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A General Strategy for the Preparation of Thalidomide-Conjugate Linkers

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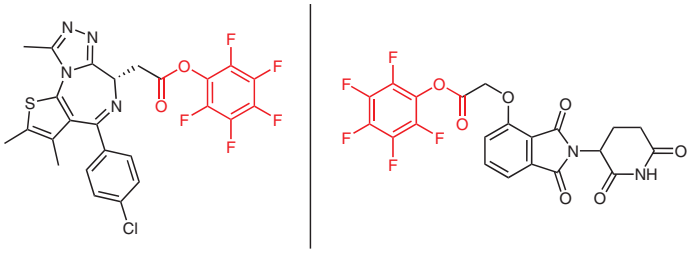
Dedicated to Victor Snieckus on the occasion of his 80th birthday



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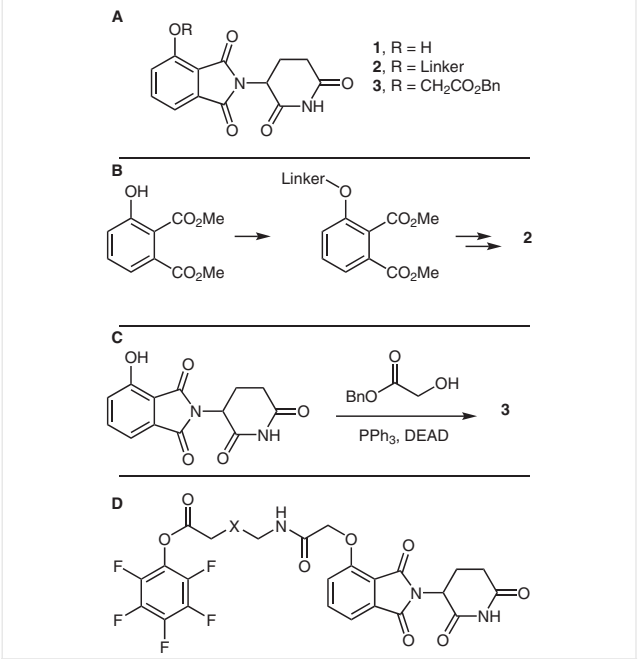
Abstract The synthesis of small-molecule linkers for installation of thalidomide-based conjugates is described. Linker properties have been recognized as vital to conjugate success in drug discovery and delivery systems. These small-molecule tethers act as linkages between molecules, can also aid in cell permeability, and act as solubilizing agents. This work shows our progress in synthesizing conjugates with a variety of linker characteristics. The adaptability and manipulation of these and other linkers holds potential in improving synthetic control of chemical connectivities toward therapeutic development.

Key words PROTAC, thalidomide, conjugates, linker, pentafluorophenyl ester

Since the seminal reports by Bradner[1] and Crews[2] on the use of thalidomide conjugation for in vivo protein degradation, our group and others have become interested in the synthetic preparation of small-molecule conjugates incorporating this moiety (Scheme 1, A).[3] As both linker length and composition have been shown to be essential for preparing functional conjugates,[1–5] we have worked to develop a sufficiently convergent synthetic strategy to prepare multiple linkers for structure–activity relationship studies.

A recent paradigm shift in proximity-directed protein degradation has seen the implementation of proteolysis targeting chimeras (PROTACs) as a means of inducing protein degradation.[4,6–8] PROTACs are small-molecule conjugates which enhance proximity between proteins of interest (POIs) through bifunctional targeting conjugates to initiate protein degradation.[4] Thalidomide derivatives have been successfully used as targeting ligands in previously published PROTAC work.[1,2,3b,9] Due to the importance of proximity enhancement for PROTACs, linker length between targeting molecules becomes vital to effective recruitment and positioning of POIs.



