Supporting Information

Functionalized Acyclic (L)-Threoninol Nucleic Acid Four-Way Junction with High Stability In Vitro and In Vivo

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General methods and reagents

Reagents and equipment

In general, commercially available reagents were purchased from Sigma-Aldrich. Cy5.5 and Cy5 NHS esters were purchased from BroadPharm. Octadecanoic acid was purchased from TCI chemicals. Unless otherwise stated all reagents were used without further purification. HPLC grade solvents were used, and anhydrous solvents were purchased in sure seal bottles with inert atmosphere or dried using MBRAUN MP SPS-800 solvent purification system. Water used for DNA or protein experiments was purified on a Milli-Q Biocel system. Unless otherwise stated reactions were carried out under argon atmosphere in flame dried glassware. NMR spectra was recorded on a Bruker Biospin GmbH Ascend with a 400 MHz magnet.

DNA oligonucleotides were purchased from Sigma-Aldrich as desalted oligonucleotides. aTNA oligonucleotides were synthesized in house on a BioAutomation Mermade-12 automated oligonucleotide synthesizer. Preloaded standard 1000 Å CPG columns were purchased from BioAutomation and used for the synthesis of aTNA oligonucleotides. Standard chemicals used in the automated oligonucleotide synthesis were purchased from Link Technologies: Deblock mix (3% trichloroacetic acid in dichloromethane), cap mix A (Tetrahydrofuran, lutidine, acetic anhydride) cap mix B (10% methylimidazole in tetrahydrofuran), ETT activator (0.25 M 5-ethyliothio-1H-tetrazole in acetonitrile), oxidizer (0.02 M iodine in tetrahydrofuran/pyridine/water). Synthesized oligonucleotides were cleaved from the solid-support using AMA (1:1 33%aq. ammonium hydroxide and 40%aq. methylamine) for 10 min at 90 °C. Oligonucleotides were purified using Agilent Top-DNA solid phase extraction columns. If needed oligonucleotides were purified by HPLC on an Agilent 1100 or 1200 series HPLC system with a Phenomenex Clarity 3u Oligo-RP 50 mm x 4.6 mm column with a gradient of acetonitrile in TEAA buffer (0.1 M, pH 7).

General gel-analysis

Native TBE-PAGE analysis was performed with the NuPage system from Thermo Scientific. Samples were prepared in Orange DNA Loading Dye (6x) from ThermoFisher and loaded onto a 4-20% TBE gel. Samples were run at 110 W for 2 h in TBE buffer.

SDS-PAGE analysis was performed using the NuPage system from Thermo Scientific. Protein samples were prepared in NuPage LDS samples buffer (4x). For reducing samples DTT was added to a final concentration of 50 mM and samples were incubated at 90 °C for 10 min. Samples were then loaded onto a 4-12% Bis-Tris gels and run for 45 min at 200 V with a Bolt MES SDS running buffer. A SeeBlue plus 2 size marker was employed in all cases.

Gels were stained for protein with SimplyBlue SafeStain (Life Technologies) Samples were visualized on a Gel-Doc EZ (Bio-Rad) or an Amersham Typhoon biomolecular imager. Image analysis was carried out in ImageQuant or ImageLab.
aTNA and DNA oligonucleotide sequences

Table S1. Sequence of oligos used.

