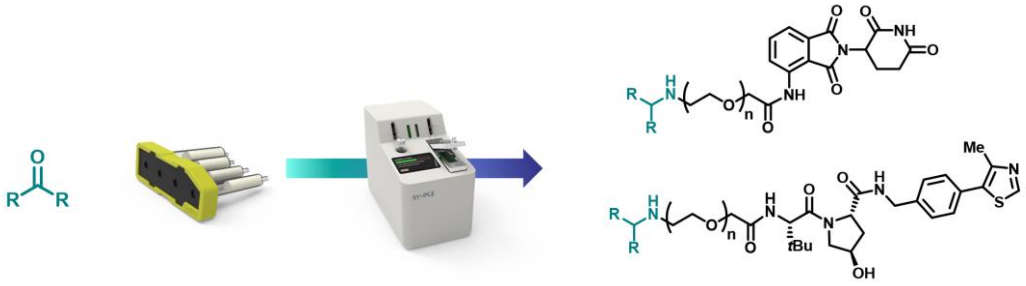
Application Note – Protein Degrader Formation

(via Reductive Amination)

Introduction

Induced protein degradation, originated from the study of hijacking the ubiquitin-proteasome system using PROteolysis TArgeting Chimeras (PROTACs), has evolved rapidly for the past two decades. Unlike the well-established strategy of protein inhibition, induced protein degradation operates as PROTACs bring the protein of interest and an E3 ligase into close proximity in the form of a ternary complex that leads to polyubiquitination and subsequent degradation by the proteasome. With the huge potential of targeting those "undruggable" proteins in the human proteome, PROTACs have drawn widespread interest across academia, pharmaceutical and biotechnology industry for the understanding of different diseases and the development of new therapeutics.

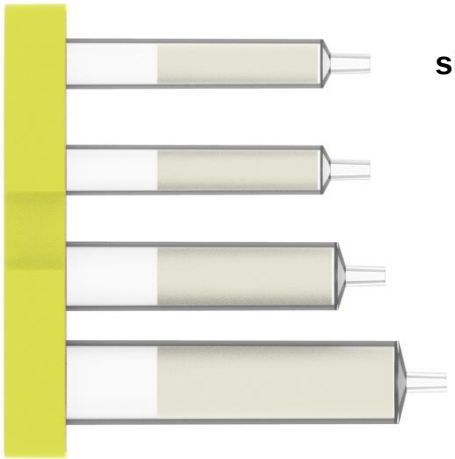
By structure, PROTACs are bifunctional molecules consisted of three key components: a target protein binding ligand, a ligand for an E3 ubiquitin ligase, and an appropriate linker connecting both. As a novel chemical modality, these molecules appear synthetically challenging as the routes are usually lengthy and tedious accompanied by stability and/or solubility issues. Overall the difficulties in intermediate handling and product purification and analysis exist as a bottleneck for quick access of PROTACs, which may further delay subsequent biological studies.



Using the approach described in this application note, the Synple Chem synthesizer offers an easy and fast automated method for assembling protein degraders through reductive amination, starting from an aldehyde or a ketone as the coupling partner.

Cartridge Contents

The cartridge contains a set of reagents to synthesize protein degraders on a scale up to 0.1 mmol.



silica-supported cyanoborohydride

silica-supported triethylamine

SCX

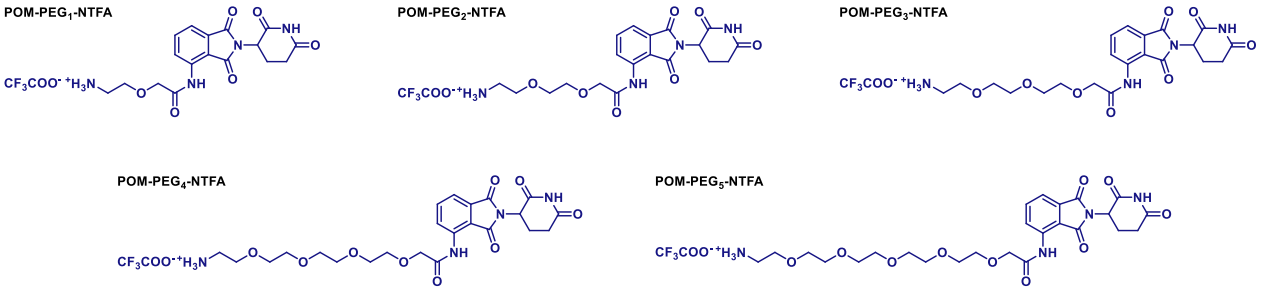
protein degrader building block

The method can be used for the following transformation:

