2. Localization of fluorescently labeled **B-13 NBD** in *S. gordonii* and *P. gingivalis* by confocal microscopy. The bacterial DNA is stained by SYTO 40 in blue and **B-13 NBD** adhered to the bacterial surface and appears in green. Bars represent 2µm.

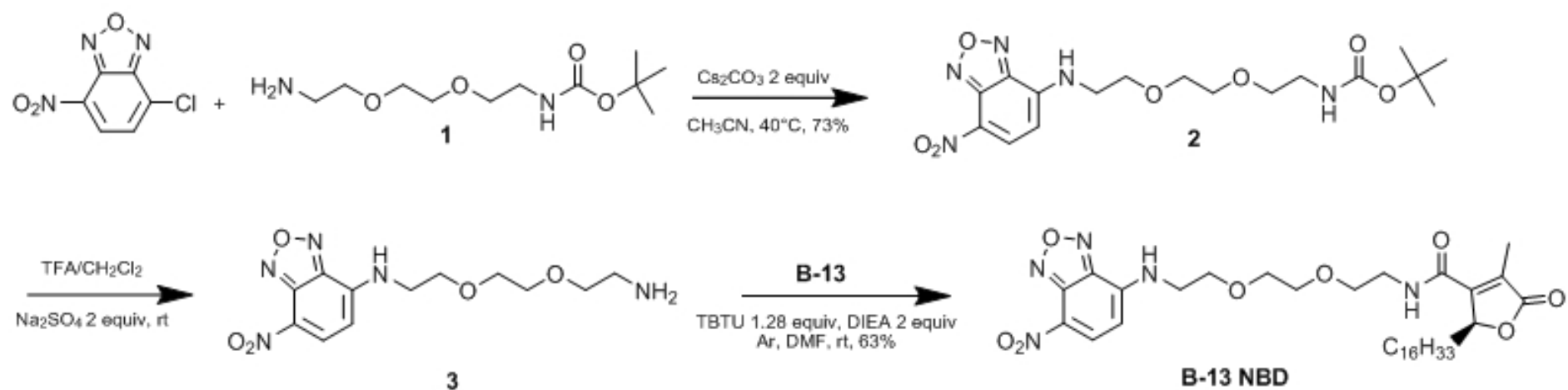
Fig. 3. Localization of **B-13** in *S. gordonii* using HPLC method. After incubation with **B-13**, *S. gordonii* was lysed, and the cell wall and cell membrane were separated from the cytoplasm by centrifugation. (a) HPLC analysis of cell wall and membrane; (b) HPLC analysis of cytoplasm. **B-13** was detected in the cell wall and membrane fraction of lysed *S. gordonii*. **B-13** as a reference (1), *S. gordonii* incubated with **B-13** for 1h (2) or 18h (3), *S. gordonii* incubated alone for 1h (4) or 18h (5).

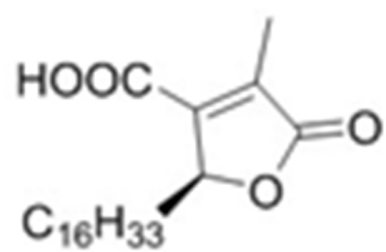
Fig. 4. Transmission electron micrographs of *S. gordonii*. **B-13**, as **B-12**, disrupted *S. gordonii* cell wall (as indicated by black arrows).

Fig. 5. *S. gordonii* cell suspension, untreated or treated with **B-13**, stained with SYTO 9 and PI, and analyzed by CLSM. Cells with intact membranes were stained with green fluorescence, whereas cells with ruptured membranes were stained with red fluorescence. The overlap of the two stains appears in orange. Bars represent 10µm.

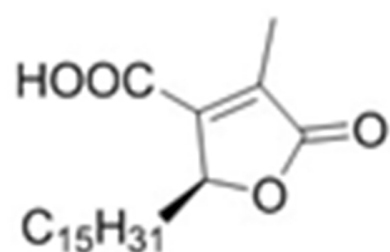
Fig. 6. Transmission electron micrographs of *P. gingivalis*. **B-13**, as **B-12**, disrupted *P. gingivalis* cell wall (as indicated by black arrows).

