Widening the Antimicrobial Spectrum of Esters of Bicyclic Amines: *In Vitro* Effect on Gram-positive *Streptococcus pneumoniae* and Gram-negative Non-Typeable *Haemophilus influenzae* biofilms

Emma Roig-Molina,^a Mirian Domenech,^b María de Gracia Retamosa,^{a,§}
Montserrat Nácher-Vázquez,^b Luis Rivas,^b Beatriz Maestro,^a
Pedro García,^{b,c} Ernesto García,^{b,c} Jesús M. Sanz^{a,b,*}

^aInstituto de Biología Molecular y Celular, Universidad Miguel Hernández, Avda
Universidad s/n, Elche-03202, Spain.

^bCentro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu, 9. 28040-Madrid, Spain

^cCentro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES),
Ramiro de Maeztu, 9. 28040-Madrid, Spain

[§] Current address: Instituto de Investigaciones Químicas (CSIC-US) and Centro de
Innovación en Química Avanzada (ORFEO-CINQA), Sevilla, Spain

Running Head: EBAs Against Pneumococcal and NTHi Biofilms

* Address correspondence to Jesús M. Sanz (jmsanz@cib.csic.es). Centro de
Investigaciones Biológicas, CSIC. c/ Ramiro de Maeztu, 9. 28040-Madrid, Spain

E.R.-M. and M.D. contributed equally to this work.

Highlights

- Esters of bicyclic amines (EBAs) destabilize the bacterial membrane
- EBAs trigger the premature autolysis of *Streptococcus pneumoniae* by LytA autolysin
- EBAs disrupt mixed biofilms formed by *S. pneumoniae* and *Haemophilus influenzae*
- Broad-spectrum effect of EBAs is useful to fight antimicrobial resistance

Abstract

Antibiotic resistance is a global current threat of increasing importance. Moreover, biofilms represent a medical challenge since the inherent antibiotic resistance of their producers demands the use of high doses of antibiotics over prolonged periods. Frequently, these therapeutic measures fail, contributing to bacterial persistence, therefore demanding the development of novel antimicrobials. Esters of bicyclic amines (EBAs), which are strong inhibitors of *Streptococcus pneumoniae* growth, were initially designed as inhibitors of pneumococcal choline-binding proteins on the basis of their structural analogy to the choline residues in the cell wall. However, instead of mimicking the characteristic cell chaining phenotype caused by exogenously added choline on planktonic cultures of pneumococcal cells, EBAs showed an unexpected lytic activity. In this work we demonstrate that EBAs display a second, and even more important, function as cell membrane destabilizers. We then assayed the inhibitory and disintegrating activity of these molecules on pneumococcal biofilms. The selected compound (EBA 31) produced the highest effect on *S. pneumoniae* (encapsulated and non-encapsulated) biofilms at very low concentrations. EBA 31 was also effective on mixed biofilms of non-encapsulated *S. pneumoniae* plus non-typeable *Haemophilus influenzae*, two pathogens frequently forming a self-produced biofilm in the human nasopharynx. These results support the role of EBAs as a promising alternative for the development of novel, broad-range antimicrobial drugs encompassing both Gram-positive and Gram-negative pathogens.

Keywords: EBAs, broad-spectrum antimicrobials, membrane permeability, mixed biofilms

Abbreviations: EBAs, Esters of bicyclic amines; *NTPn* non-typeable (non-encapsulated) pneumococci; *NTHi*, non-typeable *Haemophilus influenzae*.

1. INTRODUCTION

Infectious diseases are increasingly more difficult to control due to the global dissemination of antimicrobial (frequently multidrug) resistance (AMR). Nowadays AMR is one of the major threats in human health, aside from the associated social and economic burden. In addition, the effectiveness of antibiotics under clinical use is increasingly threatened by rising resistance and a dearth of new candidates in the pipeline. Among infectious diseases, respiratory pathologies are a leading cause of illness and death in people of all ages. In particular, lower respiratory tract infections are the leading infectious source of death, ranking as the fifth cause of mortality worldwide [1]. Nasopharynx and lungs undergo a constant and essential chemical communication with the local microbiota [2, 3]. In particular, the healthy nasopharynx is colonized by different microorganisms: encapsulated *Streptococcus pneumoniae* (pneumococcus) frequently associated with non-typeable (non-encapsulated) pneumococci (NTPn) and with strains of *Streptococcus pseudopneumoniae* and other α -hemolytic (viridans) streptococci [4], as well as with non-typeable *Haemophilus influenzae* (NTHi) to form mixed biofilms [5].

S. pneumoniae is a leading human pathogen and a major cause of severe disease, including community-acquired pneumonia, bacteremia and meningitis. Colonization begins shortly after birth, and pneumococci are carried asymptotically in the nasopharynx of healthy children [6]. Overall, the mortality rate of pneumococcal infections in the pediatric population is higher than that originated by AIDS, malaria and measles altogether [7]. Moreover, pneumococcal pneumonia caused 55.4% of deaths due to lower respiratory tract infections in all ages, being responsible of more than 1,500,000 deaths worldwide in 2015 [1]. Pneumococcal biofilms in children appear on adenoid and mucosal epithelium with recurrent middle ear infections and otitis media with effusion, and on the sinus mucosa of patients with chronic rhinosinusitis [8].

The Gram-negative NTHi is an opportunistic pathogen that colonizes the nasopharynx of near 80% of humans [9]. Colonization promotes the development of disease and produces bacterial reservoirs which facilitate person-to-person transmission. NTHi is the main bacterial cause of chronic otitis media with effusion, recurrent acute otitis media, and

acute otitis media coursing with treatment failure [10], mostly accompanied by *S. pneumoniae* and, to a lesser extent, by *Moraxella catarrhalis* and other species [10].

AMR is frequently found in diseases due to *S. pneumoniae* and *NTHi* which, as such, have been included by the World Health Organization among the 12 bacterial pathogens prioritized for the critical development of new antibiotics (http://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf). In this sense, our laboratory has proposed the choline-binding proteins (CBPs) from *S. pneumoniae* as new targets in the quest for novel antimicrobials [11-13] to overcome the current resistance threats. CBPs constitute a family of 13–16 members, present in all pneumococcal isolates, which are located at the bacterial cell wall by their specific recognition of choline residues in teichoic and lipoteichoic acids by a choline-binding module (CBM). CBPs play important roles such as adhesion to the host, daughter cell separation, autolysis and release of toxins [14]. Addition of high concentrations of choline to the medium causes the inhibition of those CBPs involved in cell separation upon division, inducing the formation of chains without any apparent loss of cell viability [15, 16]. In the search for choline analogs with higher affinities to CBPs (and therefore lower inhibitory concentrations), we found that esters of bicyclic amines (EBAs) such as atropine and ipratropium behaved as very efficient inhibitors of the enzymatic activity of some CBPs (*i.e.*, LytA, LytC and Pce) *in vitro* [11]. However, when exogenously added to pneumococcal planktonic cultures, EBAs cause cell deformations and lysis instead of the cell chaining phenotype foreseen for a choline analog, as well as a decrease in viability by several orders of magnitude [11, 13]. These observations suggested the existence of a novel antimicrobial mechanism for these compounds.

