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# Synthesis of anionic peptide nucleic acid oligomers including γ-carboxyethyl thymine monomers

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## General procedures

The commercial reagents were used without prior purification. Some solvents were purified as follows: dichloromethane (DCM) was distilled over $P_2O_5$, DMF was distilled over phthalic anhydride, THF (diethyl ether) were distilled over KOH and $LiAlH_4$.

The column chromatography was carried out using Merck silica gel 60 (0.040-0.063 mm), with the appropriate eluent. Thin-layer chromatography (TLC) was performed using Merck silica gel plates 60 $F_{254}$. The compounds on the plates were observed in UV-light (254 nm), followed by staining with 0.5% ninhydrin solution in ethanol.

The solvents were removed using rotary evaporater under reduced pressure (15 Torr). The compounds were dried *in vacuo* (2 Torr).

The NMR spectra were measured on Bruker DPX-300 NMR spectrometer. The chemical shifts are indicated in parts per million (ppm), relative to the signal of the internal standard tetramethylsilane. Coupling constants are reported in Hz. At description of $^1$H NMR spectra the multiplicity of the signals was reported as follow: s - singlet, d - doublet, dd – double doublet, t – triplet, q - quartet, m - multiplet.

The high-resolution mass spectra were measured on a micrOTOF-Q II (Bruker Daltonics GmbH, Germany). $[\alpha]_D^{20}$ values were determined using a Polarimeter AA-55 (Optical Activity Ltd, UK). Melting points were measured on a Carl Zeiss microscope.

Reverse-phase (RP) HPLC was carried out on Agilent 1100 Series (USA) to use column Nucleosile $C_{18}$ (5μ, 250×4.6 mm, 300 Å): buffer A – 0.1 M acetate ammonium; buffer B – 50% 0.1 M acetate ammonium/50% acetonitrile; flow rate 0.8 ml/min; UV 260 nm; temperature 45 °C)

The MALDI TOF spectra were measured on a Bruker Microflex (Germany). α-Cyano-4-hydroxycinnamic acid (Sigma-Aldrich, US) was used as the MALDI matrix. PNA oligomer solution was mixed with MALDI matrix solution (5 g/ml in 50% $CH_3CN$ aqueous solution) at a 1:1 ratio and momently placed on MALDI target. Every spectrum was accumulated from 200 laser shots (it was employed nitrogen laser with λ=337 nm) in reflection regime to measure positive ions (both with external and with internal calibration).

## Experimental section

### Monomer synthesis

**4-[*N*-(*tert*-Butyloxycarbonyl)amino]-5-[*N*-(2-allyloxy-2-oxoethyl)-*N*-(bromoacetyl)amino]-valeric acid benzyl ester 3.** A solution of **2** (4.78 g, 11.37 mmol) in DCM (150 ml) was cooled to 0 °C and bromoacetyl bromide (1.2 ml, 13.64 mmol) was added. After 5 min TEA (2.4 ml, 17.05 mmol) was added dropwise. The resulting solution was stirred 30 min at room temperature. The solvent was removed under reduced pressure, the residue was dissolved in ethyl acetate (100 ml) and washed with water (2×30 ml), followed by brine (2×30 ml). The organic phase was dried over $Na_2SO_4$, the solvent was removed under reduced pressure. The product was purified by column chromatography (eluent: hexane/ethyl acetate, 2:3) and dried to give 4.54 g yellow oil; yield 74%. $R_f$ 0.62 (hexane/ethyl acetate 1:1); $[\alpha]_D^{20}$ -8.3 (c 1.0, $CH_3OH$); $^1$H NMR ($CDCl_3$, 300 MHz) δ 7.43-7.29 (m, 5H), 6.01-5.84 (m, 1H), 5.41-5.21 (m, 2H), 5.16-5.09 (d, $J$ = 2.7 Hz, 2H), 4.96-4.72 (dd, $J_1$ = 8.0 Hz, $J_2$ = 7.6 Hz, 1H), 4.71-4.61 (m, 2H), 4.31-4.13 (m, 2H), 4.05-3.88 (m, 2H), 3.79 (s, 1H), 3.75-3.54 (m, 1H), 3.47-3.18 (m, 1H), 2.57-2.41 (q, $J$ = 7.2 Hz, 2H), 2.01-1.60 (m, 2H), 1.50-1.35 (d, $J$ = 1.8 Hz, 9H); $^{13}$C NMR ($CDCl_3$, 75 MHz) δ 173.0, 172.9, 168.8, 168.5, 168.2, 167.6, 155.9, 155.5, 135.8, 135.7, 131.5, 131.1, 128.6, 128.4, 128.2, 119.6, 118.8, 80.1, 79.4, 66.6, 66.5, 66.4, 66.0, 53.8, 53.4, 50.4, 50.1, 49.7, 48.9, 30.8, 30.7, 28.4, 28.3, 27.8, 26.9, 25.9, 25.6; HRMS (ESI) *m/z* calcd for $C_{24}H_{33}N_2O_7BrNa$ $[M+Na]^+$ 563.1369, found 563.1379.

