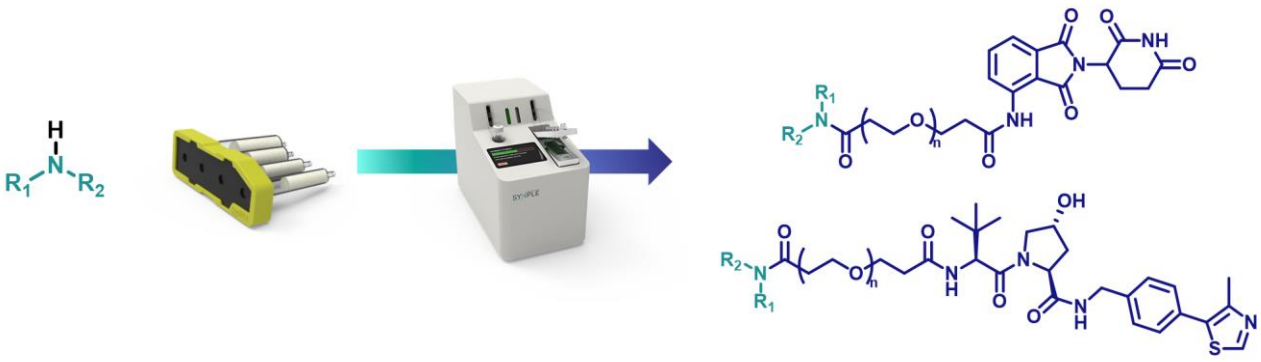
Application Note – Protein Degrader Formation

(via Amide Formation)

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.

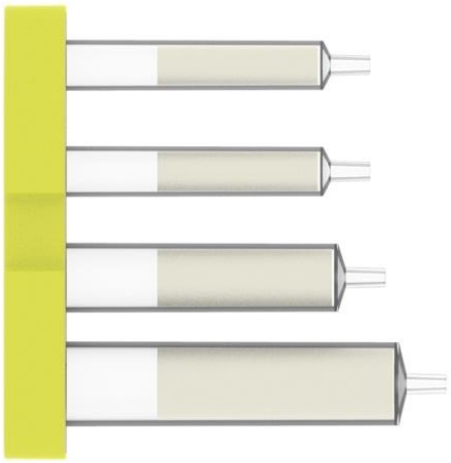
By structure, PROTACs are bifunctional molecules consisted of three key components: a target protein binding ligand, a ligand for an E3 ubiquitin ligase, and an appropriate linker connecting both. As a novel chemical modality, these molecules appear synthetically challenging as the routes are usually 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 amide formation, starting from a free alkyl amine as the coupling partner.

Cartridge Contents

The cartridge contains a set of reagents to form the protein degrader on a scale up to 0.1 mmol.



Oxyma Pure silica-supported carbodiimide

SCX (buffered)

silica-supported carbonate

protein degrader building block

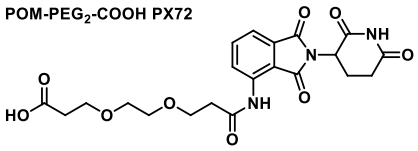
The method can be used for the following transformation:

- Synthesis of protein degrader via amide formation between a protein degrader building block and a free alkyl amine.