In this work we have investigated the molecular basis driving the antipneumococcal effect of EBAs. We focused on EBA18, EBA26, EBA27 and EBA31 compounds as the most efficient antipneumococcal molecules in a previous study and, in the particular case of EBA31 assayed *in vivo* in zebrafish models [13]. Our results indicate a membrane-destabilizing effect underlying their lethal activity. This prompted us to assess the inhibitory and/or disintegrating effect *in vitro* of these molecules both on pneumococcal

and mixed NTPn–NTHi *in vitro* biofilms, the latter accounting for a model of polymicrobial infections [17]. Our results support the notion that EBAs negatively affect biofilms of both Gram-positive and Gram-negative bacteria and may constitute a novel family of broad-range antimicrobials.

2. MATERIALS AND METHODS

2.1. EBA and chemical reagents. EBAs were synthesized by the Combinatorial Chemistry Platform in the Barcelona Scientific Park (Spain) as tertiary amines [13] (Table 1). EBA solutions were always freshly prepared in water by obtaining the corresponding hydrochloride. Solid powder was added to a 15 mM HCl aqueous solution to a final concentration of 10 mM, and then subjected to magnetic stirring for 2 hours. All other chemical reagents were purchased from Sigma-Aldrich unless otherwise stated.

2.2. Bacterial strains and growth conditions.

Pneumococcal planktonic cultures were grown without aeration in C medium [18] containing potassium phosphate buffer (33 mM, pH 8.0; CpH8 medium) eventually supplemented with 0.8 mg/ml yeast extract (C+Y medium). Optical density at 550 nm (OD₅₅₀) was monitored in a Thermo Spectronic spectrophotometer (Waltham, MA, USA). Pneumococcal biofilms were produced using *S. pneumoniae* strain R6 or *S. pneumoniae* P181, an isogenic serotype 19A transformant of R6 (Table 2). Bacterial strains were grown at 37°C in C+Y medium. Biofilm formation on 96-well polystyrene microtiter plates (Costar 3595; Corning) was obtained as previously described [19, 20]. Briefly, cells were grown in C+Y medium to an OD₅₅₀ of 0.5–0.6, harvested by centrifugation (3800 × g, 4 °C, 10 min), resuspended in an equal volume of fresh C+Y medium, and diluted 1/100; then, 200 µl containing 5 × 10⁶ colony forming unit (CFU)/ml were dispensed into 96-well polystyrene microtiter plates (Costar 3595; Corning). When glass-bottomed dishes (WillCo-dish; WillCo Wells B.V., The Netherlands) were employed, 2 ml of culture were used per well.

Table 1 Structure and characteristics of EBAs used in this study^a

Compound	Chemical structure	log <i>P</i> ^b
18		4.1
26		4.6
27		4.7
31		4.9

^a EBA identification as previously described [13]. Structures were rendered with ChemDraw 10.0 (CambridgeSoft, U.K.)

^b Logarithm of estimated partition ratio in octanol:water, calculated using the Molinspiration web utilities (<http://www.molinspiration.com/cgi-bin/properties>) (Molinspiration Cheminformatics, Nova Ulica, Slovak Republic)

For the inhibition of biofilm formation, EBAs were added to the bacteria at the beginning of the incubation in the plates or glass-bottom dishes. For dispersal experiments, biofilm formation was allowed for 5 h at 34°C (for R6) or 5 h at 37°C under 5% CO₂ (for P181). Planktonically grown cells were aspirated off and the biofilm was rinsed with sterile distilled H₂O. The resulting biofilms were treated for an additional 1 h at 37°C with various concentrations of EBAs in 20 mM sodium phosphate buffer, pH 6.9. Controls carried the same volume of buffer instead of EBA solution. The biofilms were then washed twice with sterile distilled H₂O and disaggregated by gentle pipetting and vortexing [21]. Afterwards, 10-fold dilution series were prepared in phosphate-buffered saline (PBS). The absorbance at 595 nm (*A*₅₉₅) was measured with a VersaMax microplate absorbance reader (Molecular Devices). The quantification of viable cells (planktonic and biofilm cells) was performed in blood agar plates. Colonies were counted after overnight incubation at 37°C. The susceptibility of planktonically grown R6 and P181 cells to the antibacterial agents was determined by the broth microdilution method according to CLSI guidelines [22].

2.3. Cell permeability assays. *S. pneumoniae* R6 was grown in C+Y medium at 37°C to an OD₅₅₀ of 0.5–0.6, corresponding to approximately 10⁸ CFU/ml, while NTHi 54997 was grown at 37°C under 5% CO₂ in brain-heart infusion medium supplemented with 10 µg/ml each of hemin and NAD (sBHI medium). For both microorganisms the corresponding culture was then sedimented by centrifugation (3800 × g, 4°C, 10 min). Cells were resuspended in 5 mM sodium phosphate buffer, pH 7.0, containing 280 mM sorbitol and SYTOX Green fluorophore (1 µM), to a final cell concentration of 2.5 × 10⁷ CFU/ml, and dispensed in 90 µl aliquots in a 96-well Nunc F96 MicroWell black polystyrene plate (Thermo Scientific). The plate was read in a BMG POLARstar Galaxy Microplate Reader (Offenburg, Germany) by monitoring the fluorescence in real time using an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Gain was adjusted using cells incubated with 0.1% Triton X-100 as the maximal value of permeabilization. After a stable basal fluorescence read out was obtained, 10 µl of the corresponding compound were added to each well (EBA stock solution, 1% Triton X-100 for positive control and buffer for the negative control) and incubation proceeded for 2 h. The assay was terminated by addition of 10 µl of 1% Triton X-100 to obtain the respective maximum value for each well. Experiments were performed in triplicate, and the results are expressed in percentage of fluorescence intensity with respect to the value obtained by the late addition of Triton X-100.

