PEPTIDE/PHOTOCLEAVABLE LINKER TAGGEG MOLECULES SYNTHESIS

Photocleavable tagged oligonucleotide

The peptide is synthesized on Symphony (Protein Technologies Inc.) and purified on a Delta-Pak C18 15µm 100A column (Waters). The oligonucleotide is synthesized from 3' to 5' on Expedite (Applied BioSystem). The amine function with photocleavable linker is added in 5' before cleavage and deprotection. These steps are performed using a NH_4OH 28% solution during 24 hours in the dark. The amino oligonucleotide is then purified on a Delta-Pak C18 15µm 300A column (Waters). The amino function of the oligonucleotide is coupled with a heterobifunctional reagent comprising a maleimide function. The maleimido oligonucleotide is solubilized in water and added to a 1.2 equivalent of peptide in solution. The mixture is stirred for 16 hours. The oligo-peptide conjugate is then purified on a Delta-Pak C18 15µm 300A column (Waters) and characterized by MALDI-MS (see Mass Spectrometry section).

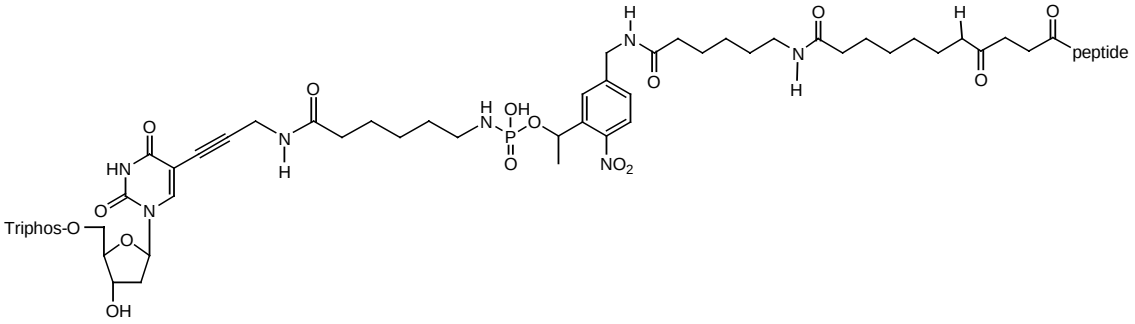
Photocleavable tagged antibody

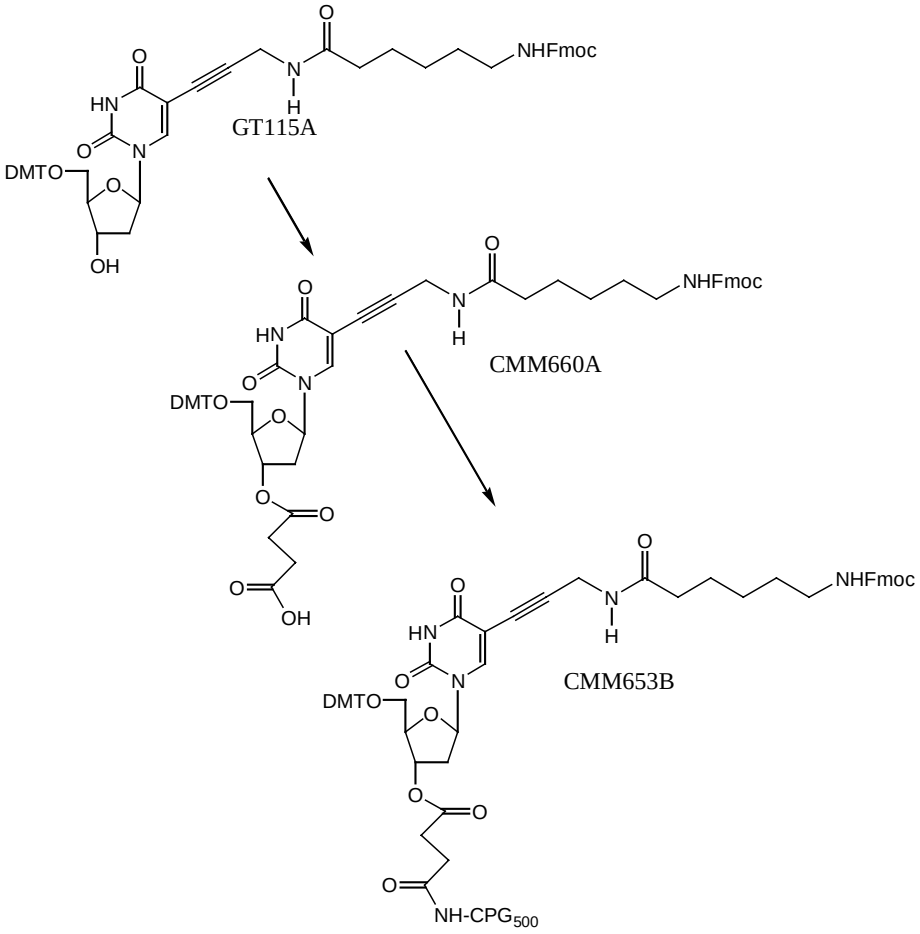
Peptides were custom made by Eurogentec S.A. using solid phase peptide synthesis (SPPS) on a 0.25 millimole (mmole) scale using Fmoc (9-fluorenylmethyloxycarbonyl amino-terminus protection) standard synthesis protocols 4 equivalent of Fmoc-AA) with double coupling reactions (twice 40minutes) using TBTU/NMM has activator on a Symphony (Rainin Instrument Co, Woburn, MA, USA) synthesizer. The photocleavable linker (4 equivalents) was introduced manually using DIPCDI/DIPEA (2 hours) as activator. Purifications were performed by RP-HPLC on a Waters (Milford, MA, USA) Delta-Pak C18 [15µm-100A-25x100mm] column using a Waters liquid chromatography system consisting of Model 600 solvent delivery pump, a Rheodine injector and a automated gradient controller (Solvent A: H2O-0.125% TFA; Solvent B: CH3CN-0.1% TFA, Gradients: 5-15% to 30-60% B in 20minutes). Detection was carried out using Model M2487 variable wavelenght UV detector connected to the Waters Millenium software control unit. The Quality Control was performed by analytical RP-HPLC on a Waters Delta-Pak C18 [5µm-100A-150x3.9mm] column (Solvent A: H2O-0.125% TFA; Solvent B: CH3CN-0.1% TFA. Gradient: 100% A to 60% B in 20minutes) using a Waters Alliance 2690 Separation Module equiped with a Waters 996 Photodiode Array Detector and by MALDI-TOF MS (see mass spectrometry section).