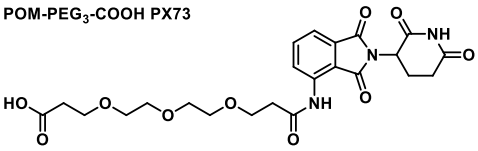
Available Degrader Building Blocks

CRBN-based building blocks

POM-PEG$_2$-COOH PX72

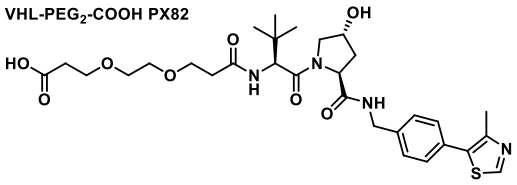


POM-PEG$_3$-COOH PX73

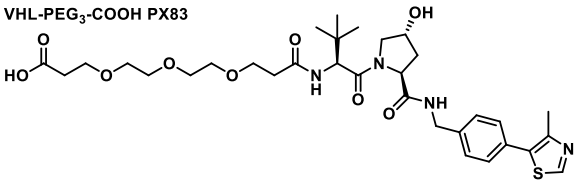


VHL-based building blocks

VHL-PEG$_2$-COOH PX82



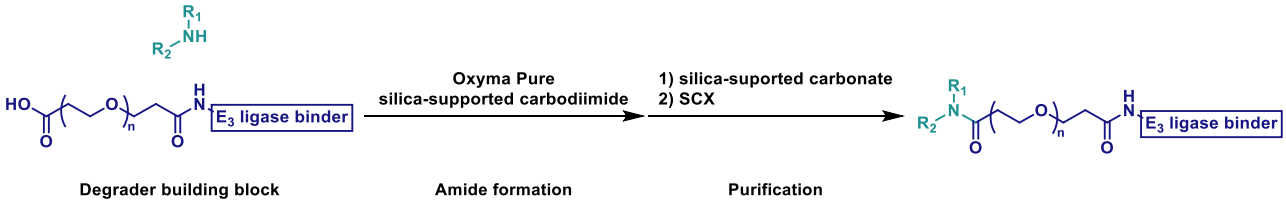
VHL-PEG$_3$-COOH PX83



Reaction Scheme

This section describes the general course of the protein degrader formation via amide formation:

The cartridge contains the degrader building block as a carboxylic acid, which is activated by the supported coupling reagent, then reacts with the desired free alkyl amine. Unreacted starting materials are removed from the crude mixture by treatment with silica-supported carbonate and SCX.



Reaction Procedure

1) Dissolution of protein degrader building block

In the first step, the solution of a free alkyl amine in anhydrous CH_2Cl_2 and MeCN is circulated through compartment 4 at 3 mL/min for 10 min to dissolve the containing protein degrader building block.

2) Amide formation

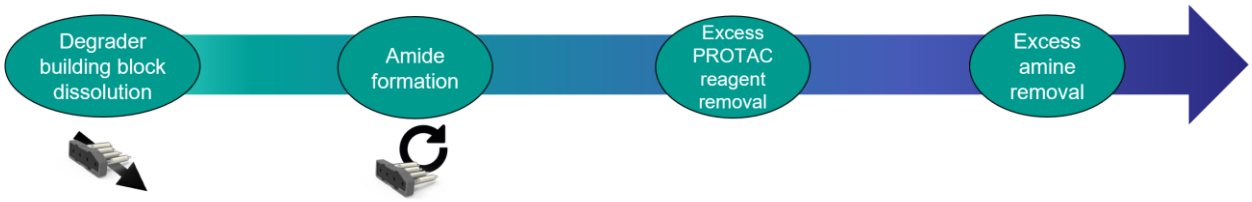
The mixture containing the free alkyl amine and the degrader building block is loaded into compartment 1 to dissolve Oxyma Pure and passed through silica-supported carbodiimide. The mixture is further circulated through compartment 1 at 3 mL/min for 4 h at room temperature.

3) Purification

The reaction mixture is loaded to compartment 3 (silica-supported carbonate) at 1 mL/min. Oxyma Pure and excess amount of carboxylic acid are scavenged in this step. Compartment 3 is further rinsed with *i*-PrOH, which goes into the vial.

The solution in the vial is further loaded way to compartment 2 (SCX) at 1 mL/min. Unreacted amine is scavenged in this step. Compartment 2 is further rinsed with *i*-PrOH, which goes into the vial.

After purification, the solution in the vial contains the protein degrader amide product.



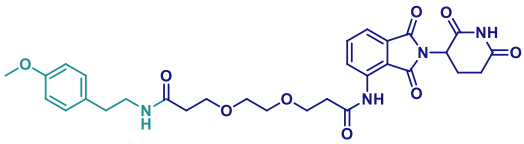
Substrate Scope

Tolerated functional groups

A large amount of different functional groups is tolerated including esters, alcohols, alkenes, ketones, carbamates, aryl halides, sulfonyl groups, heterocycles (pyridines, piperazines, morpholines, thiazoles, benzisoxazoles, etc.).

Example substrate scope (from 0.1 mmol free alkyl amine)

CRBN-based[a]



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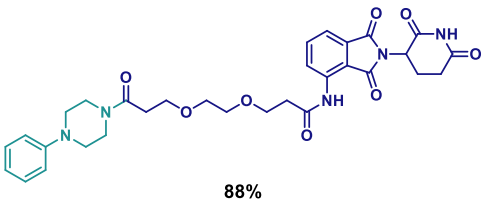

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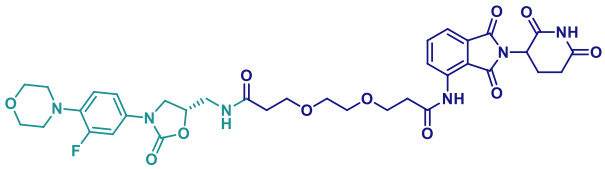