| Strand name | 5'-3' Sequence (Henholdsis '1:3' for aTNA) | Ext. (L/(mol·cm)) | Calc. mass (g/mol) | Obs. Mass (g/mol) |
|-------------|---------------------------------|------------------|-------------------|------------------|
| aTNA-A      | CGC AAT CCT GAG CAC G T         | 158200           | 5619.8            | 5619.8           |
| aTNA-B      | CGT GCT CAC CGA ATG C T         | 152900           | 5610.8            | 5610.7           |
| aTNA-C      | GCA TTC GGA CTA TGG C T         | 158800           | 5665.8            | 5665.8           |
| aTNA-D      | GCC ATA GTG GTG TGC C T         | 163900           | 5705.8            | 5705.5           |
| DNA-A       | CGC AAT CCT GAG CAC G T         | 158200           | 5154.8            | 5154.8           |
| DNA-B       | CGT GCT CAC CGA ATG C T         | 152900           | 5146.8            | 5146.8           |
| DNA-C       | GCA TTC GGA CTA TGG C T         | 158800           | 5201.8            | 5201.8           |
| DNA-D       | GCC ATA GTG GTG TGC C T         | 163900           | 5241.4            | 5241.4           |
| aTNA-A-NPEG | NPEG-CGC AAT CCT GAG CAC G T    | 158200           | 5874.9            | 5874.9           |
| aTNA-B-NPEG | N-CTG GCT CAC CGA ATG CT        | 152900           | 5789.9            | 5789.9           |
| aTNA-C-NPEG | N-PEG-GCA TTC GGA CTA TGG C T  | 158800           | 5921.9            | 5921.4           |
| aTNA-D-NPEG | N-GCC ATA GTG GTG TGC C T      | 163900           | 5884.9            | 5884.9           |
| aTNA-DC1    | CGC AAT CCA CTA TGG C T        | 155800           | 5594.8            | 5594.8           |
| DNA-DC2     | CGG TAT CAC CTA ACG C          | 159500           | 4826.2            |                  |
| aTNA-E      | TTA GAC GTG GTG TGC C T        | 164800           | 5720.9            | 5720.9           |
| aTNA-F      | GCC ATA GTA CGT CTA A T        | 167800           | 5633.8            | 5634.0           |

Bold bases are DNA nucleosides. Observed mass is only given for oligonucleotides prepared in our lab.

5'-N: Denotes Glen Research C6 amino modifier ([https://www.glenresearch.com/5-amino-modifier-c6-tfa.html](https://www.glenresearch.com/5-amino-modifier-c6-tfa.html))

5'-NPEG: Denotes Glen Research PEG amino modifier ([https://www.glenresearch.com/5-amino-modifier-teg-cephosphoramidite.html](https://www.glenresearch.com/5-amino-modifier-teg-cephosphoramidite.html))

Oligonucleotide NHS-ester modification

Amine-modified oligonucleotides were modified with NHS-esters using the same standard protocol. The method is scalable. The oligonucleotide was solubilized in water (5 µL, 15 nmol) and tetraborate buffer (0.1 M, pH 8.5, 15 µL). To this solution was added the appropriate NHS-ester (Cy5, Cy5.5, DBCO or Palmitoyl) in DMSO (15 µL, 1 mg/60 µL). If the solution turned cloudy more DMSO is added. The mixture was then shaken at 40 °C ON, whereafter the mixture was diluted with 100 µL water and then precipitated with NaOAc buffer (3 M, pH 5.2, 35 µL), glycogen (1 µL) and ethanol (96 %, 250 µL). The samples were snap frozen in liquid nitrogen and then centrifuged for 1 h at 4 °C. The resulting pellet was resolubilized in 0.1 M TEAA pH 7 buffer and then purified by RP-HPLC with a Phenomenex Clarity 3u Oligo-RP 50 mm x 4.6 mm column running a gradient of acetonitrile in TEAA buffer (0.1 M, pH 7). The product-containing fractions were then lyophilized.

Table S2. Yield and modification of NHS-ester modified oligos.

| Oligo name | Modification  | Calc. mass (g/mol) | Obs. Mass (g/mol) | Scale yield (nmol) | Yield (%) |
|------------|---------------|--------------------|-------------------|-------------------|-----------|
| aTNA-B-Cy5.5 | Cy5.5         | 6355.3             | 6354.6            | 74                | 98        |
| aTNA-D-DBCO | DBCO          | 6172.1             | 6172.3            | 28                | 97        |
| aTNA-C-Cy5  | Cy5           | 6474.2             | 6474.5            | 20                | 88        |
| aTNA-A-Palmitoyl | Palmitoyl  | 6172.1             | 6171.7            | 25                | 92        |
| aTNA-C-Palmitoyl | Palmitoyl | 6141.9             | 6156.7 (MALDI-TOF)| 25                | 77        |
CD measurement