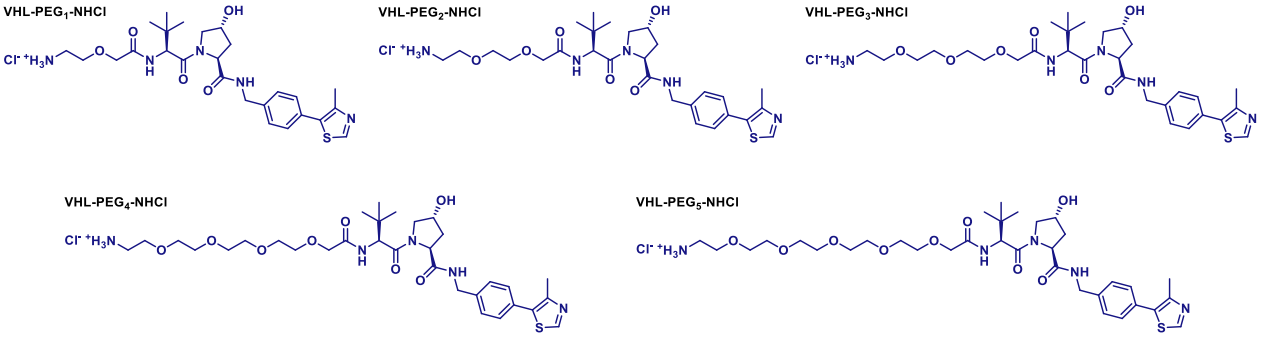
- Synthesis of protein degrader via reductive amination between a protein degrader building block and a carbonyl coupling partner (aldehyde or ketone).

Available Degrader Building Blocks

CRBN-based building blocks



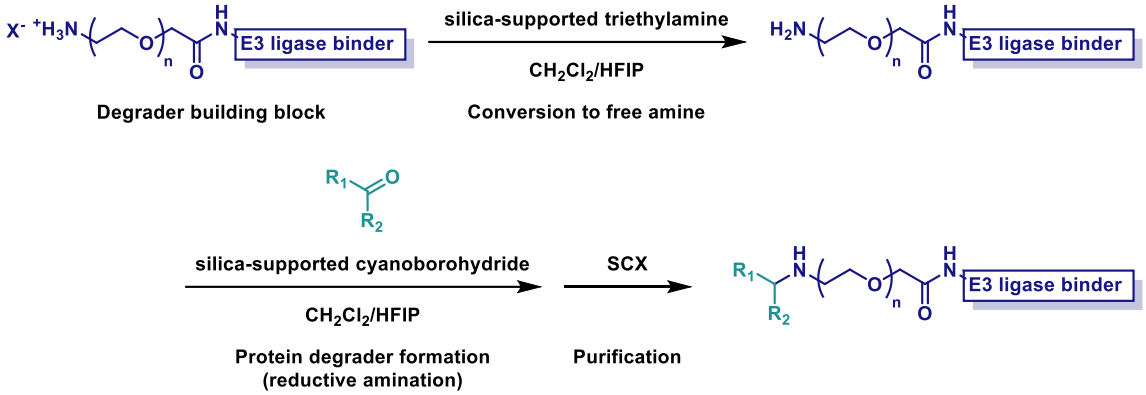
VHL-based building blocks



Reaction Scheme

This section describes the general course of the protein degrader formation by reductive amination:

The cartridge contains the protein degrader building block in the form of an amine salt. In the first step the amine salt is converted to free amine in the presence of the carbonyl coupling partner. Next, the free amine and the carbonyl coupling partner undergo a reductive amination reaction to form the protein degrader. Upon completion, the crude mixture containing the product is purified using a catch & release strategy.



