HHS Public Access

Author manuscript

Chem Commun (Camb). Author manuscript; available in PMC 2022 January 25.

Published in final edited form as:

Chem Commun (Camb). 2021 January 25; 57(8): 1026–1029. doi:10.1039/d0cc05395c.

Traceless Staudinger Ligation Enabled Parallel Synthesis of Proteolysis Targeting Chimera Linker Variants

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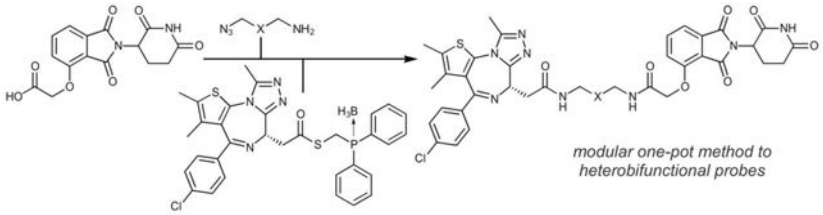
Abstract

A parallel, one-pot assembly approach to proteolysis targeting chimeras (PROTACs) is demonstrated utilizing activated esters generated *in situ*, and traceless Staudinger ligation chemistry. The method described allows for rapid structure-activity relationship studies of PROTAC linker variants. Two previously studied systems, cereblon and BRD4 degraders, are examined as test cases for the synthetic method. The two related strategies to assemble PROTAC linker variants discussed can accommodate the chromotographic separations capabilities of labs of many sizes and incorporates commercially available degrader building blocks, thereby easing synthetic entry into PROTAC chemical space.

TOC

Chemoselectivity of the traceless Staudinger ligation was leveraged to enable assembly of chimeric small-molecule linker variants in a one-pot approach.



The development of highly efficient chemical processes lies at the foundation of serialized screening systems.[1] Effectively described as Click chemistry,[2] these reactions have provided a remarkable access to small molecule diversity and has profoundly impacted our ability to prepare biological probes. Over the last decade, proteolysis targeting chimeras (PROTACs), heterobifunctional small molecules,[3] have gained recognition as a powerful tool for targeting proteins for proteosomal degradation (Fig. 1).[4] Recently, PROTACs have garnered interest due to their potency, catalytic activity, and ability to target ‘undruggable’ proteins.[5] This utility has not gone unrecognized, and was recently marked by entry into the first clinical

Electronic Supplementary Information (ESI) available: experimental procedures, spectral data and copies of select NMR spectra. See DOI: 10.1039/x0xx00000x

Conflicts of interest

There are no conflicts to declare.

trials of ARV-110 for metastatic castration-resistant prostate cancer and ARV-471 for metastatic breast cancer.[6]

First described in 2001, the PROTAC concept (Fig. S1) involves the preparation of chimeric molecules that contained two protein binding motifs that induce unnatural protein-protein interactions (PPIs).[7] While not limited to protein interactivity,[8] this concept offers a robust utility to link two pathways and molecularly rewire a cells function upon the presentation of a chimeric molecule. Developing tools to enable the preparation and screening of libraries of chimeric molecules will play a key role in our future development of concepts like PROTACs. While advances have been made to ease the synthetic entry into chimeric small molecule space,[9] preparations of linker variants remains a necessary and tedious task. One needs to consider a three-part diversification and optimization where structural variance can be introduced at the two-protein binding and linker motifs. The issue then exists as to how one can 'choreograph' these processes into a single operation, thereby streamlining the evaluation of PROTAC linker variants.

Over the last decade, our laboratory has explored the development of 4'-phosphopantetheinamide probes whose function serves as a chimeric molecule, wherein the one motif within serves to attach to a carrier protein (CP) and the second to a functional partner protein (PP).[10] In this system, a short but effective pantetheinamide linkage enables a rapid multidentate processing between the CP and multiple PP domains. During the course of these studies, we realized the importance of developing a modular synthetic approach.[11] Ultimately, we were able to convert a task that began as multistep syntheses[12] into a single 'one-pot' reaction.[13]

With modularity in mind, we envisioned a similar 'one-pot' approach that could produce PROTACs in a parallel fashion, and ideally be devoid of intermediary purifications. Developing on advances from the Raines laboratory,[14] we targeted the use of a traceless Staudinger ligation[15] as a means to introduce asymmetry through a chemoselective amide bond formation.