Membrane permeability was also assayed using 3,5-dipropylthiacarbocyanine (diSC3-5) (Thermo Fisher). The fluorescence intensity of this probe varies in response to changes in transmembrane potential [32]. Pneumococcal cultures were similarly processed as above, except that cells were resuspended in PBS buffer containing 0.6 µM diSC3-5, and subsequently dispensed in multiwell plates (90 µl) onto which 10 µl of the corresponding compound were added. Fluorescence was measured after a 1h incubation using excitation and emission wavelengths of 622 and 670 nm, respectively. Sodium deoxycholate (DOC) was added at 0.1% final concentration to register the maximum values of fluorescence.

Table 2. Bacterial strains used in this study, MICs and lytic activity of EBA 31

Strain	Characteristics	Reference	MIC (μ M) ^a	Decrease in OD ₅₅₀ (%) ^b
<i>S. pneumoniae</i>				
R6	Non-encapsulated strain derived from D39 (Hex ⁺ , <i>lytA</i> ⁺)	[23]	180 (EBA 18); 90 (EBA 26); 90 (EBA 27); 22 (EBA 31)	89
M11	R6 derivative (Hex ⁻ ; <i>lytA</i> ⁺)	[24]		
M22	M11 derivative (Nov ^r)	[25]		
M31	M11 derivative (Hex ⁻ ; Δ <i>lytA</i>)	[26]		25
R6B	R6 derivative (Hex ⁺ ; <i>lytB</i>)	[24]		78
R6C	R6 derivative (Hex ⁺ ; <i>lytC</i>)	[24]		82
P046	R6 derivative (<i>lytA lytC</i>)	[24]		2
P181	M11 transformed with 5086 DNA (serotype 19A)	[19]	32 (EBA 31)	
P233	R6 transformed with M22 DNA (Nov ^r)	[27]	16 (EBA 31)	
D39	Encapsulated strain (NCTC 07466, serotype 2) (<i>lytA</i> ⁺)	[28]		82
P095	D39 derivative (<i>lytA</i>)	[29]		20
P096	D39 derivative (<i>lytB</i>)	[30]		65
P093	D39 derivative (<i>lytC</i>)	[30]		85
P139	D39 derivative (<i>lytA lytC</i>)	[31]		14
P170	D39 derivative (<i>lytA lytB lytC</i>)	[31]		28
5086	serotype 19A; clinical isolate	[19]		
<i>H. influenzae</i>				
54997	Non-typeable clinical isolate	[27]	63 (EBA 31)	

^a MICs of the R6 strain were reported in a previous publication [13].

^b EBA 31 (100 μ M final concentration) was added to exponentially growing cultures of the indicated strains. Decrease in OD₅₅₀ compared with an untreated control of the corresponding strain after 4 h incubation at 37°C. Values are the average of three determinations.

2.4. Mixed biofilm formation assay and quantification, susceptibility testing and antibiofilm therapy. The bacterial strains used for mixed biofilms were *NTHi* 54997 and the novobiocin-resistant (Nov^r) *NTPn* P233 (Table 2). Biofilm formation was assessed with crystal violet [27]. *NTHi* 54997 was grown at 37°C in sBHI medium as described above to an OD₅₅₀ of 0.5, sedimented by centrifugation, resuspended in an equal volume of C+Y medium supplemented with hemin and NAD (15 µg/ml each) [s(C+Y) medium], and diluted 100-fold. *NTPn* P233 was grown at 37°C in C+Y medium to the same OD₅₅₀, sedimented by centrifugation, resuspended in an equal volume of s(C+Y), and diluted 100-fold. Inocula of 5×10^6 to 7×10^6 CFU of each strain were dispensed into 96-well polystyrene microtiter plates (Costar 3595; Corning) (final volume of 200 µl) and glass-bottomed dishes (WillCo-dish; WillCo Wells B.V., The Netherlands) (final volume of 2 ml). For the inhibition of biofilm formation, EBA 31 was added to the bacteria at the beginning of the incubation. For dispersal of biofilms, biofilm formation was allowed for 5 h at 37°C under 5% CO₂, then the planktonic, non-adherent cells were removed by aspiration and the biofilm was rinsed with sterile distilled H₂O. The resulting biofilms were treated with various concentrations of EBA 31 in 20 mM sodium phosphate buffer pH 6.9 for 1 additional hour at 37°C in a 5% CO₂ atmosphere. Controls were run with the same amount of buffer without EBA. The biofilms were then washed twice with sterile distilled H₂O, disaggregated as above, and viable cells (planktonic and biofilm cells) quantified in blood agar plates containing novobiocin (10 µg/ml) for *NTPn* and chocolate agar plates supplemented with 0.16 µg/ml ampicillin for *NTHi*. Colonies were counted after overnight incubation at 37°C under 5% CO₂.

2.5. Microscopic observation of biofilms and staining with HPA. For the observation of biofilms at the confocal laser scanning microscope (CLSM), biofilms were grown on glass-bottomed dishes (WillCo-dish; WillCo Wells B.V., The Netherlands) for 5–6 h at 37°C under 5% CO₂. Following incubation with EBAs, the culture medium was removed and the biofilm rinsed with sterile water to remove non-adherent bacteria. The biofilms were then stained with HPA conjugated to Alexa Fluor 488 (25 µg/ml), the *BacLight* LIVE/DEAD bacterial viability kit, or SYTO 59; all staining procedures involved

incubation for 10–20 min at room temperature in the dark. After staining, biofilms were gently rinsed with 0.5 ml of PBS. CLSM observations were made at a magnification of 63× with a Leica TCS-SP2-AOBS-UV microscope equipped with an argon ion laser. Images were analyzed with LCS software from Leica. Projections were obtained in the x – y (individual scans at 0.5 μ m intervals) and x – z (images at 6 μ m intervals) planes.