Scheme 1 4-Hydroxythalidomide derivative syntheses; X = CH_2 or $(CH_2)_2C(O)NH(CH_2)_3(OCH_2CH_2)_3$

Our initial approach to thalidomide conjugates relied on a highly linear synthesis from literature where the linker moiety needed to be introduced prior to glutarimide introduction (Scheme 1, B).[10] Our own attempts to improve the synthesis found that direct phenol alkylation of 4-hydroxy-

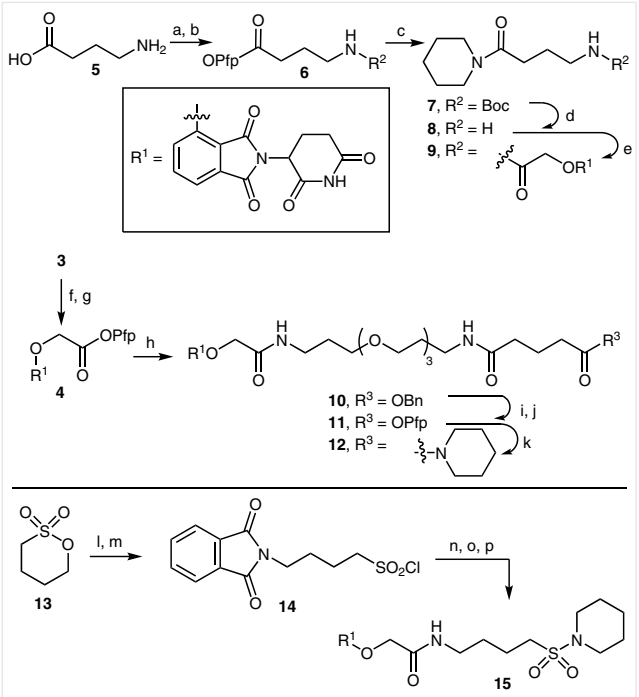
thalidomide (**1**) led to competing N-alkylation of the glutarimide moiety. We were working to make this synthesis more convergent when elegant work from Miller showed that the phenolic site of hydroxyl-thalidomide could be functionalized directly using modified Mitsunobu conditions (Scheme 1, C).[11] This advancement allowed us to synthesize our desired compounds in a much shorter time frame by making the synthesis much more convergent.

Our strategy for coupling conjugate fragments was heavily dependent on the use of pentafluorophenyl (Pfp) esters (Scheme 1, D) in order to form amide bonds, which were chosen specifically for their robust chemical properties.[12] Several other conjugation techniques, such as click chemistry[13] and selective nucleophilic aromatic substitution,[2] have also been reported in the literature.

The hydroxyl-thalidomide functionality (Scheme 1, A) suffers from poor chemoselectivity in alkylation reactions which make the synthesis of thalidomide conjugates problematic. While **1** appears stable under storage, its derivatives suffer from both acidic and basic instability resulting in cleavage of the phthalimide ring. This limitation severely restricts the chemistry compatible for conjugation. The resultant instability dictates which protecting groups could be employed based on the sensitivity of phthalimide ring opening during deprotection conditions. This undesirable reactivity is likely due to the increased electrophilicty of phthalimide carbonyls through hydrogen bonding with the phenolic hydrogen.[14] This requires the utilization of protecting-group chemistry stable to a variety of reactions and yet labile under neutral conditions, leading us to benzyl esters.

Central to this synthetic strategy was a short and facile synthesis of **3** following literature precedent. First, 3-hydroxyphthalic anhydride was reacted with 3-amino glutarimide, followed by thalidomide formation using dicyclocarbodiimide (DCC) in a 77% yield.[11] Phenol **1** was exposed to Mitsunobu reaction conditions to form the benzyl ester linkage onto the thalidomide intermediate to yield **3**. These initial steps were performed on multigram scales. Thalidomide derivative **3** was then hydrogenated to quantitatively deprotect the acid over Pd/C in DMF (Scheme 2). This reaction was easily monitored using TLC and complete conversion was observed after only one hour. The free acid was then converted into the active Pfp ester **4** using pentafluorophenyl trifluoroacetate, the products of which can be easily purified via trituration with diethyl ether.[15] Pfp ester **4** was found to be stable for prolonged storage and was then available for conjugation with a variety of amine containing compounds such as our model piperidine.[16]