The general process began with the *in situ* formation of an activated ester from one of the two proteins of interest (POI) ligands, as shown by activation of **4** by CDI (Scheme 1). This was then followed by a subsequent amide coupling with azidoamino-linkers **3a-3c**. The resulting azides **5a-5c** could then be coupled with thioester **6** to yield the second amide bond formation in a chemoselective manner through traceless Staudinger chemistry.[14,15] After engagement of the phosphine with the azide, the resulting aza-ylide intermediate[14a] is designed to undergo an intramolecular attack on the thioester, yielding **1a-1c** and **2a-2c** after hydrolysis. We tested this approach by preparing two model PROTACs.

In our first example, we examined the $CRL4^{CRBN}$ ubiquitin E3 ligase with IMiD-based ligands due to their documented risk of hydrolysis.[16] In comparison to other E3 ligase ligands,[17] the IMiD-based PROTACs pose greatest risk of hydrolysis under the Staudinger ligation conditions. This increases the likelihood of this method translating to other currently used E3 ligase ligands.[17] This was then partnered with the targeting of cereblon (CRBN) based on the recent demonstration of CRBN homodimeric PROTACs (**1a-1c**).[18]

We began by preparing thalidomide acid **7** in 6 steps with a 21% overall yield (Scheme S1),[19] which was accomplished on gram scale. Acid **7** was in turn coupled with thiol **9** to deliver borane-protected phosphine thioester **10** (Scheme 2). To our delight, **10** was obtained in high yield, and stored up to a month under dry conditions.

We then turned to explore the use of intermediate **10** as a tool to expedite the synthesis of homodimeric PROTACs. Targeting **1a**–**1c** (Scheme 2), a 55 mM stock solution the acyl imidazole was prepared by reacting **7** with 1.5 eq. of CDI in DMF. This solution was added to the respective reaction vessels containing 100 mM **3a**, **3b**, or **3c** in DMF along with 10 mol% of DMAP. After 3 h, TLC and LC/MS analyses indicated that the first bond formation reaction was complete, providing azides **8a-8c** in DMF. A 43 mM solution of thalidomide thioester **10** (1.5 eq.) was then added at room temperature followed by DABCO (4.5 eq.) as a 460 mM solution in DMF. Here, the DABCO played a key role in liberating the phosphine by forming lower energy complex with the borane.[20] This *in situ* phosphine liberation provided an excellent strategy to selectively engage reactivity as well as prevent unwanted phosphine oxidation.[21] The process was completed by the triggering of an intramolecular Staudinger ligation[14,15,21] through the addition of DABCO and heating the reaction to 40° C, affording homo-PROTACs **1a-1c**, as confirmed by LC-MS analysis (Figs. S3–S5).

While an effective strategy, this approach provided only moderate yields due in part accumulation of azides **8a**–**8c** arising from the incomplete consumption of amines **3a**–**3c** during the first amide bond formation, which ultimately reacted further with **10** to further yield additional **8a**–**8c**. While not a problem for homobifunctional **1** (ligand A=B, Scheme 1), this unwanted reactivity would scramble heterobifunctional **2** (ligand A≠B, Scheme 1) resulting in undesired mixtures of **1** and **2**. In our hands, the mixture of compounds **1a-b** and **8a-b**, respectively, proved to be inseparable on silica, and only modestly separable on reverse-phase UPLC (see Figs. S3–S5). Compound **1c** was isolated with a 10% yield.

