SUPPLEMENTAL INFORMATION

Binding of macrolide antibiotics compels the ribosome to discriminate against specific substrates based on their charge and size

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SUPPLEMENTAL FIGURES

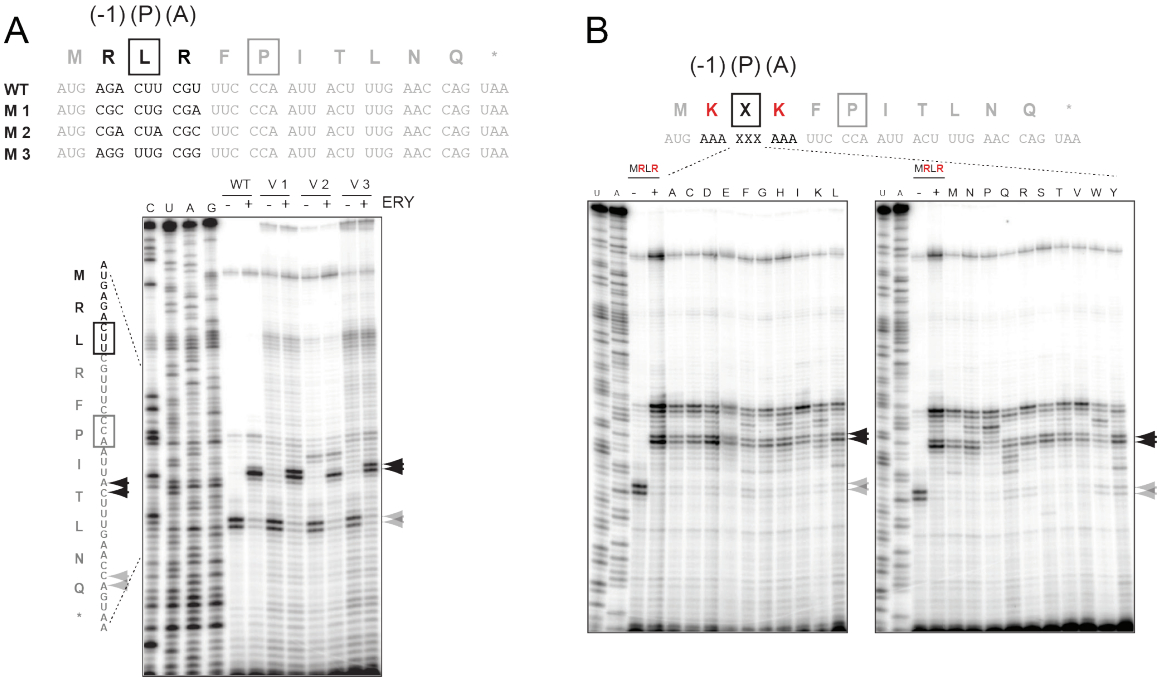


Figure S1. Related to Figure 1. Drug induced ribosome stalling in the MRLR ORF is not dependent on the mRNA or tRNA structure and identity of the third amino acid is not significant for ERY-dependent ribosome stalling in the MKLK ORF. (A) Top: Sequence of the wild type MRLR ORF and the ORFs with synonymous changes in the codons corresponding to the critical amino acid residues of the stalling motif. The wt RLR sequence is decoded by tRNAs Arg4, Leu2, Arg2; In the mutants M1-M3, the same sequence is decoded by Arg2, Leu1/Leu3, Arg2 (M1), Arg2, Leu3, Arg2, (M2), Arg5, Leu4/Leu5, Arg3 (M4). The penultimate (-1) amino acid residue of the nascent chain in the drug-stalled ribosome, the P site, and the A site residues are marked above the peptide sequence. Bottom: Toeprinting analysis of ERY dependent ribosome stalling on the wt and mutant MRLR ORFs. Black arrows indicate translation arrest at the Leu_3 codon (black box in the sequence on the side of the gel and in the top panel. Gray arrows point to the toeprint bands generated by the ribosomes, which bypassed the Leu_3 codon and were captured at the downstream Pro codon (gray box) due to the presence of mupirocin, an IleRS inhibitor. Sequencing lanes are marked. Gels are representative of two independent experiments. (B) Toeprinting analysis of ERY dependent ribosome stalling on the MKXK ORFs. Lanes marked as '-' and '+" represent the reactions with the MRLR template performed without and with ERY, respectively. The complete ORF encoding the peptide starting with the MKLK sequence is shown above the gels. Arrows are as in (A) except that in the case of the Ile_3 mutation in the MKXK peptide (lane marked 'I'), the Ile_7 codon was replaced with the Trp codon and mupirocin was substituted with indlomycin the TrpRS inhibitor. Sequencing lanes are marked. Gels are representative of two independent experiments.

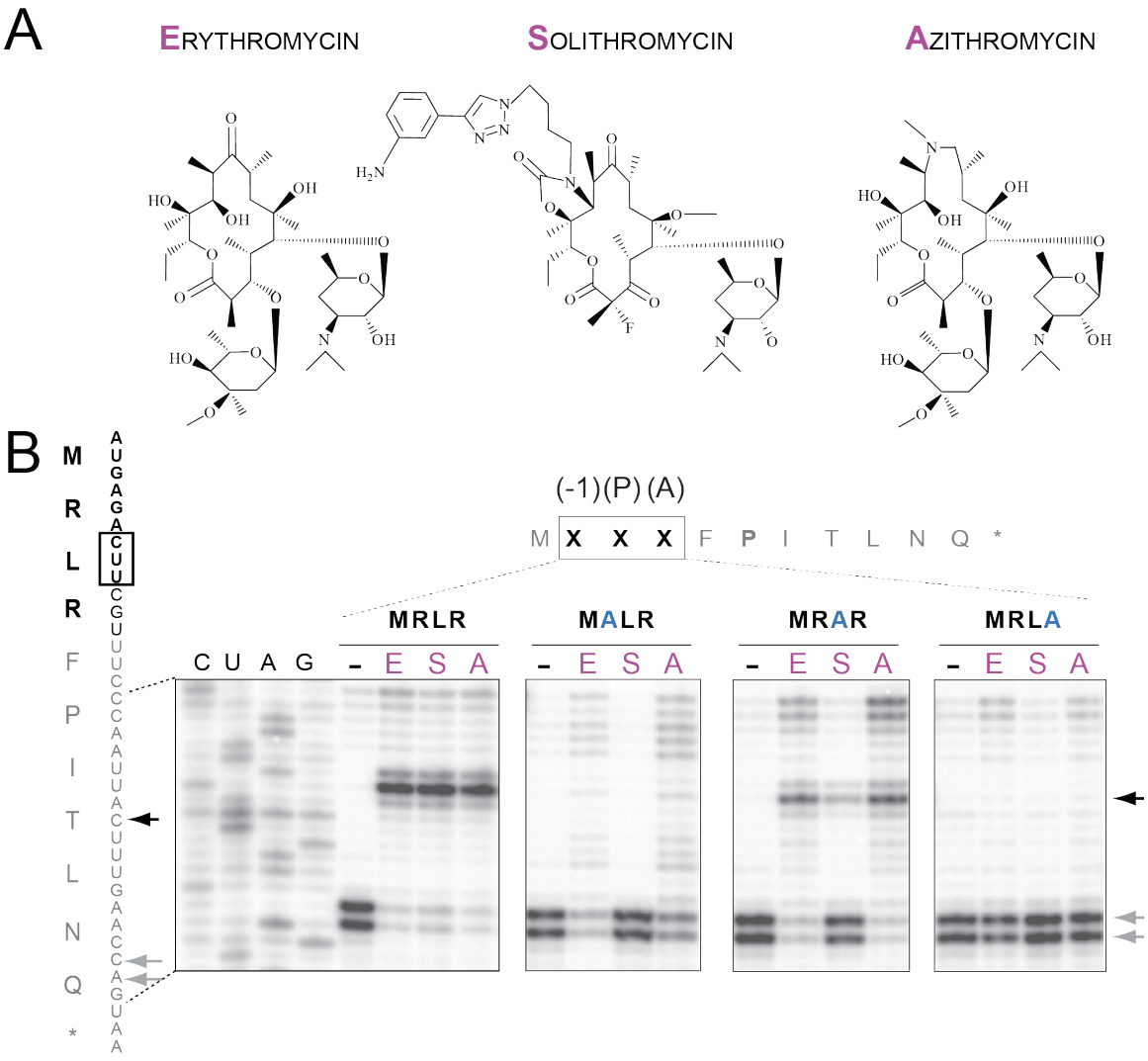


Figure S2. Related to Figure 1. Different macrolide antibiotics show similar sensitivity to the mutations of the RLR motif. (A) Chemical structures of macrolides ERY and AZI and of ketolide SOL. (B) Toeprinting analysis of drug-dependent ribosome stalling during translation of the peptides with alanine substitutions in the RLR motif. The penultimate (-1) amino acid residue of the nascent chain in the drug-stalled ribosome, the P site, and the A site residues are marked above the peptide sequence. Black arrows indicate ERY, AZI or SOL dependent translation arrest at codon 3. The toeprint bands representing ribosomes captured at the downstream Pro_5 codon due to the presence of mupirocin, an IleRS inhibitor, are indicated by gray arrows. Sequencing lanes are marked. Gels are representative of two independent experiments.