**4-[*N*-(*tert*-Butyloxycarbonyl)amino]-5-[*N*-(2-allyloxy-2-oxoethyl)-*N*-(thymine-1-yl-acetyl)amino]valeric acid benzyl ester 4.** $K_2CO_3$ (459 mg, 3.33 mmol) was added to a suspension of thymine (420 mg, 3.33 mmol) in dry DMF (30 ml). After vigorous stirring for 15 min a solution of **3** (902 mg, 1.66 mmol) in DMF (25 ml) was added to the mixture. The mixture was stirred overnight. The solvent was removed under reduced pressure, and the residue was dissolved in ethyl acetate (50 ml) and washed with water (2×15 ml), followed by brine solution (2×15 ml). The organic phase was dried over $Na_2SO_4$, the solvent was removed under reduced pressure. The product was dried to afford 840 mg white solid; yield 86%. $R_f$ 0.43 (ethyl acetate); mp 58-60 °C; $[\alpha]_D^{20}$ -1.7 (c 1.0, $CH_3OH$); $^1$H NMR ($(CD_3)_2CO$, 600 MHz) δ 10.28-10.08 (d, $J$ = 63.0 Hz, 1H), 7.49-7.29 (m, 5H), 7.26-7.17 (m, 1H), 6.19-6.11 (d, $J$ = 9.5 Hz, 1H), 6.04-5.85 (m, 1H), 5.41-5.08 (m, 4H), 4.94-4.55 (m, 4H), 4.31-4.10 (q, $J$ = 17.3 Hz, 2H), 4.01-3.78 (m, 1H), 3.65-3.35 (m, 2H), 2.60-2.40 (m, 2H), 2.01-1.85 (m, 5H), 1.48-1.33 (d, $J$ = 6.0 Hz, 9H); $^{13}$C NMR ($(CD_3)_2CO$, 150 MHz) δ 172.5, 172.4, 169.0, 168.6, 168.2, 167.6, 164.1, 164.0, 156.0, 151.1, 151.0, 141.7, 141.6, 136.7, 136.6, 132.4, 132.2, 128.4, 128.1, 128.0, 127.9, 117.8, 117.3, 109.5, 108.9, 78.5, 78.1, 65.8, 65.7, 65.6, 65.4, 65.0, 51.9, 50.8, 50.3, 48.9, 48.1, 47.7, 47.6, 30.6, 27.8, 27.7, 27.6, 27.0, 11.5, 11.4; HRMS (ESI) *m/z* calcd for $C_{29}H_{38}N_4O_9Na$ $[M+Na]^+$ 609.2536, found 609.2550.

**4-[*N*-(*tert*-Butyloxycarbonyl)amino]-5-[*N*-carboxymethyl-*N*-(thymine-1-yl-acetyl)amino]-valeric acid benzyl ester 1.** To a suspension of **6** (230 mg, 0.39 mmol) in THF (50 ml), morpholine (0.34 ml, 3.92 mmol) and $[Pd(PPh_3)_4]$ (46.2 mg, 0.04 mmol) were added under Ar. The reaction mixture was stirred for 1.5 h at room temperature. The solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate (50 ml) and washed with NaCl solution (adjusted to pH 3 with 0.1 M $KHSO_4$) (3×20 ml) and then brine (1×10 ml). The organic layer was dried over $Na_2SO_4$, the solvent was removed under reduced pressure. Ethyl ether (20

