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Development of Rapid and Facile Solid-Phase Synthesis of PROTACs via a Variety of Binding Styles

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Optimizing linker design is important for ensuring efficient degradation activity of proteolysis-targeting chimeras (PROTACs). Therefore, developing a straightforward synthetic approach that combines the protein-of-interest ligand (POI ligand) and the ligand for E3 ubiquitin ligase (E3 ligand) in various binding styles through a linker is essential for rapid PROTAC syntheses. Herein, a solid-phase approach for convenient PROTAC synthesis is presented. We designed azide intermediates with different linker lengths to which the E3 ligand, pomalidomide, is attached and performed facile PROTACs synthesis by forming triazole, amide, and urea bonds from the intermediates.

Introduction

Protein knockdown technology has attracted significant attention as a new approach in drug discovery. Proteolysis-targeting chimeras (PROTACs) and specific and nongenetic inhibitors of apoptosis protein-dependent protein erasers (SNIPERs) are representative chimeric compounds that facilitate protein degradation by hijacking the ubiquitin-proteasome system (Figure 1a).[1,2] These chimeric compounds are constructed from three components: (a) a protein-of-interest ligand (POI ligand); (b) a ligand for E3 ubiquitin ligase (E3 ligand); and (c) a linker that combines the POI and E3 ligands. Generally, small molecular ligands that bind to the target protein are used as POI ligands. The cereblon ligand (thalidomide and pomalidomide), von Hippel-Lindau ligand (VH032), and inhibitor of apoptosis protein ligand (LCL161) have been widely used as E3 ligands.[3] Alkyl and PEG chains are commonly used to link the POI and E3 ligands.[4] The mechanism of PROTACs is to recruit E3 ligase to the POI, which promotes poly-ubiquitination of the POI and subsequent proteasomal degradation. Since the first discovery of PROTACs,[5] numerous proteins have been targeted in drug discovery research. Therefore, PROTACs undoubtedly became a new modality in the pharmaceutical industry.

Discovering POI ligands that bind to target proteins with appropriate affinity is essential for improving the protein degradation activity of PROTACs. Moreover, optimizing the length and structure of the linkers ensures efficient ubiquitination by separating the two ligands by an appropriate distance. In contrast to the ongoing discovery and optimization of POI ligands and linkers, respectively, there are only a few E3 ligands that are commonly used for PROTACs, and thalidomide-type ligands (e.g. pomalidomide) are one of the more widely used building blocks for PROTAC development because they have a smaller molecular weight and are easy to synthesize when compared with other E3 ligands. Thus, screening for POI ligands and linkers is often investigated as a priority. However, these fragments are highly polar, while pomalidomide is less polar, making it difficult to handle during liquid-phase synthesis. Furthermore, PROTACs must be isolated in high purity after synthesis because they are used in biological experiments. These are some of the factors delaying the development of PROTACs.

We focused on the concept that PROTACs can be rapidly and easily synthesized by solid-phase organic synthesis (SPOS).[6] SPOS is a powerful synthetic tool used widely in combinatorial and high-throughput chemistry.[7] For drug discovery research, SPOS is used for constructing drug-like libraries from solid-phase parallel syntheses.[8,9] The first SPOS-based PROTAC synthesis has been reported by S. Krajcovicova and co-workers, in which a POI ligand was coupled with E3 ligase by alkylation of the carbonyl α-position (Figure 1b).[6] Further construction of PROTACs requires various conjugations such as *O*-/*N*-alkylation,[10] amide formation,[11] triazole