Scheme 1

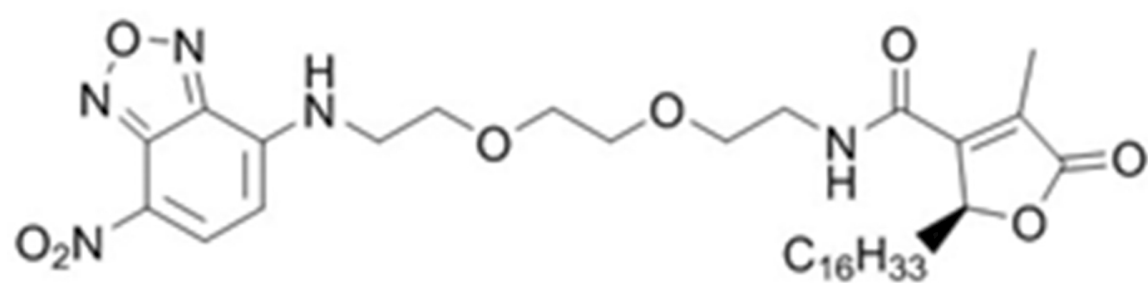




B-13



B-12



B-13 NBD

Fig. 1.

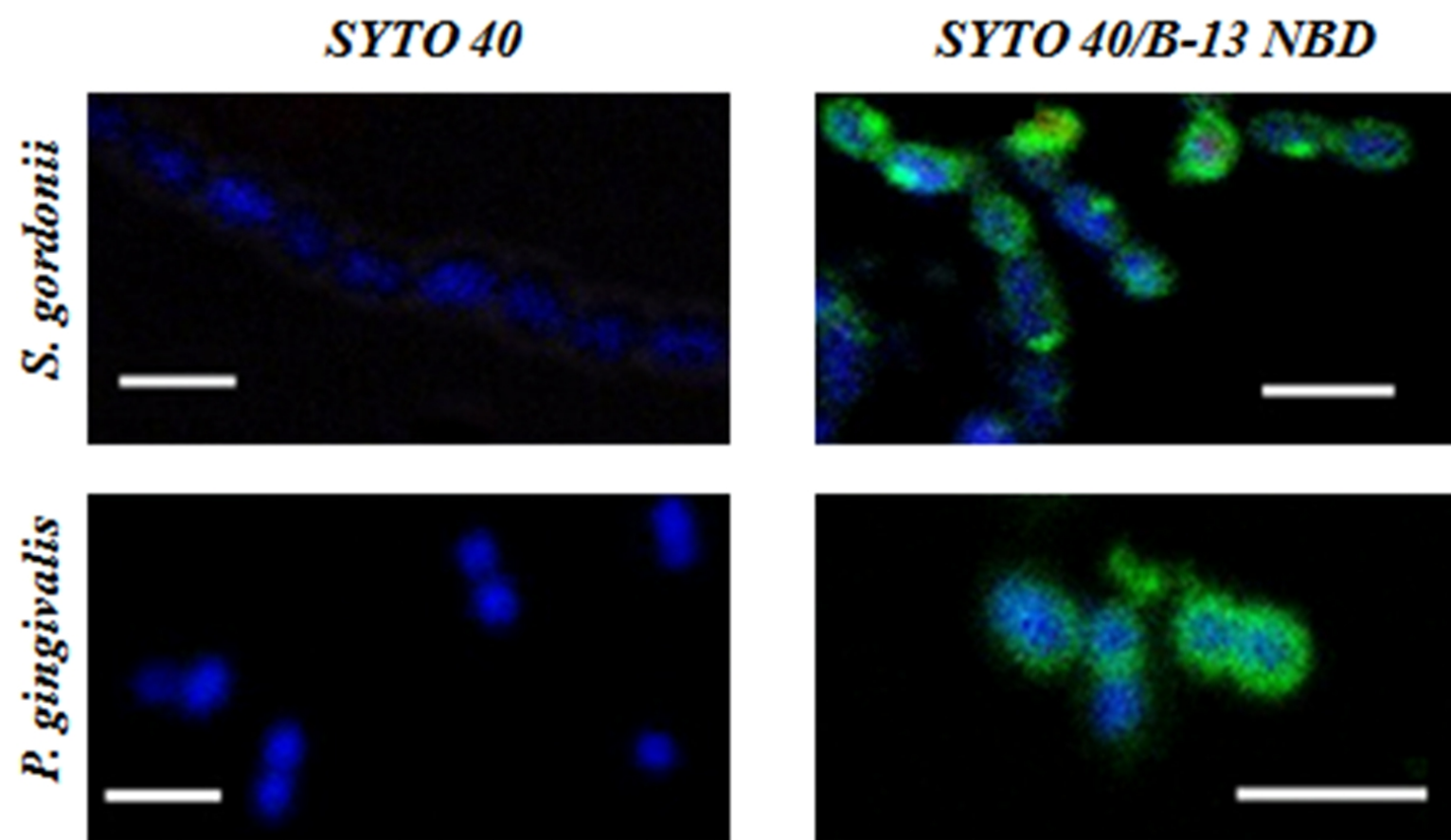