To this end, our attention shifted to the heterobifunctional bromodomain degrader dBET1[22] **2a** and its analogues **2b**–**2c** (Scheme 3). This system was chosen due to prior extensive chemical biological evaluation as exemplified by the structural studies showing ternary complex formation (Fig. S2),[23] as well as its use as a model system for other PROTAC synthetic methodological studies.[9]

Access to heterobifunctional PROTACs arose by reorganizing the approach to focus on proper choice in the thioester component. As shown in Scheme 1, (+)-JQ1 acid **11** was converted to thioester **12** (Scheme 3), which like its thalidomide counterpart **10** (Scheme 2), could be purified and stored under dry conditions.

Next, we optimized the coupling of **7** to **3b** and found improved yields with using HATU (route A, Scheme 3) over CDI (used in Scheme 2). Improving the yield of this step played a critical role in the success of the operation as it avoided aberrant reactivity and consumption of thioester **6** (Scheme 1). Starting with thalidomide acid **7** (1.3 eq.) and HATU (1.3 eq.), 110 mM linker amine **3b** (1 eq.), and 900 mM DABCO were added as solutions in DMF. This yielded azide **8b** *in situ*. Upon addition of 73 mM (+)-JQ1 thioester **12** in DMF (1 eq.), followed by warming to 40 °C, the Staudinger ligation was initiated yielding compound **2b**

in a ‘one pot’ fashion. LC-MS analyses indicated that **2b** was obtained in 48% yield (Fig. S6). However, it also indicated that purification of **2b** from this mixture (Figs. S7–S10) likely required development of precise prep-HPLC techniques, a common issue associated with PROTAC synthesis.[24]

For our purposes, we sought a method that would deliver PROTACs in a parallel fashion that would be amenable to purification *via* conventional flash chromatography. To achieve this, we returned to our reaction design and identified an improved approach (route B, Scheme 3). Here, we chose to purify azides **8a** and **8c**, and perform the Staudinger ligation[14,15,21] in parallel with a stock solution of (+)-JQ1 thioester **12**. This simplification arose from the fact that many E3-ligase ligands with linker-azides and linker-primary amines (thioester **6** can be used directly as an activated ester) can be prepared, aliquoted, and stored on gram scales (several are now commercially-available). To our delight, this method yielded compounds **2a** and **2c** in 54% and 85%, respectively, after flash column chromatography (see Supporting information). Here, we were able to repetitively add 66 mM (+)-JQ1 thioester **12** stock solution (1.2 eq.) in DMF to respective reaction vessels containing azides **8a** or **8c** (1 eq.). The Staudinger ligation[14,15,21] was then initiated by addition of 760 mM stock solution of DABCO (3.6 eq.), and heating to 40°C. This yielded compounds **2a** and **2c** in a concerted effort. Most importantly, the impurity profile did not contain aberrant homobifunctional products (Figs. S11–S12), which even in small quantities could complicate biological evaluation. Compound **2b** was later prepared and purified using this strategy with a 39% yield.

PROTAC linker design remains rather empirical, although considerable effort has been dedicated to exploring the role of linker chemistry on degrader potency and selectivity.[25] The one-pot strategy developed herein, provides an expedient approach that unites the availability of degrader building blocks, with the throughput of parallel synthesis as a means to expedite material delivery. Here, we define a practical strategy to efficiently assemble heterobifunctional small molecules. Effective desymmetrization was enabled through the chemoselectivity afforded by traceless Staudinger ligation chemistry, allowing PROTAC assembly in a single pot.

Overall, we have provided variants of the method to meet the various chromatographic capabilities of different laboratory settings. This type of strategy will enable rapid biological evaluation of PROTACs and will help ‘demystify’ the nuances of PROTAC linker chemistry by providing a platform for rapid liker diversification. Efforts are currently underway to explore other strategies, such as applications of one-pot SNAr reactions between amino terminal linkers and 4-fluoro-thalidomide,[26] and traceless Staudinger ligation chemistry[14,15,21] with water-soluble phosphines,[27] therein further reducing the chromatographic complexity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Yongxuan Su (UCSD) for mass spectral analyses and Anthony Mrse (UCSD) for assistance with acquiring NMR spectral data.