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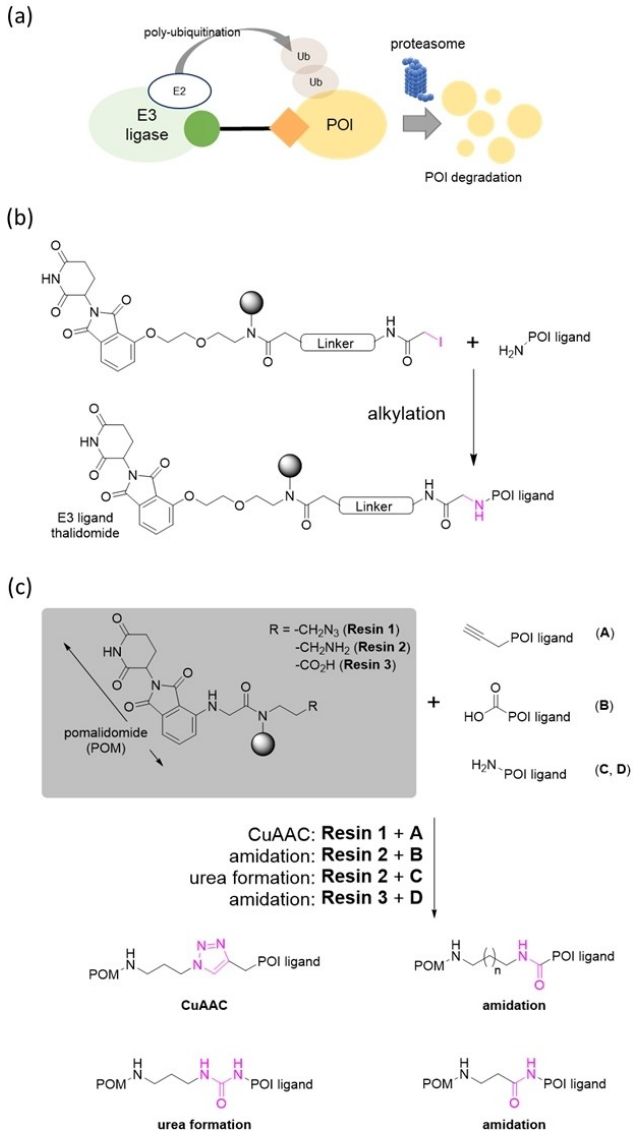


Figure 1. (a) Protein degradation mechanism of PROTACs. (b) A reported solid-phase PROTAC synthesis by alkylation. (c) Solid-phase PROTAC synthesis by various conjugation reactions (this work).