The Functionalisation with the photolinker derivatised peptide A was done as follow: a solution of 0.5mg of MBS in 300µl of DMF is added to a solution of 4mg of goat anti-rabbit IgG in 2ml of PBS and mixed for 30min. The solution is then desalted on a PD 10 column

using 50mM phosphate buffer at pH =6. To this desalted activated IgG, a solution of 1mg of the photocleavable derivatised peptide in 300µl of DMF and 1ml of PBS is added and stirred for 3h at room temperature. Afterwards, the reaction mixture is dialyzed overnight against PBS (membrane cut-off 12-14 000).

Synthesis of a dUTP-peptide conjugates with a photocleavable linker





In order to prepare this triphosphate, a Fmoc protected CPG resin was required. The succinylate was prepared from GT115A (100mg). The sample was relatively pure but contained a small amount (by TLC) of a higher running non-tritylated compound (originates from the Sonogashira reaction and does not interfere with subsequent reactions and was not visible in the NMR spectra of the sample). Since it was not possible to purify the succinate, the reaction was modified slightly. It is normal to add 2 equivalents of succinic anhydride to the reaction to get quantitative yield but if this is not removed completely, the amino residues of the cpg resin can become blocked during functionalisation. Therefore, 1.5 equivalents were used since the exact purity of the product is undetermined. The reaction did not go to completion (from TLC this was more than 50%) by comparing the intensity of the components on the TLC by UV(254nm) and the intensity of the DMT cation on treatment with HCl fumes. Since the non-succinylated product will not react, the resin was functionalised using this mixture. The resin was prepared but the loading is very low, 5.4μmolg^{-1} (180mg).

