Supplementary information

**Delivery of oligonucleotides to bone marrow to modulate ferrochelatase splicing in a mouse model of Erythropoietic Protoporphyria**

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Figure S1. Oligonucleotide analytics for *in vitro* work in COS-7 and K562 cells

Samples were analysed by LC-MS (Agilent 1200/6130 system) equipped with a Waters Acquity OST C-18 column with a gradient of MeOH in 0.4 mM HFIP, 15 mM trimethylamine; flow rate: 0.3 ml/min. The target refers to the position bound on *FECH* pre-mRNA.

| Name | Seq. 5'-3' Target | Chemistry | MW found (g/mol) | MW calc. (g/mol) | Δm (%) | UV purity (%) |
|------|-------------------|-----------|------------------|------------------|--------|--------------|
| 1    | g^9|c|ctas|^5|caagatta^5|g|c|ct | -102-120 | MOE-PS | 7547.22 | 7547 | 0.01 | 98 |
| 2    | lttagag|^5|c|ctas|^5|caaga | -94-112 | MOE-PS | 7552.98 | 7552 | 0.01 | >99 |
| 3    | ag|^9|casaatttagag|^5|c | -86-104 | MOE-PS | 7582.45 | 7582 | 0.01 | >99 |
| Name | Seq. 5'-3' | Target | Chemistry | MW found (g/mol) | MW calc. (g/mol) | Δm (%) | UV purity (%) |
|------|------------|--------|-----------|-----------------|-----------------|--------|--------------|
| 4    | agaaaaaaagcaaatttt | -78-96 | MOE-PS | 7573.13 | 7571 | 0.02 | 98          |
| 5    | aatataaaaaagaaaaagca | -70-86 | MOE-PS | 7598.62 | 7597 | 0.02 | >99         |
| 6    | ttcacattaaaaagaaaaaagaa | -62-80 | MOE-PS | 7525.52 | 7525 | 0.01 | >99         |
| Name | Seq. 5'-3'  | Target | Chemistry | MW found (g/mol) | MW calc. (g/mol) | Δm (%) | UV purity (%) |
|------|-------------|--------|-----------|----------------|------------------|--------|--------------|
| 7    | Gaaagttttctactaat | -54-72 | MOE-PS    | 7515.30        | 7514             | 0.01   | >99          |
|      |             |        |           |                |                  |        |              |
| 8    | gagaaaagttttcatttca       | -52-70 | MOE-PS    | 7555.33        | 7553            | 0.01   | >99          |
|      |             |        |           |                |                  |        |              |
| 9    | gagaaagttttcatttca       | -51-69 | Full MOE-PS | 7530.69       | 7529.8          | 0.01   | >99          |
|      |             |        |           |                |                  |        |              |
| Name | Seq. 5'-3' | Target | Chemistry | MW found (g/mol) | MW calc. (g/mol) | Δm (%) | UV purity (%) |
|------|------------|--------|-----------|------------------|------------------|--------|---------------|
| 10   | `gtgaaaatgattttctaatc` | -50-68 | Full MOE-PS | 7530.69          | 7528.8          | 0.01   | >99           |
|      | `gtgaaaatgattttctaatc` |        |           |                  |                  |        |               |
|      | `gtgaaaatgattttctaatc` |        |           |                  |                  |        |               |
|      | `gtgaaaatgattttctaatc` |        |           |                  |                  |        |               |
|      | `gtgaaaatgattttctaatc` |        |           |                  |                  |        |               |
|      | `gtgaaaatgattttctaatc` |        |           |                  |                  |        |               |
| 11   | `gtgaaaatgattttctaatc` | -49-67 | Full MOE-PS | 7531.24          | 7528.8          | 0.03   | >99           |
|      | `gtgaaaatgattttctaatc` |        |           |                  |                  |        |               |
|      | `gtgaaaatgattttctaatc` |        |           |                  |                  |        |               |
|      | `gtgaaaatgattttctaatc` |        |           |                  |                  |        |               |
|      | `gtgaaaatgattttctaatc` |        |           |                  |                  |        |               |
| 12   | `gtgaaaatgattttctaatc` | -48-66 | Full MOE-PS | 7555.40          | 7554.8          | 0.01   | >99           |
|      | `gtgaaaatgattttctaatc` |        |           |                  |                  |        |               |
|      | `gtgaaaatgattttctaatc` |        |           |                  |                  |        |               |
|      | `gtgaaaatgattttctaatc` |        |           |                  |                  |        |               |
|      | `gtgaaaatgattttctaatc` |        |           |                  |                  |        |               |