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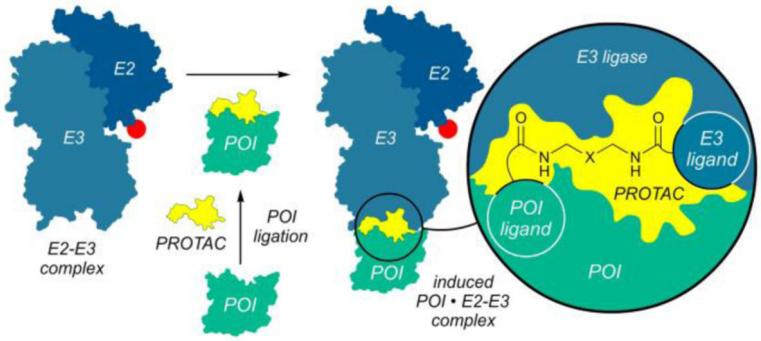
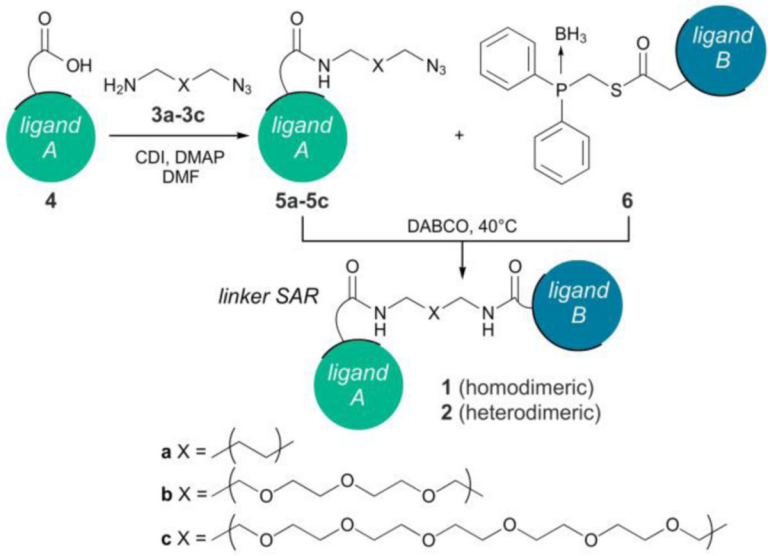