ml) was added to the residue and the suspension was cooled overnight at 4 °C. The forming precipitate was collected by filtration, washed with ethyl ether and dried to give 201 mg white solid; yield 94%. $R_f$ 0.26 (DCM/methanol/acetic acid, 9:1:0.1); mp 184-186 °C; $[\alpha]_D^{20}$ -1.3 (c 1.0, $CH_3OH$); $^1H$ NMR (DMSO-$d_6$, 300 MHz) δ 11.25 (s, 1H), 7.41-7.27 (m, 5H), 7.22 (s, 1H), 6.96 (dd, $J_1$ = 8.9 Hz, $J_2$ = 8.4 Hz, 1H), 5.13-4.99 (d, $J$ = 6.3 Hz, 2H), 4.79-4.34 (m, 2H), 3.95-3.69 (m, 2H), 3.64-3.57 (m, 1H), 3.35-3.22 (m, 1H), 3.05-2.91 (m, 1H), 2.43-2.20 (m, 2H), 1.82-1.47 (m, 5H), 1.35 (s, 9H); $^{13}C$ NMR (DMSO-$d_6$, 75 MHz) δ 173.0, 172.9, 172.8, 168.3, 164.9, 156.1, 155.9, 151.5, 142.6, 136.7, 136.6, 128.9, 128.4, 128.3, 108.5, 78.4, 78.1, 65.9, 65.8, 52.4, 51.3, 48.7, 48.4, 48.1, 30.7, 28.7, 27.4, 12.4; HRMS (ESI) *m/z* calcd for $C_{26}H_{34}N_4O_9Na$ $[M+Na]^+$ 569.2223, found 569.2221.

**Oligomer synthesis**

The polymer support (MBHA resin) was preliminarily treated before synthesis: it was let to stand in DCM for 30 min, then neutralized with 5% solution of DIEA in DCM and washed with DCM.

The PNA monomer was activated with equimolar solution of HBTU in DMF in the presence of 2 equiv. DIEA for 5 min, and then, it was added to the resin. The reaction mixture was stirred by inert gas for 2 h. After capping (the negative qualitative Kaiser's test), Boc-protected group was cleaved by TFA with adding 5% *m*-cresol for prevention of trifluoroacetelation both the growing oligomer and the polymer support. The loading of resin was determined by quantitative Kaiser's test.

The resin was sequentially washed twice with DMF/DCM (1:1) solvent mixture and DMF, after and before every stage of condensation.

Primary activation of monomer with HBTU in DMF (0.085 M) in the presence of 2 equiv. DIEA was performed to increase the condensation stage efficiency. The monomer concentration was 0.05 M. Preactivation of monomer was carried out for 5 min at room temperature. The course of reaction was controlled by qualitative test of Kaiser.

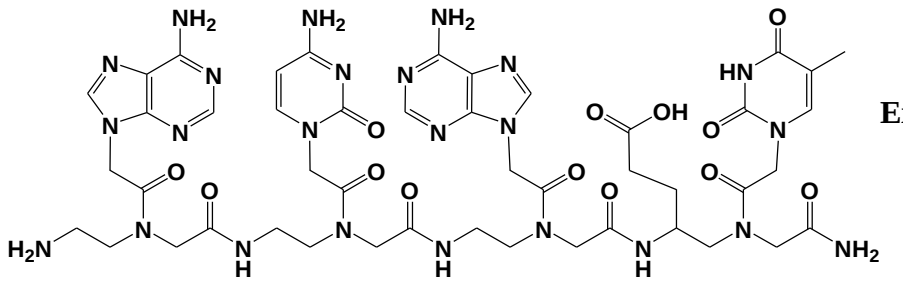
The cleavage of produced PNA oligomers from polymer support and, at the same time, deprotection of oligomers were carried out either by protocol A - "low-high TFMSA" (sequential treatment of the resin with "low TFMSA"–TFA/*m*-cresol/DMS/TFMSA (11:2:6:1, v/v/v/v) reagent mixture for 15 min at 0 ºC and "high TFMSA"–TFA/TFMSA/*m*-cresol (8:1:1, v/v/v) for 20 min at 0 ºC) or the treatment of the resin with TFA/TFMSA/TIS (3:1:0.1, v/v/v) reagent mixture B for 45 min at 0 °C with preliminary cooling of the resin to -30 °C. The solution was collected by filtration, and the resin was washed with TFA (3×2 ml). The solution was concentrated by rotor evaporator. The oligomer was precipitated from the reaction mixture by adding of absolute diethyl ether, and then it was extracted from ether with water. The water phase was lyophilizated, and then, the residue was purified by reverse-phase HPLC (RP HPLC). The structure of produced oligomer was confirmed by MALDI-TOF mass spectrometry.

