The Synthesis of "Clickable" Co-factor Analogues for use in Assay Development

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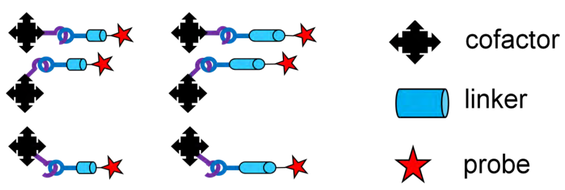
1. PROJECT INTRODUCTION

Assay development is a key step for project initiation in the early phase of drug discovery. Generation of suitable assay tools/ligands for novel targets is usually bespoke which has a high cost and time implication. Often appropriate, target specific tools are not readily accessible, particularly for novel targets.

The aim of this project was to design an assay toolbox based on frequently used co-factors and non-specific natural ligands which could be used generically across and within a range of protein families and subfamilies.

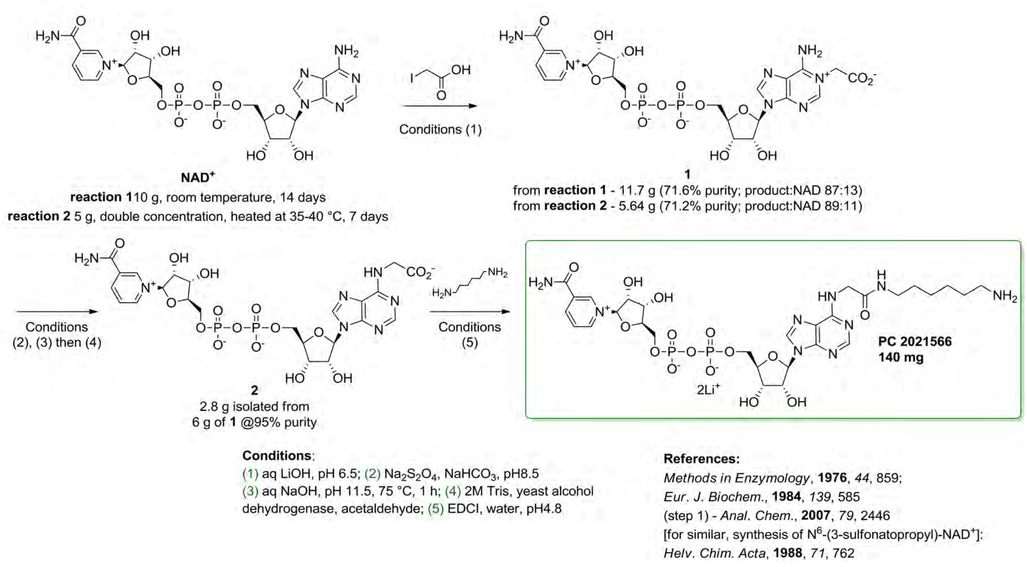
Assay tools could be assembled or attached to a suitable probe or surface (e.g. fluorescent dye; SPR sensor chip, Corning® Epic® plates) at the point of need and could make the initial assay development and validation independent of the need for bespoke tools. 'Click' chemistry was chosen as a general method for attachment as it is widely used for the formation of triazole rings under mild conditions that can be applied even in the presence of proteins.



The long-term goal was to deliver rapid testing of diverse linkers and linking groups and different sites of attachment to the co-factors.

Working closely with its client, the synthesis team successfully delivered a collection of analogues of enzyme cofactors that contained pendant acetylene units, rendering them suitable for "Click" chemistry reactions. The synthetic targets posed serious synthetic chemistry challenges, not least in the purification of very polar and water soluble compounds.

2. THE FIRST CHALLENGE – A LITERATURE RE-MAKE

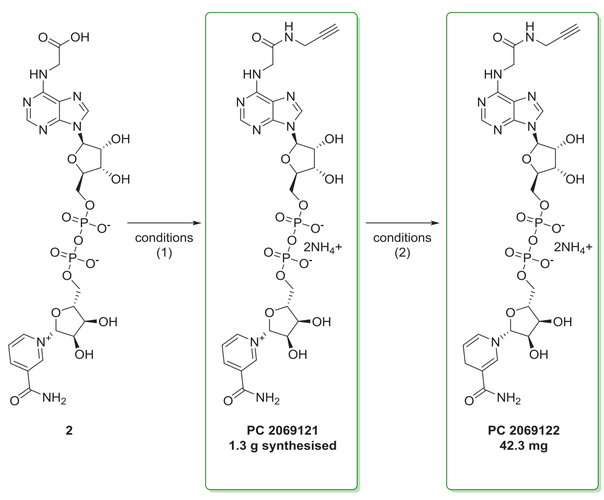


Conditions:
(1) aq LiOH, pH 6.5; (2) $Na_2S_2O_4$, $NaHCO_3$, pH8.5
(3) aq NaOH, pH 11.5, 75 °C, 1 h; (4) 2M Tris, yeast alcohol dehydrogenase, acetaldehyde; (5) EDCI, water, pH4.8