Component Molecular Absolute Relative Absorbance Absorbance
A    7528.84  7528.84  0.01  99.0%

Component Molecular Absolute Relative Absorbance Absorbance
A    7528.84  7528.84  0.01  99.0%

Component Molecular Absolute Relative Absorbance Absorbance
A    7528.84  7528.84  0.01  99.0%

Component Molecular Absolute Relative Absorbance Absorbance
A    7528.84  7528.84  0.01  99.0%

Component Molecular Absolute Relative Absorbance Absorbance
A    7528.84  7528.84  0.01  99.0%

Component Molecular Absolute Relative Absorbance Absorbance
A    7528.84  7528.84  0.01  99.0%
| Name | Seq. 5'-3' | Target | Chemistry | MW found (g/mol) | MW calc. (g/mol) | Δm (%) | UV purity (%) |
|------|------------|--------|-----------|-----------------|-----------------|--------|---------------|
| 13   | ag"c"ctgagaaatgttttc"c | -47-65 | MOE-PS    | 7556.59         | 7555            | 0.01   | >99           |
| 14   | e"c"ctgagaaatgttttc"c | -46-64 | MOE-PS    | 7555.33         | 7553            | 0.01   | >99           |
| 15   | g"c"ctgagaaatgttttc"c | -45-63 | MOE-PS    | 7580.48         | 7580            | 0.01   | >99           |
| Name | Seq. 5'-3' | Target | Chemistry | MW found (g/mol) | MW calc. (g/mol) | Δm (%) | UV purity (%) |
|------|------------|--------|-----------|------------------|------------------|--------|--------------|
| 16   | ag TAGGCGAGGCTGGAGAATTG | -44-62 | Full MOE-PS | 7588.02         | 7588.9          | 0.01   | >99          |
| 17   | tag TAGGCGAGGCTGGAGAATTG | -43-61 | Full MOE-PS | 7595.52         | 7588.9          | 0.01   | >99          |
| 18   | ttag TAGGCGAGGCTGGAGAATTG | -42-60 | Full MOE-PS | 7586.09         | 7588.9          | <0.01  | >99          |
| Name | Seq. 5’-3’ | Target | Chemistry | MW found (g/mol) | MW calc. (g/mol) | Δm (%) | UV purity (%) |
|------|------------|--------|-----------|-----------------|-----------------|--------|--------------|
| 19   | 5’cttagcag3’ctgagaaat | -41-59 | Full MOE-PS | 7586.54 | 7587.9 | 0.01 | 98.7 |

20 g’cttagcag3’ctgagaaat -40-58 Full MOE-PS 7588.83 7587.9 0.01 >99

| Name | Seq. 5’-3’ | Target | Chemistry | MW found (g/mol) | MW calc. (g/mol) | Δm (%) | UV purity (%) |
|------|------------|--------|-----------|-----------------|-----------------|--------|--------------|
| 21   | gtttagcctatgcgaagcag | none | Full MOE-PS | 7583.00 | 7579.9 | 0.04 | >99 |

