



## PNA Length Restriction of Antibacterial Activity of Peptide-PNA Conjugates in *Escherichia coli* Through Effects of the Inner Membrane

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Peptide Nucleic Acid (PNA)-peptide conjugates targeting essential bacterial genes are showing promise as antisense antimicrobials in drug discovery. Optimization has focused on selection of target genes and exact localization around the ribosome binding site, but surprisingly a length optimum around 10-12 nucleobases has been found. Addressing this observation, we have investigated the relationship between PNA-length, PNA-RNA duplex stability and antimicrobial activity in E. coli in more detail. For PNAs of identical length of ten nucleobases the expected reverse correlation between the thermal stability (Tm) of the PNA-RNA duplex and the MIC for single mismatched PNAs was found. Also the expected direct correlation between the length of the PNA and the PNA-RNA duplex stability was found. Nonetheless, 10-mer PNAs [in a 6-18 mer extension series of (KFF)<sub>3</sub>K- and (RXR)<sub>4</sub> conjugates] were the most active as antisense antimicrobials in both wild type E. coli MG1655 and AS19, suggesting that the size constraint is related to the bacterial uptake of PNA-peptide conjugates. This conclusion was supported by flow cytometry data showing higher bacterial uptake of shorter PNA fluorophore labeled conjugates. Interestingly, the size-limited uptake seems independent on outer membrane integrity (AS19), and thus the results suggest that the inner membrane limits the molecular size for peptide-PNA passage.

Keywords: antisense antimicrobials, peptide nucleic acid, peptide conjugates, bacterial envelope, Escherichia coli

## INTRODUCTION

The extensive use of antibiotics in the past half century has given rise to development of antibiotic resistant bacterial strains by a variety of mechanisms. Recently, the threat to global human health from multi-drug-resistant bacterial infections has emphasized the urgent need for discovery of new classes of antibiotics with novel molecular targets and mechanism of action. Effective antibiotics are characterized by their specificity for bacteria and thus low toxicity toward mammalian cells. Antibiotics working via an antisense mechanism, targeting essential bacterial genes is one way of ensuring high specificity. Peptide nucleic acids (PNAs) are particularly well-suited as such antimicrobial antisense agents because of their resistance to nucleases and proteases as well as favorable sequence specific RNA hybridization properties, although bacterial uptake is a general challenge in the application of PNAs (and of oligonucleotides and their analogs and mimics in

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Goltermann L, Yavari N, Zhang M, Ghosal A and Nielsen PE (2019) PNA Length Restriction of Antibacterial Activity of Peptide-PNA Conjugates in Escherichia coli Through Effects of the Inner Membrane. Front. Microbiol. 10:1032. doi: 10.3389/fmicb.2019.01032 general) as antisense agents. However, conjugation to bacteria penetrating peptides (BPPs) was discovered as a successful way to ensure increased PNA uptake and thereby efficacy (Good et al., 2001). Specifically, the (KFF)<sub>3</sub>K peptide conjugated to a PNA targeting the essential bacterial gene, *acpP*, was shown to be sequence specifically bactericidal against Escherichia coli (Good et al., 2001). Subsequently, antibacterial effects of peptide-PNA [and also of phosphordiamidate morpholino oligomer (PMO) (Geller et al., 2018)] conjugates against a range of antibiotic resistant bacterial species have been reported, e.g., multi-resistant Klebsiella pneumoniae (Kurupati et al., 2007) and Haemophilus influenzae (Otsuka et al., 2017), and Pseudomonas aeruginosa (Montagner et al., 2017) in planktonic cultures as well as biofilms. Furthermore, recent studies have identified the inner-membrane SbmA protein as a necessary transporter of some peptide-PNA conjugates (Ghosal et al., 2013). However, other carrier peptides do not require SbmA for activity, and SbmA is therefore not the only mechanism of transport across the inner membrane (Ghosal et al., 2013; Hansen et al., 2016).

Antisense efficacy relies on high sequence specific affinity for the mRNA target, and the strongest antisense effect is achieved by targeting sequences around and proximally upstream of the translation start codon (Dryselius et al., 2003). In general, increased efficacy with increased RNA target affinity, and thus length to a first approximation, is expected. However, early studies have surprisingly revealed an optimum PNA (and PMO) size of 10–12 nucleobases for antimicrobial antisense agents (Good et al., 2001; Deere et al., 2005).

It is worth noting that – analogously to the situation in eukaryotic cells – antisense agents may exert their activity via different mechanisms, broadly operating by mRNA degradation via RNaseH activation or by steric blockage of mRNA translation or processing. Antisense targeting in bacteria have almost exclusively been performed using PNA and PMO oligomers which do not activate RNaseH and therefore appear to work primarily through translation inhibition, although some reports do also indicate that mRNA decay (possibly induced by translation blockage) may result. RNA silencing occurs naturally in bacteria and these pathways may eventually also be exploited for RNA targeted antibiotic discovery (Soler Bistué et al., 2009; Good and Stach, 2011; Lopez et al., 2015).

In this study we have more systematically investigated the influence of PNA-length on the antimicrobial effect in *E. coli* using a series of peptide-PNA antibacterial antisense agents targeting *acpP* through translation inhibition with the aim of elucidating the mechanism behind the puzzling PNA length limitation. This was done for different carrier peptides, in different *E. coli* strains and in the presence or absence of the SbmA inner membrane transporter.

## MATERIALS AND METHODS

### **Strains and PNAs**

*Escherichia coli* strains MG1655, MG1655( $\Delta$ *sbmA*) (Ghosal et al., 2013), AS19 (Sekiguchi and Iida, 1967), ATCC25922( $\Delta$ *rfaG*) (Ebbensgaard et al., 2018), and *Klebsiella pneumoniae* ATCC 13883 were used throughout the study and cultured in non-cation

adjusted Muller-Hinton Broth (MHB) (Sigma-Aldrich, cat.no. 70192) at 37°C.

Peptide nucleic acids (**Table 1**) were dissolved in water and the concentration determined using a NanoDrop spectrophotometer at 260 nm. Low binding plastics (Axygen, Corning) were used throughout.

### **Tm Determination**

Thermal stability (Tm) measurements were performed on a Cary 300 Bio UV-visible spectrophotometer (Varian, Cary, NC, United States) connected to a temperature controller. Thermal melting profiles were obtained in 10 mM Na-phosphate (pH 7.0) containing 0.1 mM EDTA and 100 mM NaCl using a heating range of  $5-95^{\circ}$ C at a rate of  $0.5^{\circ}$ C/min. The melting temperature (*T*m) was determined from the maximum of the first derivative of the heating curve. Cuvettes of 1.0 cm path length and 1.0 ml volume were used for all experiments. RNA oligonucleotide 5089: 5'-AGA GUA UGA GCA CUA UCG-3' was used for all Tm experiments.

## **MIC Determination**

MIC values were determined by broth microdilution according to standard protocols with a few modifications (Cockerill et al., 2012). An overnight bacterial cell culture was diluted to approximately  $5 \times 10E5$  CFU/ml in non-cation-adjusted MHB. 190 ul bacterial solution was dispensed into a low-bind 96-well plate (Thermo-Scientific, cat.no. 260895) along with 10 ul of the test compound. The plate was incubated in a Tecan Genios plate reader at  $37^{\circ}$ C for 18 h with linear shaking, OD was measured every 20 min at 595 nm. The MIC was determined as the lowest concentration, which inhibited visible growth in the wells (OD(595 nm) < 0.1).

### **Spheroplast Preparation**

*Escherichia coli* cells were cultured in MHB overnight, diluted to OD(595 nm) = 0.3 and incubated at 37°C with shaking for 1–2 h. *E. coli* cells were harvested in exponential phase at 7000 g, 8 min at 4°C, washed twice in 0.01M Tris–HCl, pH 7.4 and then resuspended in the same buffer containing 0.5M sucrose. Then lysozyme was added to the cell suspension at a final concentration of 150 µg/ml and EDTA was added to a final concentration of 10 mM. This suspension was incubated overnight in a water bath at 37°C. Spheroplasts were gently washed three times in 0.01 M Tris–HCl with 0.5 M sucrose (Birdsell and Cota-Robles, 1967). Spheroplasts were incubated with peptide-PNA for 2 h, serially diluted with 0.5 M sucrose in 0.01 M Tris–HCl, pH 7.4, plated on LB agar plates, and colonies were counted after overnight incubation.

### **Flow Cytometry**

*Escherichia coli* cells were cultured in MHB overnight and diluted  $100 \times$  into fresh media and grown to exponential phase at  $OD_{595} = 0.2$ . The cells were pelleted and re-suspended (to a  $100 \times$  dilution) in PBS buffer containing the PNA, and then incubated for 1 h at room temperature. The cell suspension was diluted five times in PBS and profiled using an Apogee Flow Cytometer A10.