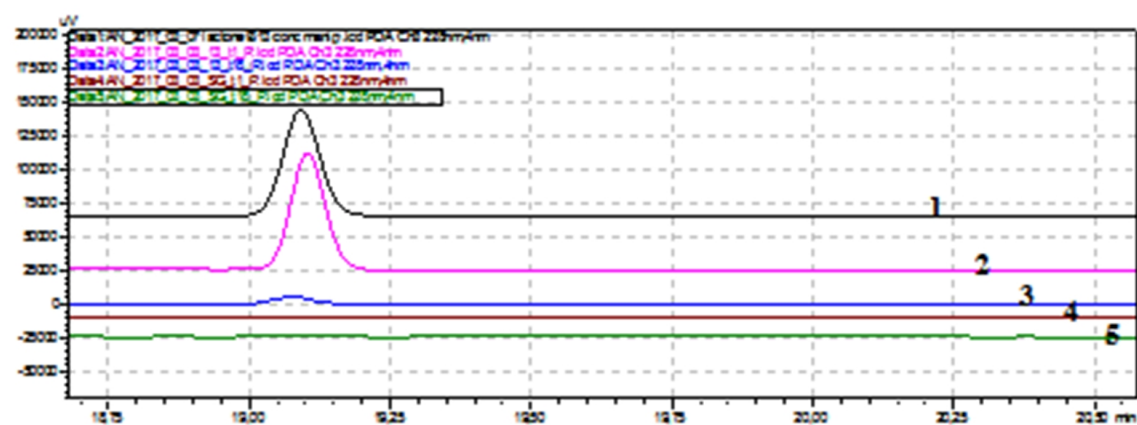


Fig. 2.

a



b

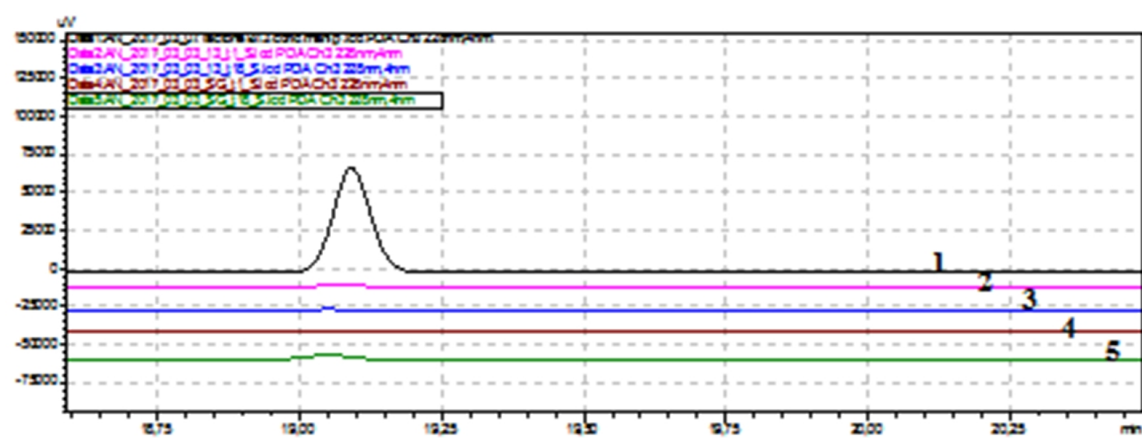


Fig. 3.