Neg Ctrl 1

22 LNA/DNA mixmer of sequence 45-63. Synthesized at Exiqon (now Qiagen).
| Name | Seq. 5’-3’ | Target | Chemistry | MW found (g/mol) | MW calc. (g/mol) | Δm (%) | UV purity (%) |
|------|------------|--------|-----------|-----------------|-----------------|--------|--------------|
| ORN1 | ag"cag"c"ctgagaatgtt"ct | -44-65 | Full MOE-PS | 8772.96 | 8770.6 | 0.03 | 98.4 |
| ORN2 | "cttag"cag"c"ctgagaatgtt"ct | -41-62 | Full MOE-PS | 8771.37 | 8770.6 | 0.01 | 98.6 |
| ORN3 | "cttag"cag"c"ctgagaatgtt"ct | -41-65 | Full MOE-PS | 9953.08 | 9952.4 | 0.01 | 99.6 |
Figure S2. Oligonucleotide analytics of CL-qPCR primers

Samples were analysed by LC-MS using conditions described above. Cy3 azide and BHQ azide moieties were coupled on oligonucleotide sequences with 2'-O-propargyl modifications according to an in-house protocol (1).
| Name | Seq. 5'-3' | Chemistry | MW found (g/mol) | MW calc. (g/mol) | Δm (%) | UV purity (%) |
|------|------------|-----------|------------------|------------------|--------|--------------|
| 29   | ACGTGGTTAGCAGCGCTGAGAA | DNA-PO | 6494.9, Na salt | 6495.3 | 0.01 | >99 |
| 30   | CACCACCCCTCCCTCCCTGATTTAACCAGAAACAT | DNA-PO Cy3 label | 9967.31 | 9966.65 | 0.01 | >99 |
| 31   | AAATGAGGGGAGGAGGAGGAGG | DNA-PO BHQ label | 5599.23 | 5598.15 | 0.02 | >99 |
| 32   | BPS-CTCAGGCTGCTAAACGGT | DNA-PO 5' BPS label | 5964.16 | 5966.06 | 0.03 | >99 |
Figure S3. Analytics of in-vivo batches of ORN1, ORN84, ORNch.

Oligonucleotide solutions were made in 0.3 M sodium acetate (ThermoFisher #AM9740) after RP-HPLC purification. Two rounds of ethanol precipitation were conducted to generate the compound as a sodium salt. Final dissolution was made in 0.9 % saline (Sigma-Aldrich #S8776), directly followed by a sterile filtration through 0.2 µm filter. An aliquot was sampled under sterile conditions to determine product concentration by $A_{260}$ measurement and purity by LC-MS (Agilent 1200/6130 system) equipped with a Waters Acquity OST C-18 column.

**ORN1**

| MW calc. (g/mol) | 8770.60 |
| MW found (g/mol) | 8772.72 |
| $\Delta m$ (%)   | 0.02   |
| UV purity (%)     | >99    |

**ORN84**

| MW calc. (g/mol) | 9859.15 |
| MW found (g/mol) | 9860.50 |
| $\Delta m$ (%)   | 0.01   |
| UV purity (%)     | >99    |

**ORNst**

| MW calc. (g/mol) | 9233.32 |
| MW found (g/mol) | 9233.10 |
| $\Delta m$ (%)   | 0.01   |
| UV purity (%)     | >99    |
Figure S4. Gel replicates for FECH endogenous splicing correction in K562 cells.

K562 cells were plated at 200,000 cells / well in a 24-well plate in 0.5 ml IMDE containing 10 % fetal bovine serum and 4 mM glutamine. Oligonucleotides were transfected using Lipofectamine™ 2000. 24 hours prior to lysis and RNA extraction, emetine was added in each desired well at a final concentration of 3 µM. RNA extraction was carried out using Qiagen RNEasy kit according to manufacturer’s instructions. cDNA was generated using PrimeScript RT kit (Takara #RR037A). PCR primers for amplification of human FECH transcripts were used. PCR products were loaded on a 2% agarose gel and bands quantified using ImageJ software. Ratios were expressed as: intensity of the aberrant amplicon band / (intensities of aberrant + correct bands). As shown for Replicate 1, no exon 3 skipping is observed over PCR amplification. Exon 3-skipped species would have a size of 213 nt (correct) and 276 nt (aberrantly spliced).
K562 cells were were plated at 100,000 cells / well in a 24-well plate in 1 ml IMDE containing 10 % fetal bovine serum and 4 mM glutamine. Oligonucleotides were transfected using Lipofectamine™ 2000. Cells were lysed 24 or 48 h after treatment. Protein concentration was assessed with the Pierce BCA protein assay kit (ThermoFisher Scientific). Samples were loaded onto 4–20% pre-cast TGX gels (BioRad) for electrophoresis. The transfer of protein onto PVDF membranes (Roche) was carried out for 90 min at 25 W. Membranes were blocked with 3% BSA and were incubated with the primary antibody overnight at 4 °C. Membranes were washed with 0.05% PBS-T and reacted with the secondary antibody for 1 h in 5% milk. Binding was detected with the ECL Prime reagent (GE Life Sciences) and quantified using the ImageJ software. Graphical output and statistical analysis (Anova) were generated with GraphPad Prism software.
Figure S6. Aberrant splicing generated by the *FECH* c.315-48T and C minigenes