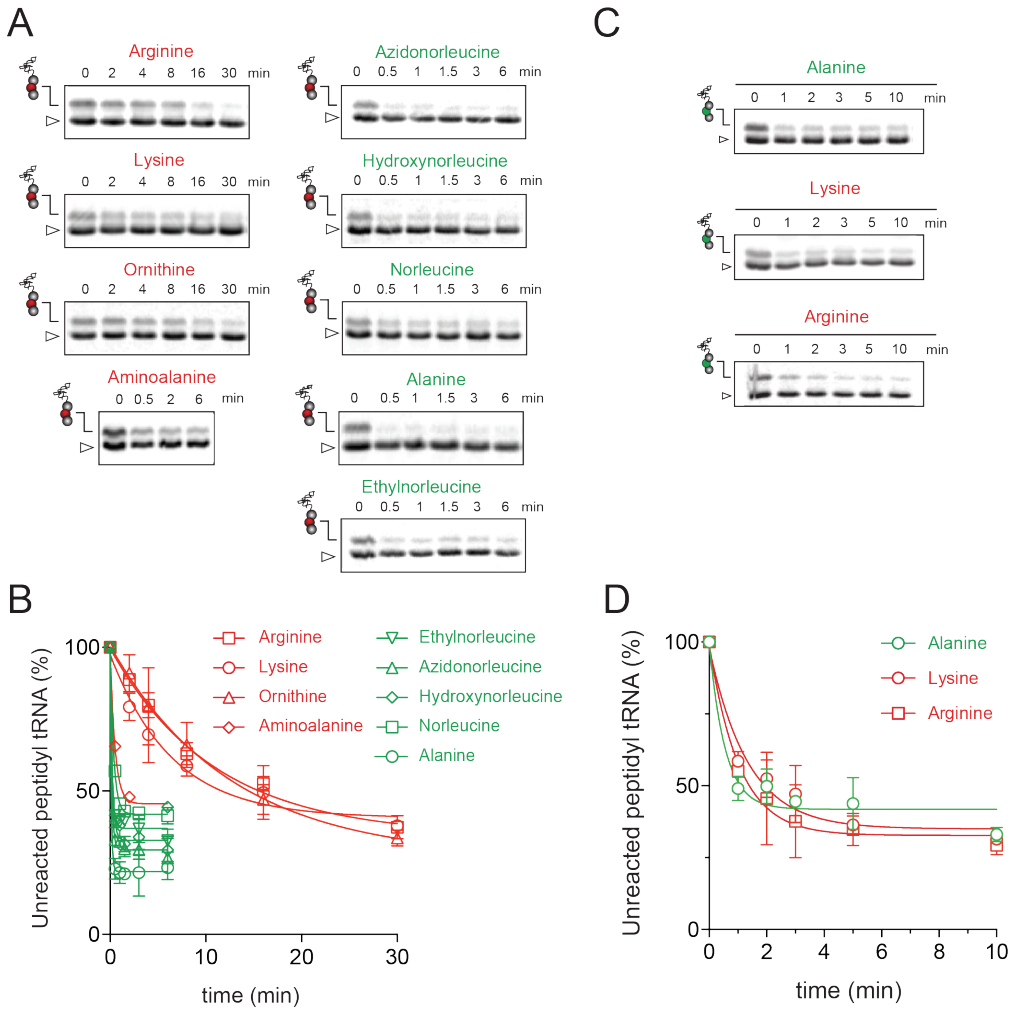
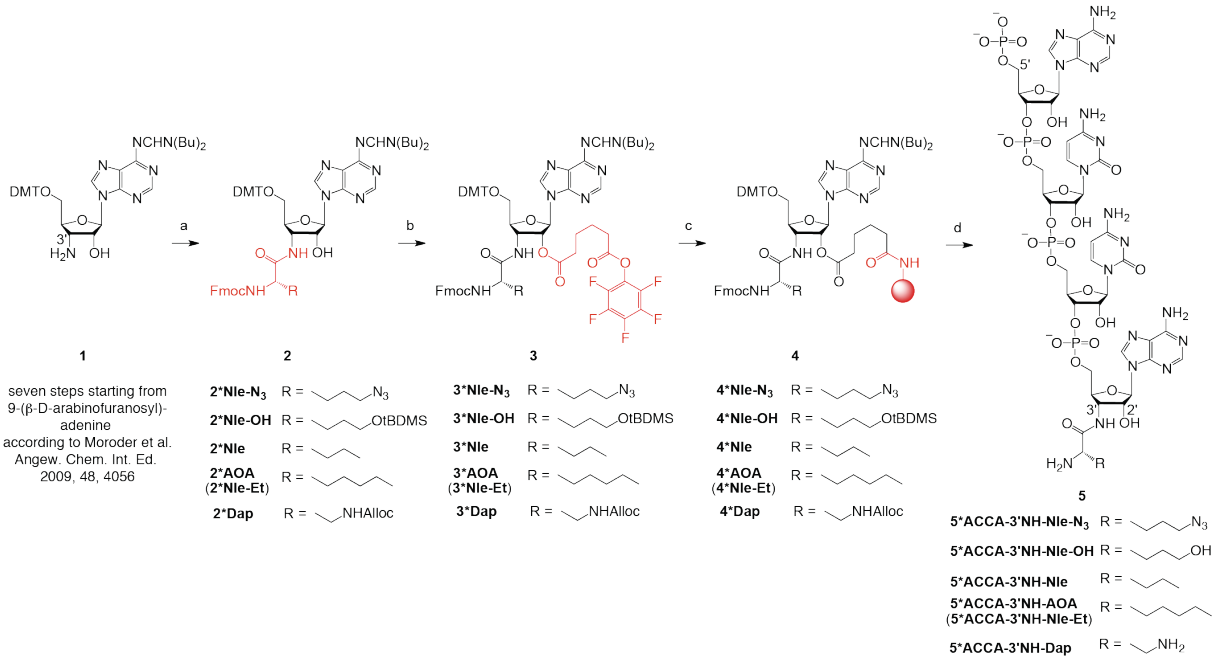


Figure S3. Related to Figure 2. Ribosome-catalyzed reaction of the model A site substrates with MRL-tRNA (A and B) or MAL-tRNA (C and D) in the absence of ERY. (A,C) Gel electrophoresis analysis of the [^{35}S] labeled MRL-tRNA^{Leu} (A) or MAL-tRNA^{Leu} (C) remaining upon reaction of the ribosome-mRNA-peptidyl-tRNA complex with the indicated acceptor substrate analogs in the absence of ERY. All the substrates conform to the general structure ACCA-N-X where X represents the amino acid. The time of incubation with the acceptor substrates is indicated above the gel lanes. Cartoons on the side of the gels indicate the band corresponding to [^{35}S] labeled MRL-tRNA^{Leu} (A) or MAL-tRNA^{Leu} (C). Triangle points to the band representing fMet-tRNA^{fMet} present in the reaction. Gels are representative of at least two independent experiments. (B,D) Quantification of the unreacted MRL-tRNA^{Leu} (B) or MAL-tRNA^{Leu} (D) over the course of the time from the corresponding gels. The amount of peptidyl-tRNA^{Leu} at 0 min was set as 100%. Error bars show deviation from the mean based on two independent experiments. The experiment with the ACCA-N-aminoalanine substrate was performed only once.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Chemical synthesis of ACCA-amino acid conjugates

The ACCA-amino acid conjugates containing 6-azido-L-norleucine, 6-hydroxy-6-norleucine, norleucine, (2*S*)-2-aminooctanoic acid (AOA) (called in the paper 'ethylnorleucine' (Nle-Et) for consistency) or L-2,3-diaminopropionic acid (Dap) (called in the paper 'amino alanine' (AAla) for consistency) were synthesized as outlined in **Scheme. S1** and described below. All the other ACCA-amino acid conjugates were synthesized following the procedures described by Moroder et al. (2009).



Scheme S1. Synthesis of solid supports and RNA-amino acid conjugates. Reaction conditions: **a**) 1.3 equiv Fmoc-amino acid-OBt, 1.5 equiv DIPEA, in *N*,*N*-dimethylformamide, r.t., 5 h, r.t., 12 h; **b**) 5 equiv of adipic acid bis(pentafluorophenyl)ester, 1 equiv DMAP in *N*,*N*-dimethylformamide/pyridine (1/1, v/v), r.t., 1 h; **c**) ~3 equiv (w/w) amino-functionalized polystyrene support (*GE Healthcare*, Custom Primer Support™ 200 Amino), ~2 equiv (w/w) pyridine, *N*,*N*-dimethylformamide, r.t., 1 day; **d**) automated RNA solid-phase synthesis, deprotection, and purification. Fmoc = *N*-(9-fluorenyl)methoxycarbonyl, Alloc = allyloxycarbonyl, Bt = Benzotriazol-1-yl, DIPEA = *N*,*N*-diisopropylethylamine, DMAP = 4-(*N*,*N*-dimethylamino)pyridine.