### TABLE 1 | Peptide-PNA-conjugates used (mm = mismatch).

2011       -       CTD ATA CTD T       10       app         4223       -       TG CTD ATA CTD T       12       app <sup>2</sup> 4224       -       CG CTD ATA CTD T       12       app <sup>2</sup> 4225       -       CG ATA GTG CTD ATA CTD T       18       app <sup>2</sup> 4226       -       CG ATA GTG CTD AGC T       10       mm for PNA202         4221       -       A GTG CTD TA CGC T       10       mm for PNA202         4521       -       GG ATA GTG CTD TA CGC T       10       mm for PNA202         4524       -       GG ATA GTG CTD TA CGC T       10       mm for PNA202         4720       H40PBjc Kog1+       CTCATACTCT       10       mm for PNA202         4721       H40PBjc Kog1+       CTCATACTCT       10       mm for PNA202         4723       H40PBjc Kog1+       CTCATACTCT       8       app <sup>2</sup> 6070       H40PBjc Kog1+       CTCATACTCT       8       app <sup>2</sup> 6108       H40PBjc Kog1+       CTCATACTCT       9       app <sup>2</sup> 6108       H40PBjc Kog1+       TG CTATACTCT       11       app <sup>2</sup>	PNA	Peptide	PNA sequence	Length	<b>Target</b> acpP	
4284-IQ CLO ALA CITO12appP4284-A GTG CTO ALA CITO14appP4286-CLO ALA CITO CITO ALA CITO18appP5881-CIC TTA CACT10mm for PMA2034891-CIC TTA CACT12mm for PMA20244892-CIC TTA CACT14mm for PMA20244892-CIC TTA CACT10mm for PMA20244892-CIC TTA CACT10mm for PMA20244892-CIC TTA CACT10mm for PMA20264721HK/RFJK-Kag1-CIC TTA CICT10mm for PMA20264722HK/RFJK-Kag1-CIC TTA CICT8appP5050HK/RFJK-Kag1-CIC TAC CICT8appP5051HK/RFJK-Kag1-CIC TAC CICT8appP5052HK/RFJK-Kag1-CIC TAC CICT10appP5053HK/RFJK-Kag1-CIC TAC CICT11appP5054HK/RFJK-Kag1-CIC TAC CICT11appP5056HK/RFJK-Kag1-CIC TAC CICT11appP5056HK/RFJK-Kag1-CIC TAC CICT14appP5057HK/RFJK-Kag1-CIC TAC ACT18appP5058HK/RFJK-Kag1-CIC TAC ACT18appP5056HK/RFJK-Kag1-CIC TAC ACT18appP5057HK/RFJK-Kag1-CIC TAC ACT18appP5058HK/RFJK-Kag1-CIC TAC ACT18mm for PMA3026<	2301	_	CTC ATA CTC T	10		
424       -       A GTG GTG ATA CTC T       18       acpP         4296       -       CG ATA GTG GTG ATA CTT T       18       acpP         4296       -       CTC TA GGC T       10       mm for PNA200         4211       -       AGT GTG TA GGC T       12       mm for PNA202         4224       -       AGT GTG TA GGC T       18       mm for PNA202         4524       -       CG ATA GTG CTO TA GAC T       18       mm for PNA202         4524       -       CG ATA GTG CTO TA GAC T       10       mm for PNA202         4720       H4WFPjK-kg1-       CTCATAGTCT       10       mm for PNA202         4723       H4WFPjK-kg1-       CTCATAGTCT       10       mm for PNA202         5073       H4WFPjK-kg1-       CTATAGTCT       8       acpP         5074       H4WFPjK-kg1-       CTATA CTC T       8       acpP         5080       H4WFPjK-kg1-       CTATA CTC T       10       acpP         5081       H4WFPjK-kg1-       CTATA CTC T       10       acpP         5084       H4WFPjK-kg1-       CTATA CTC T       10       acpP	4223	_	TG CTC ATA CTC T	12	acpP	
4286-CG ATA GTG CIC ALACIC.T16amfor PMA20303861GC TTA CAC T10mm for PMA20304521-CG TTA CAC T12mm for PMA20304524-CG ATA GTG CT TA CAC T18mm for PMA20404724H-CG ATA GTG CT TA CAC T18mm for PMA20404721HHGTC ATA GTG TT TA CAC T10mm for PMA20404722HHGTC ATA GTG TT10mm for PMA20404723HHGTC ATA GTG T6app24724HHGTC ATA GTG T8app25774HHHGTC ATA GTG T8app25785HHGTC ATA GTG T10app25886HHGTC ATA GTG T11app25887HHGTC ATA GTG T12app25888HHGTC ATA GTG T13app25884HHGTC ATA GTG T13app25885HHGTC ATA GTG T13app25886HHGTC ATA GTG T13app25886HHGTC ATA GTG T13app25886HHGTC ATA GTG T13app25886HHGTC ATA GTG T14mm for PMA20565971HHGTC ATA GTG T13app25886HHGTC ATA GTG T14mm for PMA2056 <td>4224</td> <td>-</td> <td>A GTG <u>CTC ATA CTC T</u></td> <td>14</td> <td>acpP</td>	4224	-	A GTG <u>CTC ATA CTC T</u>	14	acpP	
3961       -       CC TA CC T       10       mm for PMA201         4521       -       A GTG CTC TA QC T       12       mm for PMA223         4524       -       CG ATA GTG CTG TA QC T       14       mm for PMA224         4524       -       CG ATA GTG CTG TA QC T       16       mm for PMA205         4720       H(#FF)K-eg1-       CTGATAGTC T       10       mm for PMA206         4721       H(#FF)K-eg1-       CTGATAGTC T       10       mm for PMA206         4722       H(#FF)K-eg1-       CTGATAGTC T       10       mm for PMA206         5073       H(#FF)K-eg1-       CTGATAGTC T       8       acpP         5074       H(#FF)K-eg1-       CTGATAGTC T       11       acpP         5084       H(#FF)K-eg1-       CTGATAGTC T       11       acpP         5084       H(#FF)K-eg1-       GTGATAGTC T       11       acpP         5084       H(#FF)K-eg1-       GTGATAGTC T       10       mm for PMA206         5184       H(#FF)K-eg1-       GTGATAGTC T       11       acpP         5184       H(#FF)K-eg1-       GTGATAGTC T       10       mm for PMA3026	4226	_	CG ATA GTG <u>CTC ATA CTC T</u>	18	acpP	
4621       -       TG CTC TA CAC T       12       mm for PNA4222         4522       -       GA TG G CT TIA CAC T       14       mm for PNA4224         4524       -       GA TG G CT TIA CAC T       18       mm for PNA4226         4720       H40FFpik/eg1-       CACATACTCT       10       mm for PNA216         4721       H40FFpik/eg1-       CTCATACIGT       10       mm for PNA216         4723       H40FFpik/eg1-       CTCATACIGT       0       mm for PNA216         4723       H40FFpik/eg1-       CTCATACIGT       8       acpP         5070       H40FFpik/eg1-       CTCATACICT       8       acpP         5071       H40FFpik/eg1-       CTCATACICT       8       acpP         5080       H40FFpik/eg1-       CTATACICT       10       acpP         5081       H40FFpik/eg1-       G CTATACICT       14       acpP         5082       H40FFpik/eg1-       CTATACICT       8       mm for PNA5027         5084       H40FFpik/eg1-       CTATACICT       18       acpP         5085       H40FFpik/eg1-       CTATACICT       18       mm for PNA5027 <td>3961</td> <td>_</td> <td>CTC <b>T</b>TA C<b>A</b>C T</td> <td>10</td> <td>mm for PNA2301</td>	3961	_	CTC <b>T</b> TA C <b>A</b> C T	10	mm for PNA2301	
4622       -       A GTG GTC TI A CAC T       14       mm for PNA228         4524       -       GA CATA GTG GTC TI CAC T       18       mm for PNA228         4720       H-KFFp,K+g1-       GTC TACAT T       10       mm for PNA218         4721       H-KFFp,K+g1-       GTC TACAT GTC       10       mm for PNA218         4723       H-KFFp,K+g1-       GTC ATAGTCT       10       mm for PNA218         4723       H-KFFp,K+g1-       GTC ATA GTC T       8       app         5070       H-KFFp,K+g1-       GTC ATA GTC T       8       app         5080       H-KFFp,K+g1-       GTC ATA GTC T       11       app         5081       H-KFFp,K+g1-       GTC ATA GTC T       14       app         5082       H-KFFp,K+g1-       GTC ATA GTC T       14       app         5083       H-KFFp,K+g1-       ATA GTG GTC ATA GTC T       14       app         5164       H-KFFp,K+g1-       GTC ATA GTC T       8       mm for PNA507         5164       H-KFFp,K+g1-       GTC ATA GTC T       16       mm for PNA508         5164       H-KFFp,K+g1-       GTT ATA GTC GTC ATA GTC T       16	4521	-	TG CTC <b>T</b> TA C <b>A</b> C T	12	mm for PNA4223	
4624       -       G GATA GTG CTC TIA CAC T       18       mm for PMA2102         4720       HAKFBAK-eg1-       CTCTACTCT       10       mm for PMA2102         4721       HAKFBAK-eg1-       CTCATACTCT       10       mm for PMA2102         4723       HAKFBAK-eg1-       CTCATACTGT       10       mm for PMA2102         4723       HAKFBAK-eg1-       CTCATACTGT       6       acpP         5079       HAKFBAK-eg1-       CATA CTC T       6       acpP         5078       HAKFBAK-eg1-       CATA CTC T       9       acpP         2108       HAKFBAK-eg1-       CTCATA CTC T       11       acpP         2108       HAKFBAK-eg1-       GTCATA CTC T       12       acpP         5082       HAKFBAK-eg1-       GTCATA CTC T       12       acpP         5083       HAKFBAK-eg1-       GTCATA CTC T       13       acpP         5164       HAKFBAK-eg1-       GTCATA CTC T       16       acpP         5165       HAKFBAK-eg1-       GTCATA CTC T       18       acpP         5164       HAKFBAK-eg1-       GTCATA CTC T       11       mm for PMA50202 <t< td=""><td>4522</td><td>_</td><td>A GTG CTC <b>T</b>TA C<b>A</b>C T</td><td>14</td><td>mm for PNA4224</td></t<>	4522	_	A GTG CTC <b>T</b> TA C <b>A</b> C T	14	mm for PNA4224	
4720     H_4KFP_6K_eg1-     CACATACTCT     10     mm for PNA2108       4721     H_4KFP_6K_eg1-     CTCATACTCT     10     mm for PNA2108       4723     H_4KFP_6K_eg1-     CTCATACTCT     10     mm for PNA2108       4723     H_4KFP_6K_eg1-     CTCATACTGT     10     mm for PNA2108       5070     H_4KFP_6K_eg1-     CTCATACTGT     8     acpP       5080     H_4KFP_6K_eg1-     CTCATACTCT     9     acpP       5080     H_4KFP_6K_eg1-     CTCATACTCT     10     acpP       5080     H_4KFP_6K_eg1-     CTCATACTCT     11     acpP       5081     H_4KFP_6K_eg1-     GTCATACTCT     11     acpP       5082     H_4KFP_6K_eg1-     ATG GTCATACTCT     18     acpP       5084     H_4KFP_6K_eg1-     CG CATACTCT     18     acpP       5085     H_4KFP_6K_eg1-     CTTACACT     8     mm for PNA5078       5086     H_4KFP_6K_eg1-     CTTACACT     10     mm for PNA5087       5272     H_4KFP_6K_eg1-     CTTACACT     10     mm for PNA5087       5273     H_4KFP_6K_eg1-     CGTATACTCT     10	4524	_	CG ATA GTG CTC <b>T</b> TA C <b>A</b> C T	18	mm for PNA4226	