S. gordonii

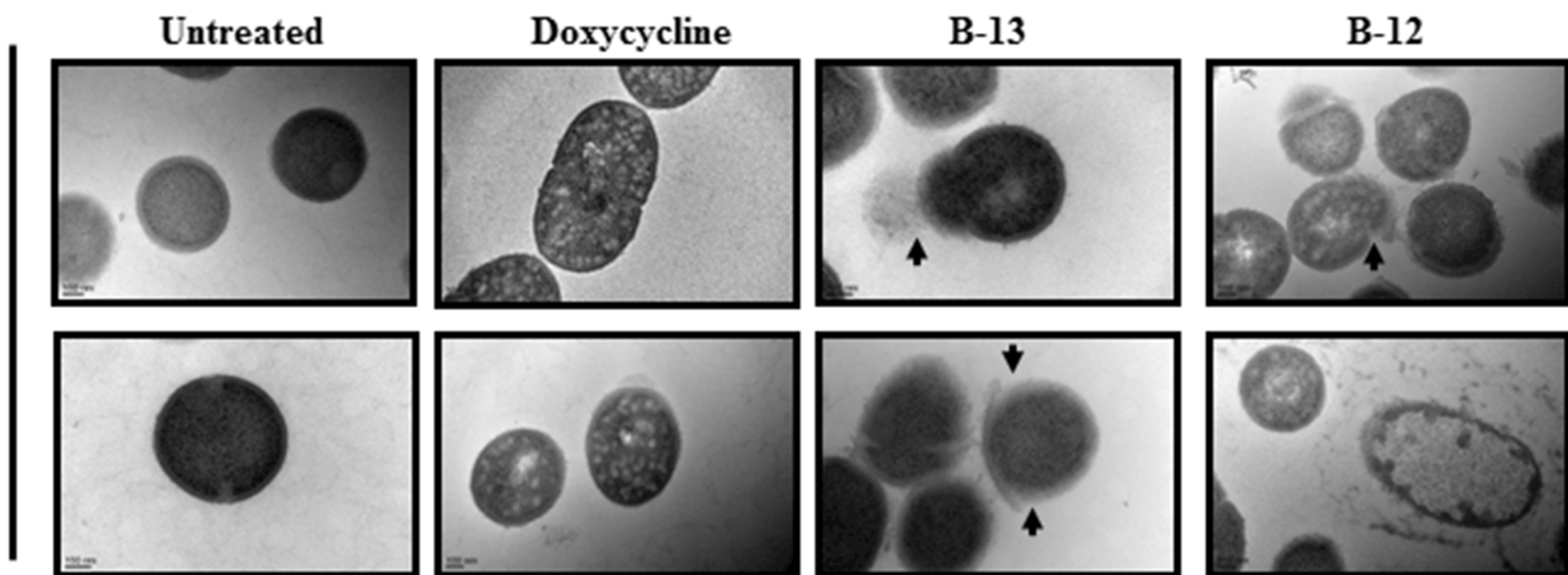


Fig. 4.