3. RESULTS

3.1. Membrane destabilization by EBAs. As stated above, EBAs show a lytic effect when added to a pneumococcal planktonic culture [11, 13], contrary to the typical cell chaining phenotype induced by standard choline analogs. In an attempt to identify whether CBPs having cell wall hydrolase activity were involved in this process, EBA 31 (the most efficient EBA tested so far) [13] was added to cultures of *S. pneumoniae* R6 or D39 strains deficient in at least one peptidoglycan hydrolase, namely LytA, LytB, and LytC (Table 2). Of note, bacterial lysis by EBAs only decreased significantly in LytA-deficient mutants, suggesting the active involvement of this autolysin in the lysis induced by EBAs. Moreover, the simultaneous presence of choline chloride (150 mM) in the R6 and D39 cultures led to a substantial reduction of EBA-induced bacterial lysis along with a significant recovery of viability (Table 3), compatible with the partial inhibition of the LytA activity [15, 16]. To check whether EBAs might induce the release of cytoplasmic LytA by promoting its translocation across the membrane, cell permeability in EBA-treated pneumococci was analyzed using the entrance of the SYTOX Green vital dye (Fig. 1). All EBAs tested (Table 1) destabilized the *S. pneumoniae* membrane to permeability levels close to those caused by the Triton X-100 detergent, being EBA 31 the compound with the fastest kinetics (Fig. 1). To further confirm the effect EBAs on the cell membrane, experiments were carried out using the fluorescent compound diSC3-5, a probe of the polarization state of the membrane [32]. Figure 1B shows that addition of EBAs causes a significant change in the fluorescence of the probe, indicative of a high degree of membrane depolarization (around 50%) with respect to the addition of sodium deoxycholate (DOC).

These results corroborate an additional activity of EBAs on membrane permeability besides the already described binding and inhibition of CBPs.

Table 3. Effect of choline chloride on the antimicrobial activity of EBA 31^a

Addition	Decrease in OD ₅₅₀ with respect to control (%)		Cell viability with respect to control (%)	
	R6	D39	R6	D39
None	89	82	< 0.001	< 0.001
Choline chloride	27	37	2.4	0.1

^a Exponentially growing cultures of the *NTPn* strain R6 received EBA 31 at 0.1 mM (final concentration). Another part of the culture was left untreated (control). Half of the treated culture also received choline chloride (at 143 mM final concentration) and bacteria were incubated for 4 h at 37°C. The values of OD₆₀₀ and bacterial viability were recorded at the beginning and at the end of the experiment. Values are the average of three independent experiments.

The chemical features of EBAs are key to explain this effect, as these molecules contain a polar moiety (harboring a tertiary amine group) esterified to a highly hydrophobic, polycyclic, aromatic structure (Table 1), prone to insertion into biological membranes. In fact, Fig. 2 shows the correlation between the physico-chemical features of tested EBAs and their antimicrobial effect on pneumococcus: the higher the hydrophobicity of the aromatic moiety (as reflected by the log *P* parameter, see Table 1), the faster kinetics on induction of membrane permeability (arbitrarily represented as the time needed to achieve 50% of maximum permeability) and the lower MIC values are displayed. In this sense, EBA 31 proved to be the optimal compound. The observed correlation between non-polarity and antibacterial performance also explains why quaternary ammonium EBAs (*i.e.*, derivatives of ipratropium), which bear a permanent positive charge, are much less efficient in triggering pneumococcal lysis than their less polar, tertiary amine counterparts, such as the EBAs tested in this study [13]. Accordingly, Figure 1 (inset) shows that addition of ipratropium (with an estimated log *P* of -1.4) to a concentration up to 200 μM barely induced any changes in membrane permeability even after a 40 min incubation.

A **B**

Figure 1. A, Evaluation of permeability of pneumococcal cells. *S. pneumoniae* R6 cells were incubated in 5 mM sodium phosphate buffer, pH 7.0, containing 280 mM sorbitol. Positive control: Triton X-100 was added at 0.1% (final concentration); negative control: only buffer added. EBAs were assayed at their respective MICs. *Inset:* Incubation with 200 μ M ipratropium bromide. Arrows indicate addition of 0.1% Triton X-100 to all samples. **B,** Depolarization of the pneumococcal membrane monitored by diSC3-5 fluorescence assays. *S. pneumoniae* R6 cells were incubated in PBS buffer. Fluorescence is related to the maximum values obtained after addition of 0.1% DOC; negative control: only buffer added. EBAs were assayed at their respective MICs.

Figure 2. Correlation between biochemical and microbiological features of EBAs. Three-dimensional plot representing the log P parameter, MICs in planktonic pneumococcal R6 cultures and time needed to reach 50% of total membrane permeability.

3.2. Effect of selected EBAs on pneumococcal biofilms. It has been previously reported that addition of choline chloride to pneumococcal cultures inhibit the formation of biofilms by blocking and/or releasing CBPs from the cell wall, without affecting the viability of the residual cells in the biofilm [24]. However, in our case, the existence of a second, membrane-destabilizing mechanism by EBAs (that is more biologically relevant than their choline-like CBP-inhibiting activity) prompted us to assay their antimicrobial effect on these biofilms. Biofilm formation by the non-encapsulated strain *S. pneumoniae* R6 was strongly inhibited upon addition of EBAs to the cultures at concentrations close to their corresponding MICs (data not shown). Moreover, the capacity of EBAs to cause biofilm disintegration was tested by monitoring both the structure of the biofilm — using CLSM — and the viability of the remaining cells. A disruption effect on the biofilm of *S. pneumoniae* R6 was observed in all cases, being EBA 31 the most effective compound (Fig. 3A). Furthermore, the membrane of non-dispersed cells was severely compromised upon incubation with EBAs (Fig. 3B), in line with the perturbation mechanism observed *in vitro* for these compounds (Fig. 1).

Figure 3. Disintegration of NTPn R6 biofilms. Biofilms were incubated with EBAs at 37°C for 1 h. Controls included biofilms incubated for 1 h in 20 mM sodium phosphate buffer, pH 6.9). **A.** The results represent the mean $\pm$ standard error of at least three independent experiments, each performed in triplicate. Error bars represent the standard error. Asterisks-marked results are statistically significant ($P < 0.05$) compared to the control. **B.** CLSM of biofilm-grown *S. pneumoniae* R6 treated with EBAs, for 1 h at 37°C. Biofilms were stained with the BacLight kit. Scale bars, 25 μ m.

The effect of the EBA 31 was further tested using biofilms formed by an encapsulated *S. pneumoniae* strain. Serotype 19A was chosen since it is still a frequent cause of severe disease despite being included in the current conjugate vaccines and, in addition, its capsular polysaccharides allow significant biofilm formation under *in vitro* conditions [19]. Addition of EBA 31 at concentrations around the MIC (Table 2), inhibited biofilm formation by *S. pneumoniae* P181 (Fig. 4A), and also disintegrated the biofilm formed by this encapsulated strain (Figs. 4B and 4C), although the decrease in viability was lower than on R6 (Fig. 3). In any case, the induction of a structurally compromised cell membrane was also detected in this strain (Fig. 4C).