Circular dichroism (CD) experiments were performed using a Jasco model J-810 instrument. The quartz cuvette (Hellma) was used for scanning the samples at path lengths of 1 mm at room temperature. The aTNA strands (5 µM each strand concentration) were dissolved in 10 mM phosphate buffer containing 100 mM sodium chloride at pH 7.4 and incubated overnight and scanned at a rate of 100 nm/min. Each CD spectrum was obtained by the average of three scans.

Figure S1. The CD was recorded for single, two, three and four aTNA strands, which showed similar spectra. The CD spectrum of 4-WJ appeared with negative Cotton effect around 282 nm and a positive Cotton effect around 259 nm. These data indicate that aTNA with two, three and four strands exhibit similar secondary structures.

LCMS verification of formation of 4WJ

The aTNA strands aTNA-A, aTNA-B, aTNA-C and aTNA-D (4 µM each strand concentration) were mixed in PBS. The formed construct was gel purified using a precasted native TBE gel. The mixture of oligoes gained was then precipitated using standard oligonucleotide precipitation with EtOH. The pellet was dissolved in water and filtered through a nylon 0.45 µm syringe filter before LC-MS measurements. The following oligoes were identified:

Table 2. Mass of oligoes found.

| Oligonucleotide | Expected mass (g/mol) | Found mass (g/mol) |
|-----------------|-----------------------|--------------------|
| aTNA 7          | 5619.8                | 5620.0             |
| aTNA 8          | 5610.8                | 5612.3             |
| aTNA 9          | 5665.8                | 5666.0             |
| aTNA 10         | 5705.8                | 5706.2             |
Chromatograms and original data from the LC-MS instrument is shown below.

aTNA 7

![Chromatogram and data](image)

| No. | Name       | PeakCour | A.Mass | SD  | R.Mass  |
|-----|------------|----------|--------|-----|---------|
| 1   | 5619.9707  | 6        | -1.00728 | 2.341 | 5619.97  |

aTNA 8

![Chromatogram and data](image)

| No. | Name       | PeakCour | A.Mass | SD  | R.Mass  |
|-----|------------|----------|--------|-----|---------|
| 1   | 5612.2785  | 6        | -1.00728 | 3.176 | 5612.28  |

aTNA 9

![Chromatogram and data](image)

| No. | Name       | PeakCour | A.Mass | SD  | R.Mass  |
|-----|------------|----------|--------|-----|---------|
| 1   | 5612.2785  | 6        | -1.00728 | 3.176 | 5612.28  |
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#### aTNA 10

| No. | Name     | PeakCour | A.Mass    | SD  | R.Mass | m/z    | Weight | Mass  |
|-----|----------|----------|-----------|-----|--------|--------|--------|-------|
| 1   | 5706.1494| 5        | -1.00728  | 0.827 | 5706.15 | 814.3 | 0.42  | 5706.31 |
| 2   | 10972    | 4        | 4.000     | 1425.39 | 0.24  | 5705.57 | 5706.31 |
| 3   | 21567    | 5        | 5.000     | 1140.28 | 0.15  | 5706.43 | 5706.31 |
| 4   | 8983     | 6        | 6.001     | 949.87  | 0.11  | 5705.29 | 5706.31 |
| 5   | 5161     | 7        | 6.998     | 814.37  | 0.08  | 5707.64 | 5706.31 |

#### aTNA 10

| No. | Name     | PeakCour | A.Mass    | SD  | R.Mass | m/z    | Weight | Mass  |
|-----|----------|----------|-----------|-----|--------|--------|--------|-------|
| 1   | 5665.9956| 4        | -1.00728  | 0.319 | 5666.00 | 115.4 | 0.21  | 5666.31 |

| No. | Int. | omic valal Atomic ve | m/z | Weight | Mass  |
|-----|------|----------------------|-----|--------|-------|
| 1   | 8879 | 4                    | 4.000 | 1415.49 | 0.42  |
| 2   | 19656| 5                    | 5.000 | 1132.28 | 0.27  |
| 3   | 19487| 6                    | 6.000 | 943.27  | 0.18  |
| 4   | 6526 | 7                    | 7.000 | 808.37  | 0.14  |