The resin was detritylated using 2% TCA/DCM washed with DCM and the process repeated until no orange colour due to the DMT cation was observed. This was then dried (suction under argon) and the resin soaked in pyr/DMF 1:3 (0.4ml) for 5 minutes before a solution of 0.1M Eckstein's reagent in dioxane was added (0.1ml). The reaction was allowed to stand for 15 minutes after which time the resin was washed (dioxane, MeCN) and dried (suction under argon). The resin was then soaked in a solution on 0.5M *bis*-(tributylammonium) pyrophosphate in anhydrous DMF and tri-*n*-butylamine for 20 minutes and the resin washed (DMF, MeCN) and dried (suction under argon). The product was oxidised (iodine/water/pyridine/THF for 30 minutes), washed (MeCN) dried (suction under argon). The Fmoc protecting group was removed (20% piperidine/DMF, 0.5ml, 20mins) and the resin washed thoroughly, (DMF, MeCN) and dried (suction under argon). This was then washed with DCI and a solution of DCI/photolabile amino linker CEP (1:1, 0.5ml) was added and the reaction was allowed to stand for 20 minutes. The solution was removed and the resin washed (MeCN) and dried (suction under argon). A mixture of cap A/cap B (1:1, 0.5ml) was added and the resin soaked for 5 minutes before removing the capping reagents and washing and drying the resin as before. The product was oxidized (I_2/THF/pyr/H_2O, 5mins) and the resin washed and dried as before. This was cleaved from the resin with cNH_4OH at room temperature for 30 min, then purified by anion exchange HPLC on a Dionex NucleoPac100 HPLC column using the following solvent system Buffer A:0.1M NH_4Cl with 10%

acetonitrile; Buffer B: 1M NH_4Cl with 10% acetonitrile; flow rate 2.5 mL/min. using 6Triphos.mth. This gave 3 fractions (A:-7mins, B:-7.9mins and C:-10.3mins). All 3 fractions were lyophilized overnight before being desalted by reverse phase HPLC Buffer A: Water; Buffer B: acetonitrile; flow rate 4 mL/min. The 3 fractions were again lyophilized overnight before being suspended in 200ul of water. M.S. showed that CMM661A pk 1 was definitely not the triphosphate but it could be either CMM661pk 2 or 3 (very similar M.S. profiles). (CMM662A was formed from CMM661A pk 2 and CMM663A was formed from CMM661A pk 3).Both samples were then used in the subsequent reaction. Bicarbonate buffer (10ul) and the maleimide NHS ester (50ul) were added to each sample and the reactions agitated overnight. The samples were diluted with milliQ water (500ul) and filtered. The samples were purified by RP-HPLC, buffer A: 0.1M TEAA, buffer B: MeCN, flow rate 4mL/min. using MeCN50.mth and the coupling of the peptide was carried out on these fractions.

Tissue preparation for *in situ* hybridization (ISH)