References:
Methods in Enzymology, **1976**, *44*, 859;
Eur. J. Biochem., **1984**, *139*, 585
(step 1) - *Anal. Chem.*, **2007**, *79*, 2446
[for similar, synthesis of N^6-(3-sulfonatopropyl)-NAD+]:
Helv. Chim. Acta, **1988**, *71*, 762

- Alkylation of NAD+ was achieved by treatment with iodoacetic acid. The pH of the reaction medium was maintained at pH6 throughout the reaction by addition of 2M LiOH. The reaction rate was greatly improved by heating at 35-40 °C. The reaction was monitored by LC and TLC (5:3 isobutyric acid:1M aq NH_3 saturated with Na_2EDTA).
- Dimroth rearrangement was achieved by first reducing to the alkali-stable reduced form of the nucleotide using sodium dithionite followed by heating to 75 °C at pH 11.5 to perform the rearrangement. Finally enzymatic re-oxidation gave **2**. Purification was achieved using Dowex AG 1X-2 resin followed by precipitation from EtOH/water.
- The EDCI mediated amide coupling was carried out in water, maintaining the reaction pH at 4.8 by addition of 1M LiOH and 1M HCl, as appropriate.

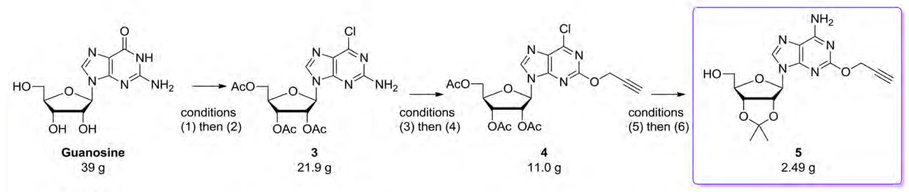
3. SYNTHESIS OF CLICKABLE NAD+ AND NADH ANALOGUES



Conditions
(1) EDCI, water, pH4.8-5.0, 2 h; (2) $Na_2S_2O_3$, 2% aq. $NaHCO_3$, 5 h (reaction was carried out in the dark)

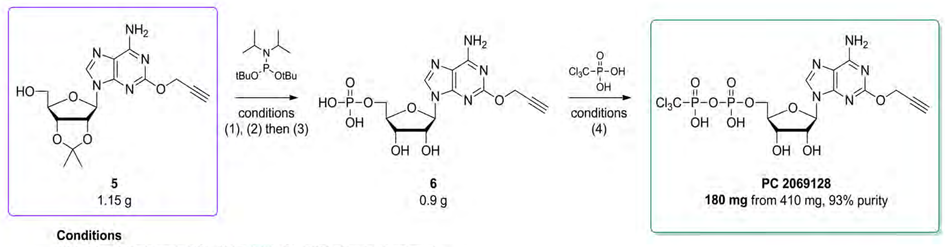
- We were able to synthesise compound **2** on multi-gram scale in 4 steps from NAD+.
- Multi-gram purification of **2** was achieved using ion-exchange chromatography (Dowex AG- IX-2).
- EDCI mediated amide coupling of **2** with propargyl amine was achieved in water at 0 °C to RT. The pH was constantly monitored and maintained at 4.8-5.0.
- **PC 2069121** was purified using ion-exchange chromatography (Sephadex-DEAE).
- Reduction to **PC 2069122** was achieved using sodium dithionite in degassed 2% aqueous sodium bicarbonate. Purification (Sephadex-DEAE) followed by freeze-drying gave the desired target.

4. SYNTHESIS OF A CLICKABLE ADP ANALOGUE



Conditions:
(1) Ac_2O, DMF, pyridine, (75%); (2) $POCl_3$, *N,N*-dimethylaniline, Et_4NCl, MeCN, 15 min (83%); (3) tert butyl nitrite, tBuOH, H_2O;
(4) propargyl bromide, Cs_2CO_3, DMF (43% two steps); (5) 10% NH_3/MeOH, 50 °C (53%); (6) acetone, pTsOH, RT (66%).

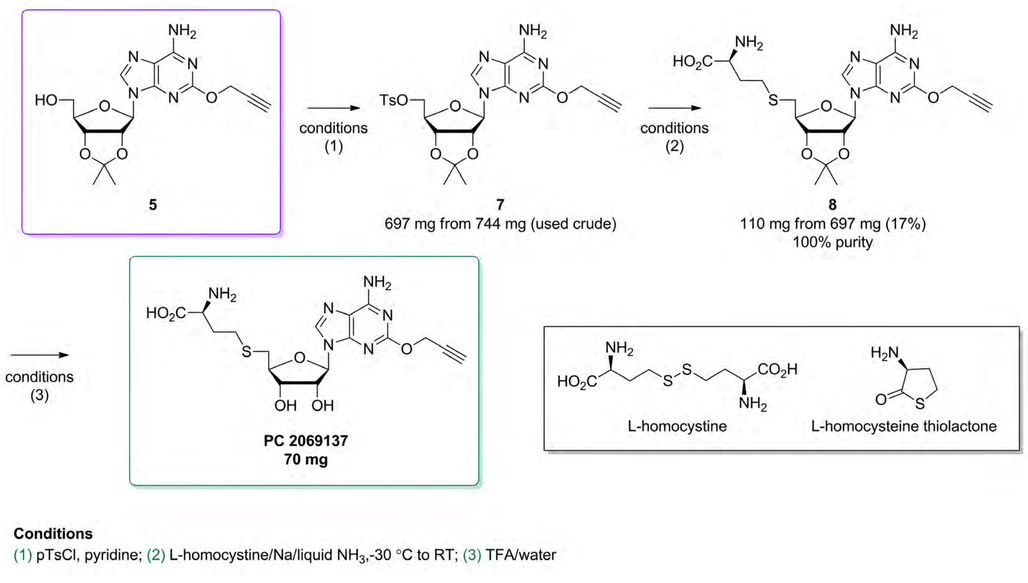
- Key intermediate **5** was made on multi-gram scale in 6 steps from guanosine using standard techniques.