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**S6**
Miscellaneous gels

Figure S2. Formation of the aTNA 4WJ analyzed by 4-20% native TBE gel electrophoresis. Required oligo for each construct was mixed in equimolar amount in PBS ON. Lane 1: aTNA-C-Cy5, Lane 2: Same as lane 1 + aTNA-D, Lane 3: Same as lane 2 + aTNA-A, Lane 4: Same as lane 3 + aTNA-B. Oligoes were incubated in PBS ON and then analyzed. Green scan is Cy5, red is SybrGold.

Figure S3. Serum stability of the 4WJ analyzed by 4-20% native TBE gel electrophoresis. Required oligo for each construct was mixed in equimolar amount in PBS. Lane 1/5: aTNA-C-Cy5, Lane 2/6: Same as lane 1 + aTNA-D, Lane 3/7: Same as lane 2 + aTNA-A, Lane 4/8: Same as lane 3 + aTNA-B. Lane 1-4 were incubated in PBS:FBS 1:1 ON at 37°C. Lane 5-8 was incubated in PBS overnight. Green scan is Cy5, red is SybrGold.
Figure S4. Stepwise formation of the aTNA 4WJ analyzed by 4-20% native TBE gel electrophoresis. Required oligo for each construct was mixed in equimolar amount in PBS ON. Lane 1: Cy5-aTNA-C, lane 2: same as lane 1 + aTNA-D, lane 3: same as lane 1 + aTNA-B, lane 4: same as lane 2 + aTNA-A, lane 5: same as lane 3 + aTNA-A, lane 6: aTNA-A, aTNA-B, Cy5-aTNA-C, aTNA-D.

Figure S5. Gel shift assay of Cy5.5-4WJ and PA-Cy5.5-4WJ. Required oligo for each construct was mixed in equimolar amount in PBS ON, and then analyzed by 4-20% native TBE gel electrophoresis. Lane 1: Cy5.5-4WJ + 50 eq. BSA, lane 2: Cy5.5-4WJ, lane 3: PA-Cy5.5-4WJ + 50 eq. BSA, lane 4: PA-Cy5.5-4WJ. Red is SybrGold scan, green is Cy5.5 scan.
Figure S6. Formation of Cy5-2Rb17c-4WJ investigated with 4-20% native TBE gel electrophoresis. Required oligo for each construct was mixed in equimolar amount in PBS for 30 min. Lane 1: aTNA-C-Cy5, lane 2: same as lane 1 + aTNA-D, lane 3: same as lane 1 + 2Rb17c-aTNA-D, lane 4: same as lane 2 + aTNA-A, lane 5: same as lane 3 + aTNA-A, lane 6: same as lane 4 + aTNA-B, lane 7: same as lane 5 + aTNA-A. Red is SybrGold scan, green is Cy5 scan.

Assembly of Cy5-2Rb17c-4WJ

Figure S7. Formation of aTNA 5WJ and aTNA 4WJ investigated with 20% native TBE gel electrophoresis. Required oligo for each construct was mixed in equimolar amount in PBS for 30 min. Lane 1 (aTNA 4WJ): aTNA-B-Cy5.5, aTNA-A, aTNA-C, aTNA-D. Lane 2(aTNA 5WJ): aTNA-B-Cy5.5, aTNA-A, aTNA-C, aTNA-E, aTNA-F. Red is SybrGold scan, green is Cy5.5 scan.

The 5WJ is assembled from five aTNA oligoes as illustrated in Figure S7 below. The sequence of the oligoes used can be found in Table S1.
Figure S8. Illustration of the assembly of the aTNA 5WJ. Five unique strands aTNA-A, aTNA-B, aTNA-C, aTNA-E and aTNA-F form the 5WJ when incubated together.