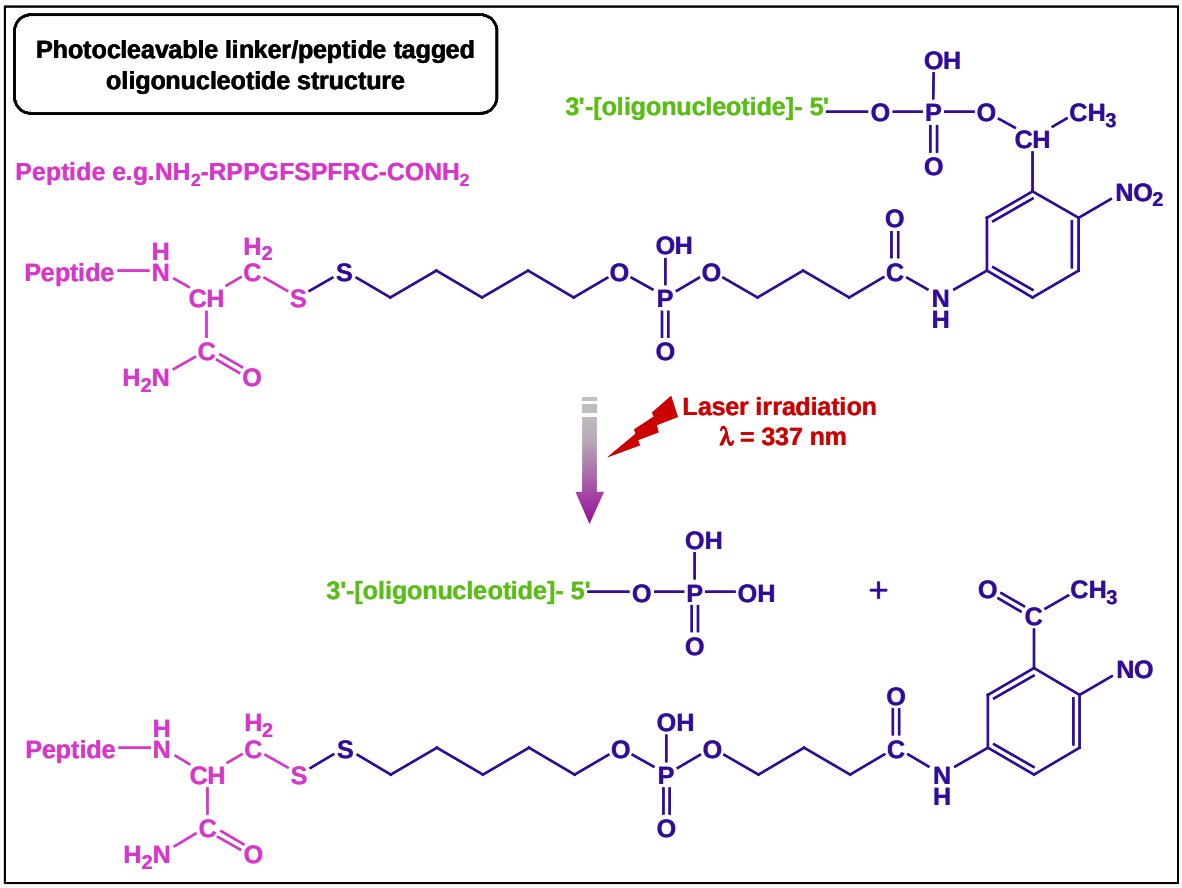
Formalin Fixed Paraffin Embedded tissues (FFPE) sections of 10 µm were transferred onto a conductive ITO-glass. Sections were stored at 30°C overnight for good adherence. Tissues were then dewaxed using xylene (2x15 minutes), and then hydrated during 5 minutes in 3 steps mixed ethanol/water baths (96°, 70°, 30°). Sections were prepared according to classical ISH protocols. Tissues were incubated in glycine 0,1M/Tris HCl buffer (pH 7.4), then treated for 15 minutes with proteinase K for protein digestion (1µg/µL in 1M/Tris HCl, 0.5M EDTA pH 8). After post-fixation with 4% paraformaldehyde (0.1M Phosphate/5mM $MgCl_2$ buffer pH 7.4) for15 minutes, a 10 minute bath with triethanolamine (0.1M pH 8) was carried out. Sections were washed with SSC 2X then ultrapure water for 5 minutes. Probes were denaturated at 100°C for 10 minutes, and after a 3 step tissue dehydratation (30°, 70°; 96°), hybridization was done for 16H at 55°C dissolving cDNA probes in hybridization buffer (Dextran sulfate 10%, formamide 50%, SSC 20X 20%, Denhardt's 100 X 10%). Tissues were incubated with Rnase (10µg/mL, 37°C 30 minutes) then rinced 10 minutes with successive SSC/2mercaptoethanol (0.07%) solutions (2X, 1X) and twice 0.5X at 55°C for 30 minutes. After rinsing slices with SSC 0.1X for 5 minutes at room temperature, one bath of ultrapure water was carried out to remove the excess of polymers. Tissues were kept drying at room temperature before MALDI matrix application.