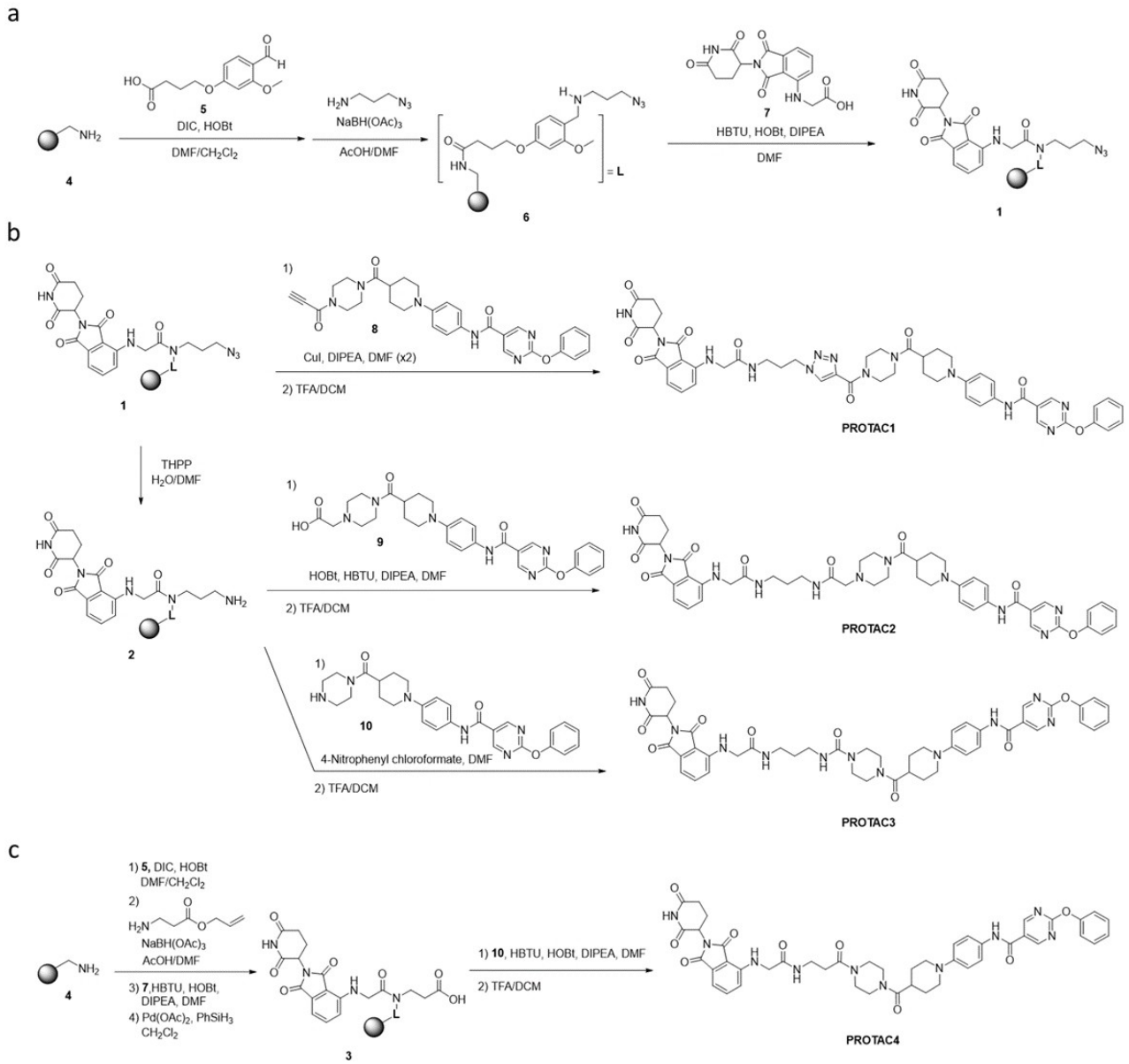
formation,[12] and ester formations.[13] Hence, in this study, we aimed to demonstrate the facile synthesis of PROTACs with various linker and ligand conjugations. Synthesizing PROTACs with diverse conjugations requires the design of a common E3 ligand bound to a linker intermediate. We focused on converting an azide group into a variety of motifs without any protection. Thus, we designed the intermediate azide-resin **1** with the E3 ligand already bonded to the alkyl linker (Figure 1c). For divergent synthesis, in addition to resin **1**, we designed amino-resin **2**, which can be synthesized by reduction of resin **1** and carboxylic acid resin **3**. These resins **1**–**3** were reacted with an alkyne (A), a carboxylic acid (B), and amines (C, D) of POI ligands to obtain amide-, triazole-, and urea-based PROTACs. Using this synthetic strategy, the E3 ligand pomalidomide and the linker were constructed during the early stages of the synthesis, and the POI ligand was introduced at a later stage. Therefore, this strategy is suitable for facile synthesis of PROTACs targeting different proteins, and PROTAC synthesis with varying lengths of the linker and POI ligands was also investigated.

Results and Discussion

We initially synthesized PROTACs targeting hematopoietic prostaglandin D synthase (H-PGDS) using 3-azidopropylamine as a three-carbon linker and TFC-007 as the POI ligand, an H-PGDS inhibitor. This PROTAC(H-PGDS) was recently reported by our group, and the results suggested that a shorter linker is ideal for its activity, which exemplifies the importance of diversifying the linker structure to improve degradation activity.[14,15] Initially, we used an aminomethyl polystyrene resin (**4**, 2.0–3.0 mmol g^{-1}, 50 mg) to acylate with 4-(4-formyl-3-methoxyphenoxy)butanoic acid (**5**) following a known method, and 3-azidopropylamine was introduced by reductive amination to afford resin **6**.[6] Progress of the reaction was monitored by the chloranil test.[16] After condensing the pomalidomide derivative (**7**) onto resin **6**, the desired intermediate resin **1** with the azide linker was obtained by cleavage from the resin, followed by LC-MS analysis.