Oligonucleotide thermal denaturing
Melting studies was performed on a Thermo Fisher Evolution 260 Bio UV-visible spectrophotometer. 2 µM of each oligo was mixed in PBS buffer and left O.N. thermal denaturing was then measured at 260 nM.

Figure S9. Thermal denaturation of aTNA 4WJ (aTNA-A, aTNA-B, aTNA-C, aTNA-D). Melting temperature: 69 °C.
Figure S10. Thermal denaturation of DNA 4WJ (DNA-A, DNA-B, DNA-C, DNA-D). Melting temperature: 31 °C.

Figure S11. Thermal denaturation of DNA duplex (DNA-DC and DNA-D). Melting temperature: 62 °C.
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Figure S12. Thermal denaturation of DNA-aTNA duplex (aTNA-D and DNA-DC2). Melting temperature: 57 °C.

Figure S13. Thermal denaturation of aTNA-aTNA duplex (aTNA-D and aTNA-DC). Melting temperature: 86 °C

**aTNA cytotoxicity study**

Human umbilical vein endothelial cells (HUVEC cells) were seeded in T-75 flasks in F-12K Medium with 5 mL 10 mg/mL heparin, 5 mL endothelial cell growth supplement and 50 mL FBS. Cells were incubated at 37 °C and 5% CO₂. Experiments were performed between passages 1 and 4. In a 96 well plate HUVEC cells were split in 1500 cells/well in 200 μL cell media. Cells were left to attach ON, and cell media was then removed. At this time different concentration and constructs (single strand, double strand or 4WJ) of aTNA was given with fresh cell media, to a final concentration of 5% PBS. Experiments were performed in triplicates. Cells were grown ON and then a MTT assay was performed using standard protocol. Absorbance was measured using a CLARIOstar microplate reader at 550 nm.
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**aTNA untargeted uptake investigation**

For flow cytometry, 200,000 cells/well (KB) were seeded in a 24 well plate and allowed to attach overnight. Cells were then treated with 100 nM Cy5 modified aTNA constructs (single stranded, double stranded or 4WJ) in fresh media (5% PBS) for 4 h at 37 °C and 5% CO$_2$. Untreated samples were treated with full cell media. After treatment cells were washed three times with PBS and detached from the well using Trypsin-EDTA (0.05%). The cells were then pelleted, and trypsin solution was removed. The cells were washed once with PBS and resuspended in PBS for analysis on a NovoCyte flow cytometer. The cells were gated for the main population and for singlets. KB cells were grown in Minimum essential medium modified (10% FBS and 5% penicillin/streptomycin) media.

**aTNA immunogenicity investigation**

Human peripheral blood mononuclear cells (PBMC) were prepared in RPMI medium (10% endotoxin-free FBS) and seeded out as 80 K cells/well in a 96 well plate. After 4 h cells were given 100 ug/mL of either Poly(I:C) aTNA-4WJ or PBS in fresh cell media (5% PBS). The cells were then incubated ON at 37 °C and 5% CO$_2$. The cells were then centrifuged, and the cell free supernatant was collected and quantified for amount of TNF-α with a human TNF-α ELISA kit from Abcam using the manufacturers protocol.