**Synthesis of tetramer ACA$^{\gamma}$T.**

The synthesis was performed to employ 100 mg MBHA resin (the loading capacity of a polymer support was 0.14 μmol·g$^{-1}$). The cleavage of obtained oligomer and its deprotection were carried out by two deblocking protocols:

A - 10.5 mg resin cooled to -30 ºC was sequentially treated with 105 µl "low-TFMSA" for 15 min and "high-TFMSA" solutions (10 µl/resin 1 mg) for 20 min at 0 ºC. After the standard work-up, the reaction mixture was analyzed by RP HPLC with buffer B gradient from 3 to 35% for 30 min. The injection volume of sample was 50 µl. The analyte retention time was 13.6 min;

B - 10.5 mg resin was preliminarily cooled to -30 ºC and 105 µl cocktail TFA/TFMSA/TIS (3:1:0.1, v/v/v) (10 µl/resin 1 mg) was added at 0 ºC. The mixture was let to stand for 45 min at 0 ºC. After the standard work out, the reaction mixture was analyzed by RP HPLC with buffer B gradient from 3 to 35% for 30 min. The injection volume of sample was 50 µl. The analyte retention time was 13.6 min; yield 31%.



**$C_{46}H_{60}N_{24}O_{13}$**
**Exact Mass: 1156,48**

**Figure S1** Structure of tetramer ACA$^{\gamma}$T.

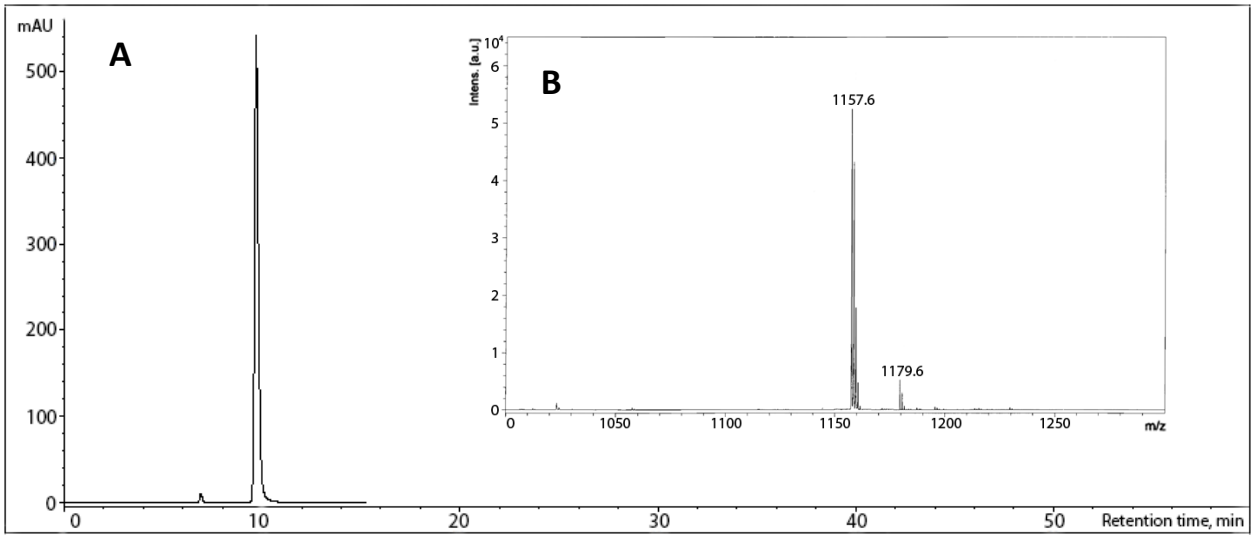



**Figure S2** (A) Analytical RP HPLC and (B) MALDI-TOF-mass-spectrum of ACA$^{\gamma}$T .

**Synthesis of hexamer CA$^{\gamma}$TCA$^{\gamma}$T.**

The synthesis was occurred to employ 80 mg MBHA resin (the loading capacity of a polymer support was 0.11 µmol·g$^{-1}$). The cleavage of obtained oligomer and its deprotection were carried out by B protocol: 10.5 mg resin was preliminarily cooled to -30 ºC and 105 µl cocktail TFA/TFMSA/TIS (3:1:0.1, v/v/v) (10 µl/resin 1 mg) was added at 0 ºC. The mixture was let to stand for 45 min at 0 ºC. After the standard work-up, the reaction mixture was analyzed by RP HPLC with buffer B gradient from 3 to 35% for 30 min. The injection volume of sample was 50 µl. The analyte retention time was 12.2 min; yield 31%.

