Synthesis of CBT-Asparagine

Coupling Reaction of 6-Amino-2-Cyanobenzothiazole and Fmoc-Asparagine-OAll

Note: We assume that the hydrophobic stacking mechanism of the side chain of the protected amino acid and the used protection group 9-fluorenylmethyloxycarbonyl (Fmoc) causes the building of aggregates. These aggregates can build a massive block during column chromatography so that it is not possible to purify the reaction mixture after the coupling reaction by this column chromatography. It is recommended to use different protection groups than Fmoc, e.g. *tert*-butyloxycarbonyl (Boc) or methyl esters (OMe).

This method is based on the synthesis of (D-Cys-Lys-CBT)$_2$ by Yuan *et al.*, 2016

- Dissolve two equivalents of Fmoc-asparagine-OAll, two equivalents of 4-methylmorpholine and two equivalents of isobutyl chloroformate in 40 mL per 1.5 g of 6-amino-2-cyanobenzothiazole (ACBT) ice-cold tetrahydrofuran (THF)
- Stir the reaction for 30 min at 0 °C
- Add one equivalent of ACBT
- Stir the reaction mixture for 2h at 0°C
- Stir the reaction mixture overnight at room temperature
- Adjudge the reaction mixture by TLC analysis
- Wash the reaction mixture three times with $NaHCO_2$, 2 times with citric acid and one time with brine in a separator
- Collect the organic layer and add $NaSO_4$ or $MgSO_4$ to remove excessive water after the washing
- Dry the washed reaction mixture under reduced pressure

Purification of the reaction mixture after the coupling reaction

- Dissolve the dried reaction mixture in ethyl acetate (EtOAc)
- Add 5 - 8 g of SiO_2 so that you get a slurry mixture
- Dry the mixture under reduced pressure to complete dryness
- Load SiO_2 onto a sintered membrane
- Flush through two times with petrol ether (PE)
- Load the dried SiO_2 loaded with the reaction mixture onto the short plug of SiO_2
- Flush through with 5 % EtOAc in PE
- Increase the polarity of the eluent step by step by 5 %. Do not increase the polarity higher than 25 %.

Note: If the previously mentioned aggregates block the small SiO_2 column collect the SiO_2 in a beaker and wash it with 100 % methanol. Repeat the purification from the first step.

- After reaching 25 % polarity, collect the SiO_2 in a beaker and wash it with 100 % methanol
- Dry the solution under reduced pressure

Removing the protection groups

Note: We used quite a lot of morpholine resulting in removing Fmoc which normally needs a higher pK_a like the pK_a of piperidine (pK_a of morpholine: rough 8, pK_a of piperidine: rough 11) and resulting in N^{γ}-cyanobenzothiazolyl-asparagine morpholine salt as variant of the product. We recommend using other protection groups like previously mentioned. This should help controlling the reaction conditions. For this case, make sure to use the right reactants for removing the protection groups.

- Dissolve one equivalent of Fmoc-CBT-Asp-OAll in THF, use 50 mL THF per 1.4 g Fmoc-CBT-Asp-OAll
- Add 0.1 equivalents of Tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) and 3 equivalents of morpholine
- Stir for 40 min at room temperature
- Adjudge by TLC
- Dry the reaction mixture under reduced pressure

Purification of the final product

- Remove excessive morpholine three times by azeotropic distillation. Therefore, dissolve the reaction mixture in EtOAc and dry it under reduced pressure.
- Suspend the product in diethyl ether and centrifuge at 5000 rcf for 6 min at 4 °C (three times)
- Discard the diethyl ether and dissolve the product in ddH_2O
- Extract the aqueous solution 3 times with EtOAc
- Collect both fractions
- Dry the organic layer under reduced pressure
- Dry the aqueous layer by lyophilization
- Most of the product from the organic layer will be the free amino acid
- Most of the product from the aqueous layer will be the morpholine salt of *N*$^{\gamma}$-cyanobenzothiazolyl-asparagine

Yuan, Y., Wang, F., Tang, W., Ding, Z., Wang, L., Liang, L., Zheng, Z., Zhang, H., and Liang, G. (2016). Intracellular Self-Assembly of Cyclic.