Agarose gel showing relative amounts of aberrant and correct transcripts from treatment (50 nM) of COS-7 cells expressing a FECH-C or a FECH-T minigene with -45-63 2'-OMe phosphorothioate SSOs complementary to the FECH-C (1) or the FECH-T (2) variants. Aberrant transcripts were stabilized with 3 μM emetine treatment. Data shows that both the C and T variants generate the aberrant transcript.

Figure S7. Status of the c.315-48 polymorphism in K562 cells: alignment of K562 gDNA sequencing data with *FECH* Human Reference Sequence (GRCh38.p13)

Crude genomic DNA was extracted using the KAPA Mouse Genotyping Kit (Merck #KK7301) as per manufacturer's instructions and was used as template for PCR amplification of the
genomic region containing the c.315-48 polymorphism using primers: Forward 5’-CTG TTG TGA ATG TGT AAA CTT AAC AC-3’; Reverse 5’-GAC AAT TCA TCC AGC AGC TTC-3’. A PCR mix containing 0.3 μl FW primer (10 μM stock), 0.3 μl RV primer (10 μM stock), 0.3 μl dNTPs mix (Promega #U1515), 2 μl 10X PCR buffer (Roche #12032902001), 0.2 μl Fast Taq DNA Polymerase (Roche #12032902001) and 14.9 μl ultrapure water was prepared and mixed with 2 μl of genomic DNA extract. PCR program: 95°C, 10min; 33 cycles (95°C, 30s – 53°C, 30s – 72°C, 45s); 68°C, 5 min. PCR products were loaded on a 2% agarose gel for analysis. PCR products were purified for DNA sequencing using Qiaquick PCR purification kit (Qiagen # 28104). Sanger sequencing was carried out using the same primers as for PCR amplification (Microsynth DNA sequencing services).

Figure S8. Calibration curves of oligonucleotide conjugates in kidney lysate
ORN1 and its conjugated forms were spiked in kidney lysate diluted at 1/750 in nanopure water and serially diluted. Chemical ligation was run for 1 h at 33°C. qPCR was run on a LC480 device (Roche) with technical triplicates for each datapoint. Cp values were calculated by the onboard software. Slopes and R² values were calculated with GraphPad Prism v7. software. PCR efficiency values were calculated with the web qPCR Efficiency calculator from ThermoFisher Scientific.
**In vivo metabolism study in mouse tissues: mass spectrometry data (Figures S9 to S12)**

Grinded tissues were suspended in 10 volumes/weight OTX Lysis Buffer, briefly needle-sonicated and centrifuged for 30 s at 14000 rpm. Supernatants were collected for oligonucleotide clean-up and directly processed with the Clarity® OTX Extraction kit (Phenomenex #KS0-9253), according to manufacturer’s procedure for tissues with two modifications: formulation of all buffers with ammonium acetate instead of sodium phosphate; and dissolution of the final eluate in 100 µl of TE buffer pH 8.0 instead of water after evaporation to dryness. Optimization of the protocol with liver samples from mice treated with cholesteryl conjugate ORN<sup>ch</sup> comprised a proteinase K pre-treatment of the tissue to ensure recovery of the oligonucleotide (this pre-treatment was used for all ORN<sup>1ch</sup>-related treatments). Samples were analysed by LC-MS (Agilent 1200/6130 system) equipped with a Waters Acquity OST C-18 column with a gradient of MeOH in 0.4 mM HFIP, 15 mM trimethylamine; flow rate: 0.3 ml/min. Deconvolution of the mass spectra returned molecular weights from which the structures of metabolites were inferred.