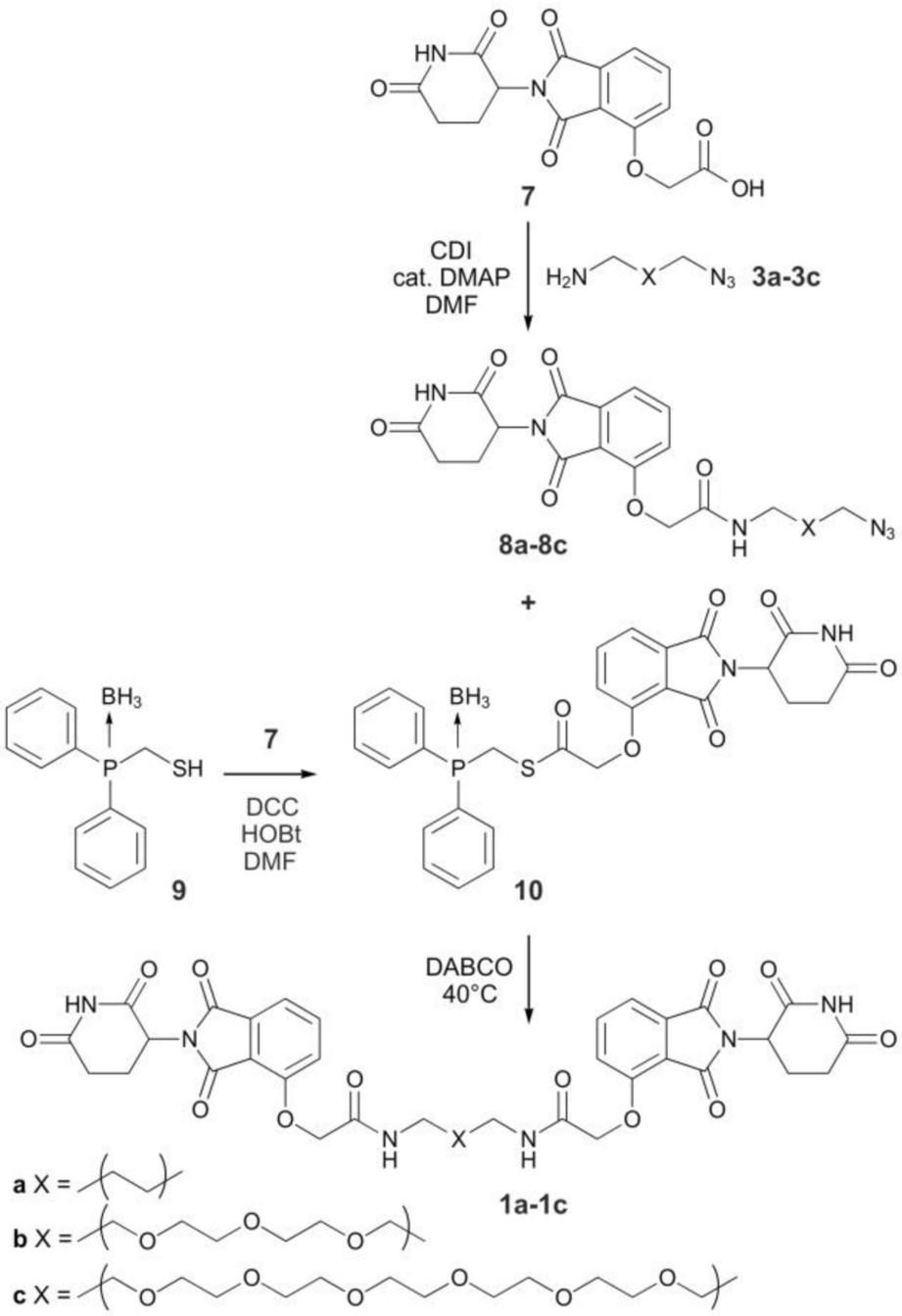
Fig. 1.

Schematic representation where a heterobifunctional molecule or PROTAC (yellow) is used to target the degradation of a protein of interest (POI, green). In this process, the PROTAC contains motifs that bind to both POI and E3 ligase (blue), yielding a ternary complex. Ubiquitin (red) can then be transferred to the POI in a proximity dependent manner, leading to proteolysis of the POI.