4721       H(KF) <sub>0</sub> K-g1-       CTCTACTCT       10       mm for PN42108         4722       H(KF) <sub>0</sub> K-g1-       CTCATACTGT       10       mm for PN42108         5077       H(KF) <sub>0</sub> K-g1-       CATACTGT       6       acpP         5078       H(KF) <sub>0</sub> K-g1-       CATACTGT       6       acpP         5079       H(KF) <sub>0</sub> K-g1-       CATACTCT       9       acpP         5080       H(KF) <sub>0</sub> K-g1-       CATACTCT       9       acpP         5082       H(KF) <sub>0</sub> K-g1-       CATACTCT       10       acpP         5083       H(KF) <sub>0</sub> K-g1-       GCTCATACTCT       14       acpP         5164       H(KF) <sub>0</sub> K-g1-       ACTGCTCATACTCT       18       acpP         5168       H(KF) <sub>0</sub> K-g1-       TAC ACT       8       mm for PN4507         5168       H(KF) <sub>0</sub> K-g1-       TAC ACT       8       mm for PN4507         5270       H(KF) <sub>0</sub> K-g1-       CTTAC ACT       10       mm for PN4508         5271       H(KF) <sub>0</sub> K-g1-       CTTAC ACT       10       mm for PN4508         5274       H(KF) <sub>0</sub> K-g1-       CTTAC ACT       10       mm for PN4508	4720	H-(KFF) <sub>3</sub> K-eg1-	CACATACTCT	10	mm for PNA2108	
4722     H(KF)s/keg1-     CTCATAGTCT     10     mm for PNA2102       4733     H(KF)s/keg1-     CTCATACTCT     10     mm for PNA2102       5070     H(KF)s/keg1-     TCATACTCT     8     acpP       5080     H(KF)s/keg1-     CATACTCT     8     acpP       5080     H(KF)s/keg1-     CTCATACTCT     9     acpP       5081     H(KF)s/keg1-     CTCATACTCT     10     acpP       5082     H(KF)s/keg1-     GTCATACTCT     11     acpP       5081     H(KF)s/keg1-     ACTG CTCATACTCT     16     acpP       5082     H(KF)s/keg1-     ACTG CTCATACTCT     16     acpP       5084     H(KF)s/keg1-     ACTG CTCATACTCT     16     acpP       5184     H(KF)s/keg1-     CTTACACT     8     mm for PNA507       5271     H(KF)s/keg1-     CTTACACT     9     mm for PNA508       5272     H(KF)s/keg1-     CTTACACT     10     mm for PNA508       5274     H(KF)s/keg1-     CTTACACT     10     mm for PNA508       5274     H(KF)s/keg1-     CGTCATACTCT     10     mm for PNA508	4721	H-(KFF) <sub>3</sub> K-eg1-	CTCTTACTCT	10	mm for PNA2108	
4723     H4KFFjsKeg1-     CTCATACTQT     10     mm for PNA210E       5077     H4KFFjsKeg1-     CATACTCT     6     acpP       5080     H4KFFjsKeg1-     CCATACTCT     9     acpP       5080     H4KFFjsKeg1-     CCATACTCT     9     acpP       5080     H4KFFjsKeg1-     CCATACTCT     10     acpP       5081     H4KFFjsKeg1-     CCATACTCT     12     acpP       5083     H4KFFjsKeg1-     CGCATACTCT     14     acpP       5086     H4KFFjsKeg1-     CGATAGTCCTACTCT     18     acpP       5086     H4KFFjsKeg1-     CGATAGTCCTACTCT     8     mm for PNA5077       5086     H4KFFjsKeg1-     CGATAGTCCTACACT     9     mm for PNA5075       5270     H4KFFjsKeg1-     CTCTACACT     10     mm for PNA5075       5271     H4KFFjsKeg1-     CTCTACACT     10     mm for PNA5062       5272     H4KFFjsKeg1-     CTCTACACT     10     mm for PNA5082       5274     H4KFFjsKeg1-     GCTCTACACT     10     mm for PNA5082       5404     H4KFFjsKeg1-     GCTCTACACT     10     mm for PNA5082 <td>4722</td> <td>H-(KFF)<sub>3</sub>K-eg1-</td> <td>CTCATA<b>G</b>TCT</td> <td>10</td> <td>mm for PNA2108</td>	4722	H-(KFF) <sub>3</sub> K-eg1-	CTCATA <b>G</b> TCT	10	mm for PNA2108	
5077       H.4KFPj.K.eg1-       TA GTC T       6       expP         5079       H.4KFPj.K.eg1-       CATA CTC T       8       axpP         5070       H.4KFPj.K.eg1-       CATA CTC T       9       axpP         2108       H.4KFPj.K.eg1-       CTC ATA CTC T       10       axpP         5080       H.4KFPj.K.eg1-       GTC ATA CTC T       11       axpP         5082       H.4KFPj.K.eg1-       GTC ATA CTC T       14       axpP         5164       H.4KFPj.K.eg1-       A GTG CTC ATA CTC T       16       axpP         5168       H.4KFPj.K.eg1-       CT CTA A CTC T       18       axpP         5398       H.4KFPj.K.eg1-       CT CTA CACT       8       mm for PNA607         5270       H.4KFPj.K.eg1-       CT CT AC ACT       9       mm for PNA607         5271       H.4KFPj.K.eg1-       CT CT TAC ACT       10       mm for PNA6082         5272       H.4KFPj.K.eg1-       CT CT TAC ACT       11       mm for PNA6082         5404       H.4KFPj.K.eg1-       CT CT TAC ACT       16       mm for PNA5082         5404       H.4KFPJ.K.eg1-       CT CT TAC ACT       16 </td <td>4723</td> <td>H-(KFF)<sub>3</sub>K-eg1-</td> <td>CTCATACT<b>G</b>T</td> <td>10</td> <td>mm for PNA2108</td>	4723	H-(KFF) <sub>3</sub> K-eg1-	CTCATACT <b>G</b> T	10	mm for PNA2108	
5079       H(KFF)K-eg1-       CATA CTC T       8       acpP         5080       H(KFF)K-eg1-       CTA ATC CT       9       acpP         5081       H(KFF)K-eg1-       CTC ATA CTC T       9       acpP         5082       H(KFF)K-eg1-       CTC ATA CTC T       10       acpP         5083       H(KFF)K-eg1-       A GTG CTC ATA CTC T       12       acpP         5164       H(KFF)K-eg1-       A GTG CTC ATA CTC T       18       acpP         5168       H(KFF)K-eg1-       CG ATA CTC CT ATA CTC T       18       acpP         5396       H(KFF)K-eg1-       CTA CACT       8       mm for PNA5077         5271       H(KFF)K-eg1-       CT TAC ACT       9       mm for PNA5087         5272       H(KFF)K-eg1-       GC TCT TAC ACT       11       mfor PNA5082         5274       H(KFF)K-eg1-       GC TCT TAC ACT       14       mm for PNA5082         5404       H(KFF)K-eg1-       A TA GTG CTT TAC ACT       14       mm for PNA5082         5404       H(KFF)K-eg1-       A GTG CTT TAC ACT       14       mm for PNA5082         5404       H(KFF)K-eg1-       A TA GTG CTT TAC ACT	5077	H-(KFF) <sub>3</sub> K-eg1-	TA CTC T	6	acpP	
5080       H4(RF)/K-eg1-       TC ATA CTC T       9       acpP         2108       H4(RF)/K-eg1-       CIC ATA CTC T       10       acpP         5082       H4(RF)/K-eg1-       G CIC ATA CTC T       11       acpP         5184       H4(RF)/K-eg1-       A GIG CIC ATA CTC T       12       acpP         5166       H4(RF)/K-eg1-       A GIG CIC ATA CTC T       14       acpP         5168       H4(RF)/K-eg1-       CI ATA CTC T       18       acpP         5168       H4(RF)/K-eg1-       CI ATA CTC T       18       acpP         5168       H4(RF)/K-eg1-       CI TAC ACT       8       mm for PNA5067         5271       H4(RF)/K-eg1-       CI TAC ACT       10       mm for PNA5062         5272       H4(RF)/K-eg1-       CI TAC ACT       11       mn for PNA5062         5274       H4(RF)/K-eg1-       CI TAC ACT       14       mf or PNA5062         5274       H4(RF)/K-eg1-       AGIG TO TAC ACT       14       mm for PNA5062         5274       H4(RF)/K-eg1-       AGIG TO TAC ACT       18       mm for PNA5062         5274       H4(RF)/K-eg1-       CG TA AGIG TO TA ACTC <td>5079</td> <td>H-(KFF)<sub>3</sub>K-eg1-</td> <td>C ATA CTC T</td> <td>8</td> <td>acpP</td>	5079	H-(KFF) <sub>3</sub> K-eg1-	C ATA CTC T	8	acpP	
2108       H-(KFP)K-eg1       CIC ATA CIC I       10       acpP         5082       H-(KFP)K-eg1       G CIC ATA CIC I       11       acpP         5083       H-(KFP)K-eg1       TG CIC ATA CIC I       12       acpP         5164       H-(KFP)K-eg1       A GIG CIC ATA CIC I       14       acpP         5168       H-(KFP)K-eg1       A GIG CIC ATA CIC I       18       acpP         5168       H-(KFP)K-eg1       CI TAC ACT       8       mm for PNA507         5270       H-(KFP)K-eg1       CI TAC ACT       9       mm for PNA507         5271       H-(KFP)K-eg1       CI TAC ACT       9       mm for PNA5087         5272       H-(KFP)K-eg1       CI TAC ACT       10       mm for PNA5087         5273       H-(KFP)K-eg1       CI TAC ACT       11       mm for PNA5082         5274       H-(KFP)K-eg1       CI TAC ACT       12       mm for PNA5082         5274       H-(KFP)K-eg1       CI CI TAC ACT       16       mm for PNA5082         5264       H-(KFP)K-eg1       CI CA TA CI CI TAC ACT       18       acpP         5264       H-(KFP)K-eg1 <t< td=""><td>5080</td><td>H-(KFF)<sub>3</sub>K-eg1-</td><td>TC ATA CTC T</td><td>9</td><td>acpP</td></t<>	5080	H-(KFF) <sub>3</sub> K-eg1-	TC ATA CTC T	9	acpP	
5082       H-(KF) <sub>3</sub> K-eg1       G CT ATA CTC T       11       acpP         5083       H-(KF) <sub>3</sub> K-eg1       TG CT ATA CTC T       12       acpP         5164       H-(KF) <sub>3</sub> K-eg1       A GT G CT ATA CTC T       14       acpP         5164       H-(KF) <sub>3</sub> K-eg1       ATA GTG CT C ATA CTC T       18       acpP         5168       H-(KF) <sub>3</sub> K-eg1       TC ATA CTC T       6       mm for PNA5077         5296       H-(KF) <sub>3</sub> K-eg1       TC TAC ACT       8       mm for PNA5075         5271       H-(KF) <sub>3</sub> K-eg1       CT TAC ACT       9       mm for PNA5082         5272       H-(KF) <sub>3</sub> K-eg1       CT CT TAC ACT       10       mm for PNA5082         5273       H-(KF) <sub>3</sub> K-eg1       CT CT TAC ACT       10       mm for PNA5082         5404       H-(KF) <sub>3</sub> K-eg1       CG TAG CT TAC ACT       11       mm for PNA5082         5404       H-(KF) <sub>3</sub> K-eg1       CG TAG CT TAC ACT       18       mm for PNA5082         5404       H-(KF) <sub>3</sub> K-eg1       CG TAG CT TAC ACT       18       mm for PNA5082         5406       H-(KF) <sub>3</sub> K-eg1       CG TAG CT TAC ACT       18       mm for PNA5082 <t< td=""><td>2108</td><td>H-(KFF)<sub>3</sub>K-eg1-</td><td>CTC ATA CTC T</td><td>10</td><td>acpP</td></t<>	2108	H-(KFF) <sub>3</sub> K-eg1-	CTC ATA CTC T	10	acpP	