**Nanobody expression**

The 2Rb17c nanobody was expressed in WK6 E. coli cells transformed with a pUltra suppressor plasmid and a pMECS expression plasmid containing the nanobody sequence with a periplasmic leader and a His-tag. Cells were streaked on agar plates containing antibiotics (ampicillin and spectinomycin) and incubated at 37 °C O/N. One distinct colony was transferred to an overnight culture consisting of 2xTY medium supplemented with antibiotics. The following day, the overnight culture was transferred to a 5 L Erlenmeyer flask containing terrific broth (TB) medium, glucose and antibiotics and grown at 37 °C under shaking. At OD600 ~0.6 4-Azido-L-phenylalanine was added, and protein expression was induced by addition of IPTG to the culture, and the cells were grown at 25 °C, shaking O/N. The bacteria were collected by centrifugation for 30 min at 4 °C, 4500 RPM. Periplasmic extraction of nanobodies was done by re-suspending and incubating the pellet in a hyperosmotic TES buffer. After centrifugation, the supernatant containing the periplasmic extract was filtered through a 0.8 µm filter, and a 0.22 µm filter before purification. Imidazole and MgCl$_2$ were added to the filtrate and the sample was purified using a HisTrap HP column on an ÅKTA Start Protein purification system (Cytiva). The collected fractions were dialyzed O/N in Slide-A-Lyzer Dialysis Cassettes (3.5K MWCO cut-off) against PBS and concentrated using Amicon Ultra-4 Centrifugal Filter Units (3K MWCO cut-off) according to the manufacturer’s guidelines.

**2Rb17h-N$_3$ aTNA modification**

To 2Rb17H in PBS (150 µM, 2 eq. 333 µL) was added DBCO modified aTNA in MQ water (aTNA-D-DBCO, 25 nmol, 50 µL). The mixture was gently shaken at 37 °C for 3 days and then purified by anion exchange (ENrich Q 5x50 mm) on a MPLC system using a salt gradient. After loading of protein-oligo conjugate the column was equilibrated with Buffer A (25 mM Tris, pH 8) for 4 column volumes, then elution of conjugate by addition of Buffer B (25 mM Tris, pH 8, 1 M NaCl) to 100% Buffer B over 10 column volumes. Excess salt was removed from the conjugate using a 5 mL HiTrap desalting column on a MPLC system with 5% PBS buffer. The product-containing fractions were collected, lyophilized and then resolubilized to 100% PBS buffer, yielding the nanobody aTNA conjugate in 64% yield. Purity was confirmed by SDS-PAGE (Figure S12).

![Figure S14](Image)
Flow cytometry of aTNA 4WJ-2Rb17h-Cy5 on SKBR3 cells

For flow cytometry, 200,000 cells/well (SKBR3 (HER2+)) were seeded in a 24 well plate and allowed to attach overnight. Cells were then treated with either 100 nM 4WJ-Cy5.5-2Rb17h or 4WJ-Cy5.5 in fresh cell media (5% PBS) for 1 h at 37 °C and 5% CO₂. Blank samples were treated with full cell media (5% PBS). For the control experiments cells were incubated with 1 µM free Rb17h for 10 min in full cell media before incubation with conjugate. After treatment cells were washed three times with PBS and detached from the well using trypsin-EDTA (0.05%). The cells were then pelleted, and the trypsin solution was removed. The cells were washed once with PBS and resuspended in PBS for analysis on a NovoCyte flow cytometer. The cells were gated for the main population and for singlets. The experiment was done in triplicates. SKBR3 cells were grown in McCoy’s 5a Medium modified (10% FBS and 5% penicillin/streptomycin) media. The plots (cell count against intensity of Cy5.5) for each sample is shown below.

![Flow cytometry data plots](image)

Figure S15. Flow cytometry data on SKBR3 (HER2+) using Rb17c-Cy5.5-4WJ or Cy5.5-4WJ. The control experiment is pretreatment of the cells with native 2Rb17c. Cells were gated for the main population, and singlets was chosen. Flow cytometry of cell count against intensity of Cy5.5 is shown for each sample.
**In vivo serum half-life studies**

The study of the circulatory half-life of palmitoylated 4WJs in mice was performed on 12-week old adult female BALB/cAnNRj mice (Janvier). The animal experiment was approved by the Animal Experiments Inspectorate under the Danish Veterinary and Food Administration Licence # 2018-15-0201-01399. All animals had free access to water and a standard rodent diet.

Samples were prepared in PBS, 100 µL with 750 pmol of Cy5.5-labeled 4WJs were administered by I.V. tail vein injection (n = 4). Blood samples were drawn from the tail of each animal at time points: 2 min, 10 min, 30 min, 2h, 6h and 24h using heparinated 20 µl capillary pipettes (Marienfeld).