Next, we performed copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC), amide formation, and urea formation reactions from the azide intermediate of resin **1**. Azide-alkyne cyclization was carried out between the azide intermediate and the TFC-007-alkyne derivative (**8**) by two cycles of coupling reaction at room temperature for 24 h in the presence of CuI (Scheme 1b). After cleavage from the resin and purification by RP-HPLC, **PROTAC 1** was isolated with 97% purity. The azide group of intermediate **1** on the resin was converted to the amino group (**2**) by the Staudinger reaction and followingly applied to amide-type PROTAC synthesis (Scheme 1b).[17] Accordingly, after reducing the azide group and subsequent condensation with TFC-007-CH_2CO_2H (**9**), the amide-type **PROTAC 2** was obtained with $>$99% purity by RP-HPLC purification. Urea formation between resin **2** and compound **10** using 4-nitrophenyl chloroformate was also achieved to obtain **PROTAC 3** with $>$95% purity. For the construction of PROTACs by amidation, it is necessary to assume the reverse type of amide formation, in which the amino group of the linker and the carboxy group of the POI ligand are swapped. Thus, another synthetic route was designed using allyl 3-aminopropanoate instead of 3-azidopropylamine for the carboxylic acid linker synthesis (Scheme 1c). Intermediate resin **3** was synthesized by condensation of compound **5** to resin **4**, reductive amination with allyl 3-aminopropanoate, condensation with compound **7**, and subsequent allyl deprotection by phenylsilane in the presence of $Pd(OAc)_2$ as the catalyst. After condensation with compound **10**, subsequent cleavage from the resin and RP-HPLC purification yielded the reverse amide-type **PROTAC 4** with 97% purity.

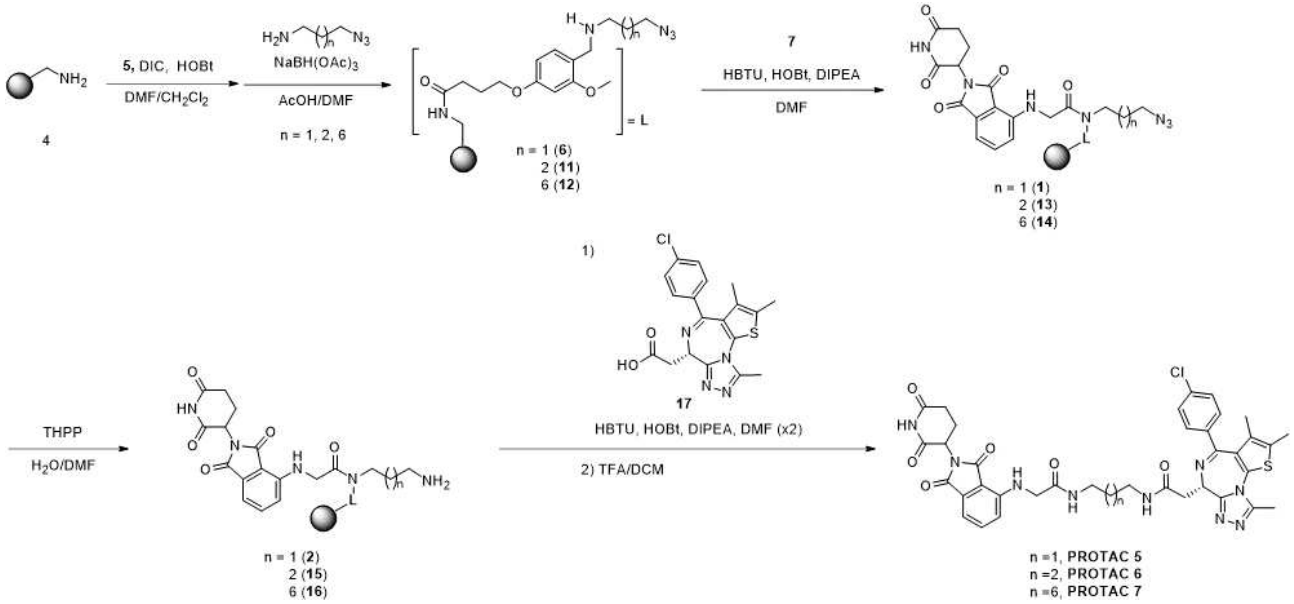
Next, we synthesized the amide-type PROTACs with different linker lengths. Because long linkers are not suitable for PROTACs(H-PGDS),[15] we chose dBET1[18] and dBET6[19] as a