**Figure S9. MS data of ORN1 in tissues**

Kidney: oligonucleotide peak at 5.448 min
Liver: Oligonucleotide peak at 5.448 min; n-1 metabolite manually integrated (black arrows)

Spleen: oligonucleotide peak at 5.556 min; n-1 metabolite manually integrated (black arrows)

Brain: no oligonucleotide detected within sensitivity limits of our equipment
Lung: oligonucleotide peak at 5.581 min

Bone marrow: oligonucleotide peak at 5.581 min
Figure S10. MS data of ORNst in tissues

Kidney: oligonucleotide peak at 5.456 min

Liver: two oligonucleotide peaks at 5.381 and 9.292 min

Peak at 5.381 min

Peak at 9.292 min (next page)
Lung: oligonucleotide peak at 5.506 min

| Component | Molecular Weight | Absolute Abundance | Relative Abundance |
|-----------|------------------|--------------------|-------------------|
| A         | 9234.86          | 149277             | 100.00            |
| B         | 9295.87          | 134666             | 22.82             |

Brain: oligonucleotide peak at 5.481 min

| Component | Molecular Weight | Absolute Abundance | Relative Abundance |
|-----------|------------------|--------------------|-------------------|
| A         | 8967.15          | 294787             | 146.00            |
| B         | 8571.97          | 28234              | 9.79              |

*** End of Report ***
Bone marrow: oligonucleotide peak at 5.597 min

Figure S11. MS data of ORN$_{84}$ in tissues

Kidney: oligonucleotide peak at 5.456 min, n-1 metabolite manually integrated (black arrows)
Liver: oligonucleotide peak at 5.331 min

Lung: oligonucleotide peak at 5.431 min
Bone marrow: oligonucleotide peak at 5.506 min

Brain: no oligonucleotide detected within sensitivity limits of our equipment

Spleen: oligonucleotide peak at 5.431 min
Figure S12. MS data of ORN\textsubscript{ch} in tissues

Kidney: two oligonucleotide peaks at 5.356 min (two main metabolites analyzed) and 11.334 min

Peak at 5.356 min

Peak at 11.34 min
Liver: two oligonucleotide peaks at 5.406 min (two main metabolites analyzed) and 10.886 min

**Peak at 5.406 min**

| Component | Molecular Weight | Absolute Abundance | Relative Abundance |
|-----------|------------------|--------------------|--------------------|
| A         | 9211.39          | 51395              | 100.00             |
| B         | 8370.38          | 40597              | 78.99              |

**Peak at 10.886 min**

| Component | Molecular Weight | Absolute Abundance | Relative Abundance |
|-----------|------------------|--------------------|--------------------|
| A         | 9393.32          | 3625731            | 100.00             |
| B         | 9377.38          | 652765             | 18.28              |
| C         | 6415.02          | 564194             | 15.62              |

***End of Report***
Spleen: oligonucleotide peak at 11.409 min

Brain: no oligonucleotide detected within sensitivity limits of our equipment

Bone marrow: two oligonucleotide peaks at 5.507 min and 11.884 min (next page)
Peak at 5.507 min

| Component | Molecular Weight | Absolute Abundance | Relative Abundance |
|-----------|------------------|--------------------|-------------------|
| A         | 8779.87          | 1966               | 100.00            |

*** End of Report ***

Peak at 11.884 min

| Component | Molecular Weight | Absolute Abundance | Relative Abundance |
|-----------|------------------|--------------------|-------------------|
| A         | 9390.91          | 32052              | 100.00            |

*** End of Report ***
Figure S13. Splicing correction generated by ORN1, ORN_{st}, ORN_{ch} and ORN_{84} in liver of Emi/wt mice.