The blood samples were scanned for fluorescent signal on an IVIS 200 instrument (Xenogen, Caliper Life Sciences, Hopkinton, MA, USA) and analyzed using Living image 4.3 software (Caliper Life Science). Spectral un-mixing of the obtained signal was performed using the system’s in-build Cy5.5 filter settings, using a non-fluorescent blood sample as control. The 2 min blood sample for each individual mouse was used as a measure of total injected sample and was used for normalization of the remaining blood samples. The clearance of the 4WJ follows a two-compartment pharmacokinetics model and a two-phase decay model was therefore used to calculate the half-life. The data points were fitted to a two-phase decay model, and the half-life was calculated in GraphPad Prism. This provided two half-life constants (half-life_{fast} and half-life_{slow}). Half-life_{fast} describes the initial uptake of the sample into organs, fatty tissue, etc. As a consequence of this uptake the amount of sample in the blood is lowered, but the actual amount of sample in the mouse is the same. Half-life_{slow} describes the actual clearance of the sample from blood, primarily through the kidney, and as it is this parameter we are interested in, this is the half-life that we have reported.

**Synthesis**

**Palmitoyl NHS-ester**

Octadecanedioic acid (2.00 g, 6.36 mmol, 1 eq.), N-hydroxysuccinimide (0.732 g, 6.36 mmol, 1 eq.) and DMAP (3.9 mg, 31.8 µmol, 0.01 eq.) were dissolved in dry THF and cooled to 0 °C with an ice bath and then DCC (1.44 g, 7.00 mmol, 1.1 eq.) was added. The reaction was left ON and allowed to warm as the ice melted. Solids were removed by filtration and volatiles were removed in vacuo. The solid was then solubilized in as little acetone as possible and left in the fridge for 5 h. Again, solids were removed and volatiles removed in vacuo. Recrystallization in MeOH yielded the desired NHS-ester as a white solid (1.16 g, 2.82 mmol, 44 %). $^1$H NMR (400 MHz, CDCl$_3$) δ 2.87 (s, 4H), 2.62 (t, $J$ = 7.5 Hz, 2H), 2.37 (t, $J$ = 7.5 Hz, 2H), 1.76 (p, $J$ = 7.5 Hz, 2H), 1.69-1.60 (m, 2H), 1.46-1.22 (m, 24 H). $^{13}$C NMR (101 MHz, CDCl$_3$) δ 182.9, 169.3, 168.8, 33.7, 31.1, 29.7, 29.7, 29.7, 29.7, 29.6, 29.5, 29.4, 29.2, 29.2, 28.9, 25.8, 24.9, 24.7.

**aTNA-G phosphoramidite**

Synthesized according to a literature procedure.[1]
aTNA-C phosphoramidite

\[
\text{HN} \quad \text{Ph} \\
\text{N} \quad \text{O} \\
\text{O} \\
\text{Ph}
\]

Synthesized according to a literature procedure.[3]

aTNA-T phosphoramidite

\[
\text{HN} \\
\text{N} \quad \text{O} \\
\text{O} \\
\text{Ph}
\]

Synthesized according to a literature procedure.[3]

aTNA-A phosphoramidite

\[
\text{HN} \\
\text{N} \quad \text{O} \\
\text{O} \\
\text{Ph}
\]

Synthesized according to a literature procedure.[3]
**SUPPORTING INFORMATION**

*N-Boc-4-azido-L-phenylalanine*

\[
\begin{array}{c}
\text{N} & \text{Boc} & \text{4-azido-L-phenylalanine} \\
\text{Synthesized according to a literature procedure.}^{[3]} \\
\end{array}
\]

4-Azido-L-phenylalanine

\[
\begin{array}{c}
\text{N} & \text{3} \\
\text{Synthesized according to a literature procedure.}^{[3]} \\
\end{array}
\]

**NMR spectra**

Palmitoyl-NHS
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