Conditions
(1) imidazole/imidazole.HCl, DMF; (2) H_2O_2 (60% after flash column);
(3) TFA/DCM/water; (4) DCC pyridine.

The key intermediate **5** was converted to the 5'-monophosphate on gram-scale by reaction with di-tert-butyl diisopropylphosphoramidite followed by oxidation. The tert-butyl phosphate intermediate was purified by flash chromatography. Global deprotection with TFA gave **6**. The monophosphate was coupled with (trichloromethyl) phosphonic acid using DCC in pyridine (Russ. J. Chem., **2006**, 542). Purification using ion-exchange chromatography (Sephadex-DEAE) gave 180 mg of the target compound.

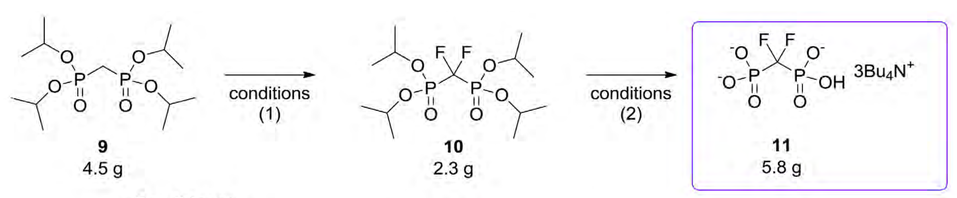
5. SYNTHESIS OF A CLICKABLE SAM ANALOGUE



Conditions
(1) pTsCl, pyridine; (2) L-homocystine/Na/liquid NH_3,-30 °C to RT; (3) TFA/water

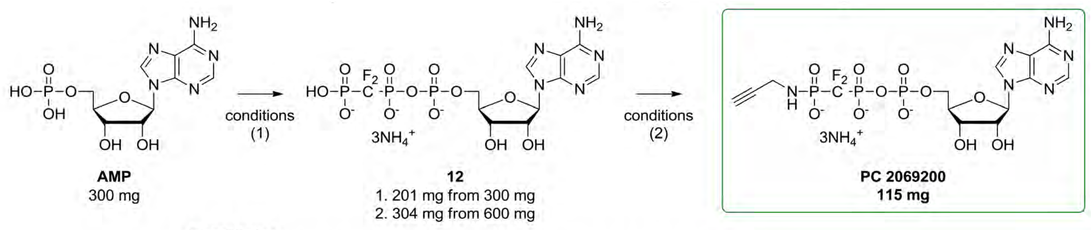
- We attempted to react **7** with L-homocysteine thiolactone in aqueous sodium hydroxide, as reported in J. Org. Chem., **1978**, 43, 5, 998, with no success.
- Reacting L-homocystine with sodium in liquid ammonia at -78°C, followed by addition of **7** and reaction at –30 °C for 12 h gave a mixture of starting material and product (for similar see J. Org. Chem., **1978**, 43, 5, 998).
- The desired product was isolated by trituration with DCM, followed by reverse phase prep. HPLC to give **8**.
- Deprotection of the acetonide with TFA followed by freeze-drying gave the desired target.

6. SYNTHESIS OF A CLICKABLE ATP ANALOGUE



Conditions:
(1) LHMDS, N-Fluorobenzenesulfonimide; (2) (a) 6M HCl, (b) 40% aq nBu_4NOH

- The intermediate difluorophosphate **11** was made on multi-gram scale using a literature procedure



Conditions:
(1) (a) TFAA, Et_3N, MeCN, (b) NMI, **11**; (2) propargyl amine, EDCI, water pH 5.65-5.85

- AMP was coupled with **11**. Purification was achieved using ion-pair chromatography [C18 reverse phase column, linear gradient of (water/tributylamine/ acetic acid) and (methanol/ tributylamine)].
- The final coupling was carried out using EDCI in water at pH 5.7. Purification was achieved using ion-exchange chromatography (Sephadex-DEAE). 115 mg was dispatched to the customer.

7. OUTCOME

Demonstration of the initial hypothesis was achieved using tool compounds for HSP90 but it was challenging to find conditions compatible with the Epic® plate. Further work is required for optimisation.

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