Reaction Procedure

1) Dissolution of protein degrader building block

In the first step, a solution of the carbonyl coupling partner in CH_2Cl_2/HFIP (1,1,1,3,3,3-hexafluoroisopropanol) is circulated through compartment 4 to dissolve the containing protein degrader building block.

2) Conversion to free amine

The solution containing the carbonyl coupling partner and the protein degrader building block is circulated through compartment 2 (silica-supported triethylamine) at 2 mL/min for 25 min, to convert the degrader building block from amine salt into free amine. Compartment 2 is further rinsed with anhydrous CH_2Cl_2, which goes into the vial.

3) Protein degrader formation (reductive amination)

The solution containing the protein degrader building block (as free amine) and the carbonyl coupling partner is circulated through compartment 1 (silica-supported cyanoborohydride) at 2 mL/min for 12 h at room temperature. When the reaction is complete, compartment 1 is rinsed with anhydrous CH_2Cl_2, which goes into the vial.

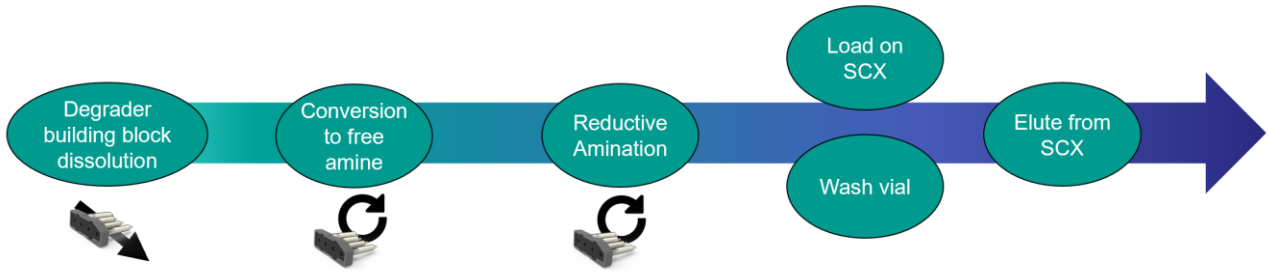
4) Purification

The reaction mixture is loaded into compartment 3 (SCX) at 2 mL/min. Compartment 3 is further rinsed with *i*-PrOH and the filtrate, which contains all the non-basic substances (formed protein degrader is basic and therefore trapped by SCX), is discarded to waste.

5) Product release

Compartment 3 (SCX) is rinsed with 2.5 M *N*,*N*-diisopropylamine in *i*-PrOH (15 mL), which goes into the vial.

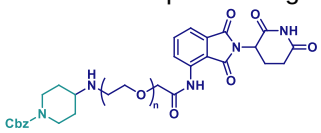
After product release, the solution in the vial contains the protein degrader product.



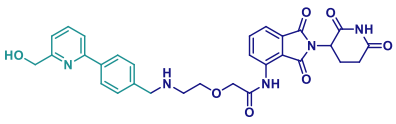
Substrate Scope

Example substrate scope

CRBN-based protein degraders



n = 1, 69%
n = 2, 71%
n = 3, 57%
n = 4, 63%
n = 5, 53%[a]



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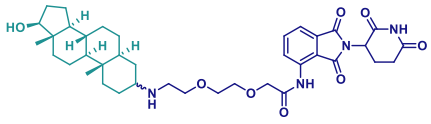

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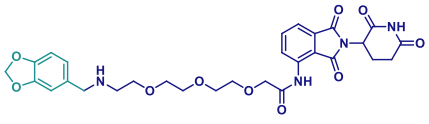
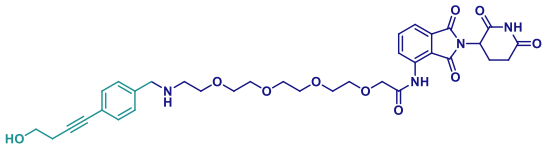


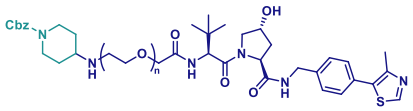
73%



70%

(a) Reaction performed at 40 °C.