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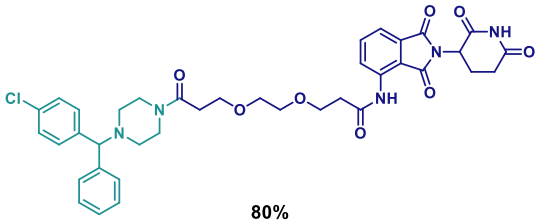

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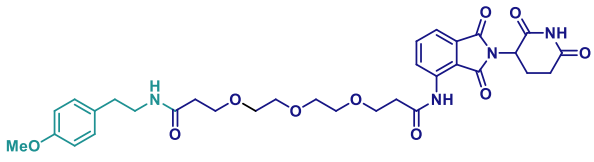
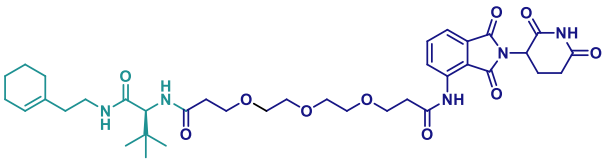
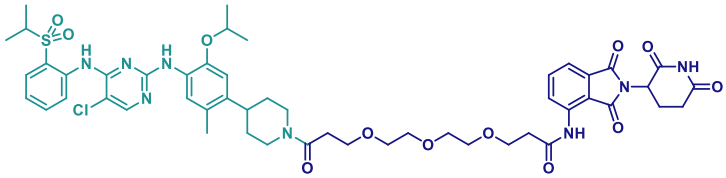


64% (62%)[b]



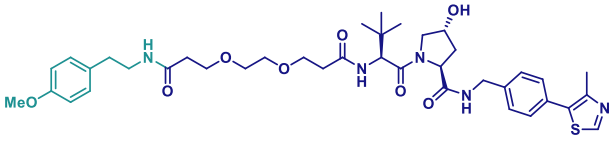
79%[c]



81%

a) products obtained in >95% purity without additional purification.
b) THF used as solvent in place of CH_2Cl_2/MeCN. Reaction time prolonged to 12 h.
c) 90% conversion of starting amine.

VHL-based[a]



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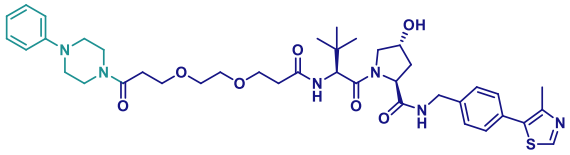

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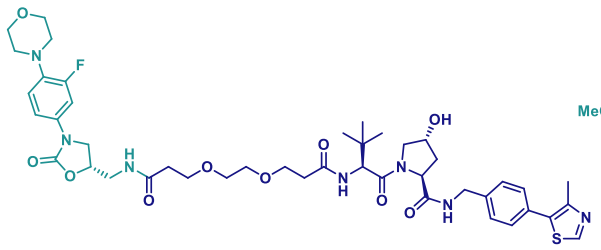

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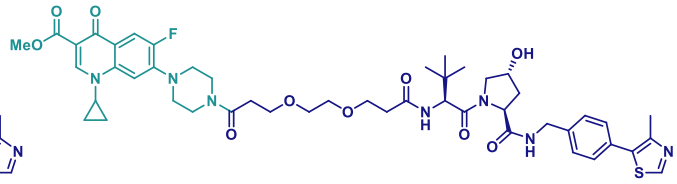

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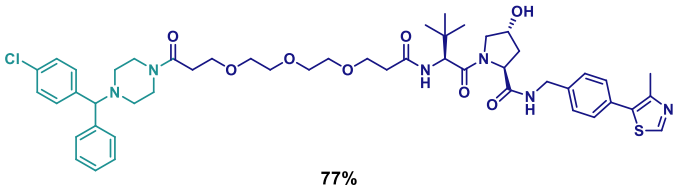

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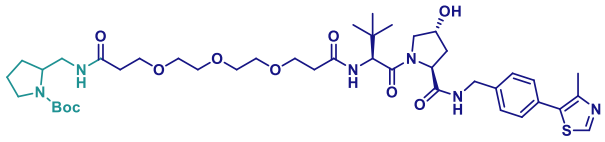
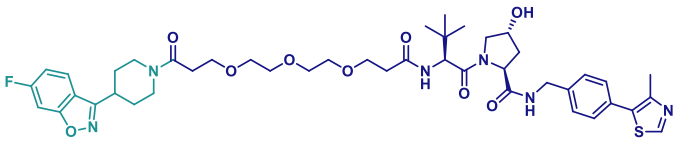


71%



57%[b]

a) products obtained in >95% purity without additional purification.
b) THF used as solvent in place of CH_2Cl_2/MeCN. Reaction time prolonged to 12 h.