3.3. Effect of EBA 31 in mixed NTPn–NTHi biofilms. After demonstrating the effectivity of EBAs to prevent and eliminate the biofilm of encapsulated and non-encapsulated pneumococcal strains, we explored whether this antimicrobial activity might be expanded to Gram-negative organisms such as NTHi. A MIC value of 63 μ M was obtained for planktonic cultures of NTHi 54997 (Table 2), demonstrating the effectivity of EBAs against this microorganism. Moreover, EBA31 induced an increase in the NTHi inner membrane permeability similar to NTPn, although with a less marked effect (Fig. 5).

In any case, this result suggests a similar bactericidal mechanism for the two organisms, which is probably exacerbated in *S. pneumoniae* due to the additional participation of the LytA autolysin (see above).

Figure 4. Effect of EBA 31 on biofilms formed by the encapsulated *S. pneumoniae* P181 strain. **A.** Inhibition of biofilm formation. *S. pneumoniae* P181 was distributed in the wells of a microtiter plate, which was then incubated for 5 h at 37°C under 5% CO₂ in the presence of EBA 31. Black and grey bars indicate growth and biofilm formation respectively. **B.** Disgregation of biofilms. Cells growing as biofilms were incubated with EBA 31 at 37°C for 1 h under 5% CO₂. The results represent the mean $\pm$ standard error of at least three independent experiments, each performed in triplicate. Error bars represent the standard error. Asterisks-marked results are statistically significant (*, $P < 0.05$; **, $P < 0.001$) compared to the control. **C.** CLSM of biofilm-grown *S. pneumoniae* P181 treated with EBA 31, for 1 h at 37°C under 5% CO₂. Biofilms were stained with the BacLight kit. Scale bars, 25 μm.

Figure 5. Evaluation of permeability of *H. influenzae* cells. NTHi 54997 cells were incubated in 5 mM sodium phosphate buffer, pH 7.0, containing 280 mM sorbitol. Positive control: Triton X-100 was added at 0.1% (final concentration); negative control: only buffer added. EBA31 was assayed at 1× MIC (63 μM) and 2× MIC (126 μM). Arrow indicates addition of 0.1% Triton X-100 to all samples.

The effect of EBA 31 on NTPn P233 and mixed NTPn P233–NTHi 54997 biofilms was then examined. A significant reduction was observed in the formation of mixed biofilms at concentrations around the respective MICs (Table 2, Fig. 6A): EBA 31 at 55 μM was sufficient to completely inhibit the formation of the biofilm generated by *S. pneumoniae* P233, whereas the cell viability of NTHi 54997 in mixed biofilms decreased in more than 90% (Fig. 6B). EBA 31 also showed a disintegrating and bactericidal effect on mixed biofilms. Incubation of preformed NTPn P233–NTHi 54997 biofilms with this compound at 220 μM caused reductions in the viability of 2 and 3 log units for NTHi 54997 and NTPn P233 strain respectively, whereas at 550 μM, EBA 31 increased cell death in both cases, with practical eradication of the population (Fig. 6C). Figure 6D shows the distribution of the populations that form the biofilm using SYTO 59 together with the *Helix pomatia* agglutinin HPA, which recognizes the αGlcNAc residues of the cell wall teichoic and lipoteichoic acids of *S. pneumoniae* [27, 33]. Moderate concentrations of EBA 31 (55 μM) caused the selective eradication of *S. pneumoniae*, whereas higher concentrations (220 μM) exerted a clear bactericidal effect on both species.

4. DISCUSSION

AMR is considered by WHO as one of the most serious concerns for global health (<http://www.who.int/mediacentre/factsheets/fs194/en/>). This situation has promoted the search for alternatives to the antibiotics currently in use. In this sense, previous results of our group identified a novel type of molecules, namely esters of bicyclic amines (EBAs) as efficient antipneumococcal compounds capable of arresting bacterial growth and of conferring protection from infection in zebrafish models with MICs at the micromolar range [13], similar to other antibiotics. However, although EBAs were initially designed as pneumococcal CBP inhibitors to induce a cell chaining phenotype (and, indeed, they demonstrate a specific inhibition of the LytA autolysin *in vitro* [13]), here they are instead responsible of bacterial lysis with a prominent involvement of LytA, according to the results obtained from mutants deficient in one or more CBP cell-wall hydrolases (Table 2). This apparent paradox suggested an alternative antibacterial mechanism by EBAs to be elucidated. The increase in pneumococcal cell permeability by EBAs (Fig. 1) points to a membrane disruption activity closely related to the hydrophobic nature and dimension of their respective polycyclic, aromatic moieties (Fig. 2). The extent of membrane damage probably allows the uncontrolled release of cytoplasmic LytA and the triggering of the premature autolysis of the culture. This hypothesis would explain that the membrane-destabilizing mechanism induced by EBAs is predominant over any theoretical inhibition of LytA by the compounds. In fact, 100 μ M EBA 31 is needed to account for 50% inhibition of LytA *in vitro* [13], whereas as low as 22 μ M (the MIC), is sufficient to display a clear bactericidal effect. All these results prompted us to test the effect of EBAs on pneumococcal biofilms.

Figure 6. Effect of EBA 31 on mixed *NTPn* P233–*NTHi* 54997 biofilms. **A.** EBA 31 was added to the initial biofilm at the indicated concentrations and incubated for 6 h at 37°C under 5% CO₂ to allow formation of the biofilm. Black and grey bars indicate growth and biofilm formation respectively. **B.** Inhibition of mixed biofilm formation. Cell viability from the mixed biofilm in the presence of EBA 31 (55 μM) is indicated. The results represent the mean ± standard error of at least 3 independent experiments, each performed in triplicate. Asterisks-marked results are statistically significant (*, $P < 0.05$; **, $P < 0.001$) compared to the control. **C.** Disintegration of mixed biofilms. Cell viability is displayed after incubating the biofilm in the presence of 220 or 550 μM of EBA 31 for 1 h at 37°C under 5% CO₂. The results represent the mean ± standard error of at least 3 independent experiments, each performed in triplicate. Asterisks-marked results are statistically significant (*, $P < 0.05$; **, $P < 0.001$) compared to the control. **D.** CLSM image of the distribution of the populations in mixed *NTPn*–*NTHi* biofilms. The mixed biofilms were stained with a combination of SYTO 59 (green) and HPA lectin conjugated with Alexa Fluor 488 (pink). *First row:* untreated control; *Second row,* inhibition of biofilm formation by EBA 31 (55 μM). The compound was added to the culture at the beginning of the biofilm formation and incubated for 6 h at 37°C in 5% CO₂. *Third row,* disruption of biofilm by EBA 31 (220 μM). Magnification bar = 25 μm.