First-generation linkers consisted of four carbon linkages following a system which had shown promising results in recently published work.[1] The primary amine of the simple building block, γ-aminobutyric acid (**5**) was Boc protected (Scheme 2). The remaining free acid was then transformed to reactive Pfp ester **6** using pentafluorophenyl trifluroacetate. Compound **6** was then coupled to piperidine under basic conditions to afford compound **7**. Intermediate **7** was exposed to trifluoroacetic acid (TFA) to liberate the terminal amine which was then exposed to **4**,[17] affording **9** in two hours at ambient temperature.[18]



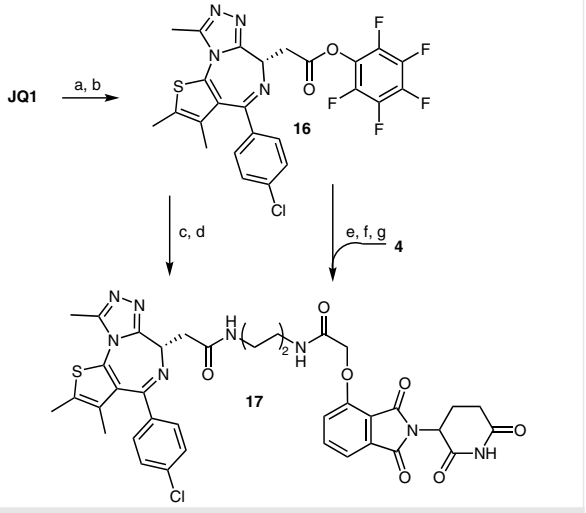
Scheme 2 Model conjugation of thalidomide linkers with secondary amine model compound - piperidine. *Reagents and conditions*: a) Boc_2O, DMAP, 99%; b) $CF_3C(O)OC_6F_5$, DIPEA, 78%; c) piperidine, DIPEA, quantitative; d) TFA, 98% e) **4**, DIPEA, 75%; f) H_2, Pd/C; g) $CF_3C(O)OC_6F_5$, DIPEA, 79% (2 steps); h) $NH_2CH_2CH_2CH_2(OCH_2CH_2)_3CH_2NHC(O)(CH_2)_3C(O)OBn$, DIPEA, 79%; i) H_2, Pd/C; j) $CF_3C(O)OC_6F_5$, DIPEA, 74% (2 steps); k) piperidine, DIPEA, 79%; l) potassium phthalimide; m) $SOCl_2$ 22% (2 steps); n) piperidine, Et_3N, 53%; o) hydrazine, 94%; p) **4**, DIPEA, 47%.

Our second generation of linkers was designed to investigate the synthetic differences in employing longer linkers with more polar functionalities, inspired by recent publications.[1,2,3b,9] We looked to derivatize the polyethylene glycol (PEG) diamine 4,7,10-trioxa-1,13-tridecanediamine. While this linker was considerably longer than the four-carbon alkyl linker, it was inherently much more water-soluble. Succinic anhydride was opened using benzyl alcohol and NaH to afford a monoprotected diacid. The remaining acid was then converted into an acid chloride in situ. The PEG diamine was first mono-Boc protected and then exposed to the activated acid to afford the desired intermediate consisting of two different protected terminal functionalities.