VHL-based protein degraders



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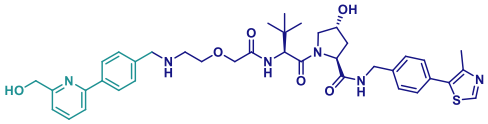
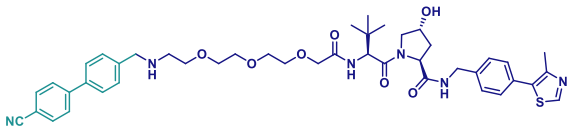


76%



54%

(a) Reaction performed at 40˚C

Identified Chemistry Limitation

Synple automated protein degrader formation exploits reductive amination to generate protein degraders from carbonyl coupling partners. Therefore, some reported limitation in reductive amination reactions applies, for examples, reactions are sluggish with conjugated ketones. Sterically hindered ketones also react more slowly and may result in low conversions.

Insoluble starting materials
The carbonyl coupling partner (aldehyde or ketone) shall be soluble in the reaction solvent (CH_2Cl_2/HFIP = 3:1) when the sample is prepared initially. Insoluble materials will lead to low or no conversion, in the worst case, may cause damage to the synthesizer.

Acid sensitive groups
Acid sensitive functional groups, such as *tert*-butoxycarbonyl (Boc) groups, acetals or silyl ethers, may undergo partial cleavage during the purification step due to the acidity of SCX. This can be avoided by disabling the SCX purification step (see Reaction Parameter Editing).

Excess/unreacted starting materials
Purification of the generated protein degrader is achieved with a catch & release strategy, namely the basic protein degrader product is caught by SCX while all non-basic species are washed off, then the product is released by washing SCX with a basic solution. When the degrader building block (as free amine) does not react fully, it will be caught over SCX together with the protein degrader product. Similarly, when the chosen carbonyl coupling partner contains basic moieties, it will be caught and released together with the protein degrader product. In either case, an additional purification is necessary to obtain the pure protein degrader product.

Double reductive amination
When an aldehyde is used as the carbonyl coupling partner, double reductive amination may occur in a small amount due to a secondary reaction between the formed protein degrader (secondary amine) and the aldehyde. In this case, an additional purification is necessary to obtain the pure protein degrader product. To minimize this side reaction, it is suggested not to use an aldehyde in an excess amount.

Reaction Parameter Editing

Editing parameters:

Parameter 1	Reaction time for reduction (seconds)
Parameter 2	Amount of solvent for elution from “catch & release” resin: In case of very polar substrates more solvent could be required to wash of the last bit of product from the catch & release resin. Therefore, the value can be increase. To calculate the input value multiply the volume in mL by 600. For example the value 9000 is equivalent to 15 mL (Maximum value 12000)

Enabling and Disabling parts:

Part 1:
Purification step
The purification step of the sequence can be disabled. In case of very acid sensitive functional groups the purification might not be suitable. The machine will then provide the reaction product in solution in the reaction vial after the reduction step.

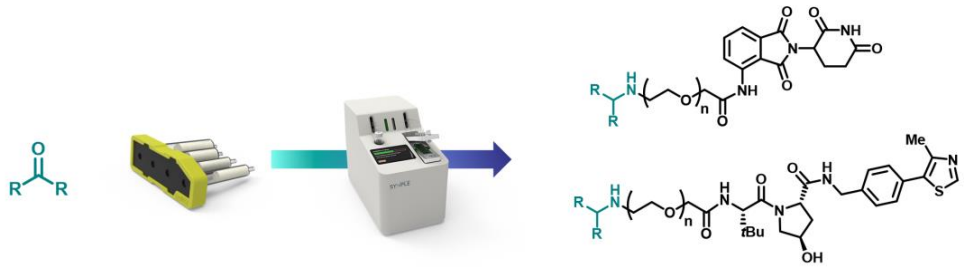
Reaction Planning

Solubility
The carbonyl starting material shall be soluble in the reaction solvent (CH_2Cl_2/HFIP = 3/1).

Scale
The Synple automated protein degrader formation is suitable for a scale up to 0.1 mmol as the cartridge contains 0.1 mmol of the protein degrader building block. When the carbonyl coupling partner is a ketone, excess amount can be used to improve both yield and purity of the crude protein degrader product. When the carbonyl coupling partner is an aldehyde, it is recommended to use 1.0 equiv of the aldehyde (0.1 mmol) to avoid the side reaction of double reductive amination (see Identified Chemistry Limitation).