A biofilm is the natural form of growth for many bacterial species. Moreover it is believed that more than 60% of human bacterial infections, and up to 80% of those that become chronic, involve microbial growth in biofilms [34]. The medical importance of biofilms lies in the significant decrease of antibiotic susceptibility under this type of growth [35]. In fact, antibiotic-susceptible strains form thicker biofilms than resistant ones [36], and the antibiotic concentrations needed to eradicate biofilm-forming bacteria can be up to 1,000 times higher than those required to eliminate planktonic microorganisms [37]. In an interesting, related study, Vandeveld *et al* [38] have previously reported the effect of ipratropium as a disassembling agent *in vitro* for aged pneumococcal biofilms, although no molecular mechanism was detailed for this event and no significant effect on cellular viability was detected either unless employed in cooperation with other antibiotics. Lack of bactericidal effect in this case could be due, at least in part, to the low concentrations of ipratropium used by these authors (around 4 μ M), which are unable to induce appreciable changes in planktonic cell viability [11] and/or in membrane permeability (no detected effect at 200 μ M - see Fig. 1, inset), as well as/or to the different protocols followed for biofilm formation. Rather, in the present study we have focused on tertiary amine, polyaromatic EBAs due to their most efficient antipneumococcal activity on planktonic cultures [13]. Tested EBAs were capable of inhibiting and disintegrating biofilms formed by the NTPn R6 strain (Fig. 3), as well as those of an isogenic R6 transformant expressing the 19A capsular polysaccharide (Fig. 4), causing a strong perturbation of the cell membrane (according to the *BacLight* staining procedure) concomitant with a decrease in viability, although we cannot rule out the possibility that EBAs also negatively affect biofilm structure by displacing some pneumococcal CBPs from the cell wall and/or the biofilm extracellular space [24, 39, 40].

The promising results with pneumococcal biofilms, together with the unveiled membrane-destabilizing mechanism of EBAs, prompted us to check the effect of these molecules on the Gram-negative pathogen *H. influenzae*. In fact, *S. pneumoniae* and NTHi are the predominant ottopathogens associated with otitis media as identified through bacterial culture [10] and frequently form mixed biofilms. Taking advantage of the *in vitro*

mixed *NTPn*–*NTHi* biofilm development procedure recently developed [27], we tested the antimicrobial effect of EBA 31 and showed that this compound also demonstrated inhibiting and disintegrating properties in this system (Fig. 6). Although the bactericidal effect of EBA 31 was higher for *S. pneumoniae* than for *H. influenzae* cells, it should be remarked that the EBAs tested in this study derive from a screening process strictly based on antipneumococcal properties [11, 13], and that pneumococcal lysis is boosted by the release of the LytA autolysin (Table 2), whereas it is likely that effects on *NTHi* arise basically from membrane perturbation effects (Fig. 5).

5. CONCLUSIONS

We show here the existence of a second mechanism governing the antimicrobial activity of EBAs based on the severe destabilization of the bacterial cell membrane, leading, in the case of *S. pneumoniae*, to premature autolysis. Moreover, EBAs inhibit and disintegrate monospecies biofilms formed by pneumococci, as well as mixed *NTPn*–*NTHi* systems. These results broaden the potential application of EBAs as antimicrobials targeted both to Gram-positive and Gram-negative microorganisms, and pave the way for the rational design of new variants with enhanced membrane-inserting, and therefore antimicrobial, properties.

ACKNOWLEDGMENTS

We thank M. T. Seisdedos and G. Elvira for their help with CLSM, and S. Ruiz and M. Garzón for skillful technical assistance.

Funding: this work was supported by grants from the Spanish Ministry of Economy, Industry and Competitiveness (BIO2013-47684-R, BIO2016-79323-R and SAF2017-88664-R), and the RETICS-FEDER RICET RD16/0027/0010. The CIBER de Enfermedades Respiratorias (CIBERES) is an initiative of the Spanish Instituto de Salud Carlos III.

REFERENCES

- [1] Global Burden of Disease Cancer Collaborators, Estimates of the global, regional, and national morbidity, mortality, and aetiologies of lower respiratory tract infections in 195 countries: a systematic analysis for the Global Burden of Disease Study 2015, *Lancet Infect. Dis.*, 17 (2017) 1133-1161.
- [2] M.R. Redinbo, The microbiota, chemical symbiosis, and human disease, *J. Mol. Biol.*, 426 (2014) 3877-3891.
- [3] Y. Belkaid, T.W. Hand, Role of the microbiota in immunity and inflammation, *Cell*, 157 (2014) 121-141.
- [4] C.D. Doern, C.A. Burnham, It's not easy being green: the viridans group streptococci, with a focus on pediatric clinical manifestations, *J. Clin. Microbiol.*, 48 (2010) 3829-3835.
- [5] A. Tikhomirova, S.P. Kidd, *Haemophilus influenzae* and *Streptococcus pneumoniae*: living together in a biofilm, *Pathog. Dis.*, 69 (2013) 114-126.
- [6] J.N. Weiser, The pneumococcus: why a commensal misbehaves, *J. Mol. Med. (Berl.)* 88 (2010) 97-102.
- [7] I. Rudan, K.L. O'Brien, H. Nair, L. Liu, E. Theodoratou, S. Qazi, I. Luksic, C.L. Fischer Walker, R.E. Black, H. Campbell, G. Child Health Epidemiology Reference, Epidemiology and etiology of childhood pneumonia in 2010: estimates of incidence, severe morbidity, mortality, underlying risk factors and causative pathogens for 192 countries, *J Glob Health*, 3 (2013) 010401.
- [8] M. Domenech, E. García, M. Moscoso, Biofilm formation in *Streptococcus pneumoniae*, *Microb. Biotechnol.* 5 (2012) 455-465.
- [9] A.R. Foxwell, J.M. Kyd, A.W. Cripps, Nontypeable *Haemophilus influenzae*: pathogenesis and prevention, *Microbiol. Mol. Biol. Rev.* 62 (1998) 294-308.
- [10] C.C. Ngo, H.M. Massa, R.B. Thornton, A.W. Cripps, Predominant bacteria detected from the middle ear fluid of children experiencing otitis media: a systematic review, *PLoS One*, 11 (2016) e0150949.
- [11] B. Maestro, A. González, P. García, J.M. Sanz, Inhibition of pneumococcal choline-binding proteins and cell growth by esters of bicyclic amines, *FEBS J.* 274 (2007) 364-376.
- [12] N. Silva-Martín, M.G. Retamosa, B. Maestro, S.G. Bartual, M.J. Rodes, P. García, J.M. Sanz, J.A. Hermoso, Crystal structures of CbpF complexed with atropine and ipratropium reveal clues for the design of novel antimicrobials against *Streptococcus pneumoniae*, *Biochim. Biophys. Acta*, 1840 (2014) 129-135.
- [13] M.G. Retamosa, R. Diez-Martinez, B. Maestro, E. García-Fernández, B. de Waal, E.W. Meijer, P. García, J.M. Sanz, Aromatic esters of bicyclic amines as antimicrobials against *Streptococcus pneumoniae*, *Angew. Chem. Int. Ed. Engl.*, 54 (2015) 13673-13677.