Scheme 1. Solid-phase synthesis of PROTACs(H-PGDS). (a) Synthesis of intermediate resin **1**. (b) Synthesis of **PROTACs 1**–**3** from resin **1**. (c) Synthesis of **PROTAC 4** using resin **3**. Abbreviations: DIC: *N,N'*-diisopropylcarbodiimide; HOBt: 1-hydroxybenzotriazole; HBTU: 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium 3-oxide hexafluorophosphate; DIPEA: *N,N*-diisopropylethylamine; TFA: trifluoroacetic acid.

synthetic target. These are typical PROTACs with alkyl linkers that contain JQ1 as a POI ligand and degrade the target protein, bromodomain-containing 4 (BRD4).[20] A number of PROTACs targeting BRD4 have been reported for cancer therapy.[21] Most of them contain an alkyl linker,[18,19,22] while others are based on a PEG linker,[23] a photoswitchable azobenzene type,[24] or dihydropyrazine[25] formed by cyclization of *trans*-cyclo-octene with tetrazine. In the reported dBET1 and dBET6, thalidomide was used as the E3 ligand, but the C–O bond of thalidomide is possibly more unstable in buffer (pH 7.4) and human plasma than the C–N bond of pomalidomide.[26] Therefore, we decided to synthesize the pomalidomide-type dBET (**PROTACs 5**–**7**, Scheme 2). For the synthesis of **PROTACs 5**–**7**, in addition to 3-azidopropylamine, 4-azidobutan-1-amine, and 8-azidooctan-1-amine were synthesized separately and used as starting linkers (Scheme S4, Supporting Information). The synthetic route was the same as that used for **PROTAC 2**, with each linker attached to resin **4** to obtain resins **6**, **11**, and **12**. Subsequent condensation with compound **7** gave the azide intermediates **1**, **13**, and **14**, which upon further reduction afforded resins **2**, **15**, and **16**. Finally, (+)-JQ1-CO_2H (**17**) was coupled to the resins to obtain the target **PROTACs 5**–**7** with $>99\%$ purity.

We have successfully constructed azide-alkyne cycloadditions and amide bonds, which are used widely in synthesizing PROTACs, with efficient pathways and reaction conditions suitable for solid-phase synthesis. Thus, the rapid and straightforward synthesis of PROTACs by the solid-phase method improves the convenience of complicated PROTACs syntheses



Scheme 2. Solid-phase synthesis of **PROTACs**(BRD4) **5**–**7**. Abbreviation: THPP: tris(hydroxypropyl)phosphine.

by the liquid-phase method. In the reported solid-phase PROTAC synthesis,[6] the E3 ligand is bound to the Fmoc-protected amine, followed by Fmoc deprotection and finally the introduction of the POI ligand. In contrast, our method uses the azide moiety, which has high reaction tolerance as the binding motif for POI ligand linkage, and we succeeded in diversifying the linker and omitting the protection and deprotection steps.

Finally, we evaluated the degradation activity of the synthesized PROTACs against their respective target proteins by western blotting. The degradation activity of **PROTACs 1**, **2**, and **4** against H-PGDS was evaluated in KU812 cells. **PROTAC 1** with the triazole moiety was found to have the highest degradation activity against H-PGDS and comparable to the reported **PROTAC(HPGDS)-1** (Figure 2a). In contrast, **PROTAC 4**, which has an inverted amide bond, showed the lowest degradation activity. The degradation activity of **PROTACs 5**–**7** against BRD4 was evaluated in MV4-11 cells, and **PROTAC 7** with the longest linker showed the highest activity, which was higher than the reported dBET1 activities (Figures 2b and S1).