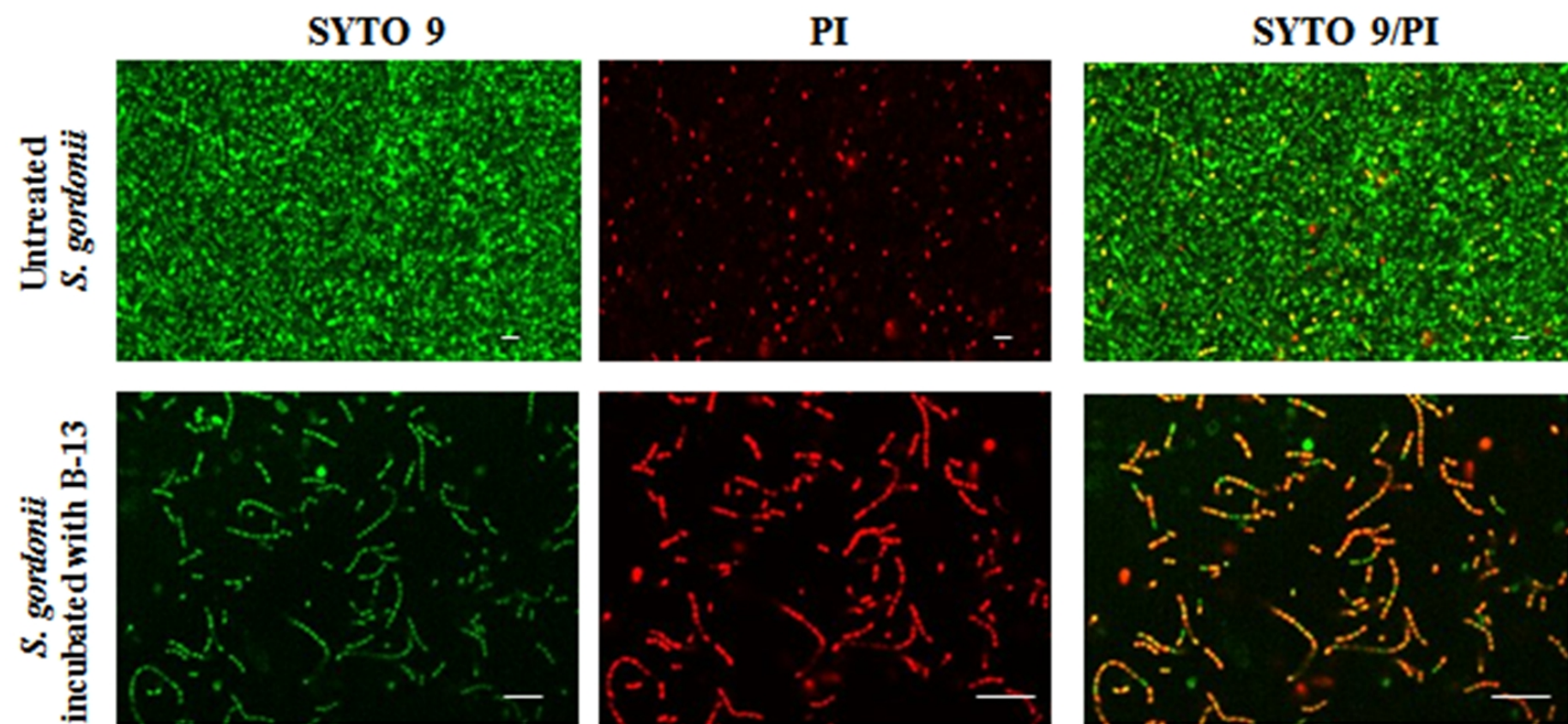


Fig. 5.

Table 1. MIC and MBC of butyrolactones against *P. gingivalis* by broth microdilution

Compound	MIC (µg/mL)	MBC (µg/mL)
B-1	9.38	37.5
B-2	37.5	150
B-3	4.69	37.5
B-4	4.69	75
B-5	37.5	75
B-6	2.34	75
B-7	75	>i
B-8	9.38	150
B-9	37.5	150
B-10	0.073	9.38
B-11	0.586	4.69
B-12	0.037	1.17
B-13	0.293	0.586
Doxycycline	0.13	>i

>i: greater than the initial concentration

Table 2. Percentage of damaged bacterial cells upon treatment with butyrolactones or doxycycline (enumerated in the transmission electron micrographs).

	<i>S. gordonii</i>	<i>P. gingivalis</i>
B-12	58.5±1.3	92.5±2.8
B-13	55.7±1.6	86.1±3.1
Doxycycline	32.6±2.6	66.4±1.2