Tolerance of air and/or moisture
Protein degrader formation using Synple Chem synthesizer is insensitive toward air and moisture. As some protein degraders are highly hygroscopic, it is recommended that the cartridge shall be used directly after opening. Leaving the cartridge open for prolonged time may lead to lower yield and/or purity of the protein degrader product.

Sample Preparation

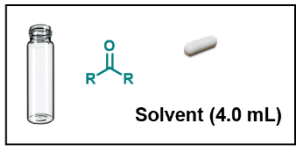


Precaution
To ensure a successful reaction in the Synple Chem synthesizer, automated CH_2Cl_2 wash shall be run before setting up a protein degrader formation reaction.

Setup

Components for sample preparation:

- Vial
- Aldehyde or ketone (0.1 mmol)
- Stirbar
- CH_2Cl_2 (3.0 mL, >99.8%, amylene stabilized)
  HFIP (1.0 mL, 99.9%)



Modifying the sequence

1) The standard reaction time of Synple automated protein degrader formation is 12 h. The reaction time can be shortened if needed (see Reaction Parameter Editing). Generally, for PEG_1, PEG_2 and PEG_3 protein degrader building blocks, high conversion (>80%) can be reached even within 3-5 hours of reaction time.

2) POM-PEG$_5$-NTFA and VHL-PEG$_5$-NHCl are less reactive than other protein degrader building blocks. To reach a good conversion, we suggest to set the vial and cartridge temperature to 40 °C (see Reaction Parameter Editing).

Machine Solvents for the use with protein degrader formation cartridge

Please connect the following solvent to the color-coded solvent lines:

	S1: CH_2Cl_2, 99.8%, anhydrous, 50 ppm amylene stabilized
	S2: –
	S3: *i*-PrOH, ≥99.8%
	S4: *N*,*N*-diisopropylamine (175 mL, ≥99.8%) in *i*-PrOH (325 mL, ≥99.8%)
	S5: –

Machine Cleaning after Protein Degrader Formation (via Reductive Amination)

1) Run automated CH_2Cl_2 wash right after the protein degrader formation reaction.
2) If any solid particles are observed in the lines after the protein degrader formation reaction, run automated *i*-PrOH wash (select automated MeOH wash on the touchscreen), then an automated CH_2Cl_2 wash.

Miscellaneous

Stability of CRBN-based protein degrader
The pomalidomide core in CRBN-based protein degraders can be unstable in protic solvents. Its half-life in MeOH at 40 °C is approximately 30 min and the side product is generated by ring opening of the cyclic imide. This often results in an additional peak when a CRBN-based protein degrader is injected into an HPLC or LC-MS using MeOH as eluent. Such molecules also show, to some extent, instability in aqueous media. In fact, substantial degradation was observed if a CRBN-based protein degrader is left for 24 hours at physiological conditions (37 °C, pH 7.4), while it proved to be stable at lower pH. Therefore, we suggest to avoid keeping any CRBN-based protein degraders in aqueous or alcoholic solution for long time.

see: Bricelj, A.; Dora Ng, Y. L.; Ferber, D.; Kuchta, R.; Müller, S.; Monschke, M.; Wagner, K. G.; Krönke, J.; Sosič, I.; Gütschow, M.; et al. Influence of Linker Attachment Points on the Stability and Neosubstrate Degradation of Cereblon Ligands. *ACS Med. Chem. Lett.* **2021**, *12* (11), 1733–1738. Link.

Solvent Consumption and Run Time

SEQUENCE RUNTIME	
Reaction Sequence	**Time**
PROTAC formation amine (via reductive amination)	15 h 49 min
PROTAC formation amide (via amide formation)	5 h 14 min

SOLVENT COMSUMPTION FOR PROTAC AMINE	
For Reaction Setup	**Amount**
Dichloromethane (CH_2Cl_2)	3 mL
Hexafluoroisopropanol (HFIP)	1 mL
Machine Solvents	
Dichloromethane (CH_2Cl_2)	39 mL
Methanol (MeOH)	30 mL
Diisopropylamine (DIPA) – Isopropanol mixture (13:7)	19 mL