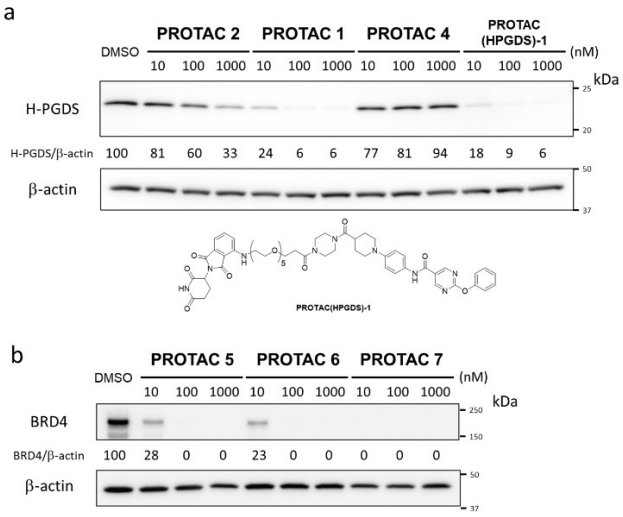


Figure 2. (a) H-PGDS protein reduction activity of **PROTACs 1, 2**, and **4** in *K*U812 cells. (b) BRD4 protein reduction activity of **PROTACs 5**–**7** in MV4-11 cells.

Conclusion

In summary, we have rapidly and easily synthesized PROTACs by a solid-phase method using various conjugation reactions. Triazole, amide, urea, and reverse amide formation reactions were exploited to synthesize PROTACs (H-PGDS). We have also synthesized pomalidomide derivatives of dBET as PROTACs with different alkyl-linker lengths and have shown that they exhibit potent degradation activity. The solid-phase PROTAC synthetic method presented in this study should facilitate the rapid development of PROTACs targeting various proteins.

Experimental Section

General Information

All chemicals were purchased from Sigma-Aldrich Co. LLC, Kanto Chemicals Co. Inc., Tokyo Chemical Industry Co. Ltd., Wako Pure Chemical Industries Ltd., and were used without further purification. Reactions were followed by thin-layer chromatography (TLC) (60 F254, Merck), and spots were visualized by UV irradiation with a handheld UV lamp (254/365 nm) (UVP) and iodine vapor or ninhydrin reagent. Silica gel for column chromatography was Kanto Chemical 60 N (spherical, neutral), NH silica gel (Chromatrex NH-DM1020, Fuji Silicia), or packed columns for medium pressure column chromatography (Hi-Flash column / Inject column Yamazen). Resin: Aminomethyl polystyrene resin (50 mg, loading 2.5 mmol g^{-1}). Reagent for acetaldehyde/chloranil tests: 2%

acetaldehyde in *N,N*-dimethylformamide (DMF), 2% chloranil in DMF. ^{1}H and ^{13}C NMR spectra were measured on an ECZ 600R spectrometer (JEOL) using deuterated solvents. Chemical shift values (ppm) were corrected for residual solvent signals as internal standards [DMSO-d_6: 2.50 for ^{1}H NMR, 39.5 for ^{13}C NMR; CD_3OD: 3.30 for ^{1}H NMR, 49.0 for ^{13}C NMR; $CDCl_3$: 7.26 for ^{1}H NMR, 77.2 for ^{13}C NMR]. The splitting modes of the signals are as follows (singlet (s), doublet (d), triplet (t), quartet (q), double of doublets (dd), multiplet (m), broad (br)). High-resolution mass spectrometry (HRMS) was measured by electrospray ionization using Shimadzu IT-TOF MS (Shimadzu).

Solid-Phase Organic Synthesis

Procedure for loading of the amide linker and 3-azidopropylamine onto a resin (synthesis of resin 6)

Loading of amide linker: Aminomethyl polystyrene resin (50 mg, loading 2.5 mmol g^{-1}) was swollen in CH_2Cl_2 (2 mL) for 30 min, washed with DMF (3×2 mL), neutralized in DMF/piperidine (5:1, 2 mL) for 30 min and washed with DMF (3×2 mL). Backbone amide linker of 4-(4-formyl-3-methoxyphenoxy)butanoic acid (90 mg, 0.375 mmol), 1-hydroxybenzotriazole (HOBt, 51 mg, 0.375 mmol) were dissolved in DMF/CH_2Cl_2 (1:1, 2 mL, *v/v*) and *N,N'*-diisopropylcarbodiimide (DIC, 59 μL, 0.375 mmol) was added. The resulting solution was added to resin. The reaction slurry was shaken at room temperature overnight, followed by wash with DMF (3×2 mL) and CH_2Cl_2 (3×2 mL). Acetaldehyde/Chloranil test confirmed the absence of amino groups.