5083       H-(KF) <sub>3</sub> K-eg1-       TG CIC ATA CTC T       12       acpP         5164       H-(KF) <sub>3</sub> K-eg1-       A GTG CTC ATA CTC T       14       acpP         5166       H-(KF) <sub>3</sub> K-eg1-       CG ATA GTG CTC ATA CTC T       18       acpP         5168       H-(KF) <sub>3</sub> K-eg1-       CG ATA GTG CTC ATA CTC T       18       acpP         5396       H-(KF) <sub>3</sub> K-eg1-       CT TAC ACT       6       mm for PNA5077         5271       H-(KF) <sub>3</sub> K-eg1-       CT TAC ACT       8       mm for PNA5087         5272       H-(KF) <sub>3</sub> K-eg1-       CT TAC ACT       10       mm for PNA5082         5273       H-(KF) <sub>3</sub> K-eg1-       CT CT TAC ACT       11       mm for PNA5082         5274       H-(KF) <sub>3</sub> K-eg1-       CG CT TAC ACT       12       mm for PNA5082         5404       H-(KF) <sub>3</sub> K-eg1-       CG ATG TG CT TAC ACT       18       mm for PNA5082         5404       H-(KF) <sub>3</sub> K-eg1-       CG ATA GT GC TT TAC ACT       18       mm for PNA5082         5408       H-(KF) <sub>3</sub> K-eg1-       CG ATA GT GT TAC ACT       18       mm for PNA5082         5408       H-(R-Aba) <sub>6</sub> -(P-Ala)-       CG CT ATA CTC T       18       acpP	5082	H-(KFF) <sub>3</sub> K-eg1-	G CTC ATA CTC T	11	acpP	
5164       H(KFF)K-eg1-       A GTG CTC ATA CTC T       14       acpP         5166       H(KFF)K-eg1-       ATA GTG CTC ATA CTC T       16       acpP         5168       H(KFF)K-eg1-       CG ATA CTC T       18       acpP         5286       H(KFF)K-eg1-       TCA CACT       6       mm for PNA507         5270       H(KFF)K-eg1-       CT TAC ACT       8       mm for PNA507         5271       H(KFF)K-eg1-       CT TAC ACT       10       mm for PNA5062         5272       H(KFF)K-eg1-       CT TAC ACT       11       mm for PNA5062         5273       H(KFF)K-eg1-       CT TAC ACT       14       mm for PNA5062         5274       H(KFF)K-eg1-       CG TT TAC ACT       14       mm for PNA5062         5404       H(KFF)K-eg1-       AG TG CT TAC ACT       14       mm for PNA5166         5406       H(KFF)K-eg1-       CG ATAG TGC TG TAC ACT       18       mm for PNA5166         5408       H(K-FNK)K-eg1-       CG ATAG TGC TG TAC ACT       18       acpP         5203       H(K-FNK)K-eg1-       CG ATAG TGC TG TAC ACT       18       acpP         5204       H(R-ANK)K-(R-Ala)-	5083	H-(KFF) <sub>3</sub> K-eg1-	TG CTC ATA CTC T	12	acpP	
5166       HKRFjkkeg1-       ATA GTG CTC ATA CTC T       16       appP         5188       HKRFjkkeg1-       CG ATA GTG CTC ATA CTC T       18       acpP         5396       HKRFjkkeg1-       TAC ACT       6       mm for PNA5077         5270       HKRFjjkkeg1-       CT TAC ACT       8       mm for PNA5076         5271       HKRFjjkkeg1-       CT TAC ACT       9       mm for PNA5062         5272       HKRFjjkkeg1-       CT TAC ACT       10       mm for PNA5062         5273       HKRFjjkkeg1-       GC TOT TAC ACT       11       mm for PNA5062         5274       HKRFjjkkeg1-       AG GT CT TAC ACT       12       mm for PNA5062         5274       HKRFjjkkeg1-       AG GT GT TAC ACT       18       mm for PNA5062         5404       HKRFjjkkeg1-       AG GT GT TAC ACT       18       mm for PNA5166         5408       HKRFjjkkeg1-       AG GT GT CT TAC ACT       18       acpP         4227       HR-Ambje/G-Ala)-       TG CT ATA CTC T       12       acpP         4228       HR-Ambje/G-Ala)-       AG GT GT GT ATA CTC T       18       acpP         4229       HR-Ambje/G-Ala)-<	5164	H-(KFF) <sub>3</sub> K-eq1-	A GTG CTC ATA CTC T	14	acpP	
5168 $H(KFP)_{3}K \cdot eg1$ -     CG ATA GTG CIC ATA CTC T     18 $acp^{P}$ 5396 $H_{(KFP)_{3}K \cdot eg1}$ -     TAC ACT     6     mm for PNA5077       5270 $H_{(KFP)_{3}K \cdot eg1}$ -     CT TAC ACT     8     mm for PNA5075       5271 $H_{(KFP)_{3}K \cdot eg1}$ -     CT TAC ACT     9     mm for PNA5085       5272 $H_{(KFP)_{3}K \cdot eg1}$ -     CT TAC ACT     10     mm for PNA5085       5273 $H_{(KFP)_{3}K \cdot eg1}$ -     GC TCT TAC ACT     11     mm for PNA5085       5274 $H_{(KFP)_{3}K \cdot eg1}$ -     GG CTCT TAC ACT     14     mm for PNA5164       5404 $H_{(KFP)_{3}K \cdot eg1}$ -     CG ATA GTG CTCT TAC ACT     18     mm for PNA5164       5406 $H_{(KFP)_{3}K \cdot eg1}$ -     CG ATA GTG CTCT TAC ACT     18     mm for PNA5164       5408 $H_{(KP)_{3}K \cdot eg1}$ -     CG ATA GTG CTCT TAC ACT     18     mm for PNA5164       5408 $H_{(KP)_{3}K \cdot eg1}$ -     CG ATA GTG CTCT TAC ACT     18     mm for PNA5164       5404 $H_{(R+Atw)_{6} \cdot (P,Ala)}$ -     ATG GTG CTA ACTC T     10     acpP       4227 $H_{(R-Atw)_{6} \cdot (P,Ala)}$ -     ATG GTG CTA ACTC T     16     acpP	5166	H-(KFF) <sub>3</sub> K-eq1-	ATA GTG CTC ATA CTC T	16	acpP	
Number of the term of	5168	H-(KFF) <sub>3</sub> K-eq1-	CG ATA GTG CTC ATA CTC T	18	acpP	
The Arby, Go       CITAC ACT       8       mm for PNA507G         5270       H-(KF), K-eg1-       TOT TAC ACT       9       mm for PNA508G         5271       H-(KF), K-eg1-       C TOT TAC ACT       9       mm for PNA508G         5272       H-(KF), K-eg1-       C TOT TAC ACT       10       mm for PNA508G         5273       H-(KF), K-eg1-       G C TOT TAC ACT       11       mm for PNA508G         5274       H-(KF), K-eg1-       A G TG C TOT TAC ACT       14       mm for PNA508G         5404       H-(KF), K-eg1-       A GTG C TT TAC ACT       18       mm for PNA508G         5406       H-(KF), K-eg1-       CG ATA GTG C TT TAC ACT       18       mm for PNA508G         5408       H-(R-Anb, e, Ala)-       CTC ATA CTC T       10       acpP         4227       H-(R-Anb, e, Ala)-       A GTG CTC ATA CTC T       10       acpP         4228       H-(R-Anb, e, G-Ala)-       A GTG CTC ATA CTC T       18       acpP         4229       H-(R-Anb, e, G-Ala)-       C G TA A GTG CTC ATA CTC T       18       acpP         3987       H-(R-Anb, e, G-Ala)-       C G TA GTG CT TAC ACT       10       mm for PNA3986 <t< td=""><td>5396</td><td>H-(KFF)<sub>3</sub>K-eq1-</td><td>TAC <b>A</b>CT</td><td>6</td><td>, mm for PNA5077</td></t<>	5396	H-(KFF) <sub>3</sub> K-eq1-	TAC <b>A</b> CT	6	, mm for PNA5077	
S271H_KFF)3K-6g1-TOT TAC ACT9mm for PNASOBC5272H-(KFF)3K-6g1-C TCT TAC ACT10mm for PNASOBC5273H-(KFF)3K-6g1-GC TCT TAC ACT11mm for PNASOBC5274H-(KFF)3K-6g1-TGC TCT TAC ACT12mm for PNASOBC5404H-(KFF)3K-6g1-AG TGC TCT TAC ACT14mm for PNASOBC5406H-(KFF)3K-6g1-AG TGC TCT TAC ACT16mm for PNASOBC5408H-(KFF)3K-6g1-CGA TAG TGC TCT TAC ACT18mm for PNASOBC3986H-(R-Ahx)6-(β-Ala)-CTC ATA CTC T10acpP4227H-(R-Ahx)6-(β-Ala)-TG CIC ATA CTC T14acpP4228H-(R-Ahx)6-(β-Ala)-TG CIC ATA CTC T16acpP4229H-(R-Ahx)6-(β-Ala)-CG ATA GTG CTC ATA CTC T18acpP4220H-(R-Ahx)6-(β-Ala)-CTC TAC ACT10mm for PNA398C4500H-(R-Ahx)6-(β-Ala)-CTC TAC ACT10mm for PNA398C4500H-(R-Ahx)6-(β-Ala)-CTC TAC ACT10mm for PNA398C4500H-(R-Ahx)6-(β-Ala)-CTC TAC ACT10mm for PNA42224501H-(R-Ahx)6-(β-Ala)-CTC TAC ACT10mm for PNA42264502H-(R-Ahx)6-(β-Ala)-CTC TAC ACT10mm for PNA42264503H-(R-Ahx)6-(β-Ala)-CG ATA GTG CTT TAC ACT16mm for PNA42264504H-(R-Ahx)6-(β-Ala)-CG ATA GTG CT TAC ACT16mm for PNA42264503H-(R-Ahx,6)-Ala)-CG ATA GTG	5270	H-(KFF) <sub>3</sub> K-eq1-		8	mm for PNA5079	
5272H(KFP)K-eg1-C TCT TAC ACT10mm for PNA210E5273H-(KFP)K-eg1-GC TCT TAC ACT11mm for PNA210E5274H-(KFP)K-eg1-TGC TCT TAC ACT12mm for PNA50825404H-(KFP)K-eg1-AG TGC TCT TAC ACT14mm for PNA51645406H-(KFP)K-eg1-A TAG TGC TCT TAC ACT16mm for PNA51645408H-(KFP)K-eg1-CGA TAG TGC TCT TAC ACT18mm for PNA51645408H-(KFP)K-eg1-CGA TAG TGC TCT TAC ACT18mm for PNA51645408H-(R-Atx)b_(-G-Ala)-CTC ATA CTC T10acpP4227H-(R-Atx)b_(-G-Ala)-TG CTC ATA CTC T14acpP4228H-(R-Atx)b_(-G-Ala)-CG CT ATA CTC T16acpP4229H-(R-Atx)b_(-G-Ala)-CT CT TAC ACT18acpP4230H-(R-Atx)b_(-G-Ala)-C TCT TAC ACT10mm for PNA396E4500H-(R-Atx)b_(-G-Ala)-C TCT TAC ACT10mm for PNA396E4500H-(R-Atx)b_(-G-Ala)-C TCT TAC ACT10mm for PNA396E4500H-(R-Atx)b_(-G-Ala)-CGA TAG TGC TCT TAC ACT10mm for PNA42254501H-(R-Atx)b_(-G-Ala)-CGA TAG TGC TT TAC ACT18mm for PNA42264502H-(R-Atx)b_(-G-Ala)-CGA TAG TGC TT TAC ACT18mm for PNA42264503H-(R-Atx-R)A_A-Atx-(B-Ala)-CGA TAG TGC TT TAC ACT18mm for PNA42264504H-(R-Atx-R)A_A-Atx-(B-Ala)-CGA TAG TGC TT TAC ACT14acpP <tr< td=""><td>5271</td><td>H-(KFF)<sub>3</sub>K-eg1-</td><td>TC<b>T</b> TAC <b>A</b>CT</td><td>9</td><td>mm for PNA5080</td></tr<>	5271	H-(KFF) <sub>3</sub> K-eg1-	TC <b>T</b> TAC <b>A</b> CT	9	mm for PNA5080	