Chemical synthesis of solid supports 4

General remarks. Reagents were purchased in the highest available quality from commercial suppliers (Sigma-Aldrich, Acros, IRIS Biotech GmbH) and used without further purification. Organic solvents for reactions were dried overnight over freshly activated molecular sieves (4Å). The reactions were carried out under argon atmosphere. ^{1}H and ^{13}C spectra were recorded on a Bruker DRX 300 MHz spectrometer. Chemical shifts (δ) are reported relative to tetramethylsilane (TMS) referenced to the residual proton signal of the deuterated solvent DMSO-d_6 (2.50 ppm for ^{1}H NMR spectra and 39.52 ppm for ^{13}C spectra). The following abbreviations were used to denote

multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet, b = broad. Signal assignments were based on ^{1}H-^{1}H-COSY and ^{1}H-^{13}C-HSQC experiments. MS experiments were performed on a Finnigan LCQ Advantage MAX ion trap instrumentation (Thermo Fisher Scientific) with an electrospray ion source. Samples were analyzed in the positive- or negative-ion mode. Reaction control was performed via analytical thin-layer chromatography (TLC, Macherey-Nagel) with fluorescent indicator. Spots were further visualized using cerium molybdate or anisaldehyde staining reagents. Column chromatography was carried out on silica gel 60 (70-230 mesh). Custom Primer SupportTM 200 Amino was purchased from GE Healthcare. Derivatized amino acids Fmoc-L-Nle(6-N_3)-OH (also called Fmoc-L-Lys(N3)-OH), Fmoc-L-Nle(6-OtBDMS)-OH, Fmoc-L-Nle-OH and Fmoc-L-2Aoc-OH (also called Fmoc-L-2Aoc-OH) were purchased from *Iris Biotech GmbH*. Solid supports **4** containing other amino acids (L-Lys, L-Orn, L-Glu, etc.,) were prepared as described by Moroder et al. (2009).

N^{6}-*[(Di*-n-*butylamino)methylene]-3'-[*N-*(9-fluorenyl)methoxycarbonyl-6-azido*-L-*norleucinyl]amino-3'-deoxy-5'*-O-*(4,4'-dimethoxytrityl)*-D-*adenosine* (**2*Nle-N_3**). Fmoc protected L-6-azidonorleucine (100 mg, 0.26 mmol) was dissolved in DMF (10 mL) followed by addition of *O*-(benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HBTU, 96 mg, 0.26 mmol), 1-hydroxybenzotriazole hydrate (HOBt, 39 mg, 0.26 mmol) and *N,N*-diisopropylethylamine (DIPEA, 52 µL, 0.30 mmol). After 3 minutes of activation, 3'-amino-*N*^{6}-[(di-*n*-butylamino)methylene]-3'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-D-adenosine **1** (Geiermann et al., 2015) (140 mg, 0.20 mmol, in 1 mL DMF) was added and the mixture was stirred overnight at room temperature. Then, the solvent was evaporated, the residue dissolved in CH_2Cl_2 and washed consecutively with half-saturated aqueous $NaHCO_3$ solution, 5% citric acid solution, and saturated aqueous NaCl solution. The organic layer was dried (Na_2SO_4), evaporated, and the crude product was purified via SiO_2 chromatography yielding 162 mg of compound **2*Nle-N_3** as white foam (75 %).
^{1}H NMR (300 MHz, $CDCl_3$) δ 9.04 (s, 1H, HC=N(6)), 8.47 (s, 1H, H-C(2)), 8.17 (s, 1H, H-C(8)), 7.71 (m, 2H, HC(ar)), 7.53 (m, 2H, HC(ar)), 7.38-7.16 (m, 13H, HC(ar) and $CDCl_3$), 6.91 (s, 1H, NH(3')), 6.77 (d, 4H, J = 9.0, CH(ar)), 6.02 (s, 1H, H-C(1')), 5.50 (d, 1H, J = 7.5, H-N(Nle)), 4.69 (s, 2H, H-C(2') and H-C(3')), 4.32 (m, 3H, H-C(4') and O-CH_2(Fmoc)), 4.17 (m, 2H, H-C(9, Fmoc) and CH(α, Nle)), 3.72 (s, 6H, 2xOCH_3(DMT)), 3.67 (m, 2H, N($CH_2CH_2CH_2CH_3$)), 3.45-3.30 (m, 4H, H_2C(5') and N($CH_2CH_2CH_2CH_3$)), 3.16 (t, 2H, J = 6.7, H_2C-N_3), 1.69-1.26 (m, 14H, N($CH_2CH_2CH_2CH_3$)$_2$ and 3xCH_2), 1.00-0.91 (m, 6H, N($CH_2CH_2CH_2CH_3$)$_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.3, 162.7, 160.5, 159.0 (HC=N(6)), 156.4, 152.4 (C(2)), 150.5, 144.5, 143.8, 143.7, 141.4, 139.6, 135.7, 130.2 (C(ar)), 128.3 (C(ar)), 128.0 (C(ar)), 127.2 (C(ar)), 125.1 (C(ar)), 120.1 (C(ar)), 113.3 (C(ar)), 91.4 (C(1')), 86.6, 83.2 (C(4')), 74.7 (C(2')), 67.3 (O-CH_2(Fmoc)), 63.0 (C(5')), 55.3 (2xOCH_3(DMT)), 55.0 (C(α, Nle)), 52.0 (C(3') and N($CH_2CH_2CH_2CH_3$)$_2$)), 51.1 (CH_3-N_3), 47.2 (CH(Fmoc)), 45.4 (N($CH_2CH_2CH_2CH_3$)$_2$), 38.7, 36.6, 32.4 (CH_2), 31.1 (CH_2), 29.4 (CH_2), 28.5 (CH_2), 22.7 (CH_2), 20.3 (CH_2), 19.9 (CH_2), 14.0 (CH_3), 13.8 (CH_3). ESI-MS (m/z): $[M+H]^+$ calcd for $C_{61}H_{70}N_{11}O_8$, 1084.54; found 1084.57.

N^{6}-*[(Di-n-butylamino)methylene]-3'-[*N-*(9-fluorenyl)methoxycarbonyl-6-azido*-L-*norleucinyl]amino-3'-deoxy-5'-O-(4,4'-dimethoxytrityl)-2'-O-[1,6-dioxo-6-(pentafluorophenyloxy)hexyl]*-D-*adenosine* (**3*Nle-N_3**). To a solution of compound **2*Nle-N_3** (162 mg, 0.15 mmol) in DMF (1.5 mL) and pyridine (1.0 mL) was added DMAP (20 mg, 0.16 mmol) and bis(pentafluorophenyl) adipate (143 mg, 0.30 mmol). The mixture was stirred for one hour followed by evaporation of the solvents. The crude product was

purified via SiO_2 chromatography (CH_2Cl_2/acetone, 7/3) yielding 112 mg of compound **3*Nle-N_3** as white foam (55%).
^{1}H NMR (300 MHz, $CDCl_3$) δ 8.96 (s, 1H, HC=N(6)), 8.49 (s, 1H, H-C(2)), 8.09 (s, 1H, H-C(8)), 7.75 (m, 2H, H-C(ar)), 7.55 (m, 2H, H-C(ar)), 7.40-7.19 (m, 13H, H-C(ar) and $CDCl_3$), 6.77 (d, 4H, J = 8.6, H-C(ar)), 6.77 (d, 1H, J = 7.1, H-N(3')), 6.15 (d, 1H, J = 2.9, H-C(1')), 5.82 (m, 1H, H-C(2')), 5.33-5.22 (m, 2H, H-N(Nle) and H-C(3')), 4.49-4.35 (m, 2H, O-CH_2(Fmoc)), 4.20 (m, 2H, H-C(4') and H-C(9, Fmoc)), 4.03 (m, C-H(α, Nle)), 3.76 (s, 6H, OCH_3(DMT)), 3.64 (t, 2H, J = 6.1, N($CH_2CH_2CH_2CH_3$)$_2$), 3.45-3.35 (m, 4H, H-C(5') and N($CH_2CH_2CH_2CH_3$)$_2$), 3.21-3.16 (m, 2H, N_3-CH_2), 2.58 and 2.40 (s, 2H, $OOCCH_2CH_2CH_2CH_2COO$), 1.74-1.46 (m, 12H, N($CH_2CH_2CH_2CH_3$)$_2$ and $OOCCH_2CH_2CH_2CH_2COO$ and 3 x CH_2(Nle)), 1.39-1.26 (m, 4H, N($CH_2CH_2CH_2CH_3$)$_2$), 0.97-0.88 (m, 6H, N($CH_2CH_2CH_2CH_3$)$_2$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.6, 169.5, 162.9, 159.9, 158.7 (HC=N(6)), 156.5, 152.8 (C(2)), 151.0, 144.4, 143.7, 141.4 (C(8)), 140.1, 135.6, 135.5, 130.2 (C(ar)), 129.3 (C(ar)), 128.3, 128.0, 127.2, 127.0 (C(ar)), 125.9 (C(ar)), 125.0, 124.9 (C(ar)), 120.2 (C(ar)), 113.3 (C(ar)), 87.7 (C(1')), 86.8, 82.4 (C(4')), 75.3 (C(2')), 67.2 (O-CH_2(Fmoc)), 63.1 (C(5')), 55.3 (2xOCH_3), 54.8 (C(α, Nle)), 52.1, 51.1 (N_3-CH_2), 50.6 (C(3')), 47.2 (HC(Fmoc)), 45.4 (N($CH_2CH_2CH_2CH_3$)$_2$)), 38.7, 36.7, 33.3 ($OOCCH_2CH_2CH_2CH_2COO$), 32.9 ($OOCCH_2CH_2CH_2CH_2COO$), 31.6 (CH_2), 31.4 (CH_2), 31.0 (CH_2), 29.3 (CH_2), 28.5 (CH_2), 24.0 (CH_2), 23.9 (CH_2), 20.2 (CH_2), 19.9 (CH_2), 13.9 (2x CH_3). ESI-MS (m/z): $[M+H]^+$ calcd for $C_{73}H_{76}N_{11}O_{11}$, 1378.57; found 1378.56.