The amine pole was then deprotected using TFA, and the newly formed primary amine was reacted with **4** to form intermediate **10** which could also be easily purified by

trituration with diethyl ether. The remaining benzyl ester was hydrogenated over Pd/C in two hours to afford the free acid which was exposed to pentafluorophenyl trifluoroacetate to form the respective Pfp ester **11** in one pot (Scheme 2). Intermediate **11** was reacted with piperidine to afford conjugate **12** in a 79% yield.[23]

To expand the scope of linkages beyond simple amide bonds, sulfonamides were also explored. A Gabriel synthesis of 1,4-butane sultone (**13**) was performed, opening the ring to produce a straight chain linker with already established sulfonate and protected amine terminal motifs. The sulfonate salt was then converted into sulfonyl chloride **14**, which proved to be stable to flash chromatography (Scheme 2). Previous attempts to open the sultone ring were successfully performed using NH_4OH to yield a straight chain linker with a free primary amine which was Boc-protected. However, attempts at forming the sulfonyl chloride afforded low yields (6%), due to rapid intramolecular recyclization of the monoprotected amine. The Gabriel synthesis was employed as a means of introducing a doubly protected terminal amine in one step. Sulfonyl chloride **14** was then exposed to the model reaction conditions with piperidine to afford the desired sulfonamide in moderate yield. The phthalimide ring was then cleaved with hydrazine monohydrate to liberate the free terminal amine, which was reacted with **4** to yield conjugate **15**.[24]



Scheme 3 Syntheses of known PROTAC **17**. *Reagents and conditions*: a) HCO_2H, quantitative;[19] b) $CF_3C(O)OC_6F_5$, DIPEA, 56%;[20] c) 1,4-diaminobutane, DIPEA; d) **4**, DIPEA, 48% over two steps;[21] e) **4**, $H_2N(CH_2)_4NHC(O)OC(CH_3)_3$, DIPEA, 81%; f) TFA, quantitative;[11] g) DIPEA, **16**, 81%.[22]

In order to showcase the versatility of **4** in our synthetic strategy we employed this approach to synthesize a PROTAC reported by the Bradner group (Scheme 3).[1] We developed two novel synthetic routes for forming **17** via the Pfp ester of JQ1 (**16**). As this approach minimizes the amount of byproduct formation, this strategy allows purification by flash chromatography instead of HPLC as previously reported.[1]

Conjugates **9**, **12**, **15**, and **17** were successfully formed through a synthetic route with clear advantages over previous thalidomide-containing syntheses. One of the most beneficial characteristics of this linker strategy is the use of pentafluorophenyl trifluoroacetate in order to form respective Pfp esters. The ease of purification associated with simple trituration eliminates issues associated with isolation of highly polar compounds. In addition to the ease of synthesis and purification, this linker strategy can easily be tailored to accommodate a broad scope of targeting molecules. The adaptability of this synthetic strategy lends itself to the diverse linkers required for PROTAC synthesis. By varying the length, hydrophobic properties, as well as linkage identity we have developed a strategy for conjugating a broad scope of target molecules through a variety of functional groups. In vitro structure activity relationship studies are currently underway.

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Supporting Information

Supporting information for this article is available online at https://doi.org/10.1055/s-0036-1588539.

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(15) **General Procedure for the Synthesis of 4**
A solution of **3** (0.691 g, 1.64 mmol) in DMF (15 mL) was stirred with (Pd/C (0.04 g, 10 mol%) under hydrogen for 1.5 h, filtered, and concentrated in vacuo. The crude product (0.148 g, 0.45 mmol) was then redissolved in DMF (6 mL), DIPEA (0.118 g, 0.16 mL, 0.92 mmol) was added with stirring, and the solution was cooled to 0 °C. Pentafluorophenyl trifluoroacetate (0.187 g, 0.12 mL, 0.67 mmol) was then added with stirring. The reaction mixture was allowed to come to ambient temperature for 2 h. The mixture was concentrated in vacuo, and purified by trituration with Et_2O to afford 0.175 g (79%) of **4**.
^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.99 (s, 1 H), 7.76 (dd, *J* = 8.4, 7.3 Hz, 1 H), 7.63 (dd, *J* = 7.4, 0.7 Hz, 1 H), 7.26 (dd, *J* = 8.5, 0.8 Hz, 1 H), 5.34 (d, *J* = 1.4 Hz, 2 H), 5.04–4.97 (m, 1 H), 2.98–2.74 (m, 3 H), 2.22–2.14 (m, 1 H). ESI-HRMS: *m/z* calcd for $[C_{21}H_{11}F_5N_2O_7 + Na]^+$: 521.0379; found: 521.0368.