Loading of 3-azidopropylamine: The resin intermediate was swollen in CH_2Cl_2 (2 mL) for 30 min, and washed with DMF (3×2 mL). The solution of 3-azidopropylamine (38 mg, 0.375 mmol) in DMF/AcOH (10:1, 2 mL, *v/v*) was added to resin and shaken overnight at room temperature. $NaBH(OAc)_3$ (80 mg, 0.375 mmol) in DMF/AcOH (20:1, 2 mL, *v/v*) was added to the reaction mixture for 4 h, followed by washing with DMF (3×2 mL). Acetaldehyde/Chloranil test confirmed the absence of amino groups.

Procedure for loading of pomalidomide onto a resin (synthesis of resin 1): Resin **6** (50 mg, loading 2.5 mmol g^{-1}) was swollen in CH_2Cl_2 (2 mL) for 30 min, and washed with DMF (3×2 mL). The mixture of compound **7** (124 mg, 0.375 mmol), HOBt (61 mg, 0.375 mmol), 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium 3-oxide hexafluorophosphate (HBTU, 143 mg, 0.375 mmol) and *N,N*-diisopropylethylamine (DIPEA, 97 mg, 0.75 mmol) in DMF was added to the resin and shaken overnight at room temperature, followed by washing with DMF (3×2 mL).

Procedure for reduction of the azide group of resin 1 (synthesis of resin 2): Resin **1** (50 mg, loading 2.5 mmol g^{-1}) was swollen in CH_2Cl_2 (2 mL) for 30 min, and washed with DMF (3×2 mL). Tris(hydroxypropyl)phosphine (THPP, 78 mg, 0.375 mmol) was dissolved in water/DMF (1:9, 2 mL, *v/v*) and added to the resin shaken overnight at room temperature, followed by washing with DMF (3×2 mL).

The resin **15** and **16** were synthesized from resin **4** in the same manner as resin **2**, using 4-azidobutan-1-amine for resin **15** and 8-azidooctan-1-amine for resin **16** instead of 3-azidopropylamine.

Procedure for the synthesis of PROTAC 1 by CuAAC: Resin **1** (50 mg, loading 2.5 mmol g^{-1}) was swollen in CH_2Cl_2 (2 mL) for 30 min, and washed with DMF (3×2 mL). The mixture of **8** (202 mg, 0.375 mmol), CuI (24 mg, 0.125 mmol) and *N,N*-diisopropylethylamine (97 mg, 0.75 mmol) in DMF was added to the resin and shaken for 24 h at room temperature, followed by washing with DMF (3×2 mL). After performing the above coupling reaction twice, the resin was suspended in a cleavage cocktail (50% trifluoroacetic acid (TFA) and 50% CH_2Cl_2) at room temperature for 1 h. The cleavage cocktail with combined wash was evaporated under a stream of nitrogen, the crude products were dissolved in MeOH (3 mL) and purified by HPLC (gradient: 40–80% MeCN-H_2O, 30 min) to give **PROTAC 1** (2.4 mg) in 1% yield.

Procedure for the synthesis of PROTAC 2 by amidation: Resin **2** (50 mg, loading 2.5 mmol g^{-1}) was swollen in CH_2Cl_2 (2 mL) for 30 min, and washed with DMF (3×2 mL). The mixture of compound **9** (204 mg, 0.375 mmol), HOBt (124 mg, 0.375 mmol), HBTU (143 mg, 0.375 mmol) and *N,N*-diisopropylethylamine (97 mg, 0.75 mmol) in DMF was added to the resin and shaken overnight at room temperature, followed by washing with DMF (3×2 mL). The resin was suspended in a cleavage cocktail (50% TFA and 50% CH_2Cl_2) at room temperature for 1 h. The cleavage cocktail with combined wash was evaporated under a stream of nitrogen, the crude products were dissolved in MeOH (3 mL) and purified by HPLC (gradient: 10–90% MeCN-H_2O containing 0.1% TFA, 30 min) to give **PROTAC 2** (2.1 mg) in 1% yield.