DMTO-rA$^{3'\text{-}NH}$-(N-Fmoc-6-N_3-Nle) solid support (**4*Nle-N_3**). Compound **3*Nle-N_3** (112 mg, 0.083 mmol) was dissolved in dry DMF (2.0 mL) and pyridine (15 µL) was added. To this solution, amino-functionalized support (*GE Healthcare*, *Custom Primer Support^{TM} 200 Amino*, 300 mg) was added, and the suspension was agitated for 20 hrs at room temperature. Subsequently, the beads were collected on a Büchner funnel and washed with DMF, methanol, and CH_2Cl_2. For capping of unreacted amino groups, the beads were treated with a mixture of solution A (0.2 M phenoxy acetic anhydride in THF, 10 mL) and solution B (0.2 M *N*-methyl imidazole, 0.2 M *sym*-collidine in THF, 10 mL) and agitated for 10 min at room temperature. The suspension was filtrated again, the beads were washed with THF, methanol and CH_2Cl_2, and dried under vacuum. Loading of the support **4*Nle-N_3** was 40 µmol/g.

N^6-*[(Di*-n-*butylamino)methylene]-3'-[*N-*(9-fluorenyl)methoxycarbonyl-6*-O-tert.*butyldimetylsilyloxy*-L-*norleucinyl]amino-3'-deoxy-5'*-O-*(4,4'-dimethoxytrityl)*-D-*adenosine* (**2*Nle-OH**). *N*-Fluorenylmethoxycarbonyl-*O-tert.*butyldimetylsilyl-6-hydroxy-L-norleucine (144 mg, 0.30 mmol) was dissolved in DMF (3 mL) followed by addition of *O*-(benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HBTU, 113 mg, 0.30 mmol), 1-hydroxybenzotriazole hydrate (HOBt, 46 mg, 0.30 mmol) and *N,N*-diisopropylethylamine (DIPEA, 60 µL, 0.34 mmol). After 3 minutes of activation, 3'-amino-*N*^{6}-[(di-*n*-butylamino)methylene]-3'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-D-adenosine **1** (Geiermann et al., 2015) (162 mg, 0.23 mmol, in 1 mL DMF) was added and the mixture was stirred overnight at room temperature. Then, the solvent was evaporated, the residue dissolved in CH_2Cl_2 and washed consecutively with half-saturated aqueous $NaHCO_3$ solution, 5% citric acid solution, and saturated aqueous NaCl solution. The organic layer was dried (Na_2SO_4), evaporated and the crude product was purified via SiO_2 chromatography yielding 240 mg of compound **2*Nle-OH** as white foam (90 %).
^{1}H NMR (300 MHz, $CDCl_3$) δ 9.10 (s, 1H, HC=N(6)), 8.45 (s, 1H, H-C(2)), 8.16 (s, 1H, H-C(8)), 7.72 (m, 2H, H-C(ar)), 7.56 (m, 2H, H-C(ar)), 7.34-7.18 (m, 13H, H-C(ar) and $CDCl_3$), 6.89 (b, 1H, HN(3')), 6.77 (d, 4H, J = 8.2, HC(ar)), 6.00 (s, 1H, H-C(1'), 5.50 (d,

1H, J = 6.3, H-N(Nle)), 4.76 (m, 1H, H-C(2')), 4.68 (s, 1H, H-C(3'), 4.35 (m, 3H, H-C(4') and O-CH_2(Fmoc)), 4.18 (t, 2H, J = 6.9, H-C(α, Fmoc) and H-C(9, DMT)), 3.74 (s, 8H, 2x OCH_3(DMT) and OCH_2(Nle)), 3.64-3.61 (m, 2H, $N(CH_2CH_2CH_2CH_3)_2$), 3.55 (m, 2H, H-C(5')), 3.38 (m, 2H, $N(CH_2CH_2CH_2CH_3)_2$), 1.63 (m, 4H, $N(CH_2CH_2CH_2CH_3)_2$), 1.59-1.41 (m, 6H, 3xH_2C(Nle)), 1.37 (m, 4H, $N(CH_2CH_2CH_2CH_3)_2$), 0.98-0.89 (m, 6H, $N(CH_2CH_2CH_2CH_3)_2$), 0.86 (s, 9H, 3xCH_3(TBDMS)), 0.02 (s, 6H, 2xSi-CH_3(TBDMS)). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.6, 159.2, 158.6 (HC=N(6)), 156.5, 152.0 (C(2)), 150.6, 144.5, 143.9, 141.4, 139.9 (C(8)), 135.8, 130.2 (C(ar)), 128.3 (C(ar)), 127.9 (C(ar)), 127.2 (C(ar)), 125.2 (C(ar)), 120.1 (C(ar)), 113.3 (C(ar)), 91.3 (C(1')), 86.7, 83.7 (C(4')), 74.7 (C(2')), 67.3 (OCH_2(Fmoc)), 62.9 (C(5')), 55.3 (C(α, Nle) and 2x OCH_3), 52.2 (C(3') and $N(CH_2CH_2CH_2CH_3)_2$), 47.2 (CH(Fmoc)), 45.5 (OCH_2(Nle)), 38.7, 32.4 and 31.1 and 29.3 ($N(CH_2CH_2CH_2CH_3)_2$ and CH_2(Nle)), 26.1 (3x CH_3(TBDMS)), 22.1 and 20.4 and 19.9 ($N(CH_2CH_2CH_2CH_3)_2$ and CH_2(Nle)), 18.4, 14.1 ($N(CH_2CH_2CH_2CH_3)_2$), 13.8 ($N(CH_2CH_2CH_2CH_3)_2$), -5.2 (2x Si-CH_3). ESI-MS (m/z): $[M+H]^+$ calcd for $C_{67}H_{85}N_8O_9Si$, 1173.62; found 1173.55.

N^6-*[(Di-n-butylamino)methylene]-3'-[*N-*(9-fluorenyl)methoxycarbonyl-6-*O-tert.*butyldimetylsilyloxy-*L-*norleucinyl]amino-3'-deoxy-5'-O-(4,4'-dimethoxytrityl)-2'-O-[1,6-dioxo-6-(pentafluorophenyloxy)hexyl]-*D-*adenosine* (**3*Nle-OH 6**). To a solution of compound **2*Nle-OH** (230 mg, 0.20 mmol) in DMF (2.5 mL) and pyridine (2.0 mL) was added DMAP (26 mg, 0.22 mmol) and bis(pentafluorophenyl) adipate (188 mg, 0.39 mmol). The mixture was stirred for one hour followed by evaporation of the solvents. The crude product was purified via SiO_2 chromatography (CH_2Cl_2/acetone, 7/3) yielding 167 mg of compound **3*Nle-OH** as white foam (58%).
^{1}H NMR (300 MHz, $CDCl_3$) δ 8.96 (s, 1H, HC=N(6)), 8.49 (s, 1H, H-C(2)), 8.08 (s, 1H, H-C(8)), 7.74 (d, 2H, J = 7.4, H-C(ar)), 7.54 (d, 2H, J = 7.2, H-C(ar)), 7.41-7.19 (m, 13H, H-C(ar) and $CDCl_3$), 6.76 (d, 4H, J = 8.6, H-C(ar)), 6.50 (b, 1H, H-N(3')), 6.16 (d, 1H, J = 3.0, H-C(1')), 5.83 (m, 1H, H-C(2')), 5.20 (m, 2H, H-C(3') and H-N(Nle)), 4.47-4.26 (m, 3H, O-CH_2(Fmoc) and H-C(9, Fmoc)), 4.18 (m, 1H, H-C(4')), 3.99 (m, 1H, H-C(α, Nle)), 3.75 (s, 6H, 2xOCH_3(DMT)), 3.64 (m, 2H, $N(CH_2CH_2CH_2CH_3)_2$), 3.56 (t, 2H, J = 6.3 (OCH_2(Nle)), 3.45 (m, 2H, H-C(5')), 3.38 (t, 2H, J = 7.2, $N(CH_2CH_2CH_2CH_3)_2$), 2.57 (s, 2H, ($OOCCH_2CH_2CH_2CH_2COO$)), 2.41 (s, 2H, ($OOCCH_2CH_2CH_2CH_2COO$)), 1.78-1.28 (m, 18H, 3xCH_2(Nle) and ($OOCCH_2CH_2CH_2CH_2COO$) and $N(CH_2CH_2CH_2CH_3)_2$), 0.93 (m, 6H, $N(CH_2CH_2CH_2CH_3)_2$), 0.88 (s, 9H, 3xCH_3(TBDMS)), 0.04 (s, 6H, 2xSi-CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.9, 171.6, 169.4, 160.0, 158.7 (HC=N(6)), 152.9 (C(2)), 151.1, 144.4, 143.7, 141.4 140.0 (C(8)), 139.6, 135.7, 130.2 (C(ar)), 129.3 (C(ar)), 128.4 (C(ar)), 128.0 (C(ar)), 127.2 (C(ar)), 127.0 (C(ar)), 125.9 (C(ar)), 125.0 (C(ar)), 120.2 (C(ar)), 113.3 (C(ar)), 87.5 (C(1')), 86.9, 82.6 (C(4')), 75.2 (C(2')), 67.3 (OCH_2(Fmoc)), 63.3 (C(5')), 62.8 ($N(CH_2CH_2CH_2CH_3)_2$), 55.3 (C(α, Nle) and 2xOCH_3(DMT)), 52.0 ($N(CH_2CH_2CH_2CH_3)_2$), 50.6 (C(3')), 47.2 (CH(Fmoc)), 45.4 (OCH_2(Nle)), 38.7, 33.3 and 32.9 ($OOCCH_2CH_2CH_2CH_2COO$), 32.4-29.4 ($OOCCH_2CH_2CH_2CH_2COO$ and/or CH_2(Nle) and/or $N(CH_2CH_2CH_2CH_3)_2$), 26.1 (2x CH_3(TBDMS)), 24.0-18.5 ($OOCCH_2CH_2CH_2CH_2COO$ and/or CH_2(Nle) and/or $N(CH_2CH_2CH_2CH_3)_2$), 13.9 and 13.8 (2x CH_3), 1.1, -5.2 (2x Si-CH_3). ESI-MS (m/z): $[M+H]^+$ calcd for $C_{79}H_{92}F_5N_8O_{12}Si$, 1467.65; found 1467.63.