2273 $H(KFP)_{3}K-eg1-$ GC TCT TAC ACT11mm for PNA50825274 $H(KFP)_{3}K-eg1-$ TGC TCT TAC ACT12mm for PNA50825404 $H(KFP)_{3}K-eg1-$ AG TGC TCT TAC ACT14mm for PNA51645406 $H(KFP)_{3}K-eg1-$ A TAG TGC TCT TAC ACT16mm for PNA51665408 $H(KFP)_{3}K-eg1-$ CGA TAG TGC TCT TAC ACT18mm for PNA51665408 $H(KFP)_{3}K-eg1-$ CGA TAG TGC TCT TAC ACT10 $acpP$ 2227 $H(R-Anx)_{6}(\beta,Ala)-$ CTC ATA CTC T12 $acpP$ 4228 $H(R-Anx)_{6}(\beta,Ala)-$ A GTG CTC ATA CTC T14 $acpP$ 4229 $H(R-Anx)_{6}(\beta,Ala)-$ A GTG CTC ATA CTC T16 $acpP$ 2230 $H(R-Anx)_{6}(\beta,Ala)-$ C G TATA GTG CTC ATA CTC T18 $acpP$ 3987 $H(R-Anx)_{6}(\beta,Ala)-$ C TCT TAC ACT10mm for PNA39864500 $H(R-Anx)_{6}(\beta,Ala)-$ C TCT TAC ACT10mm for PNA42224501 $H(R-Anx)_{6}(\beta,Ala)-$ C TCT TAC ACT14mm for PNA42254502 $H(R-Anx)_{6}(\beta,Ala)-$ C GCT TAC ACT16mm for PNA42254503 $H(R-Anx)_{6}(\beta,Ala)-$ C GCT TAC ACT18mm for PNA42264504 $H(R-Anx)_{6}(\beta,Ala)-$ C GCT ATA CTC T16mm for PNA42254503 $H(R-Anx)_{6}(\beta,Ala)-$ G G TCA TA CTC T18mm for PNA42254504 $H(R-Anx)_{6}(\beta,Ala)-$ G G TCA TA CTC T18 $acpP$ 4246 $H(R-Anx,R)_{4},Anx(\beta,Ala)-$ G G TCA TA CTC T <td>5272</td> <td>H-(KFF)<sub>3</sub>K-eg1-</td> <td>C TC<b>T</b> TAC <b>A</b>CT</td> <td>10</td> <td>mm for PNA2108</td>	5272	H-(KFF) <sub>3</sub> K-eg1-	C TC <b>T</b> TAC <b>A</b> CT	10	mm for PNA2108	
$C_{1}$ $C_{1}$ $C_{2}$ $C_{1}$ $C_{2}$ $C_{2}$ $C_{1}$ $C_{2}$ <	5273	H-(KFF) <sub>3</sub> K-eq1-	GC TC <b>T</b> TAC <b>A</b> CT	11	mm for PNA5082	
AG TGC TCT TAC ACT14mm for PNA51645404 $H_{(KFP)_3K-eg1}$ .AG TGC TCT TAC ACT16mm for PNA51665408 $H_{(KFP)_3K-eg1}$ .CGA TAG TGC TCT TAC ACT18mm for PNA51665408 $H_{(R-Atx)_6-(B-Ala)-}$ CTC ATA CTC T10 $acpP$ 2227 $H_{(R-Atx)_6-(B-Ala)-}$ CTC ATA CTC T12 $acpP$ 4228 $H_{(R-Atx)_6-(B-Ala)-}$ AG TG CTC ATA CTC T16 $apP$ 4229 $H_{(R-Atx)_6-(B-Ala)-}$ ATA GTG CTC ATA CTC T16 $apP$ 4230 $H_{(R-Atx)_6-(B-Ala)-}$ CT CT TAC ACT18 $acpP$ 3987 $H_{(R-Atx)_6-(B-Ala)-}$ CT CT TAC ACT10mm for PNA39864500 $H_{(R-Atx)_6-(B-Ala)-}$ CT CT TAC ACT12mm for PNA39864500 $H_{(R-Atx)_6-(B-Ala)-}$ CT CT TAC ACT16mm for PNA42274501 $H_{(R-Atx)_6-(B-Ala)-}$ CT CT TAC ACT16mm for PNA42264502 $H_{(R-Atx)_6-(B-Ala)-}$ AG TG CT TAC ACT16mm for PNA42264503 $H_{(R-Atx)_6-(B-Ala)-}$ CG ATA GTG CT TAC ACT18mm for PNA42264503 $H_{(R-Atx)_6-(B-Ala)-}$ CG ATA GTG CT TAC ACT18mm for PNA42264246 $H_{(R-Atx)_6-(B-Ala)-}$ CG CT ATA CTC T10 $acpP$ 4246 $H_{(R-Atx)_6-(A-Ala)-}$ CG ATA GTG CT ATA CTC T16 $apP$ 4248 $H_{(R-Atx)_6-Ala)-}$ AG TG CTC ATA CTC T16 $apP$ 4288 $H_{(R-Atx)_6-Ala)-}$ ATA GTG CT CATA CTC T16 $ap$	5274	H-(KFF) <sub>3</sub> K-eq1-	TGC TC <b>T</b> TAC <b>A</b> CT	12	mm for PNA5083	
HerHerA TAG TGC TCT TAC ACT16mm for PNA51665406HerHerCGA TAG TGC TCT TAC ACT18mm for PNA51663986HerHerAnaloCTC ATA CTC T10acpP4227HerHerAnaloGTC ATA CTC T12acpP4228HerAnalo-A GTG CTC ATA CTC T14acpP4229HerAnalo-A GTG CTC ATA CTC T16acpP4230HerAnalo-C G ATA GTG CTC ATA CTC T18acpP3987HerAnalo-C G TT AC ACT10mm for PNA39864500HerAnalo-C G TT TAC ACT10mm for PNA42274501HerAnalo-A GTG CTCT TAC ACT14mm for PNA42264502HerAnalo-C GA TAG TGC TT TAC ACT16mm for PNA42264503HerAnalo-A TAG TGC TCT TAC ACT18mm for PNA42264503HerAnalo-CGA TAG TGC TT TAC ACT18mm for PNA42264503HerAnalo-CGA TAG TGC TT TAC ACT10acpP4246HerAnalo-CTC ATA CTC T10acpP4247HerAnalo-Analo-CTC ATA CTC T14acpP4248HerHerAnalo-ATA GTG CTC ATA CTC T18acpP4248HerHerAnalo-CG ATA GTG CTC ATA CTC T16acpP4288HerHerAnalo-CG ATA GTG CTC ATA CTC T16acpP <td>5404</td> <td>H-(KFF)<sub>3</sub>K-eg1-</td> <td>AG TGC TC<b>T</b> TAC <b>A</b>CT</td> <td>14</td> <td>mm for PNA5164</td>	5404	H-(KFF) <sub>3</sub> K-eg1-	AG TGC TC <b>T</b> TAC <b>A</b> CT	14	mm for PNA5164	
H-(KFF) <sub>8</sub> (K-eg1-CGA TAG TG C TC TAC ACT18mm for PNA516E3986H-(R-Ahx) <sub>6</sub> -(β-Ala)-CTC ATA CTC T10 $acpP$ 4227H-(R-Ahx) <sub>6</sub> -(β-Ala)-TG CTC ATA CTC T12 $acpP$ 4228H-(R-Ahx) <sub>6</sub> -(β-Ala)-A GTG CTC ATA CTC T14 $acpP$ 4229H-(R-Ahx) <sub>6</sub> -(β-Ala)-ATA GTG CTC ATA CTC T16 $acpP$ 4230H-(R-Ahx) <sub>6</sub> -(β-Ala)-CG ATA GTG CTC ATA CTC T18 $acpP$ 3987H-(R-Ahx) <sub>6</sub> -(β-Ala)-C G ATA GTG CTC ATA CTC T10mm for PNA39864500H-(R-Ahx) <sub>6</sub> -(β-Ala)-C T CT TAC ACT12mm for PNA32864501H-(R-Ahx) <sub>6</sub> -(β-Ala)-AG TGC TCT TAC ACT14mm for PNA42274501H-(R-Ahx) <sub>6</sub> -(β-Ala)-AG TGC TCT TAC ACT16mm for PNA42264502H-(R-Ahx) <sub>6</sub> -(β-Ala)-CGA TAG TGC TCT TAC ACT18mm for PNA42264503H-(R-Ahx) <sub>6</sub> -(β-Ala)-CGA TAG TGC TCT TAC ACT10 $acpP$ 4246H-(R-Ahx) <sub>6</sub> -(β-Ala)-CTC ATA CTC T10 $acpP$ 4246H-(R-Ahx) <sub>6</sub> -(β-Ala)-CTC ATA CTC T12 $acpP$ 4247H-(R-Ahx) <sub>6</sub> -(Ala)-AG TG CTC ATA CTC T16 $acpP$ 4248H-(R-Ahx-R) <sub>4</sub> -Ahx-(β-Ala)-CG ATA GTG CTC ATA CTC T16 $acpP$ 4248H-(R-Ahx-R) <sub>4</sub> -Ahx-(β-Ala)-CG ATA GTG CTC ATA CTC T16 $acpP$ 4248H-(R-Ahx-R) <sub>4</sub> -Ahx-(β-Ala)-CG ATA GTG CTC ATA CTC T16 $acpP$ 6311H-(KFF) <sub>3</sub> K-Gy8(DDIPY)-TA CTC T	5406	H-(KFF) <sub>3</sub> K-eq1-	A TAG TGC TC <b>T</b> TAC <b>A</b> CT	16	mm for PNA5166	
3986H-(R-Ahx) <sub>0</sub> -(B-Ala)-CTC ATA CTC T10 $acpP$ 4227H-(R-Ahx) <sub>0</sub> -(B-Ala)-TG CTC ATA CTC T12 $acpP$ 4228H-(R-Ahx) <sub>0</sub> -(B-Ala)-A GTG CTC ATA CTC T14 $acpP$ 4229H-(R-Ahx) <sub>0</sub> -(B-Ala)-ATA GTG CTC ATA CTC T16 $acpP$ 4230H-(R-Ahx) <sub>0</sub> -(B-Ala)-CG ATA GTG CTC ATA CTC T18 $acpP$ 3987H-(R-Ahx) <sub>0</sub> -(B-Ala)-C TCT TAC ACT10mm for PNA39864500H-(R-Ahx) <sub>0</sub> -(B-Ala)-TGC TCT TAC ACT12mm for PNA42274501H-(R-Ahx) <sub>0</sub> -(B-Ala)-AG TGC TCT TAC ACT14mm for PNA42254502H-(R-Ahx) <sub>0</sub> -(B-Ala)-AG TGC TCT TAC ACT16mm for PNA42264503H-(R-Ahx) <sub>0</sub> -(B-Ala)-CGA TAG TGC TCT TAC ACT18mm for PNA42264099H-(R-Ahx-R) <sub>A</sub> -Aha-(B-Ala)-CGC ATA CTC T10 $acpP$ 4246H-(R-Ahx-R) <sub>A</sub> -Aha-(B-Ala)-TG CTC ATA CTC T14 $acpP$ 4247H-(R-Ahx-R) <sub>A</sub> -Aha-(B-Ala)-A GTG CTC ATA CTC T16 $acpP$ 4248H-(R-Ahx-R) <sub>A</sub> -Aha-(B-Ala)-A GTG CTC ATA CTC T16 $acpP$ 4248H-(R-Ahx-R) <sub>A</sub> -Ahx-(B-Ala)-A GTG CTC ATA CTC T16 $acpP$ 4288H-(R-Ahx-R) <sub>A</sub> -Ahx-(B-Ala)-A GTG CTC ATA CTC T16 $acpP$ 4288H-(R-Ahx-R) <sub>A</sub> -Ahx-(B-Ala)-CG ATA GTG CTC ATA CTC T16 $acpP$ 5631H-(KFP) <sub>3</sub> K-eg1-Oys(BODIPY)-TA CTC T6 $acpP$ 5491H-(KFP) <sub>3</sub> K-eg1-Oys(BODIPY)-A CTC T<	5408	H-(KFF) <sub>3</sub> K-eq1-	CGA TAG TGC TC <b>T</b> TAC <b>A</b> CT	18	mm for PNA5168	
4227H-(B-Ahx)g-(B-Ala)-TG CTC ATA CTC T12 $acpP$ 4228H-(B-Ahx)g-(B-Ala)-A GTG CTC ATA CTC T14 $acpP$ 4229H-(B-Ahx)g-(B-Ala)-ATA GTG CTC ATA CTC T16 $acpP$ 4230H-(B-Ahx)g-(B-Ala)-CG ATA GTG CTC ATA CTC T18 $acpP$ 987H-(B-Ahx)g-(B-Ala)-C TCT TAC ACT10mm for PNA39864500H-(B-Ahx)g-(B-Ala)-TGC TCT TAC ACT12mm for PNA42274501H-(B-Ahx)g-(B-Ala)-TGC TCT TAC ACT14mm for PNA42254502H-(B-Ahx)g-(B-Ala)-AG TGC TCT TAC ACT16mm for PNA42254503H-(B-Ahx)g-(B-Ala)-CGA TAG TGC TCT TAC ACT18mm for PNA42264503H-(B-Ahx)g-(B-Ala)-CGA TAG TGC TT TAC ACT18mm for PNA42264503H-(B-Ahx)g-(B-Ala)-CGA TAG TGC TT TAC ACT10 $acpP$ 4246H-(B-Ahx-(B-Ala)-CGA TAG TGC TT10 $acpP$ 4246H-(B-Ahx-(B-Ala)-TG CTC ATA CTC T14 $acpP$ 4247H-(B-Ahx-(B-Ala)-A GTG CTC ATA CTC T16 $acpP$ 4248H-(B-Ahx-R)_4 -Ahx-(B-Ala)-A GTG CTC ATA CTC T16 $acpP$ 4248H-(B-Ahx-R)_4 -Ahx-(B-Ala)-CG ATA GTG CTC ATA CTC T18 $acpP$ 4288H-(B-Ahx-R)_4 -Ahx-(B-Ala)-CG ATA GTG CTC ATA CTC T16 $acpP$ 4288H-(R-Ahx-R)_4 -Ahx-(B-Ala)-CG ATA GTG CTC ATA CTC T18 $acpP$ 5631H-(KFF)_3K-C9(GDDIPY)-TA CTC T6 $acpP$ <	3986	H-(R-Ahx) <sub>6</sub> -(β-Ala)-	CTC ATA CTC T	10	acpP	
4228H-(R-Ahx)6-(B-Ala)-A GTG CTC ATA CTC T14 $acpP$ 4229H-(R-Ahx)6-(B-Ala)-ATA GTG CTC ATA CTC T16 $acpP$ 4230H-(R-Ahx)6-(B-Ala)-CG ATA GTG CTC ATA CTC T18 $acpP$ 3987H-(R-Ahx)6-(B-Ala)-C TCT TAC ACT10mm for PNA39864500H-(R-Ahx)6-(B-Ala)-TGC TCT TAC ACT12mm for PNA42274501H-(R-Ahx)6-(B-Ala)-AG TGC TCT TAC ACT14mm for PNA42264502H-(R-Ahx)6-(B-Ala)-AG TGC TCT TAC ACT16mm for PNA42264503H-(R-Ahx)6-(B-Ala)-CGA TAG TGC TCT TAC ACT18mm for PNA42304503H-(R-Ahx)6-(B-Ala)-CGA TAG TGC TCT TAC ACT10 $acpP$ 4246H-(R-Ahx)6-(B-Ala)-CTC ATA CTC T10 $acpP$ 4247H-(R-Ahx-R)4-Ahx-(B-Ala)-TG CTC ATA CTC T14 $acpP$ 4248H-(R-Ahx-R)4-Ahx-(B-Ala)-CG ATA GTG CTC ATA CTC T16 $acpP$ 4248H-(R-Ahx-R)4-Ahx-(B-Ala)-CG ATA GTG CTC ATA CTC T18 $acpP$ 4288H-(R-Ahx-R)4-Ahx-(B-Ala)-CG ATA GTG CTC ATA CTC T18 $acpP$ 4288H-(R-Ahx-R)4-Ahx-(B-Ala)-CG ATA GTG CTC ATA CTC T18 $acpP$ 4281H-(KFF)3K-c91-C93(BODIPY)-TA CTC T6 $acpP$ 5631H-(KFF)3K-c91-C93(BODIPY)-AG TG CTC ATA CTC T10 $acpP$ 5649H-(KFF)3K-c91-C93(BODIPY)-AG TG CTC ATA CTC T14 $acpP$ 5629H-(KFF)3K-c91-C93(BODIPY)-AG TG CTC ATA CT	4227	H-(R-Ahx) <sub>6</sub> -(β-Ala)-	TG CTC ATA CTC T	12	acpP	
H-(R-Ahx)6-(β-Ala)-     ATA GTG CTC ATA CTC T     16     acpP       4230     H-(R-Ahx)6-(β-Ala)-     CG ATA GTG CTC ATA CTC T     18     acpP       3987     H-(R-Ahx)6-(β-Ala)-     C TCT TAC ACT     10     mm for PNA3986       4500     H-(R-Ahx)6-(β-Ala)-     TGC TCT TAC ACT     12     mm for PNA4227       4501     H-(R-Ahx)6-(β-Ala)-     AG TGC TCT TAC ACT     14     mm for PNA4228       4502     H-(R-Ahx)6-(β-Ala)-     AG TGC TCT TAC ACT     16     mm for PNA4228       4503     H-(R-Ahx)6-(β-Ala)-     CGA TAG TGC TCT TAC ACT     16     mm for PNA4228       4503     H-(R-Ahx)6-(β-Ala)-     CGA TAG TGC TCT TAC ACT     18     mm for PNA4228       4099     H-(R-Ahx,R)4-Ahx-(β-Ala)-     CGC ATA CTC T     10     acpP       4246     H-(R-Ahx-R)4-Ahx-(β-Ala)-     TG CTC ATA CTC T     12     acpP       4247     H-(R-Ahx-R)4-Ahx-(β-Ala)-     AG TG CTC ATA CTC T     14     acpP       4248     H-(R-Ahx-R)4-Ahx-(β-Ala)-     AG TG CTC ATA CTC T     16     acpP       4248     H-(R-Ahx-R)4-Ahx-(β-Ala)-     CG ATA GTG CTC ATA CTC T     16     acpP       4288     H-(R-Ahx-R)4-Ah	4228	$H-(R-Ahx)_{6}-(\beta-Ala)-$	A GTG CTC ATA CTC T	14	acpP	
