Protocols for Hydrophobic modifications of ssDNA strands

Content

1- Palmitate and arachidic acid modification

To attach the hydrophobic modifications to the oligonucleotides, those were ordered with a 5' amino group that reacts with the carboxylic group present in the molecule that contains the hydrophobic groups. This is an N-acylation reaction, and its general mechanism is depicted in the following figure. The reaction is not spontaneous, and so the activators HATU and HOAt are required.

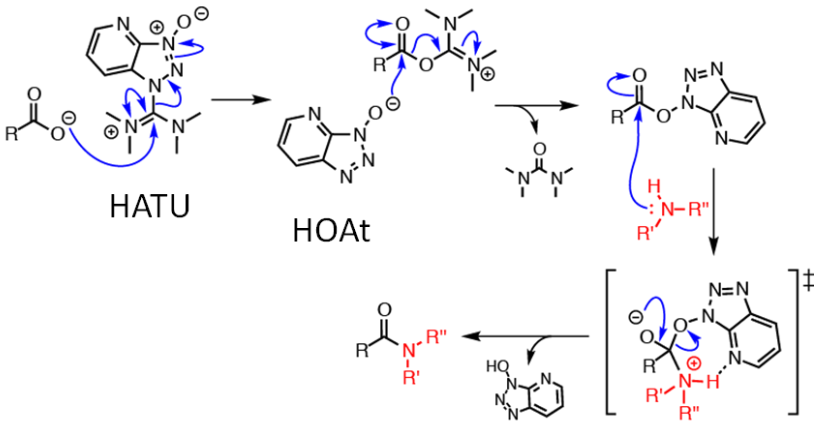

Figure 1. Scheme of the N-acylation reaction used to incorporate the hydrophobic modifications in the oligonucleotides. An amino group present in the oligonucleotide reacts with the carboxylic group present in the molecule containing the hydrophobic groups with the help of HATU and HOAt activators.

Oligonucleotides still attached to the CPG beads were ordered from Biomers, with the 5'-amino-C6-modifier and the Monomethoxytrityl (MMT) group still on. The nucleotide sequence was the following:

5'- CGCGGATGGCGATGCGCGCAC-3'

The amino-C6-modifier confers the amino group necessary for the attachment of the hydrophobic modifications.

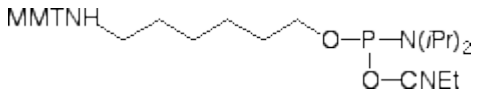

Figure 2. Amino C6 modifier

The MMT group was then removed and the Fmoc-L-dap(Palmitate)-OH molecule (Cas nº 724785-41-5) was introduced, carrying the palmitic acid, and the carboxylic group that will react with the amino group present in the oligonucleotide. With a similar reaction, the amino group from this molecule was deprotected from the Fmoc molecule, and the arachidic acid (Cas nº 506309) was introduced in this position.

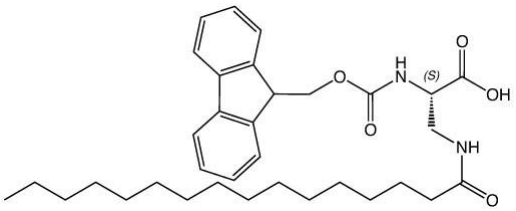

Figure 3. Fmoc-L-dap(Palm)-OH Cas Nº 724785-41-5

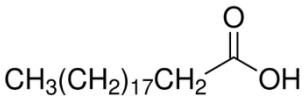

Figure 4. Arachidic acid Cas Nº 506309

The steps were the following:

1. Weighing of reagents and dissolving in DMF.
    - HATU 6.8 mg in 40µl DMF
    - HOAT 2.18 mg in 20µl DMF
    - Fmoc-L-DAP(Palm)-OH 12.3 mg in 100µl DMF
2. Let dissolve in Ultrasonic bath for 5’
3. First mix HATU + HOAT, then add Fmoc-L-dap(Palm)-OH. Incubate 1h RT under agitation (meanwhile steps 4-6)
4. Wash beads with 1mL Deblock (dissolved in DCM) to remove MMT group. Trityl is yellowish, repeat until solution is clear.
5. Wash beads 3x with DCM
6. Let beads dry, if necessary using vacuum centrifuge
7. After the 1h reaction, add the mixture to the beads + 60µl TEA 1M. Let react for 5h or ON at 30ºC.
8. Wash beads with DMF until the solution appears clear.
9. Wash 3x with DCM, let dry or vacuum dry.
10. Weighing of reagents to couple arachidic acid:

- HATU 6.8 mg in 40µl DMF
- HOAT 2.18 mg in 20µl DMF
- Arachidic acid 6.25 mg in 100µl DMF

11. Let dissolve in Ultrasonic bath for 5’
12. First mix HATU + HOAT, then add arachidic acid. Incubate 1h RT under agitation (meanwhile steps 13-14)
13. Wash the beads 3x with diperidine for the Fmoc decouping.
14. Wash 3x with DCM, let dry or vacuum dry.
15. Add reagent mixture + 60µl TEA.
16. Let react ON at 30ºC under agitation.
17. Wash beads 3x with DMF, 3x with DCM and let dry or vacuum dry.

To release the oligos from the beads and also remove the protective groups from the bases ammonia was added and DNA was let dissolve while beads precipitate. Then ammonia was evaporated in vacuum and the modified oligos were solubilized with water. The modified oligo was purified by HPLC and its purity was analyzed my mass spectrometry.

2- Cholesterol modification

For cholesterol modification, the oligonucleotides were synthesized on a solid support where cholesterol was previously attached. The sequence was the following:

5’- CGCGGATGGCGATA-3’

In this case it is shorter for the modification to be slightly separated from the walls of the channel. To release the oligos from the beads and also remove the protective groups from the bases ammonia was added and DNA was let dissolve while beads precipitate. Then ammonia was evaporated in vacuum and the modified oligos were solubilized with water. The modified oligo was purified by HPLC and its purity was analyzed my mass spectrometry.

3- HPLC purification

The reverse phase columns were kept at a temperature of 50ºC throughout the purification. The aqueous phase used was TEAA 100mM and the organic phase was acetonitrile. A gradient of the organic phase was used from 10% to 100% acetonitrile in a time interval of 20 minutes. In the case of the palmitate-arachidic acid modification, this wasn’t sufficient for the product to elute so a gradient with methanol.

4- Mass spectrometry

Samples were analyzed with an electrospray mass spectrometry device with a

quadrupole detection system coupled to a UPLC (ultrahigh pressure liquid chromatography) purification system.

All the protocols for the hydrophobic modifications were provided by Francesco Reddavide, currently working in Yixing Zhang's lab, B-CUBE, Dresden.

5- Electrophoresis for hydrophobic modification incorporation analysis

Gel preparation

1. An agarose 1% gel was prepared in a TBE 0,5X solution
2. The solution was heated up until boiling and let cool down until 55ºC
3. A magnesium chloride 1M solution was added to reach a concentration of 12mM.

Sample preparation

1. The samples were diluted with folding buffer with a concentration of 12mM $MgCl_2$ and 100ng of DNA were loaded in each well.
2. Samples were mixed with an orange G-based loading dye in a ration 1:5.

Gel running and staining

Gel was runned with a running buffer consisting of TBE 0,5X with 12mM $MgCl_2$, at 70V during 4 hours. The electrophoresis cell was maintained surrounded by ice for the system not to heat up, and the buffer was changed and the electrodes cleaned after 2h. The gel was then stained with Ethidium bromide during 20' for the further visualization under UV light.

Protocol provided by Alexander Ohmann, currently working in Stefan Diez's lab, in B-CUBE, Dresden.