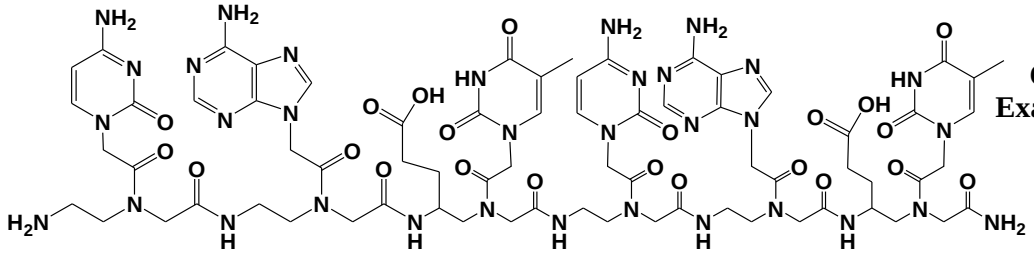



**Figure S3** Structure of hexamer CA$^{\gamma}$TCA$^{\gamma}$T.

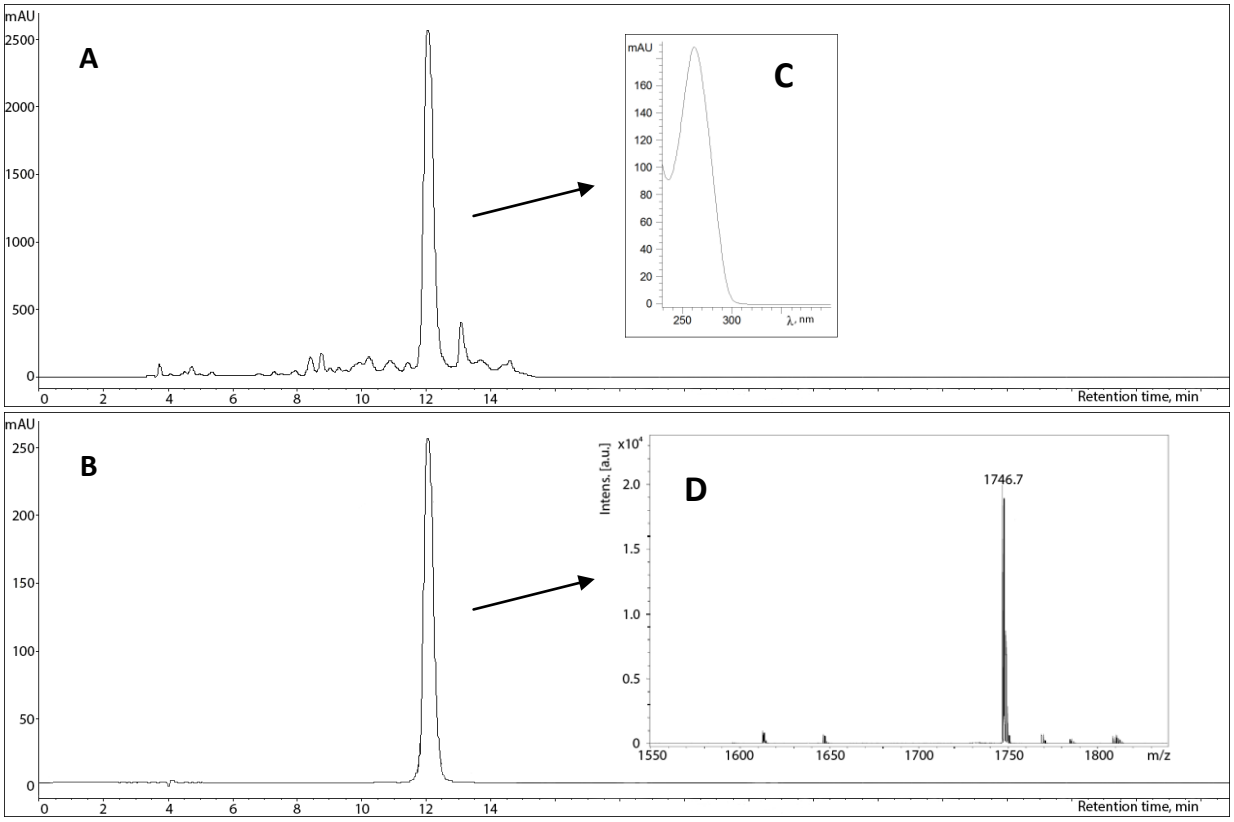



**Figure S4** RP HPLC profile and MALDI-TOF-MS spectrum of reaction mixture of CA$^{\gamma}$TCA$^{\gamma}$T (A) and analytical RP HPLC profile of hexamer (B), (C) UV-and (D) MALDI TOF MS spectra of target oligomer.