4230     H-(R-Ahx) <sub>6</sub> -(β-Ala)-     CG ATA GTG CTC ATA CTC T     18     acpP       3987     H-(R-Ahx) <sub>6</sub> -(β-Ala)-     C TCT TAC ACT     10     mm for PNA3986       4500     H-(R-Ahx) <sub>6</sub> -(β-Ala)-     TGC TCT TAC ACT     12     mm for PNA4227       4501     H-(R-Ahx) <sub>6</sub> -(β-Ala)-     AG TGC TCT TAC ACT     14     mm for PNA4228       4502     H-(R-Ahx) <sub>6</sub> -(β-Ala)-     A TAG TGC TCT TAC ACT     16     mm for PNA4228       4503     H-(R-Ahx) <sub>6</sub> -(β-Ala)-     CGA TAG TGC TCT TAC ACT     18     mm for PNA4228       4503     H-(R-Ahx) <sub>6</sub> -(β-Ala)-     CGA TAG TGC TCT TAC ACT     18     mm for PNA4228       4503     H-(R-Ahx-R) <sub>4</sub> -Ahx-(β-Ala)-     CTC ATA CTC T     10     acpP       4246     H-(R-Ahx-R) <sub>4</sub> -Ahx-(β-Ala)-     CTC ATA CTC T     12     acpP       4246     H-(R-Ahx-R) <sub>4</sub> -Ahx-(β-Ala)-     A GTG CTC ATA CTC T     14     acpP       4247     H-(R-Ahx-R) <sub>4</sub> -Ahx-(β-Ala)-     A GTG CTC ATA CTC T     14     acpP       4248     H-(R-Ahx-R) <sub>4</sub> -Ahx-(β-Ala)-     A GTG CTC ATA CTC T     16     acpP       4288     H-(R-Ahx-R) <sub>4</sub> -Ahx-(β-Ala)-     CG ATA GTG CTC ATA CTC T     18     acpP	4229	H-(R-Ahx) <sub>6</sub> -(β-Ala)-	ATA GTG CTC ATA CTC T	16	acpP	
3987H-(R-Ahx) <sub>6</sub> -( $\beta$ -Ala)-C TCT TAC ACT10mm for PNA39864500H-(R-Ahx) <sub>6</sub> -( $\beta$ -Ala)-TGC TCT TAC ACT12mm for PNA42274501H-(R-Ahx) <sub>6</sub> -( $\beta$ -Ala)-AG TGC TCT TAC ACT14mm for PNA42284502H-(R-Ahx) <sub>6</sub> -( $\beta$ -Ala)-A TAG TGC TCT TAC ACT16mm for PNA42284503H-(R-Ahx) <sub>6</sub> -( $\beta$ -Ala)-CGA TAG TGC TCT TAC ACT18mm for PNA42304099H-(R-Ahx-R) <sub>4</sub> -Ahx-( $\beta$ -Ala)-CTC ATA CTC T10 $acpP$ 4246H-(R-Ahx-R) <sub>4</sub> -Ahx-( $\beta$ -Ala)-TG CTC ATA CTC T12 $acpP$ 4247H-(R-Ahx-R) <sub>4</sub> -Ahx-( $\beta$ -Ala)-A GTG CTC ATA CTC T16 $acpP$ 4248H-(R-Ahx-R) <sub>4</sub> -Ahx-( $\beta$ -Ala)-A GTG CTC ATA CTC T16 $acpP$ 4288H-(R-Ahx-R) <sub>4</sub> -Ahx-( $\beta$ -Ala)-CG ATA GTG CTC ATA CTC T18 $acpP$ 5631H-(KFF) <sub>3</sub> K-eg1-Cys(BODIPY)-TA CTC T6 $acpP$ 5491H-(KFF) <sub>3</sub> K-eg1-Cys(BODIPY)-A GTG CTC ATA CTC T10 $acpP$ 5629H-(KFF) <sub>3</sub> K-eg1-Cys(BODIPY)-A GTG CTC ATA CTC T14 $acpP$	4230	H-(R-Ahx) <sub>6</sub> -(β-Ala)-	CG ATA GTG CTC ATA CTC T	18	acpP	
4500     H-(R-Ahx) <sub>θ</sub> -(β-Ala)-     TGC TCŢ TAC ACT     12     mm for PNA4227       4501     H-(R-Ahx) <sub>θ</sub> -(β-Ala)-     AG TGC TCŢ TAC ACT     14     mm for PNA4228       4502     H-(R-Ahx) <sub>θ</sub> -(β-Ala)-     A TAG TGC TCŢ TAC ACT     16     mm for PNA4228       4503     H-(R-Ahx) <sub>θ</sub> -(β-Ala)-     CGA TAG TGC TCŢ TAC ACT     18     mm for PNA4228       4503     H-(R-Ahx-R) <sub>4</sub> -Ahx-(β-Ala)-     CGC ATA GTG C TCŢ TAC ACT     10     acpP       4246     H-(R-Ahx-R) <sub>4</sub> -Ahx-(β-Ala)-     TG CTC ATA CTC T     10     acpP       4247     H-(R-Ahx-R) <sub>4</sub> -Ahx-(β-Ala)-     TG CTC ATA CTC T     14     acpP       4248     H-(R-Ahx-R) <sub>4</sub> -Ahx-(β-Ala)-     A GTG CTC ATA CTC T     16     acpP       4248     H-(R-Ahx-R) <sub>4</sub> -Ahx-(β-Ala)-     CG ATA GTG CTC ATA CTC T     16     acpP       4288     H-(R-Ahx-R) <sub>4</sub> -Ahx-(β-Ala)-     CG ATA GTG CTC ATA CTC T     18     acpP       5631     H-(KFF) <sub>3</sub> K-eg1-Cys(BODIPY)-     TA CTC T     6     acpP       5491     H-(KFF) <sub>3</sub> K-eg1-Cys(BODIPY)-     CTC ATA CTC T     10     acpP       5629     H-(KFF) <sub>3</sub> K-eg1-Cys(BODIPY)-     A GTG CTC ATA CTC T     14     acnP <td>3987</td> <td>H-(R-Ahx)<sub>6</sub>-(β-Ala)-</td> <td>C TC<b>T</b> TAC <b>A</b>CT</td> <td>10</td> <td>, mm for PNA3986</td>	3987	H-(R-Ahx) <sub>6</sub> -(β-Ala)-	C TC <b>T</b> TAC <b>A</b> CT	10	, mm for PNA3986	
4501H-(R-Ahx)6-(β-Ala)-AG TGC TCT TAC ACT14mm for PNA42284502H-(R-Ahx)6-(β-Ala)-A TAG TGC TCT TAC ACT16mm for PNA42284503H-(R-Ahx)6-(β-Ala)-CGA TAG TGC TCT TAC ACT18mm for PNA42284099H-(R-Ahx-R)4-Ahx-(β-Ala)-CTC ATA CTC T10acpP4246H-(R-Ahx-R)4-Ahx-(β-Ala)-TG CTC ATA CTC T12acpP4247H-(R-Ahx-R)4-Ahx-(β-Ala)-A GTG CTC ATA CTC T14acpP4248H-(R-Ahx-R)4-Ahx-(β-Ala)-A GTG CTC ATA CTC T16acpP4288H-(R-Ahx-R)4-Ahx-(β-Ala)-CG ATA GTG CTC ATA CTC T18acpP5631H-(KFF)3K-eg1-Cys(BODIPY)-TA CTC T6acpP5629H-(KFF)3K-eg1-Cys(BODIPY)-A GTG CTC ATA CTC T10acpP	4500	H-(R-Ahx) <sub>6</sub> -(β-Ala)-	TGC TC <b>T</b> TAC <b>A</b> CT	12	mm for PNA4227	
4502H-(R-Ahx)6-( $\beta$ -Ala)-A TAG TGC TCT TAC ACT16mm for PNA42294503H-(R-Ahx)6-( $\beta$ -Ala)-CGA TAG TGC TCT TAC ACT18mm for PNA42304099H-(R-Ahx-R)4-Ahx-( $\beta$ -Ala)-CTC ATA CTC T10acpP4246H-(R-Ahx-R)4-Ahx-( $\beta$ -Ala)-TG CTC ATA CTC T12acpP4247H-(R-Ahx-R)4-Ahx-( $\beta$ -Ala)-A GTG CTC ATA CTC T14acpP4248H-(R-Ahx-R)4-Ahx-( $\beta$ -Ala)-A GTG CTC ATA CTC T16acpP4288H-(R-Ahx-R)4-Ahx-( $\beta$ -Ala)-CG ATA GTG CTC ATA CTC T18acpP5631H-(KFF)3K-eg1-Cys(BODIPY)-TA CTC T6acpP5491H-(KFF)3K-cys(BODIPY)-A GTG CTC ATA CTC T10acpP5629H-(KFF)3K-eg1-Cys(BODIPY)-A GTG CTC ATA CTC T14acnP	4501	H-(R-Ahx) <sub>6</sub> -(β-Ala)-	AG TGC TC <b>T</b> TAC <b>A</b> CT	14	mm for PNA4228	
4503H-(R-Ahx)6-( $\beta$ -Ala)-CGA TAG TGC TCT TAC ACT18mm for PNA423C4099H-(R-Ahx-R)4-Ahx-( $\beta$ -Ala)-CTC ATA CTC T10acpP4246H-(R-Ahx-R)4-Ahx-( $\beta$ -Ala)-TG CTC ATA CTC T12acpP4247H-(R-Ahx-R)4-Ahx-( $\beta$ -Ala)-A GTG CTC ATA CTC T14acpP4248H-(R-Ahx-R)4-Ahx-( $\beta$ -Ala)-ATA GTG CTC ATA CTC T16acpP4288H-(R-Ahx-R)4-Ahx-( $\beta$ -Ala)-CG ATA GTG CTC ATA CTC T18acpP5631H-(KFF)3K-eg1-Cys(BODIPY)-TA CTC T6acpP5629H-(KFF)3K-eg1-Cys(BODIPY)-A GTG CTC ATA CTC T10acpP	4502	H-(R-Ahx) <sub>6</sub> -(β-Ala)-	A TAG TGC TC <b>T</b> TAC <b>A</b> CT	16	mm for PNA4229	
4099H-(R-Ahx-R) <sub>4</sub> -Ahx-( $\beta$ -Ala)-CTC ATA CTC T10acpP4246H-(R-Ahx-R) <sub>4</sub> -Ahx-( $\beta$ -Ala)-TG CTC ATA CTC T12acpP4247H-(R-Ahx-R) <sub>4</sub> -Ahx-( $\beta$ -Ala)-A GTG CTC ATA CTC T14acpP4248H-(R-Ahx-R) <sub>4</sub> -Ahx-( $\beta$ -Ala)-ATA GTG CTC ATA CTC T16acpP4288H-(R-Ahx-R) <sub>4</sub> -Ahx-( $\beta$ -Ala)-CG ATA GTG CTC ATA CTC T18acpP5631H-(KFF) <sub>3</sub> K-eg1-Cys(BODIPY)-TA CTC T6acpP5491H-(KFF) <sub>3</sub> K-cg1-Cys(BODIPY)-CTC ATA CTC T10acpP5629H-(KFF) <sub>3</sub> K-eg1-Cys(BODIPY)-A GTG CTC ATA CTC T14acnP	4503	H-(R-Ahx) <sub>6</sub> -(β-Ala)-	CGA TAG TGC TC <b>T</b> TAC <b>A</b> CT	18	mm for PNA4230	
4246H-(R-Ahx-R)_4-Ahx-( $\beta$ -Ala)-TG <u>CTC ATA CTC T</u> 12acpP4247H-(R-Ahx-R)_4-Ahx-( $\beta$ -Ala)-A GTG <u>CTC ATA CTC T</u> 14acpP4248H-(R-Ahx-R)_4-Ahx-( $\beta$ -Ala)-ATA GTG <u>CTC ATA CTC T</u> 16acpP4288H-(R-Ahx-R)_4-Ahx-( $\beta$ -Ala)-CG ATA GTG <u>CTC ATA CTC T</u> 18acpP5631H-(KFF)_3K-eg1-Cys(BODIPY)-TA CTC T6acpP5491H-(KFF)_3K-cys(BODIPY)-CTC ATA CTC T10acpP5629H-(KFF)_3K-eg1-Cys(BODIPY)-A GTG CTC ATA CTC T14acnP	4099	H-(R-Ahx-R)₄-Ahx-(β-Ala)-	CTC ATA CTC T	10	acpP	
4247H-(R-Ahx-R) <sub>4</sub> -Ahx-( $\beta$ -Ala)-A GTG CTC ATA CTC T14 $acpP$ 4248H-(R-Ahx-R) <sub>4</sub> -Ahx-( $\beta$ -Ala)-ATA GTG CTC ATA CTC T16 $acpP$ 4288H-(R-Ahx-R) <sub>4</sub> -Ahx-( $\beta$ -Ala)-CG ATA GTG CTC ATA CTC T18 $acpP$ 5631H-(KFF) <sub>3</sub> K-eg1-Cys(BODIPY)-TA CTC T6 $acpP$ 5491H-(KFF) <sub>3</sub> K-cys(BODIPY)-CTC ATA CTC T10 $acpP$ 5629H-(KFF) <sub>3</sub> K-eg1-Cys(BODIPY)-A GTG CTC ATA CTC T14 $acnP$	4246	H-(R-Ahx-R) <sub>4</sub> -Ahx-(β-Ala)-	TG CTC ATA CTC T	12	acpP	
4248H-(R-Ahx-R) <sub>4</sub> -Ahx-( $\beta$ -Ala)-ATA GTG CTC ATA CTC T16 $acpP$ 4288H-(R-Ahx-R) <sub>4</sub> -Ahx-( $\beta$ -Ala)-CG ATA GTG CTC ATA CTC T18 $acpP$ 5631H-(KFF) <sub>3</sub> K-eg1-Cys(BODIPY)-TA CTC T6 $acpP$ 5491H-(KFF) <sub>3</sub> K-Cys(BODIPY)-CTC ATA CTC T10 $acpP$ 5629H-(KFF) <sub>3</sub> K-eg1-Cys(BODIPY)-A GTG CTC ATA CTC T14 $acnP$	4247	H-(R-Ahx-R)₄-Ahx-(β-Ala)-	A GTG CTC ATA CTC T	14	acpP	
4288H-(R-Ahx-R) <sub>4</sub> -Ahx-(β-Ala)-CG ATA GTG CTC ATA CTC T18acpP5631H-(KFF) <sub>3</sub> K-eg1-Cys(BODIPY)-TA CTC T6acpP5491H-(KFF) <sub>3</sub> K-Cys(BODIPY)-CTC ATA CTC T10acpP5629H-(KFF) <sub>3</sub> K-eg1-Cys(BODIPY)-A GTG CTC ATA CTC T14acnP	4248	H-(R-Ahx-R)₄-Ahx-(β-Ala)-	ATA GTG CTC ATA CTC T	16	acpP	
5631H-(KFF) <sub>3</sub> K-eg1-Cys(BODIPY)-TA CTC T6acpP5491H-(KFF) <sub>3</sub> K-cys(BODIPY)-CTC ATA CTC T10acpP5629H-(KFF) <sub>3</sub> K-eg1-Cys(BODIPY)-A GTG CTC ATA CTC T14acpP	4288	H-(R-Ahx-R)₄-Ahx-(β-Ala)-	CG ATA GTG CTC ATA CTC T	18	acpP	
5491       H-(KFF) <sub>3</sub> K-Cys(BODIPY)-       CTC ATA CTC T       10       acpP         5629       H-(KFF) <sub>3</sub> K-eq1-Cys(BODIPY)-       A GTG CTC ATA CTC T       14       acoP	5631	H-(KFF) <sub>3</sub> K-ea1-Cvs(BODIPY)-	TA CTC T	6	acpP	
5629 H-(KFF) <sub>3</sub> K-eq1-Cvs(BODIPY)- A GTG CTC ATA CTC T 14 accoP	5491	H-(KFF) <sub>3</sub> K-Cys(BODIPY)-	CTC ATA CTC T	10	acpP	
	5629	H-(KFF) <sub>3</sub> K-eq1-Cvs(BODIPY)-	A GTG CTC ATA CTC T	14	, acpP	