DMTO-r$A^{3'\text{-}NH}$-(N-Fmoc-6-OtBDMS-Nle) solid support (**4*Nle-OH**). Compound **3*Nle-OH** (167 mg, 0.11 mmol) was dissolved in dry DMF (3.0 mL) and pyridine (21 µL) was added. To this solution, amino-functionalized support (*GE Healthcare*, *Custom Primer Support™ 200 Amino*, 400 mg) was added, and the suspension was agitated for 20 hrs at room temperature. Subsequently, the beads were collected on a Büchner funnel and washed

with DMF, methanol, and CH_2Cl_2. For capping of unreacted amino groups, the beads were treated with a mixture of solution A (0.2 M phenoxy acetic anhydride in THF, 10 mL) and solution B (0.2 M *N*-methyl imidazole, 0.2 M *sym*-collidine in THF, 10 mL) and agitated for 10 min at room temperature. The suspension was filtrated again, the beads were washed with THF, methanol and CH_2Cl_2, and dried under vacuum. Loading of the support **4*Nle-OH** was 75 µmol/g.

N^6-*[(Di-*n*-butylamino)methylene]-3'-[*N*-(9-fluorenyl)methoxycarbonyl*-L-*norleucinyl]amino-3'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)*-D-*adenosine* (**2*Nle**). Fmoc protected L-6-azidonorleucine (79 mg, 0.22 mmol) was dissolved in DMF (3 mL) followed by addition of *O*-(benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HBTU, 81 mg, 0.22 mmol), 1-hydroxybenzotriazole hydrate (HOBt, 27 mg, 0.17 mmol) and *N,N*-diisopropylethylamine (DIPEA, 45 µL, 0.26 mmol). After three minutes of activation, 3'-amino-N^6-[(di-*n*-butylamino)methylene]-3'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-D-adenosine **1** (Geiermann et al., 2015) (122 mg, 0.17 mmol, in 1 mL DMF) was added and the mixture was stirred overnight at room temperature. Then, the solvent was evaporated, the residue dissolved in CH_2Cl_2 and washed consecutively with half-saturated aqueous $NaHCO_3$ solution, 5% citric acid solution, and saturated aqueous NaCl solution. The organic layer was dried (Na_2SO_4), evaporated and the crude product was purified via SiO_2 chromatography yielding 116 mg of compound **2*Nle** as white foam (65%).
^{1}H NMR (300 MHz, $CDCl_3$) δ 9.04 (s, 1H, HC=N(6)), 8.48 (s, 1H, H-C(2)), 8.14 (s, 1H, H-C(8)), 7.72 (m, 1H, H-C(ar)), 7.54 (m, 2H, H-C(ar)), 7.36-7.16 (m, 13H, H-C(ar) and $CDCl_3$), 6.83 (s, 1H, H-N(3')), 6.76 (d, 4H, J = 8.5, H-C(ar)), 5.99 (s, 1H, H-C(1')), 5.39 (d, 1H, J = 7.3, H-N(Nle)), 4.77 (m, 1H, H-C(2')), 4.66 (m, 1H, H-C(3')), 4.34 (m, 3H, O-CH_2(Fmoc) and H-C(4')), 4.18 (m, 2H, H-C(α, Nle) and H-C(9, Fmoc)), 3.75 (s, 6H, 2xOCH_3(DMT)), 3.69 (m, 2H, N($CH_2CH_2CH_2CH_3$)$_2$), 3.39 (m, 4H, H-C(5') and N($CH_2CH_2CH_2CH_3$)$_2$), 1.66 (m, 6H, H_2C(β, Nle) and N($CH_2CH_2CH_2CH_3$)$_2$), 1.38 (m, 4H, N($CH_2CH_2CH_2CH_3$)$_2$), 1.26 (m, 4H, 2xH_2C(Nle)), 0.94 (m, 6H, N($CH_2CH_2CH_2CH_3$)$_2$), 0.84 (t, 3H, J = 6.6, H_3C(Nle)). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.6 (HC=N(6)), 160.5, 158.9, 158.6, 156.4, 152.5 (C(2)), 150.7, 144.5, 143.9, 143.8, 141.4, 139.6 (C(8)), 135.8, 135.7, 130.2 (C(ar)), 128.3 (C(ar)), 128.0 (C(ar)), 127.9 (C(ar)), 127.2 (C(ar)), 126.9 (C(ar)), 125.2 (C(ar)), 120.1 (C(ar)), 113.3 (C(ar)), 91.4 (C(1')), 86.6, 83.6 (C(4')), 74.7 (C(2')), 67.3 (O-CH_2(Fmoc)), 63.4 (C(5')), 55.3 (C(α, Nle) and 2xOCH_3(DMT)), 52.4 (C(3')), 52.1 (N($CH_2CH_2CH_2CH_3$)$_2$), 47.2 (CH(Fmoc)), 46.3, 45.4 (N($CH_2CH_2CH_2CH_3$)$_2$), 32.5 (N($CH_2CH_2CH_2CH_3$)$_2$ and CH_2(Nle)), 31.1 (N($CH_2CH_2CH_2CH_3$)$_2$), 29.4, 27.7 (CH_2(Nle)), 22.4 (CH_2(Nle)), 20.3 (N($CH_2CH_2CH_2CH_3$)$_2$), 19.9 (N($CH_2CH_2CH_2CH_3$)$_2$), 14.0, 14.0 (N($CH_2CH_2CH_2CH_3$)$_2$), 13.8 (CH_3(Nle), 11.0. ESI-MS (m/z): $[M+H]^+$ calcd for $C_{61}H_{71}N_8O_8$, 1043.54; found 1043.55.

N^6-*[(Di-n-butylamino)methylene]-3'-[*N*-(9-fluorenyl)methoxycarbonyl*-L-*norleucinyl]amino-3'-deoxy-5'-O-(4,4'-dimethoxytrityl)-2'-O-[1,6-dioxo-6-(pentafluorophenyloxy)hexyl]*-D-*adenosine* (**3*Nle**). To a solution of compound **2*Nle** (62 mg, 0.06 mmol) in DMF (1.5 mL) and pyridine (1.0 mL) was added DMAP (7 mg, 0.06 mmol) and bis(pentafluorophenyl) adipate (89 mg, 0.19 mmol). The mixture was stirred for one hour followed by evaporation of the solvents. The crude product was purified via SiO_2 chromatography (CH_2Cl_2/acetone, 7/3) yielding 36 mg of compound **2*Nle** as white foam (46%).
^{1}H NMR (300 MHz, $CDCl_3$) δ 8.97 (s, 1H, HC=N(6)), 8.48 (s, 1H, H-C(2)), 8.01 (s, 1H, H-C(8)), 7.73 (m, 2H, HC(ar)), 7.75 (m, 2H, HC(ar)), 7.40-7.18 (m, 13H, HC(ar) and $CDCl_3$), 6.75 (d, 4H, J = 7.8, HC(ar)), 6.54 (m, 1H, HN(3')), 6.14 (d, 1H, J = 2.6, HC(1')), 5.83 (m, 1H, HC(2')), 5.27 (m, 1H, HN(Nle)), 5.20 (m, 1H, HC(3')), 4.43 (m, 2H, OCH_2(Fmoc)),

4.18 (m, 2H, HC(4') and HC(9, Fmoc)), 4.01 (m, 1H, HC(α, Fmoc)), 3.75 (s, 6H, 2xOCH_3(DMT)), 3.67 (m, 2H, N($CH_2CH_2CH_2CH_3$)$_2$), 3.45-3.35 (m, 4H, HC(5') and N($CH_2CH_2CH_2CH_3$)$_2$), 2.57 (m, 2H, OOC$CH_2CH_2CH_2CH_2$COO), 2.39 (m, 2H, OOC$CH_2CH_2CH_2CH_2$COO), 1.65 (m, 8H, CH_2(AOA) and OOC$CH_2CH_2CH_2CH_2$COO and N($CH_2CH_2CH_2CH_3$)$_2$), 1.36 (m, 2H, N($CH_2CH_2CH_2CH_3$)$_2$), 1.23 (m, 4H, 2xCH_2(Nle)), 0.93 (q, 6H, N($CH_2CH_2CH_2CH_3$)$_2$), 0.84 (t, 3H, CH_3(Nle)). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.1, 171.6, 169.6, 169.5, 162.7, 160.3, 158.6 (HC=N(6)), 158.5, 156.5, 153.1 (C(2)), 151.3, 144.5, 143.8, 141.4, 140.0 (C(8)), 135.7, 130.2 (C(ar)), 128.4 (C(ar)), 127.9 (C(ar)), 127.2 (C(ar)), 125.1 (C(ar)), 120.1 (C(ar)), 113.3 (C(ar)), 87.5 (C(1')), 86.8, 82.5 (C(4')), 75.2 (C(2')), 67.2 (OCH_2), 63.4 (C(5')), 55.3 (C(α, Nle) and 2xOCH_3(DMT)), 52.0 (N($CH_2CH_2CH_2CH_3$)$_2$), 50.7 (C(3')), 47.2 (CH(Fmoc)), 46.3, 45.3 (N($CH_2CH_2CH_2CH_3$)$_2$), 36.6, 34.7 (OOC$CH_2CH_2CH_2CH_2$COO), 33.3 (OOC$CH_2CH_2CH_2CH_2$COO), 32.9, 31.6, 31.1 (CH_2(Nle)), 29.4 (2x OOC$CH_2CH_2CH_2CH_2$COO), 27.8 (CH_2(Nle)), 25.7, 24.0 and 23.9 (N($CH_2CH_2CH_2CH_3$)$_2$), 22.4 (CH_2(Nle)), 20.3 (N($CH_2CH_2CH_2CH_3$)$_2$), 19.9, 14.0 (N($CH_2CH_2CH_2CH_3$)$_2$), 13.8 (CH_3(Nle)), 8.8. ESI-MS (m/z): $[M+H]^+$ calcd for $C_{75}H_{82}F_5N_8O_{11}$, 1337.57; found 1337.46.