Procedure for the synthesis of PROTAC 3 by urea formation: Resin **2** (50 mg, loading 2.5 mmol g^{-1}) was swollen in CH_2Cl_2 (2 mL) for 30 min, and washed with DMF (3×2 mL). 4-Nitrophenyl chloroformate was dissolve in THF (1 mL) and stirred at −40 °C for 30 min and then added to the resin with *N,N*-diisopropylethylamine (97 mg, 0.75 mmol) and stirred for 24 h at room temperature. The mixture of compound **10** (182 mg, 0.375 mmol), *N,N*-diisopropylethylamine (97 mg, 0.75 mmol) and 4-dimethylaminopyridine (DMAP, 10 mg, 0.075 mmol) in THF/DMF (1 mL) was added to the resin and stirred for 16 h at room temperature, followed by washing with DMF (3×2 mL). The resin was suspended in a cleavage cocktail (50% TFA and 50% CH_2Cl_2) at room temperature for 1 h. The cleavage cocktail with combined wash was evaporated under a stream of nitrogen, the crude products were dissolved in MeOH (3 mL) and purified by HPLC (gradient: 40–80% MeCN-H_2O, 30 min) to give **PROTAC 3** (3.3 mg) in 1% yield.

Procedure for the synthesis of carboxylic acid resin 3: Resin **3** was synthesized same manner as resin **1** via compound **5** condensation to the resin **4**, reductive amination, compound **7** condensation, and following allyl deprotection by $Pd(OAc)_2$ catalyst with phenylsilane. The procedure for allyl deprotection is as follows; Resin after the compound **7** condensation (50 mg, loading 2.5 mmol g^{-1}) was swollen in CH_2Cl_2 (2 mL) for 30 min, and washed with DMF (3×2 mL). The mixture of palladium(II) acetate (5.6 mg, 0.025 mmol) and phenylsilane (270.5 mg, 2.5 mmol) in CH_2Cl_2 was added to the resin and shaken overnight at room temperature, followed by washing with DMF (3×2 mL) for 2 times.

Procedure for the synthesis of PROTAC 4 by amidation: Resin **3** (50 mg, loading 2.5 mmol g^{-1}) was swollen in CH_2Cl_2 (2 mL) for 30 min, and washed with DMF (3×2 mL). The mixture of compound **10** (182.5 mg, 0.375 mmol), HOBt (124 mg, 0.375 mmol), HBTU (143 mg, 0.375 mmol) and *N,N*-diisopropylethylamine (97 mg, 0.75 mmol) in DMF was added to the resin and shaken overnight at room temperature, followed by washing with DMF (3×2 mL). The resin was suspended in a cleavage cocktail (50% TFA and 50% CH_2Cl_2) at room temperature for 1 h. The cleavage cocktail with combined wash was evaporated under a stream of nitrogen, the crude products were dissolved in MeOH (3 mL) and purified by HPLC (gradient: 30–70% MeCN-H_2O, 30 min) to give **PROTAC 4** (1.2 mg) in 1% yield.

Procedure for the synthesis of PROTACs 5–7: The **PROTACs 5–7** were synthesized in the same manner as **PROTAC 2** synthesis by using compound **17** instead of compound **7**.

PROTAC 5: The crude product was dissolved in MeOH (3 mL) and purified by HPLC (gradient: 40–70% MeCN-H_2O, 30 min) to give **PROTAC 5** (9.3 mg) in 10% yield.

PROTAC 6: The crude product was dissolved in MeOH (3 mL) and purified by HPLC (gradient: 10–90% MeCN-H_2O, 30 min) to give **PROTAC 6** (1.5 mg) in 2% yield.

PROTAC 7: The crude product was dissolved in MeOH (3 mL) and purified by HPLC (gradient: 40–80% MeCN-H_2O, 30 min) to give **PROTAC 7** (3.2 mg) in 2% yield.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Keywords: BRD4 · H-PGDS · PROTAC · protein degradation · solid-phase synthesis

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