- [14] B. Maestro, J.M. Sanz, Choline binding proteins from *Streptococcus pneumoniae*: a dual role as enzybiotics and targets for the design of new antimicrobials, *Antibiotics* (Basel) 5 (2016) 21.
- [15] T. Briese, R. Hakenbeck, Interaction of the pneumococcal amidase with lipoteichoic acid and choline, *Eur. J. Biochem.* 146 (1985) 417-427.
- [16] S. Giudicelli, A. Tomasz, Attachment of pneumococcal autolysin to wall teichoic acids, an essential step in enzymatic wall degradation, *J. Bacteriol.* 158 (1984) 1188-1190.
- [17] T. Bjarnsholt, The role of bacterial biofilms in chronic infections, *APMIS* 121 (2013) 1-51.
- [18] S. Lacks, R.D. Hotchkiss, A study of the genetic material determining an enzyme in *Pneumococcus*, *Biochim. Biophys. Acta*, 39 (1960) 508-518.
- [19] M. Domenech, L. Araújo-Bazán, E. García, M. Moscoso, *In vitro* biofilm formation by *Streptococcus pneumoniae* as a predictor of post-vaccination emerging serotypes colonizing the human nasopharynx, *Environ. Microbiol.* 16 (2014) 1193-1201.
- [20] M. Domenech, E. García, M. Moscoso, Versatility of the capsular genes during biofilm formation by *Streptococcus pneumoniae*, *Environ. Microbiol.* 11 (2009) 2542-2555.
- [21] M. Domenech, E. Ramos-Sevillano, E. García, M. Moscoso, J. Yuste, Biofilm formation avoids complement immunity and phagocytosis of *Streptococcus pneumoniae*, *Infect. Immun.* 81 (2013) 2606-2615.
- [22] CaLSI, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, Approved standard-ninth edition: M07-A9 ed. Clinical and Laboratory Standards Institute, Wayne, PA 19087, USA, 2012.
- [23] J. Hoskins, W.E. Alborn, Jr., J. Arnold, L.C. Blaszcak, S. Burgett, B.S. DeHoff, S.T. Estrem, L. Fritz, D.J. Fu, W. Fuller, C. Geringer, R. Gilmour, J.S. Glass, H. Khoja, A.R. Kraft, R.E. Lagace, D.J. LeBlanc, L.N. Lee, E.J. Lefkowitz, J. Lu, P. Matsushima, S.M. McAhren, M. McHenney, K. McLeaster, C.W. Mundy, T.I. Nicas, F.H. Norris, M. O'Gara, R.B. Peery, G.T. Robertson, P. Rockey, P.M. Sun, M.E. Winkler, Y. Yang, M. Young-Bellido, G. Zhao, C.A. Zook, R.H. Baltz, S.R. Jaskunas, P.R. Rosteck, Jr., P.L. Skatrud, J.I. Glass, Genome of the bacterium *Streptococcus pneumoniae* strain R6, *J. Bacteriol.* 183 (2001) 5709-5717.
- [24] M. Moscoso, E. García, R. López, Biofilm formation by *Streptococcus pneumoniae*: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion, *J. Bacteriol.* 188 (2006) 7785-7795.
- [25] C. Ronda, J.L. García, R. López, Characterization of genetic transformation in *Streptococcus oralis* NCTC 11427: expression of the pneumococcal amidase in *S. oralis* using a new shuttle vector, *Mol. Gen. Genet.* 215 (1988) 53-57.
- [26] J.M. Sanchez-Puelles, C. Ronda, J.L. García, P. García, R. López, E. García, Searching for autolysin functions. Characterization of a pneumococcal mutant deleted in the *lytA* gene, *Eur. J. Biochem.* 158 (1986) 289-293.
- [27] M. Domenech, E. García, N-acetyl-L-cysteine and cysteamine as new strategies against mixed biofilms of nonencapsulated *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae*, *Antimicrob. Agents Chemother.* 61 (2017), e01992-16.