DMTO-rA$^{3'\text{-}NH}$-(N-Fmoc-Nle) solid support (**4*Nle**). Compound **3*Nle** (36 mg, 0.03 mmol) was dissolved in dry DMF (1.5 mL) and pyridine (5 µL) was added. To this solution, amino-functionalized support (*GE Healthcare*, *Custom Primer Support^{TM} 200 Amino*, 200 mg) was added, and the suspension was agitated for 20 hrs at room temperature. Subsequently, the beads were collected on a Büchner funnel and washed with DMF, methanol, and CH_2Cl_2. For capping of unreacted amino groups, the beads were treated with a mixture of solution A (0.2 M phenoxy acetic anhydride in THF, 10 mL) and solution B (0.2 M *N*-methyl imidazole, 0.2 M *sym*-collidine in THF, 10 mL) and agitated for 10 min at room temperature. The suspension was filtrated again, the beads were washed with THF, methanol and CH_2Cl_2, and dried under vacuum. Loading of the support **4*Nle** was 40 µmol/g.

N^6-*[(Di*-n-*butylamino)methylene]-3'-[(2S)*-N-*(9-fluorenyl)methoxycarbonyl-2-amino-octanamido]-3'-deoxy-5'*-O-*(4,4'-dimethoxytrityl)*-D-*adenosine* (**2*AOA**). *(2S)-N*-(9-fluorenyl)methoxycarbonyl-2-aminooctanic acid (83 mg, 0.20 mmol) was dissolved in DMF (3 mL) followed by addition of *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU, 66 mg, 0.17 mmol), 1-hydroxybenzotriazole hydrate (HOBt, 27 mg, 0.17 mmol) and *N,N*-diisopropylethylamine (DIPEA, 35 µL, 0.20 mmol). After 3 minutes of activation, 3'-amino-*N*^{6}-[(di-*n*-butylamino)methylene]-3'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-D-adenosine **1** (Geiermann et al., 2015) (95 mg, 0.13 mmol, in 1 mL DMF) was added and the mixture was stirred overnight at room temperature. Then, the solvent was evaporated, the residue dissolved in CH_2Cl_2 and washed consecutively with half-saturated aqueous $NaHCO_3$ solution, 5% citric acid solution, and saturated aqueous NaCl solution. The organic layer was dried (Na_2SO_4), evaporated and the crude product was purified via SiO_2 chromatography yielding 62 mg of compound **2*AOA** as white foam (43%).
^{1}H NMR (300 MHz, $CDCl_3$) δ 9.05 (s, 1H, HC=N(6)), 8.49 (s, 1H, H-C(2)), 8.17 (s, 1H, H-C(8)), 7.71 (m, 2H, H-C(ar)), 7.54 (m, 2H, H-C(ar)), 7.39-7.14 (m, 13H, H-C(ar)), 6.87 (m, 1H, H-N(3')), 6.75 (d, 4H, J = 8.4, H-C(ar)), 6.01 (s, 1H, H-C(1')), 5.48 (d, 1H, J = 7.4, H-N(AOA)), 4.79 (m, 1H, H-C(2')), 4.69 (m, 1H, H-C(3')), 4.36 (m, 3H, OCH_2(Fmoc) and H-C(4')), 4.18 (m, 2H, H-C(α, AOA) and H-C(9, Fmoc)), 3.74 (s, 6H, OCH_3(DMT)), 3.72-3.60 (m, 2H, N($CH_2CH_2CH_2CH_3$)$_2$), 3.48-3.36 (m, 4H, N($CH_2CH_2CH_2CH_3$)$_2$ and H-C(5')), 1.62 (m, 6H, N($CH_2CH_2CH_2CH_3$)$_2$) and CH_2(AOA)), 1.38-1.29 (m, 6H, N($CH_2CH_2CH_2CH_3$)$_2$ and CH_2(AOA)), 1.24 (br, 6H; 3xCH_2(AOA)), 0.97-0.83 (m, 9H,

3xCH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 173.1, 160.7, 158.8 (HC=N(6)), 156.5, 152.5 (C(2)), 150.8, 144.7, 144.1, 141.5 139.8 (C(8)), 136.0, 130.2, 128.3, 128.0, 127.2, 125.2, 120.1 113.3, 91.4(C(1')), 86.8, 83.7 (C(4')), 74.9 (C(2')), 67.4 (O-CH_2(Fmoc)), 63,5 (C(5')), 55.4 (C(α, AOA) and 2xOCH_3(DMT)), 52.5 (C(3')), 52.1 (N($CH_2CH_2CH_2CH_3$)$_2$) , 47.3, 46.5 (CH(Fmoc)), 45.5 (N($CH_2CH_2CH_2CH_3$)$_2$), 38.9, 32.9, 31.8 and 31.2 (N($CH_2CH_2CH_2CH_3$)$_2$ and CH_2(AOA)), 29.4 and 29.1 (N($CH_2CH_2CH_2CH_3$)$_2$ and CH_2(AOA)), 25.7 (CH_2(AOA)), 22.7 (CH_2(AOA)), 20.4 and 20.0 (N($CH_2CH_2CH_2CH_3$)$_2$), 14.3-13.9 (N($CH_2CH_2CH_2CH_3$)$_2$ and CH3(AOA)), 8.9. ESI-MS (m/z): $[M+H]^+$ calcd for $C_{63}H_{75}N_8O_8$, 1071.57; found 1071.44.

N^6*-[(Di-n-butylamino)methylene]-3'-[(2S)*-N-*(9-fluorenyl)methoxycarbonyl-2-amino-octanamido]-3'-deoxy-5'-O-(4,4'-dimethoxytrityl)-2'-O-[1,6-dioxo-6-(pentafluorophenyloxy)hexyl]*-D-*adenosine* (**3*AOA**). To a solution of compound **2*AOA** (62 mg, 0.06 mmol) in DMF (1.5 mL) and pyridine (1.0 mL) was added DMAP (7 mg, 0.06 mmol) and bis(pentafluorophenyl) adipate (89 mg, 0.19 mmol). The mixture was stirred for one hour followed by evaporation of the solvents. The crude product was purified via SiO_2 chromatography (CH_2Cl_2/acetone, 7/3) yielding 36 mg of compound **3*AOA** as white foam (46%).
^{1}H NMR (300 MHz, $CDCl_3$) δ 8.99 (s, 1H, HC=N(6)), 8.49 (s, 1H, H-C(2)), 8.08 (s, 1H, H-C(8)), 7.75 (m, 2H, H-C(ar)), 7.56 (m, 2H, H-C(ar)), 7.41-7.16 (m, 13H, H-C(ar)), 6.78 (d, 4H, J = 8.7, H-C(ar)), 6.52 (br, 1H, H-N(3')), 6.16 (d, 1H, J = 3.1, H-C(1)), 5.83 (m, 1H, H-C(2')), 5.24 (m, 2H, H-C(3') and H-N(AOA)), 4.47-4.29 (m, 2H, O-CH_2(Fmoc)), 4.18 (m, 2H, H-C(4') and H-C(9, Fmoc)), 4.01 (m, 1H, H-C(α, AOA)), 3.75 (s, 6H, 2xO-CH_3(DMT)), 3.64 (m, 2H, N($CH_2CH_2CH_2CH_3$)$_2$), 3.45-3.36 (m, 4H, H_2C(5') and N($CH_2CH_2CH_2CH_3$)$_2$), 2.57 (s, 2H, OOC$CH_2CH_2CH_2CH_2$COO), 2.41 (s, 2H, OOC$CH_2CH_2CH_2CH_2$COO), 1.65-1.57 (m, 8H, CH_2(AOA) and OOC$CH_2CH_2CH_2CH_2$COO and N($CH_2CH_2CH_2CH_3$)$_2$), 1.39-1.29 (m, 4H, N($CH_2CH_2CH_2CH_3$)$_2$), 1.28-1.19 (m, 8H, CH_2(AOA)), 0.97-0.84 (m, 9H, 3xCH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.0, 171.6, 169.5, 159.9, 158.7 (HC=N(6)), 156.5, 152.7 (C(2)), 151.1, 144.4, 143.7, 141.4 (C(8)), 140.1, 139.6, 135.6 130.2 (C(ar)), 129.3, 128.4(C(ar)), 128.0, 127.2, 127.0, 125.9, 125.0 (C(ar)), 120.1 (C(ar)), 113.3 (C(ar)), 87.6 (C(1')), 86.8, 82.5 (C(4')), 75.2 (C(2')), 67.2 (OCH_2(Fmoc)), 63.2 (C(5')), 55.3 (C(α, Nle) and 2xOCH_3(DMT)), 52.1 (N($CH_2CH_2CH_2CH_3$)$_2$), 50.6 (C(3')), 47.2 (HC(Fmoc)), 45.4 (N($CH_2CH_2CH_2CH_3$)$_2$), 33.3 (OOC$CH_2CH_2CH_2CH_2$COO), 32.9 (OOC$CH_2CH_2CH_2CH_2$COO), 31.7 (CH_2(AOA)), 31.1 (OOC$CH_2CH_2CH_2CH_2$COO), 29.3, 29.0 (2xCH_2(AOA)), 25.7 (CH_2(AOA)), 24.0, 23.8 (N($CH_2CH_2CH_2CH_3$)$_2$), 22.7 (CH_2(AOA)), 20.2, 19.9 (N($CH_2CH_2CH_2CH_3$)$_2$), 14.1-13.7 (3xCH_3). ESI-MS (m/z): $[M+H]^+$ calcd for $C_{75}H_{82}F_5N_8O_{11}$, 1365.60; found 1366.48.