Identified Chemistry Limitation

Reactants
At present, the reaction has not been optimized for weakly nucleophilic amines, e.g. aryl amines. Hindered amines react smoothly, but increasing the reaction time is recommended in such cases.

Amine salts
At present, amine salts cannot be used in this reaction and converting the amine salt to the corresponding free amine is required before starting the reaction. A protocol to allow using amine salts is under development.

Insoluble starting materials
The starting free alkyl amine must be soluble in the reaction solvent (anhydrous CH_2Cl_2/MeCN = 1/1) when the sample is prepared initially. Anhydrous THF can be used in place of CH_2Cl_2/MeCN, but reaction time shall be increased to 12h (43200s, see Reaction Parameter Editing). Insoluble materials will lead to low or no conversion and, in the worst case, may cause damage to the synthesizer.

Protic solvents
Protic solvents (i.e. MeOH, EtOH, *i*-PrOH...) are not suitable for this reaction, since they will react with the degrader building block to give the correspondent esters.

Acidic functional groups

Acidic functional groups, such as carboxylic, sulfonic or phosphoric acids, will cause the product to be trapped over silica-supported carbonate during the purification. Acidic phenols can also be partially or completely trapped.

Reaction Parameter Editing

Editing parameters:

Parameter 1	Reaction time for amide bond formation (seconds)

Enabling and Disabling parts:

Part 1:
Purification step
The purification step of the sequence can be disabled in case the product can be trapped by the carbonate resin (see Identified Chemistry Limitation – Acidic functional groups)

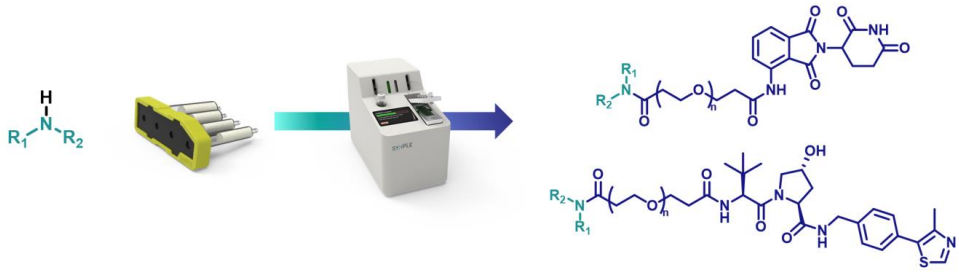
Reaction Planning

Solubility
The amine starting material shall be soluble in the reaction solvent (anhydrous CH_2Cl_2/MeCN = 1/1 or, in alternative, anhydrous THF).

Scale
Protein degrader formation (via amide formation) using Synple Chem synthesizer is suitable for a scale up to 0.1 mmol as the cartridge contains 0.1 mmol of the protein degrader building block.

Tolerance of air and/or moisture
Protein degrader formation (via amide formation) using Synple Chem synthesizer is insensitive toward air, however anhydrous solvents (CH_2Cl_2, MeCN, THF) shall be used to minimize any potential hydrolysis of active intermediates.

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. Any residual alcoholic solvents in the system can lead to formation of the corresponding ester as side product.

Setup
Components for sample preparation:

- Vial
- Free alkyl amine (0.1 mmol)
- Stirbar

- Anhydrous CH_2Cl (2.0 mL, 99.5%)
  Anhydrous MeCN (2.0 mL, 99.9%)
  Sonication may help dissolving insoluble materials.

Modifying the sequence
The standard reaction time for Synple automated protein degrader synthesis is 4 h (14400 s). The reaction time can be prolonged if needed (see Reaction Parameter Editing). Prolonged reaction time is recommended when using THF as solvent, and is generally suggested when hindered amines are used.

Machine Solvents for the use with protein degrader cartridges
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: –
	S5: –

Machine Cleaning after Protein Degrader Formation (via Amide Formation)

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) first, followed by 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 AMIDE	
For Reaction Setup	**Amount**
Dichloromethane (CH_2Cl_2)	3 mL
Hexafluoroisopropanol (HFIP)	1 mL
Machine Solvents	
Dichloromethane (CH_2Cl_2)	10 mL
Methanol (MeOH)	11 mL