RNA was extracted from liver tissue as described in the Methods section. For semi-quantitative RT-PCR; PCR primers specific for amplification of Emi exon 3-exon 4 transcripts were used: Forward 5'- GAG ACC TCA TGA CAC TTC-3', Reverse 5'- GGG GAT CCG CCT CCA ATC-3'. A PCR mix containing 1.25 μl FW primer (10 μM stock), 1.25 μl RV primer (10 μM stock), 0.125 μl dNTPs mix (Promega #U1515), 0.25 μl 5X Q5 Polymerase Buffer (NEB #M0491), 0.25 μl Q5 DNA Polymerase (NEB #M0491) and 15.75 μl ultrapure water was prepared and mixed with 1 μl of cDNA solution. PCR program: 98°C, 30s; 33 cycles (98°C, 10s – 59°C, 20s – 72°C, 10s); 72°C, 2 min. PCR products were loaded on a 2% agarose gel and bands quantified using ImageJ software. Ratios were expressed as: intensity of the aberrant amplicon band / (intensities of aberrant + correct bands). Statistical analysis (multiple t-tests) were conducted with GraphPad Prism v7. Software (* = p <0.05). Data are shown on the graph as average ratio values ± s.e.m.
Table S1. Library of 10-nt ORNs for minigene screening

Oligonucleotides were produced and characterized as described earlier. Sequences are fully modified MOE-PS chemistry (\(mC\) refers to 5-methyl cytidine).

| Name | Seq. 5’-3’ | Target | MW calc. (g/mol) | MW found (g/mol) | \(\Delta m\) (%) | UV purity (%) |
|------|------------|--------|-----------------|-----------------|----------------|--------------|
| 33   | A\(^m\)CT\(^m\)CAATAAA | -75-66 | 3916.6          | 3917.6          | 0.02           | 93           |
| 34   | T\(^m\)CTA\(^m\)CT\(^m\)CAAT | -72-63 | 3888.5          | 3889.2          | 0.02           | 99           |
| 35   | TTTT\(^m\)CTA\(^m\)CT\(^m\)C | -69-60 | 3870.4          | 3871.6          | 0.03           | 98           |
| 36   | ATGTTTT\(^m\)CTA | -66-57 | 3906.5          | 3905.3          | 0.03           | 97           |
| 37   | GAAATGTTTTT | -63-54 | 3941.5          | 3940.8          | 0.02           | >99          |
| 38   | TGAGAAATGT | -60-51 | 3975.6          | 3974.6          | 0.03           | 98           |
| 39   | T\(^m\)CTA\(^m\)CT\(^m\)CAAT | -57-48 | 3973.6          | 3975.1          | 0.04           | 97           |
| 40   | G\(^m\)CAG\(^m\)C\(^m\)CTGAG | -54-45 | 3979.6          | 3979.9          | 0.01           | >99          |
| 41   | TTAG\(^m\)CAG\(^m\)C\(^m\)CT | -51-42 | 3929.5          | 3929.2          | 0.01           | >99          |
| 42   | AG\(^m\)CTTAG\(^m\)CAG | -48-39 | 3964.6          | 3965.6          | 0.02           | >99          |
| 43   | T\(^m\)C\(^m\)CAG\(^m\)CTTAG | -45-36 | 3929.5          | 3929.8          | 0.01           | >99          |
| 44 Neg Ctrl 4 | TAT\(^m\)CATT\(^m\)CAG\(^m\)C | Scrambled | 3888.5          | 3888.9          | 0.01           | >99          |
Supplementary methods

Deprotection and purification conditions for lead oligonucleotide ORN1
The CPG was suspended in fresh 25% ammonia (Sigma Aldrich) and shaken at 45°C for 22 hours. The CPG was filtered, washed with 50% (aq) EtOH and the liquid fractions were evaporated to dryness prior to dissolution in ultrapure water. After brief centrifugation, the crude DMT-ON oligonucleotide was purified by RP-HPLC (Agilent) with a reverse phase column XBridge OST C18 with a gradient of acetonitrile in 0.1 M aqueous triethylammonium acetate buffer, pH 8. Fractions containing the product were evaporated to dryness and redissolved for detritylation in 50% (aq) acetic acid. Detritylation was allowed to run under moderate shaking for 1 hour at RT prior to evaporation to dryness and a second round of RP-HPLC purification with the same system. Fractions containing pure product were combined and evaporated to dryness prior to redissolution in ultrapure water.