The PNAs were synthesized as previously reported (Good et al., 2001; Ghosal et al., 2013). eg1, 8-amino-3,6-dioxaoctanoic acid; Ahx, 6-aminohexanoic acid; Cys, cysteine; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene. Underlined: 10 nucleobase common motif. Bold and underlined: mismatched nucleobases.

### TABLE 2 | MIC-values.

(KFF) <sub>3</sub> K	PNA5079 8-mer		PNA5080 9-mer		PNA2108 10-mer		PNA5082 11-mer		PNA5083 12-mer	
	Match	mm	Match	mm	Match	mm	Match	mm	Match	mm
MG1655	4	16	2–4	16	0.5–1	16	4	16	4	16
MG1655(∆sbmA)	16	16–32	16	16	8–16	16–32	16	16	16	16
AS19	0.5	2	0.5	2	0.125	1–2	0.5	2	0.5	2
ATCC25922 (Δ <i>rfa</i> G)	0.25	>8	nd	nd	0.125	4	>0.25	>1	1–2	1–2
Klebsiella pneumoniae	8–16	>32	nd	nd	2	>32	nd	nd	8	>32

MIC values (µM) of 8–12-mer match or mismatch (italicized) anti-acpP PNA conjugate. nd, Not determined.

### RESULTS

# MIC-PNA Length Relation of (KFF)<sub>3</sub>K-PNA Conjugates

Exploiting the most commonly used E. coli gene target as well as delivery BPP, the antimicrobial effect of a series of 8-18 mer (KFF)<sub>3</sub>K-PNA conjugates against the acpP target in E. coli as well as the Tm of their duplexes with complementary RNA was determined (Table 2 and Supplementary Figure S1). The results (Figure 1A) revealed a direct correlation between antimicrobial activity and Tm for PNAs shorter than 10 nucleobases, while a reverse correlation was seen for PNAs longer than 10, thus showing an activity optimum at 10 nucleobases against E. coli MG1655 in agreement with previous reports (Good et al., 2001; Deere et al., 2005). Importantly, when analogous mismatch PNA constructs were tested, low activity and no length dependence was found (Table 2 and Supplementary Figure S2); thus corroborating the conclusion that the antimicrobial activity is indeed caused by an antisense mechanism of action also for the PNAs of different lengths. This observation extended to Klebsiella pneumonia (Table 2) in which the acpP sequence target is conserved demonstrating that the length constraint is not limited to *E. coli*.