DMTO-rA$^{3'-NH}$-(N-Fmoc-AOA) solid support (**4*AOA**). Compound **3*AOA** (36 mg, 0.03 mmol) was dissolved in dry DMF (1.5 mL) and pyridine (5 µL) was added. To this solution, amino-functionalized support (*GE Healthcare*, *Custom Primer Support^{TM} 200 Amino*, 200 mg) was added, and the suspension was agitated for 20 hrs at room temperature. Subsequently, the beads were collected on a Büchner funnel and washed with DMF, methanol, and CH_2Cl_2. For capping of unreacted amino groups, the beads were treated with a mixture of solution A (0.2 M phenoxy acetic anhydride in THF, 10 mL) and solution B (0.2 M *N*-methyl imidazole, 0.2 M *sym*-collidine in THF, 10 mL) and agitated for 10 min at room temperature. The suspension was filtrated again, the beads were washed with THF, methanol and CH_2Cl_2, and dried under vacuum. Loading of the support **4*AOA** was 45 µmol/g.

N^6-*[(Di*-n-*butylamino)methylene]-3'-[3*-N-allyloxycarbonyl-*2*-N-*(9-fluorenyl)methoxycarbonyl*-L-*2,3-diaminopropionyl]amino-3'-deoxy-5'*-O-*(4,4'-dimethoxytrityl)*-D-*adenosine* (**2*Dap**). 3-*N*-Allyloxycarbonyl-2-*N*-fluorenylmethoxycarbonyl-L-2,3-diaminopropionic acid (26 mg, 63 µmol) was dissolved in DMF (1 mL) followed by addition of *O*-(benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium hexafluorophosphate (HBTU, 25 mg, 66 µmol), 1-hydroxybenzotriazole hydrate (HOBt, 8 mg, 52 µmol) and *N,N*-diisopropylethylamine (DIPEA, 13 µL, 75 µmol). After 10 minutes of activation, 3'-amino-N^6-[(di-*n*-butylamino)methylene]-3'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-D-adenosine **1** (Geiermann et al., 2015) (35 mg, 49 µmol, in 1 mL DMF) was added and the mixture was stirred overnight at room temperature. Then, 1 mL of water was added and stirring was continued for further 10 minutes. All volatiles were evaporated, the residue dissolved in CH_2Cl_2 and washed consecutively with half-saturated aqueous $NaHCO_3$ solution, and saturated aqueous NaCl solution. The organic layer was dried (Na_2SO_4), evaporated and the crude product was purified via SiO_2 chromatography, eluting with a gradient from 1 to 5 % of methanol in dichloromethane, yielding 23 mg of compound **2*Dap** as white foam (42 %).
^{1}H NMR (300 MHz, d6-DMSO) δ 8.93 (s, 1H, HC=N(6)), 8.40 (s, 1H, H-C(2)), 8.34 (s, 1H, H-C(8)), 8.00 (m, 1H, H-C(ar)), 7.90 - 7.87 (m, 2H, H-C(ar)), 7.70 - 7.68 (m, 2H, H-C(ar)), 7.44 - 7.12 (m, H-C(ar)), 6.77 (m, 4H, H-C(ar)), 6.04 (s, 1H, H-C(1')), 5.86 (m, 1H, H-C(allyl)), 5.25 - 5.09 (m, 2H, H=C(allyl)), 4.75 (m, 1H, H-C(2')), 4.42 (m, 2H, CH_2(allyl)), 4.25 - 4.18 (m, H-C(α), CH_2(Dap), H-C(Fmoc), H-C(3')), 3.69 (s, 7H, 2 x OMe(DMT) and H-C(4')), 3.59 (m, 2H, $N(CH_2CH_2CH_2CH_3)_2$), 3.45 (m, 2H, $N(CH_2CH_2CH_2CH_3)_2$), 3.25 - 3.20 (m, 4H, CH_2(Fmoc), 2 x H-C(5')), 1.60 (m, 2H, $N(CH_2CH_2CH_2CH_3)_2$), 1.32 (m, 2H, $N(CH_2CH_2CH_2CH_3)_2$), 1.24 (m, 4H, $N(CH_2CH_2CH_2CH_3)_2$)), 0.92 (m, 6H, $N(CH_2CH_2CH_2CH_3)_2$)) ppm. ESI-MS (m/z): $[M+H]^+$ calcd for $C_{62}H_{70}N_9O_{10}$, 1100.52; found 1100.27.

DMTO-rA$^{3'\text{-}NH}$-(3-N-Alloc-2-N-Fmoc-Dap) solid support (**4*Dap**). Compound **2*Dap** (23 mg, 21 µmol) and bis(pentafluorophenyl) adipate (20 mg, 42 µmol) were dissolved in dry DMF (1.5 mL) and pyridine (1.0 mL) and then DMAP (3 mg, 25 µmol) was added to form **3*Dap** in situ. After 2 hours of agitation at room temperature, amino-functionalized support (*GE Healthcare*, *Custom Primer Support^{TM} 200 Amino*, 125 mg) was added, and the suspension was further agitated for 3 days at room temperature. Subsequently, the beads were collected on a Büchner funnel and washed with DMF, methanol and CH_2Cl_2. For capping of unreacted amino groups, the beads were treated with a mixture of solution A (0.2 M phenoxy acetic anhydride in THF, 10 mL) and solution B (0.2 M *N*-methyl imidazole, 0.2 M *sym*-collidine in THF, 10 mL) and agitated for 10 min at room temperature. The suspension was filtrated again, the beads were washed with THF, acetonitrile and CH_2Cl_2, and dried under vacuum. Loading of the support **4*Dap** was 20 µmol/g.

RNA solid-phase synthesis, deprotection and purification

*Automated synthesis on solid supports **4***. The 5'-p-ACC moiety was assembled on an *ABI 392 Nucleic Acid Synthesizer* following standard synthesis protocols using 2'-*O*-[(Triisopropylsilyl)oxy]methyl (TOM) protected nucleoside phosphoramidites (Pitsch et al., 2001; Micura, 2002) and the above described solid supports **4**. Detritylation (120 s): dichloroacetic acid/1,2-dichloroethane (4/96); coupling (120 s): phosphoramidites (0.1 M in acetonitrile, 130 µL) were activated with benzylthiotetrazole (0.3 M in acetonitrile, 180

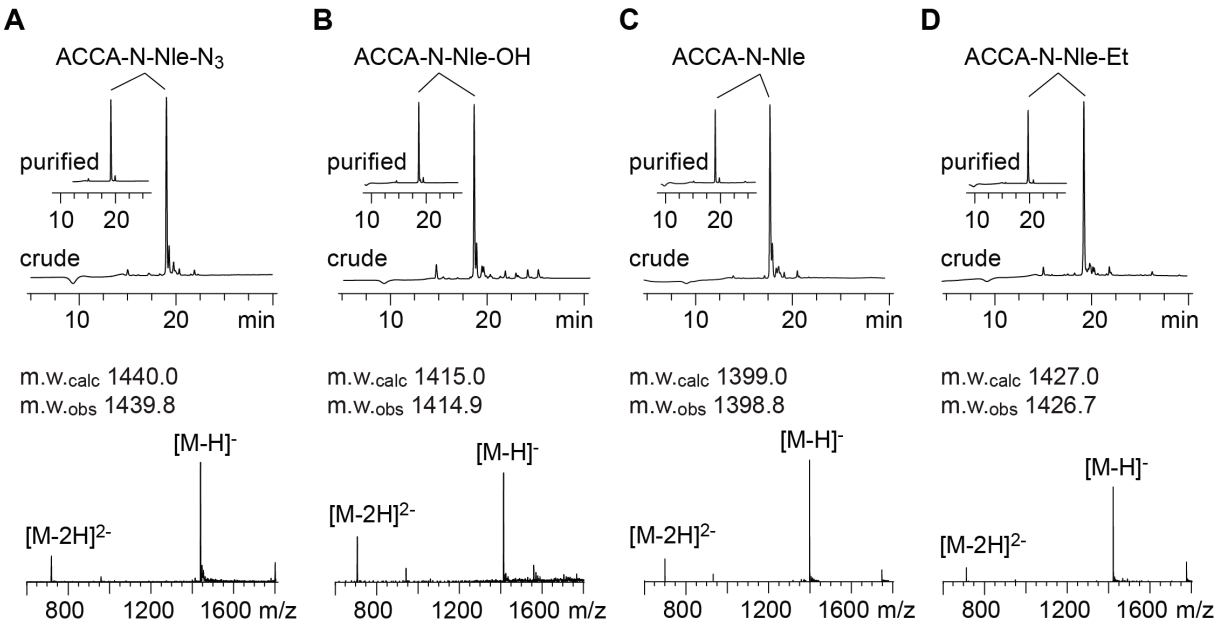
µL); capping (2 x 10 s, Cap A/Cap B = 1/1): Cap A: phenoxyacetic anhydride (0.2 M in THF), Cap B: *N*-methyl imidazole (0.2 M), sym-collidine (0.02 M) in THF; oxidation (20 s): I_2 (0.2 M) in THF/pyridine/H_2O (35/10/5). Nucleoside phosphoramidites, benzylthiotetrazole, and capping solutions were dried over activated molecular sieves (4 Å) overnight.