Conjugation on support, deprotection and purification of stearic acid conjugate ORNst
The amino C6 modifier (Glen Research #10-1906) was prepared at 0.1M; synthesis was conducted with same parameters as above. The 5’ MMT group was deprotected by flushing 3 x 10 ml of Deblock solution through the CPG. The CPG was washed with 2 x 10 ml of acetonitrile and allowed to react with a solution of 160 mg stearic NHS ester and 80 µl DIPEA in 8 ml DMF for 30 min. After washing with 2 x 10 ml of acetonitrile, standard deprotection and purification protocols were applied as for naked compound ORN1, however only one round of RP-HPLC was conducted.

Deprotection of maleimide-protected form of ORN1 and retro Diels Alder uncaging for conjugation to thiol ligands
The 5’-Maleimide modifier (Glen Research #10-1938) was prepared at 0.1M; synthesis was conducted with same parameters as above. The use of the 5’-maleimide modifier was so far successful with Pac-DNA amidites (2,3) and protocol fine-tuning was required to achieve in the case of MOE oligonucleotides in one hand full deprotection of the nucleobases and cleavage from support, while keeping in the other integrity of the 5’ protected maleimide modification. With final conditions, the CPG was suspended in fresh 25% ammonia (Sigma Aldrich) and shaken at 35°C for 18 hours. For the same reaction time, t temperatures ≥ 40°C led to unacceptable maleimide degradation; temperatures ≤ 30°C to uncomplete nucleobase deprotection. Purification protocol was done as described for ORN1 but only with one round of RP-HPLC. The 5’-protected maleimide oligonucleotide was analyzed by LC-MS and stored at -20°C at this stage in water; only required amounts were engaged whenever needed for uncaging of the 5’-maleimide and conjugation. Retro-Diels Alder reaction was conducted using a modified protocol from the literature (3): the 5’-maleimide intermediate was prepared at a concentration of 100 µM in water and microwave-irradiated for 90 minutes at 90°C, followed by immediate concentration in a Speedvac until ≈ 90% volume
reduction was obtained. Evaporation to dryness was avoided. LC-MS injection was routinely carried out to control quality of the retro Diels-Alder deprotection prior to conjugation.

**Thiocholesteryl conjugate preparation ORN<sub>ch</sub> from the uncaged maleimide intermediate**

A solution of thiocholesterol (Sigma-Aldrich #136115) was prepared in THF at 10 mg/ml and was added to the concentrated solution of maleimide intermediate in water to reach 10 molar eq. of thiocholesterol. The final solvent ratio was adjusted if necessary to get a ratio THF/H<sub>2</sub>O of 3:1. The mixture was degassed by argon bubbling and microwave-irradiated for 25 min at 30°C. RP-HPLC purification was then carried out as for compound ORN1, but with one round of RP-HPLC only.

**CD84-binding peptide conjugate preparation ORN<sub>84</sub> from the uncaged maleimide intermediate**

A solution of peptide (Genscript) was prepared in water at 10 mg/ml and was added to the concentrated solution of maleimide intermediate in water to reach 7 molar eq. of peptide. The reaction was buffered with TEAA at pH 7 and allowed to run for 30 minutes at RT. Product ORN<sub>84</sub> was purified by RP-HPLC on the same system as for ORN1 but with milder conditions: 4 ml/min, 40°C and a gradient of acetonitrile in 0.1 M TEAA buffer pH 6.8.

**References**

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