## Correlation Between PNA–RNA Duplex Stability and Antibacterial Activity

From the results of a previous sequence target optimization study, a general relationship between antibacterial efficacy and PNA-DNA duplex stability was not apparent (Dryselius et al., 2003). However, due to confounding effects of mRNA target position, a direct comparison of these two parameters requires the location of the mRNA target to remain constant while only changing the antisense oligomer (e.g., in terms of nucleobase sequence). By introducing single mismatches at different positions in a well-described antisense peptide-PNA conjugate, and exploiting the differential effect of mismatch position on duplex destabilization, we interrogated the correlation between PNA/RNA affinity, as measured by thermal melting, and the antimicrobial effect of the PNA. In this case a clear, direct correlation between PNA/RNA duplex stability (melting temperature) and the antisense efficacy (as measured by MIC) was

observed (Figure 1B and Supplementary Table S1) (except for one outlier, which must be caused by an unexplained biological effect). Therefore the above described effect of PNA length on antibacterial activity (Figure 1A) follows the expected duplex stability between 8 and 10 nucleobases, but reverses above 10 nucleobases. Consequently, the decreasing activity as the PNA is extended beyond 10 nucleobases must be due to another phenomenon, of which length dependent decreased uptake would be an obvious candidate, as previously proposed but not experimentally supported (Good et al., 2001).

### **PNA Length Effects in Envelope Mutants**

The length dependence was also studied in *E. coli* AS19 and  $\Delta rfaG$ , which because of a compromised LPS barrier are inherently more sensitive toward antisense PNAs, and antibiotics in general (Good and Nielsen, 1998). The AS19 strain has a not fully characterized truncated LPS structure (Sekiguchi and Iida, 1967), while the  $\Delta rfaG$  strain lacks the entire outer LPS core (Ebbensgaard et al., 2018). Similar to the results obtained with the MG1655 strain, the (KFF)<sub>3</sub>K-PNA with a PNA-length of 10 nucleobases showed the lowest MIC value, and any decrease or increase in PNA-length reduced the growth inhibitory effect of the (KFF)<sub>3</sub>K-PNA (**Table 2**). This suggests that the inner membrane (or the peptidoglycan cell wall) is a significant contributor to the length constraints. In order to eliminate the effect of the carrier peptide, we also tested the length dependence





### TABLE 3 | MIC-values.

Naked PNA	PNA2301	10-mer	PNA4223	12-mer	PNA4224	14-mer
AS19	2	>32	4	16	32	>32
MIC values	(1. M) of	10 14 mor	match or	micmatch	(italiaizad)	anti aonP

MIC values ( $\mu$ M) of 10–14-mer match or mismatch (italicized) anti-acpP PNA in AS19.

of naked PNA in the AS19-strain. Increasing the PNA length from 10- to 12- and 14-mer reduced activity in AS19 (**Table 3** and **Supplementary Figure S3**), indicating that even without a carrier peptide, increasing the PNA length impairs antibacterial efficacy.

### Effect of the SbmA Transporter

The antibacterial activity of (10-mer) (KFF)<sub>3</sub>K-PNA is dependent on the inner membrane transporter SbmA for activity (Ghosal et al., 2013). Therefore, a reduced uptake of longer PNAs could be due to length (size) limiting transport efficacy of SbmA. As expected, none of the (8- to 12-mers) (KFF)<sub>3</sub>K-PNAs exhibited any antisense related antibacterial activity in the MG1655( $\Delta sbmA$ ) strain since match and mismatch compounds showed no significant difference in MIC values (Table 2). However, it has previously been demonstrated that the (KFF)<sub>3</sub>K-peptide can be substituted with other peptides [such as  $H-(R-Ahx)_6-(\beta-Ala)$  or  $H-(R-Ahx-R)_4-Ahx-(\beta-Ala)$ ] resulting in PNA conjugates that do not require SbmA for bacterial uptake (Ghosal et al., 2013; Hansen et al., 2016). Thus, we investigated whether the length effect was limited to the (KFF)<sub>3</sub>K peptide by measuring the MIC values of the anti-acpP-PNA conjugated to the  $H-(R-Ahx)_6-(\beta-Ala)$  or the H-(R-Ahx-R)<sub>4</sub>-Ahx-( $\beta$ -Ala) peptide, respectively. Again, exceeding a PNA length of 10 nucleobases reduced the MIC value significantly (Table 4), indicating that this effect is not limited to the (KFF)<sub>3</sub>K-PNA conjugate. Combined, these results suggest that PNA length is important for transport through SbmA as well as for transport via an SbmAindependent pathway.

### Effect of PNA on E. coli Spheroplasts

In an effort to completely abolish any contribution of the outer membrane, the effect of anti-*acpP*-PNAs of different lengths on *E. coli* spheroplasts revival was measured. Spheroplasts were isolated and incubated with peptide-PNA conjugates of different lengths, and survivors were determined by plating (**Figure 2**). The 10-mer was clearly the



most efficient in reducing bacterial survival among the 6-, 10- and 14-mer (KFF)<sub>3</sub>K-PNAs (**Figure 2A**). Analogously, the 10-mer H-(R-Ahx-R)<sub>4</sub>-Ahx-( $\beta$ -Ala)-PNA showed higher activity than the longer PNAs (**Figure 2B**), and a reverse activity/length relation was seen in line with the data obtained with intact cells.

## Determination of Peptide-PNA Uptake by Flow Cytometry

In order to physically monitor the uptake of the peptide-PNAconjugates of different PNA length, we constructed BODIPYlabeled variants, which could be traced using flow cytometry. Although inclusion of fluorophores generally hampers cellular activity (increases MIC) (unpublished results), the 10-mer retained higher activity compared to the 6-mer and 14mer, respectively (Figure 3). Although the major part of the bacterial population contained only a limited amount of PNA (Figure 3A), a fraction with a very significant uptake of the green BODIPY-labeled PNA could be detected as a tail of higher fluorescence on the histogram (Figure 3B). Similar population heterogeneity of uptake at low concentration of antimicrobial peptides has previously been reported (Pérez-Peinado et al., 2018). Analyzing the fraction of green cells reveals a reverse correlation between the PNA length and bacterial cellular uptake, although the conjugates containing the 6-mer and 14-mer PNA show similar activity (MIC).

<b>TABLE 4  </b> MIC values (μM) of H-(R-Ahx) <sub>6</sub> -(β-Ala)-PNA and H-(R-Ahx-R) <sub>4</sub> -Ahx-(β-Ala)-PNA from 10-mer to 18-mer.								
H-(R-Ahx-R)₄-Ahx-(β-Ala)-	PNA3986 10-mer	PNA4227 12-mer	PNA4228 14-mer	PNA4229 16-mer	PNA4230 18-mer			
MG1655	1–2	8	>32	>32	>32			
MG1655(∆ <i>sbmA</i> )	1	4	>32	>32	>32			
AS19	0.5/2*	1/1–2*	2/nd*	2/2*	2/2-4*			
H-(R-Ahx) <sub>6</sub> -(β-Ala)-	PNA4099 10-mer	PNA4246 12-mer	PNA4247 14-mer	PNA4248 16-mer	PNA4288 18-mer			
MG1655	1	8	>16	>16	>16			
MG1655(∆ <i>sbmA</i> )	1	4	>32	>32	>32			

\*Mismatch PNA.



Thus by compensating for the different uptake efficiency, a very rough estimate of the intracellular efficacy of the 6-, 10and 14-mer PNA indicates a 1:3:10 ratio between these. This supports the hypothesis that longer PNAs are less efficiently taken up thereby limiting their otherwise higher intrinsic potency, and that the optimum for a 10-mer PNA reflects a balance between cellular uptake efficiency and intrinsic antisense activity.

## DISCUSSION

Uptake of antimicrobial agents through the Gram-negative outer membrane is determined by their size, symmetry and hydrophobicity. In general, small ( < 600 Da) hydrophilic or amphiphilic molecules, such as beta-lactams and some quinolones, penetrate the outer membrane through porins (Hancock and Bell, 1988; Nikaido, 2003), while certain larger antibiotics may be able to enter through nutrient transporters (Braun et al., 2001).

Larger and more hydrophobic compounds (e.g., aminoglycosides) are generally taken up much slower through a self-promoted pathway in which the compounds accumulate on the cell surface until reaching a critical concentration at which, pores form in the outer membrane allowing a surge of antibiotic to enter (Hancock and Bell, 1988; Richter and Hergenrother, 2019).

Other compounds, such as the bacteriocins enter by an energy dependent pathway using the tolA or tonB systems (Nikaido, 2003). Highly hydrophobic compounds are usually ineffective against Gram-negatives except for deep rough mutants in which these antibiotics can diffuse through the lipid bilayer, also known as the hydrophobic pathway (Nikaido, 1976; Hancock and Bell, 1988). Furthermore, perturbation of the LPS-layer such as that found in rough or deeprough mutants generally increases the sensitivity toward hydrophobic antibiotics while reducing the sensitivity to selected hydrophilic antibiotics, probably because of rearrangement of the membrane and reduction of the number of porins (Sen and Nikaido, 1991).

The PNA-peptide conjugates having masses of around 5 kDa are significantly larger than conventional antibiotics and also somewhat larger than most naturally derived antimicrobial peptides. It is therefore unlikely that porins are involved in transport over the outer membrane. This is supported by the fact that no porin mediated resistance mechanisms have yet been identified for PNA-peptide conjugates.

The BPP-PNAs differ from membrane-disruptive cationic antimicrobial peptides by having a cytoplasmic target and therefore must also cross the peptidoglycan cell wall and the inner membrane. Other antibiotic classes such as the aminoglycosides, the macrolides and some quinolones are most likely sufficiently hydrophobic to pass through the inner membrane by passive diffusion, and the cell wall appears permeable for particles up to at least 2 nm (Demchick and Koch, 1996). Hydrophilic molecules such as most antimicrobial peptides with intracellular targets utilize transporters in the inner membrane for translocation (such as SbmA) (Paulsen et al., 2016). We have previously described a similar mechanism for certain peptide-PNA conjugates, which do exploit the SbmA-transporter for passage. For PNAs conjugated to arginine-rich carrier peptides, however, it remains unclear how the inner membrane is traversed. No porin or transporter mutants have yet been identified, which could provide resistance toward PNAs conjugated to arginine-rich peptides. This suggests that no single non-essential gene product is responsible for the uptake and/or that multiple pathways exist for entry into the cytoplasm for these compounds, and the mechanism may rely on local disturbance/disruption of the lipid bilayer. Finally, it is unlikely that the peptidoglycan cell wall constitutes a size barrier for the longer PNA conjugates, as particles of 2 nm, i.e., much wider than the diameter of the PNA oligomer, transverse freely through the cell wall (Demchick and Koch, 1996).

### CONCLUSION

The present results clearly show that antisense potency of PNApeptide conjugates in *E. coli* exhibits an optimum around a target size of 10 nucleobases, and that this optimum is due to opposing PNA length effects on mRNA binding affinity versus efficiency of bacterial uptake. Interestingly, the size-limited uptake is independent of the delivery peptide and the data indicates that the size limitation may predominantly be ascribed to restrictions of inner membrane passage. Thus further studies elucidating the detailed molecular mechanism for bacterial uptake is warranted in order to understand the details of the uptake mechanism as well as to facilitate rational approaches for design of novel delivery vehicles that may relax this size-limitation thereby allowing the development of longer and thus higher potency bacterial antisense agents.

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### **AUTHOR CONTRIBUTIONS**

PN and LG designed the experiments. LG, NY, AG, and MZ performed the experiments. LG and PN wrote the manuscript.

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## SUPPLEMENTARY MATERIAL

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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