- [28] J.A. Lanie, W.L. Ng, K.M. Kazmierczak, T.M. Andrzejewski, T.M. Davidsen, K.J. Wayne, H. Tettelin, J.I. Glass, M.E. Winkler, Genome sequence of Avery's virulent serotype 2 strain D39 of *Streptococcus pneumoniae* and comparison with that of unencapsulated laboratory strain R6, *J. Bacteriol.* 189 (2007) 38-51.
- [29] E. Ramos-Sevillano, A. Urzainqui, S. Campuzano, M. Moscoso, F. Gonzalez-Camacho, M. Domenech, S. Rodríguez de Córdoba, F. Sánchez-Madrid, J.S. Brown, E. García, J. Yuste, Pleiotropic effects of cell wall amidase LytA on *Streptococcus pneumoniae* sensitivity to the host immune response, *Infect. Immun.* 83 (2015) 591-603.
- [30] E. Ramos-Sevillano, M. Moscoso, P. García, E. García, J. Yuste, Nasopharyngeal colonization and invasive disease are enhanced by the cell wall hydrolases LytB and LytC of *Streptococcus pneumoniae*, *PLoS One*, 6 (2011) e23626.
- [31] E. Ramos-Sevillano, Enfermedad neumocócica invasiva: mecanismos moleculares de patogenicidad y protección. Ph. D. Thesis, Universidad Complutense de Madrid (2013).
- [32] M. Wu, E. Maier, R. Benz, R.E. Hancock, Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*, *Biochemistry*, 38 (1999) 7235-7242.
- [33] M. Domenech, E. García, Fluorescence imaging of *Streptococcus pneumoniae* with the *Helix pomatia* agglutinin (HPA) as a potential, rapid diagnostic tool, *Front. Microbiol.* 8 (2017) 1333.
- [34] R.D. Wolcott, G.D. Ehrlich, Biofilms and chronic infections, *JAMA*, 299 (2008) 2682-2684.
- [35] K. Lewis, Persister cells, *Annu. Rev. Microbiol.* 64 (2010) 357-372.
- [36] R. Camilli, A. Pantosti, L. Baldassarri, Contribution of serotype and genetic background to biofilm formation by *Streptococcus pneumoniae*, *Eur. J. Clin. Microbiol. Infect. Dis.* 30 (2011) 97-102.
- [37] R.M. Donlan, J.W. Costerton, Biofilms: survival mechanisms of clinically relevant microorganisms, *Clin. Microbiol. Rev.* 15 (2002) 167-193.
- [38] N.M. Vandeveld, P.M. Tulkens, G.G. Muccioli, F. Van Bambeke, Modulation of the activity of moxifloxacin and solithromycin in an *in vitro* pharmacodynamic model of *Streptococcus pneumoniae* naive and induced biofilms, *J. Antimicrob. Chemother.* 70 (2015) 1713-1726.
- [39] M. Domenech, E. García, A. Prieto, M. Moscoso, Insight into the composition of the intercellular matrix of *Streptococcus pneumoniae* biofilms, *Environ. Microbiol.* 15 (2013) 502-516.
- [40] M. Domenech, S. Ruiz, M. Moscoso, E. García, *In vitro* biofilm development of *Streptococcus pneumoniae* and formation of choline-binding protein-DNA complexes, *Environ. Microbiol. Rep.* 7 (2015) 715-727.

FIGURE LEGENDS

Figure 1. A, Evaluation of permeability of pneumococcal cells. *S. pneumoniae* R6 cells were incubated in 5 mM sodium phosphate buffer, pH 7.0, containing 280 mM sorbitol. Positive control: Triton X-100 was added at 0.1% (final concentration); negative control: only buffer added. EBAs were assayed at their respective MICs. *Inset:* Incubation with 200 μ M ipratropium bromide. Arrows indicate addition of 0.1% Triton X-100 to all samples. **B,** Depolarization of the pneumococcal membrane monitored by diSC3-5 fluorescence assays. *S. pneumoniae* R6 cells were incubated in PBS buffer. Fluorescence is related to the maximum values obtained after addition of 0.1% DOC; negative control: only buffer added. EBAs were assayed at their respective MICs

Figure 2. Correlation between biochemical and microbiological features of EBAs. Three-dimensional plot representing the log *P* parameter, MICs in planktonic pneumococcal R6 cultures and time needed to reach 50% of total membrane permeability.

Figure 3. Disintegration of NTPn R6 biofilms. Biofilms were incubated with EBAs at 37°C for 1 h. Controls included biofilms incubated for 1 h in 20 mM sodium phosphate buffer, pH 6.9). **A.** The results represent the mean $\pm$ standard error of at least three independent experiments, each performed in triplicate. Error bars represent the standard error. Asterisks-marked results are statistically significant ($P < 0.05$) compared to the control. **B.** CLSM of biofilm-grown *S. pneumoniae* R6 treated with EBAs, for 1 h at 37°C. Biofilms were stained with the BacLight kit. Scale bars, 25 μ m.

Figure 4. Effect of EBA 31 on biofilms formed by the encapsulated *S. pneumoniae* P181 strain. **A.** Inhibition of biofilm formation. *S. pneumoniae* P181 was distributed in the wells of a microtiter plate, which was then incubated for 5 h at 37°C under 5% CO₂ in the presence of EBA 31. Black and grey bars indicate growth and biofilm formation respectively. **B.** Disgregation of biofilms. Cells growing as biofilms were incubated with EBA 31 at 37°C for 1 h under 5% CO₂. The results represent the mean $\pm$ standard error of at least three independent experiments, each performed in triplicate. Error bars represent the standard error. Asterisks-marked results are statistically significant (*, $P < 0.05$; **, $P < 0.001$) compared to the control. **C.** CLSM of biofilm-grown *S. pneumoniae* P181 treated with EBA 31, for 1 h at 37°C under 5% CO₂. Biofilms were stained with the BacLight kit. Scale bars, 25 μ m.

Figure 5. Evaluation of permeability of *H. influenzae* cells. NTHi 54997 cells were incubated in 5 mM sodium phosphate buffer, pH 7.0, containing 280 mM sorbitol. Positive control: Triton X-100 was added at 0.1% (final concentration); negative control: only buffer added. EBA31 was assayed at 1 $\times$ MIC (63 μ M) and 2 $\times$ MIC (126 μ M). Arrow indicates addition of 0.1% Triton X-100 to all samples.

Figure 6. Effect of EBA 31 on mixed NTPn P233–NTHi 54997 biofilms. **A.** EBA 31 was added to the initial biofilm at the indicated concentrations and incubated for 6 h at 37°C under 5% CO₂ to allow formation of the biofilm. Black and grey bars indicate growth and

biofilm formation respectively. **B.** Inhibition of mixed biofilm formation. Cell viability from the mixed biofilm in the presence of EBA 31 (55 μ M) is indicated. The results represent the mean $\pm$ standard error of at least 3 independent experiments, each performed in triplicate. Asterisks-marked results are statistically significant (*, $P < 0.05$; **, $P < 0.001$) compared to the control. **C.** Disintegration of mixed biofilms. Cell viability is displayed after incubating the biofilm in the presence of 220 or 550 μ M of EBA 31 for 1 h at 37°C under 5% CO₂. The results represent the mean $\pm$ standard error of at least 3 independent experiments, each performed in triplicate. Asterisks-marked results are statistically significant (*, $P < 0.05$; **, $P < 0.001$) compared to the control. **D.** CLSM image of the distribution of the populations in mixed NTP_n–NTH_i biofilms. The mixed biofilms were stained with a combination of SYTO 59 (green) and HPA lectin conjugated with Alexa Fluor 488 (pink). *First row*: untreated control; *Second row*, inhibition of biofilm formation by EBA 31 (55 μ M). The compound was added to the culture at the beginning of the biofilm formation and incubated for 6 h at 37°C in 5% CO₂. *Third row*, disruption of biofilm by EBA 31 (220 μ M). Magnification bar = 25 μ m.