(16) No decomposition observed after 5 weeks at –20 °C.

(17) **General Procedure for Coupling to 4**
To a solution of corresponding free amine (1 equiv) in DMF was added DIPEA (3 equiv) under stirring. A solution of **4** (1.1 equiv) in DMF was added to the reaction mixture at ambient temperature. After 2 h the mixture was concentrated in vacuo and purified by silica gel flash chromatography to afford the title compound.

(18) **2-[(2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)oxy]-*N*-[4-oxo-4-(piperidin-1-yl)butyl]acetamide (9)**
Purified by silica gel flash chromatography (5% MeOH in $CHCl_3$) to afford 0.119 g (75%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.70 (s, 1 H), 7.75 (dd, *J* = 8.4, 7.3 Hz, 1 H), 7.71 (t, *J* = 5.6 Hz, 1 H), 7.57 (dd, *J* = 7.4, 0.7 Hz, 1 H), 7.23 (dd, *J* = 8.5, 0.7 Hz, 1 H), 5.00–4.92 (m, 1 H), 4.73–4.60 (m, 2 H), 3.58–3.52 (m, 2 H), 3.45 (ddd, *J* = 14.7, 7.4, 6.0 Hz, 1 H), 3.41–3.33 (m, 3 H), 2.98–2.70 (m, 3 H), 2.42–2.36 (m, 2 H), 2.22–2.15 (m, 1 H), 1.97–1.88 (m, 2 H), 1.66–1.60 (m, 2 H), 1.59–1.49 (m, 4 H). ^{13}C NMR (400 MHz, $CDCl_3$): δ = 170.80, 170.42, 168.04, 166.89, 166.54, 154.97, 137.01, 133.61, 120.51, 118.56 117.68, 77.20, 69.00, 49.42, 46.61, 42.78, 39.12, 31.34, 30.66, 26.48, 25.55, 24.84, 24.54, 22.73. EIS-HRMS: *m/z* calcd for $[C_{24}H_{28}N_4O_7 + H]^+$: 485.2031; found: 485.2047.

(19) **2-{4-(4-Chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl}acetic Acid (JQ1-Acid)**
JQ1 (0.05g, 0.1099 mmol) was dissolved in formic acid (4.5 mL) and stirred for 4 d. The solvent was removed in vacuo to afford a fine yellow powder. The product was used without purification.

(20) **Perfluorophenyl 2-{4-(4-Chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl}acetate (16)**
To a solution of JQ1-acid (0.055 g, 0.136 mmol) in DMF (2 mL) was added DIPEA (0.17 mL, 0.123 g, 0.952 mmol) and pentaflurophenyl trifluoroacetate (0.05 mL, 0.076 g, 0.272 mmol). The solution was stirred for 1 h and solvent was removed in vacuo. The residue was purified by silica gel flash chromatography (EtOAc/hexanes/THF = 1:1:1) to afford 0.0432 g (56%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.49–7.34 (m, 4 H), 4.71 (dd, *J* = 9.5, 4.6 Hz, 1 H), 4.08 (dd, *J* = 16.9, 9.5 Hz, 1 H), 3.91 (dd, *J* = 16.9, 4.6 Hz, 1 H), 2.73 (s, 3 H), 2.45 (d, *J* = 0.9 Hz, 3 H), 1.73 (d, *J* = 0.9 Hz, 3 H). ^{13}C NMR (151 MHz, $CDCl_3$): δ = 167.62, 164.38, 154.65, 150.13, 141.90 140.26, 138.70, 137.03, 136.30, 132.22, 131.00, 130.97, 130.26, 129.83, 128.76, 53.57, 36.39, 14.40, 13.11, 11.85. HRMS (MALDI): *m/z* calcd for $[C_{25}H_{16}ClF_5N_4O_2S + H]^+$: 567.0675; found: 567.0771.