Deprotection of the 5'-p-ACCA$^{3'NH}$-amino acid conjugates. For conjugates synthesized on solid support **4*Dap**: *Allyl deprotection* (for supports **4*Nle-N$_3$**, **4*Nle-OH**, **4*Nle**, and **4*Nle-Et**, deprotection starts directly with Step A): The solid support was transfered into an Eppendorf tube, dried in high vacuum and purged with argon. Dry and degassed dichloromethane (1.0 mL) was added, followed by borane dimethylamine complex (35 mg). After ten minutes $Pd(P[Ph]_3)_4$ (35 mg) was added. The reaction was agitated and kept under argon for 3 hours. The solid support was filtered and treated with a solution of sodium diethyldithiocarbamate (0.5% in DMF, 5 x 2 mL) and washed with DMF and dichloromethane.
Step A) *Fmoc deprotection*. The solid support was treated with a solution of 20 % piperidine in acetonitrile (10 mL, 10 min), washed with acetonitrile and dried (can be done in the ABI synthesis column). Step B) *Acyl deprotection and cleavage from the solid support*. In an Eppendorf tube, the beads were treated with equal volumes of methylamine in ethanol (8 M, 0.5 mL) and methylamine in H_2O (40%, 0.5 mL) were added. After 6 h shaking at room temperature the supernatant was filtered and evaporated to dryness. Step C) *2'-O-TOM and 6-OtBDMS-Nle deprotection*. The obtained residue was treated with TBAF·3 H_2O in THF (1 M, 1 mL) overnight at room temperature. The reaction was quenched by the addition of triethylammonium acetate (TEAA) (1 M, pH 7.4, 1 mL). After reducing the volume of the solution, it was applied on a size-exclusion chromatography column (*GE Healthcare*, HiPrep 26/10 Desalting, 2.6 x 10 cm, Sephadex G25). By eluating with H_2O, the conjugate-containing fractions were collected, evaporated to dryness, and the residue was dissolved in H_2O (1 mL). Analysis of the crude products was performed by anion-exchange chromatography on a Dionex DNAPac PA-100 column (4 x 250 mm) at 60°C. Flow rate: 1 mL min^{-1}; eluent A: 25 mm Tris·HCl (pH 8.0), 6 M urea; eluent B: 25 mM Tris·HCl (pH 8.0), 0.5 M $NaClO_4$, 6 M urea; gradient: 0–60 % B in A within 45 min or 0–40 % B in A within 30 min, UV detection at λ = 260 nm.

Purification of the 5'-p-ACCA$^{3'NH}$-amino acid conjugates. The crude conjugate was purified on a semipreparative Dionex DNAPac PA-100 column (9 x 250 mm) at 60 °C with flow rate of 2 mL min^{-1} (for eluents see above). Fractions containing the conjugate were loaded on a C18 SepPak Plus cartridge (Waters/Millipore), washed with 0.1–0.15 M $(Et_3NH)^+HCO_3^-$, H_2O, and eluted with H_2O/CH_3CN (1:1). Conjugate-containing fractions were evaporated to dryness and dissolved in H_2O (1 mL). The quality of the purified conjugate was analyzed by analytical anion-exchange chromatography and their molecular weights were confirmed by LC-ESI mass spectrometry. Yields were determined by UV photometrical analysis of conjugate solutions. The final compound was dissolved in water to achieve ~50 mM concentration for stock solutions and later used for soaking.

Characterization of RNA–amino acid conjugates is presented in the figure below which shows: Anion-exchange HPLC traces (top) of: (A) ACCA-3′ NH-Nle-N_3, (B) ACCA-3′ NH-Nle-OH, (C) ACCA-3′ NH-Nle, and (D) ACCA-3′ NH-Nle-Et and the corresponding LC–ESI mass spectra (bottom). HPLC conditions: Dionex DNAPac column (4 × 250 mm), 80 °C, 1 mL min–1, 0–60%

buffer B in buffer A within 45 min; buffer A: Tris-HCl (25 mM), urea (6 M), pH 8.0; buffer B: Tris-HCl (25 mM), urea (6 M), NaClO4 (0.5 M), pH 8.0.



SUPPLEMENTAL TABLE: DNA primers used in the biochemical experiments

Primer name	Primer sequence
T7	TAATACGACTCACTATAGGG
NV1	GGTTATAATGAATTTTGCTTATTAAC
MRLR-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATA TGAGACTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MALR –I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATA TGGCACTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MCLR –I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATA TGTGCCTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MDLR –I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GGATCTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MELR –I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GGAACTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MFLR –I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATA TGTTTCTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MGLR –I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GGGTCTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MHLR –I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATA TGCACCTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MILR –W-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GATTCTTCGTTTCCCATGGACTTTGAACCAGTAAGTGATAG
MKLR –I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAAACTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MLLR –I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATA TGCTTCTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MMLR –I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GATGCTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MNLR –I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAACCTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MPLR –I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATA TGCCACTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MQLR –I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GCAGCTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MSLR –I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATA TGTCACTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MTLR –I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GACACTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MVLR –I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GGTACTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MWLR –I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GTGGCTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MYLR –I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATA TGTATCTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRAR-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGAGCACGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRCR-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGATGCCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRDR-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGAGATCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRER-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT

	GAGAGAACGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRFR-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGATTTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRGR-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGAGGTCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRHR-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGACACCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRIR-W-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGAATTCGTTTCCCATGGACTTTGAACCAGTAAGTGATAG
MRKR-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGAAAACGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRMR-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGAATGCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRNR-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGAAACCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRPR-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGACCACGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRQR-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGACAGCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRRR-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGACGGCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRSR-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGATCACGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRTR-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGAACACGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRVR-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGAGTACGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRWR-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGATGGCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRYR-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGATATCGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRLA-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGACTTGCATTCCCAATTACTTTGAACCAGTAAGTGATAG
MRLC-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGACTTTGCTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRLD-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGACTTGATTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRLE-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGACTTGAATTCCCAATTACTTTGAACCAGTAAGTGATAG
MRLF-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGACTTTTTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRLG-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGACTTGGTTTCCCAATTACTTTGAACCAGTAAGTGATAG
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MRLL-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGACTTCTTTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRLM-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGACTTATGTTCCCAATTACTTTGAACCAGTAAGTGATAG

MRLN-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGACTTAACTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRLP-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGACTTCCATTCCCAATTACTTTGAACCAGTAAGTGATAG
MRLQ-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGACTTCAGTTCCCAATTACTTTGAACCAGTAAGTGATAG
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MRLV-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGACTTGTATTCCCAATTACTTTGAACCAGTAAGTGATAG
MRLW-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGACTTTGGTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRLY-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGACTTTATTTCCCAATTACTTTGAACCAGTAAGTGATAG
MRLR-Mut-rev	GGTTATAATGAATTTTGCTTATTAACGATAGAATTCTATCACTTAC TGGTTCAAA
MDLD-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GGATCTTGATTTCCCAATTACTTTGAACCAGTAAGTGATAG
MELE-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GGAACTTGAATTCCCAATTACTTTGAACCAGTAAGTGATAG
RLR-V1-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GCGCCTGCGATTCCCAATTACTTTGAACCAGTAAGTGATAG
RLR-V2-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GCGACTACGCTTCCCAATTACTTTGAACCAGTAAGTGATAG
RLR-V3-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAGGTTGCGGTTCCCAATTACTTTGAACCAGTAAGTGATAG
MKAK-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAAGGCAAAATTCCCAATTACTTTGAACCAGTAAGTGATAG
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MKEK-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAAGGAAAAATTCCCAATTACTTTGAACCAGTAAGTGATAG
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MKHK-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAAGCACAAATTCCCAATTACTTTGAACCAGTAAGTGATAG
MKIK-W-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAAGATTAAATTCCCATGGACTTTGAACCAGTAAGTGATAG
MKKK-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAAGAAGAAATTCCCAATTACTTTGAACCAGTAAGTGATAG
MKLK-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAAGCTTAAATTCCCAATTACTTTGAACCAGTAAGTGATAG
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MKQK-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAAGCAGAAATTCCCAATTACTTTGAACCAGTAAGTGATAG
MKRK-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAAGCGTAAATTCCCAATTACTTTGAACCAGTAAGTGATAG
MKSK-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAAGTCAAAATTCCCAATTACTTTGAACCAGTAAGTGATAG
MKTK-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAAGACAAAATTCCCAATTACTTTGAACCAGTAAGTGATAG
MKVK-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAAGGTAAAATTCCCAATTACTTTGAACCAGTAAGTGATAG
MKWK-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAAGTGGAAATTCCCAATTACTTTGAACCAGTAAGTGATAG
MKYK-I-fwd	TAATACGACTCACTATAGGGCTTAAGTATAAGGAGGAAAAAATAT GAAGTATAAATTCCCAATTACTTTGAACCAGTAAGTGATAG

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