Tissue preparation for immunocytochemistry (ICC)

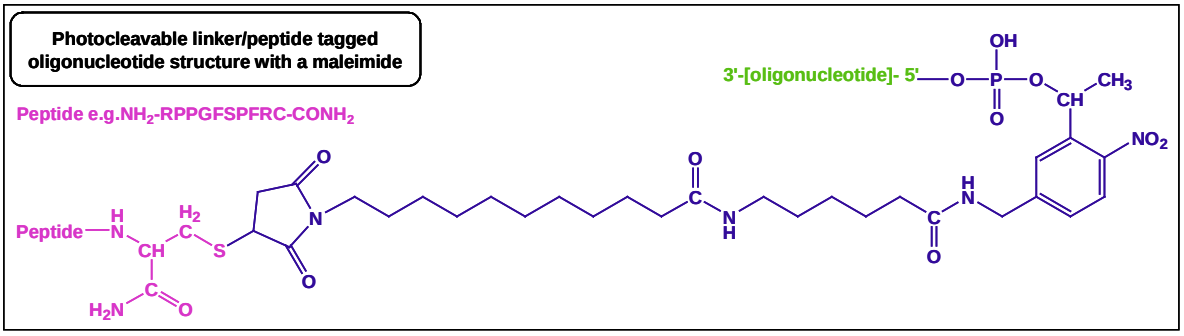
Adult male Wistar rats weighing 250-350 g (animal welfare accreditation by the French ministry of the agriculture N° 04860) maintained under standard care were used. Animals were sacrificed by decapitation and immediately dissected to remove the brain. Frozen sections of 10 µm were performed on a cryostat and immediately transferred onto a conductive Indium-Tin Oxide (ITO) glass (Bruker Daltonics, Wissenbourg, France). After drying sections for 5 minutes at room temperature, tissues were heated at 80°C for 20 seconds to allow for good adherence on slides. Rat brain sections were incubated at room temperature with 500 µL buffer (0.1M PBS / 1% BSA / 1% normal goat serum / 0.05% triton X100) for 30 minutes. The same buffer was used to dilute carboxypeptisase D (CPD) antibody (1/400), and incubation was performed overnight at 4°C. After 3 times washing in PBS, sections were incubated with peroxidase-conjugated secondary antibody (anti-rabbit IgG 1/100 developed in goat; Jackson Immunoresearch Inc. Europe LTD) or FITC-conjugated secondary antibody (anti-rabbit IgG 1/100 developed in goat; Jackson Immunoresearch Inc. Europe LTD) or using photocleavable tagged antibody (1/100) for 80 minutes at room temperature. After another 3 washing steps in PBS buffer, the sections for peroxydase ICC were incubated in chloronaphtol with 0.05% H_2O_2 for detection. Reaction was stopped with several PBS and ultrapurewater baths. For FITC ICC, slices were prepared using phenylenediamine in glycerol. For photocleavable tagged antibody, tissues were rinced 3 times for 5 minutes with ultrapure water to remove salts, and sections were kept drying at room temperature before matrix application. Tissues were then, compared using microscopy.