(21) **2-{4-(4-Chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl}-*N*-[4-(2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl]oxy}acetamido)butyl]acetamide (17)**
To a solution of 1,4-diaminobutane (0.03 g, 0.371 mmol) in DMF (2 mL) was added DIPEA (0.065 mL, 0.048 g, 0.371 mmol) and **16** (0.021 g, 0.0371 mmol). The mixture was stirred for 1.5 h, concentrated in vacuo, and redissolved in DMF (1 mL). DIPEA (0.026 mL, 0.019 g, 0.0148 mmol) and **4** (0.028 g, 0.0557 mmol) were added and stirred overnight, The mixture was concentrated in vacuo and purified by silica gel flash chromatography (5–10% MeOH in $CHCl_3$) to afford 0.014 g (48%) of the title compound as a mixture of diastereomers. ^{1}H NMR (600 MHz, MeOD): δ = 8.34–8.29 (*m, 1 H), 8.12 (*q, *J* = 5.7 Hz, 1 H), 7.80 (dd, *J* = 8.4, 7.3 Hz, 1 H), 7.52 (d, *J* = 7.3 Hz, 1 H), 7.45–7.38 (m, 5 H), 5.10 (ddd, *J* = 12.4, 5.5, 3.1 Hz, 1 H), 4.77 (d, *J* = 1.7 Hz, 2 H), 4.63 (ddd, *J* = 9.1, 5.4, 1.1 Hz, 1 H), 3.44–3.32 (m, 4 H), 3.30–3.25 (m, 2 H), 2.86–2.77 (m, 1 H), 2.73–2.65 (m, 5 H), 2.43 (d, *J* = 2.5 Hz, 3 H), 2.10 (dddd, *J* = 10.7, 8.0, 4.9, 2.6 Hz, 1 H), 1.70–1.60 (m, 7 H); * exchangeable protons. ^{13} NMR (151 MHz, $CDCl_3$): δ = 174.4, 172.77, 172.68, 171.30, 171.27, 169.91, 168.24, 167.79, 166.24, 166.21, 157.00, 156.29, 152.19, 138.23, 138.09, 137.95, 134.87, 133.53, 133.18, 132.04, 132.02, 131.96, 131.31, 129.78, 121.90, 121.87, 119.37, 118.00, 69.57, 69.55, 55.23, 50.55, 50.54, 40.21, 40.19, 40.09, 40.06, 39.84, 38.88, 38.86, 32.14, 32.12, 27.77, 27.66, 27.64, 23.63, 23.61, 14.41, 12.92, 11.62. HRMS (MALDI): *m/z* calcd for $[C_{38}H_{37}ClN_8O_7S + H]^+$ = 785.2267; found: 785.2232.

(22) **2-{4-(4-Chlorophenyl)-2,3,9-trimethyl-6*H*-thieno[3,2-*f*][1,2,4]triazolo[4,3-*a*][1,4]diazepin-6-yl}-*N*-[4-(2-{[2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl]oxy}acetamido)butyl]acetamide (17)**
To a solution of the free amine **4** linker (0.021 g, 0.052 mmol) in DMF (1.5 mL) was added DIPEA (0.09 mL, 0.067 g, 0.5 mmol) and **16** (0.022 g, 0.039 mmol) and stirred overnight. The reaction mixture was stirred with K_2CO_3 (0.021 g) for 30 min, filtered, concentrated in vacuo, and purified by silica gel flash chromatography (5–10% MeOH in $CHCl_3$) to afford 0.025 g (81%) of the title compound as a mixture of diastereomers. Data are identical to ref. 22.