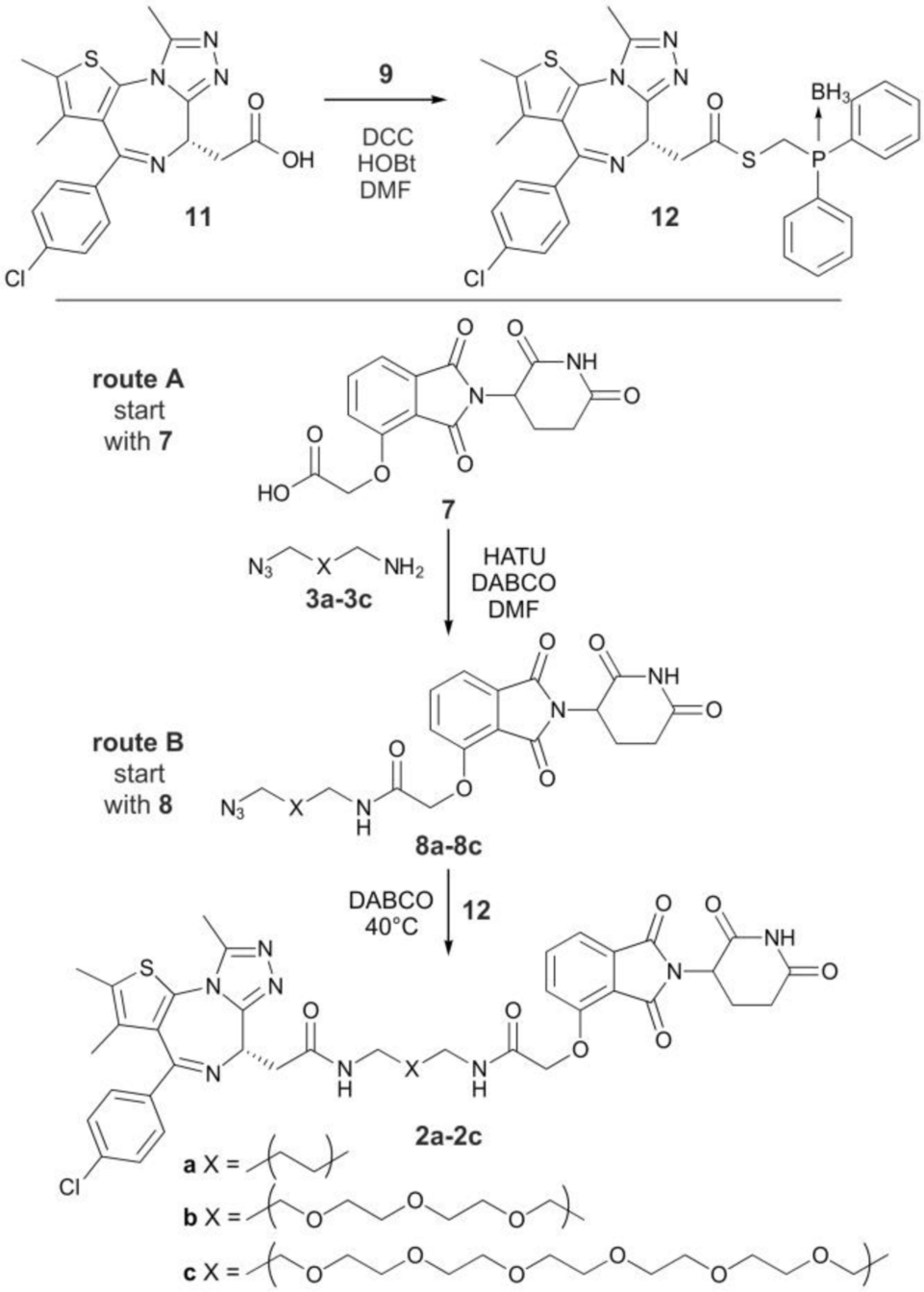
Scheme 1.

The 'one-pot' PROTAC assembly approach begins with conversion of a carboxylic acid functional group **4** to its an acyl-imidazolate *in situ* followed by coupling to amines **3a**–**3c**. The resulting azides **5a-5c** are then coupled with thioester **6**, yielding bifunctional molecules **1a-1c** and **2a-2c**. We chose to use *N,N*-carbonyldiimidizole (CDI) for our initial study due to the ease of by-product removal, however other coupling reagents may be used.



Scheme 2.

Application to homobifunctional PROTACs. Three PROTACs **1a**–**1c** were assembled in a one-pot fashion beginning with thalidomide acid **7**. Compound **10** was synthesized and isolated from thiol **9** prior to the one-pot procedure. Intermediates **8a**–**8c** were formed *in situ* and were not isolated.



Scheme 3.

Application to heterobifunctional PROTACs. Three heterodimeric PROTACs **2a**–**2c** were assembled in a one-pot fashion beginning with **7** (route A) or **8** (route B). The choice of coupling reagent should be optimized for each system in order to consume the linker amine **3** and avoid aberrant reactivity and consumption of thioester **6** (Scheme 1). This process can be conducted on analytical scales and evaluated prior to use. One-pot procedure beginning with **7** may require HPLC purification, however starting with a collection of degrader building blocks **8** (commercially available) enables purification via standard flash column

chromatography. Both methods are conducive to parallel synthesis, thereby producing all linker variants in a concerted effort from a stock solution of thioester **6** (Scheme 1).