PEPTIDE/PHOTOCLEAVABLE LINKER TAGGEG MOLECULES STRUCTURES

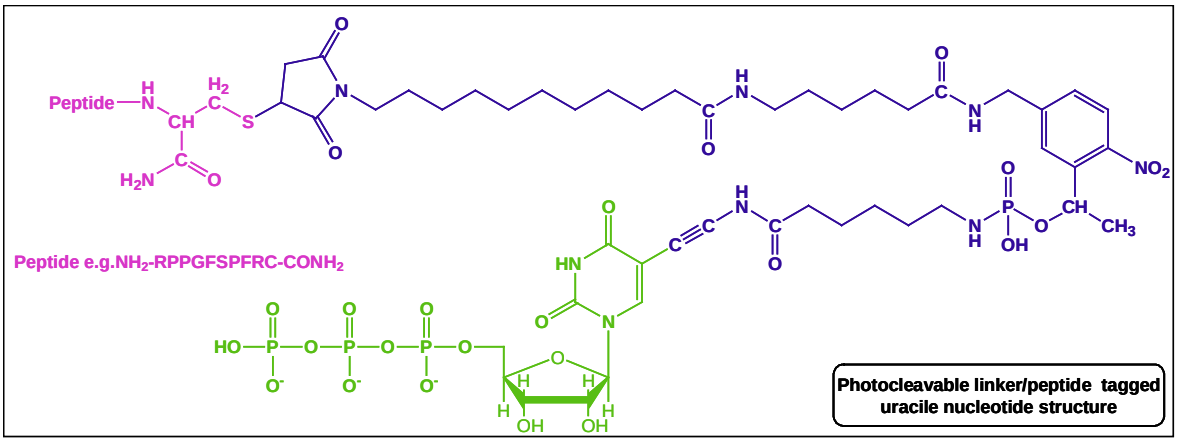
STRUCTURE 1



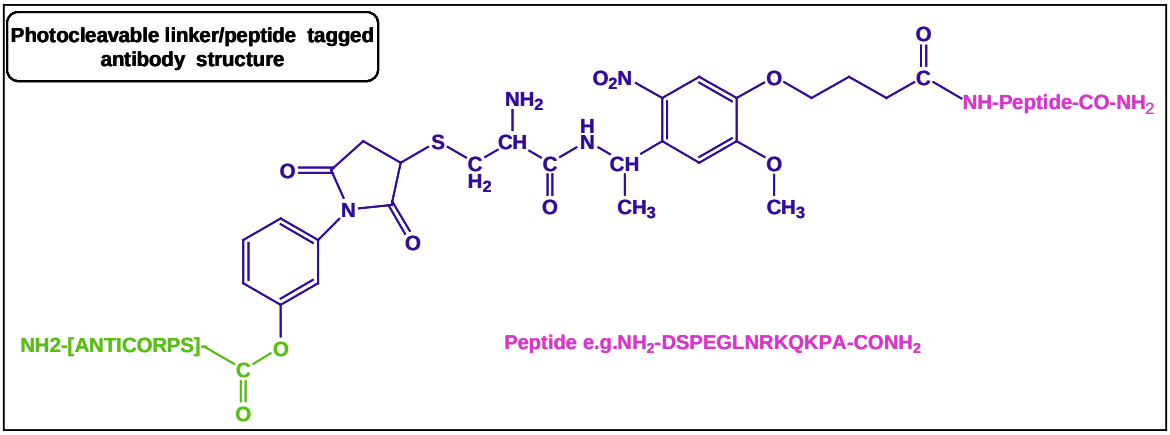
STRUCTURE 2


Photocleavable linker/peptide tagged oligonucleotide structure with a maleimide
Peptide e.g.NH2-RPPGFSPFRC-CONH2
3'-[oligonucleotide]- 5'
OH
O
P
O
O
CH
CH3
NO2
Peptide
H
N
CH
H2
C
S
C
H2N
O
O
N
O
O
C
N
H
H
N
C
O

STRUCTURE 3


Peptide
Peptide e.g.NH2-RPPGFSPFRC-CONH2
NO2
Photocleavable linker/peptide tagged uracile nucleotide structure

STRUCTURE 4


Photocleavable linker/peptide tagged antibody structure
NH2-[ANTICORPS]-
Peptide e.g.NH2-DSPEGLNRKQKPA-CONH2
NH-Peptide-CO-NH2