(23) ***N*-(1-{[2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl]oxy}-2-oxo-7,10,13-trioxa-3-azahexadecan-16-yl)-4-oxo-4-(piperidin-1-yl)butanamide (12)**
Purified by flash chromatography (5% MeOH in $CHCl_3$) to afford 0.011 g (79%) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$): δ = 9.43 (s, 1 H), 7.74 (dd, *J* = 8.4, 7.4 Hz, 1 H), 7.60 (t, *J* = 5.8 Hz,

1 H), 7.55 (dd, *J* = 7.5, 0.7 Hz, 1 H), 7.21 (dd, *J* = 8.4, 0.6 Hz, 1 H), 6.66 (t, *J* = 5.6 Hz, 1 H), 5.03–4.96 (m, 1 H), 4.65 (d, *J* = 2.7 Hz, 2 H), 3.67–3.64 (m, 4 H), 3.64–3.61 (m, 2 H), 3.58 (tt, *J* = 6.5, 2.7 Hz, 3 H), 3.53 (td, *J* = 5.7, 1.8 Hz, 4 H), 3.50–3.44 (m, 2 H), 3.43–3.39 (m, 2 H), 3.31 (qd, *J* = 6.4, 4.9 Hz, 2 H), 2.91–2.75 (m, 3 H), 2.66 (td, *J* = 6.7, 1.3 Hz, 2 H), 2.49 (t, *J* = 6.8 Hz, 2 H), 2.18–2.13 (m, 1 H), 1.87 (p, *J* = 6.4 Hz, 2 H), 1.82 (s, 1 H), 1.75 (q, *J* = 6.3 Hz, 2 H), 1.62 (td, *J* = 6.9, 4.9 Hz, 2 H), 1.58–1.48 (m, 4 H).^{13}C NMR (600 MHz, $CDCl_3$): δ = 172.57, 171.34, 170.13, 168.33, 166.69, 166.62, 154.61, 136.93, 133.62, 119.68, 117.33, 70.41, 70.40, 70.15, 70.06, 69.39, 68.73, 68.22, 49.35, 46.40, 42.90, 37.31, 36.49, 31.59, 31.46, 29.23, 28.97, 28.80, 26.30, 25.54, 24.48, 22.69. ESI-HRMS: *m/z* calcd for $[C_{34}H_{47}N_5O_{11}$ + Na]$^+$: 724.3163; found: 724.3168.

(24) **2-{[2-(2,6-Dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl]oxy}-*N*-[4-(piperidin-1-ylsulfonyl)butyl]acetamide (15)**
Purified by flash chromatography (5% MeOH in $CHCl_3$) to afford 0.016 g (47%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.08 (s, 1 H), 7.75 (dd, *J* = 8.4, 7.4 Hz, 1 H), 7.67 (s, 1 H), 7.56 (dd, *J* = 7.3, 0.7 Hz, 1 H), 7.20 (dd, *J* = 8.4, 0.7 Hz, 1 H), 5.05–4.98 (m, 1 H), 4.65 (d, *J* = 2.3 Hz, 2 H), 3.43 (sept, *J* = 6.9 Hz, 2 H), 3.29–3.12 (m, 4 H), 2.97–2.89 (m, 3 H), 2.89–2.71 (m, 2 H), 2.23–2.14 (m, 1 H), 1.99–1.87 (m, 2 H), 1.74 (dt, *J* = 14.4, 6.9 Hz, 2 H), 1.67–1.53 (m, 6 H). ^{13}C NMR (400 MHz, $CDCl_3$): δ = 170.82, 168.00, 166.95, 166.55, 154.65, 137.10, 133.55, 120.08, 118.46, 117.64, 77.21, 68.49, 49.38, 48.51, 46.64, 38.44, 31.40, 28.27, 25.67, 23.81, 22.57, 20.55. ESI-HRMS: *m/z* calcd for $[C_{24}H_{30}N_4O_8S$ + H]$^+$: 535.1857; found: 535.1853.