

## Efforts Toward Elucidating Thalidomides Molecular Target: An Expedient Synthesis of the First Thalidomide Biotin Analogue

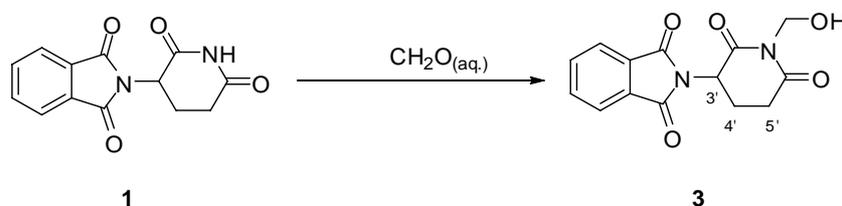
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**General:** All reactions were performed in flame dried glassware under an argon atmosphere unless stated otherwise. All reactions involving heating were immediately placed in an oil bath preheated to the specified temperature. Thin layer chromatography (TLC) was performed on Merck silica gel 60 F<sub>254</sub>, pre-coated aluminium sheets. Visualisation of developed plates was achieved through the use of a 254 nm or 365 nm UV lamp or staining with phosphomolybdic acid. Column chromatography was performed using silica gel 60 (0.063-0.200 mm), as supplied by Merck, with the eluents indicated. Proton (<sup>1</sup>H) and carbon (<sup>13</sup>C) NMR spectra were acquired on either a Bruker Advance (AV) 500 Spectrometer (500.13 MHz, 125.8 MHz, for <sup>1</sup>H and <sup>13</sup>C, respectively) at 25°C or a Bruker Advance (AV) 600 Spectrometer (600.1 MHz and 150.9 MHz for <sup>1</sup>H and <sup>13</sup>C, respectively) at 25°C. For <sup>1</sup>H and <sup>13</sup>C, CDCl<sub>3</sub> and [D<sub>6</sub>]acetone were used as solvents. Chemical shift are reported on a δ scale in ppm. Signals are quoted as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br (broad). <sup>13</sup>C assignments were aided with the use of DEPT135 analysis.<sup>23</sup> Mass Spectra were acquired on a VG AutoSpec instrument through electron impact ionisation (EI). HRMS was performed with a resolution of approximately 10,000. Infrared (IR) spectra were recorded with a PerkinElmer Spectrum One Spectrometer FT-IR spectrometer. Samples were analysed as thin films on NaCl discs, CHCl<sub>3</sub> solution between NaCl plates or pressed KBr plates.

### (*R,S*)-2-(1-(hydroxymethyl)-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (**3**)



(*R,S*)-2-(1-(hydroxymethyl)-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (**3**) was prepared following the procedure of Hess *et al.*<sup>14</sup> (*R,S*)-Thalidomide (**1**) (200 mg, 0.774 mmol) was added to an aqueous formaldehyde solution (1.55 mL, 35%) and stirred at reflux until the mixture became homogenous. The reaction mixture was then cooled to room temperature and

stirred for 24 h. After further cooling in an ice bath for 30 minutes, the mixture was filtered and the ensuing precipitate washed with an aqueous formaldehyde solution (3.5%, 2 x 5 mL) to afford the title compound **3** (165 mg, 74%) as a white solid. The spectral data for this compound matches that reported in the literature.<sup>14</sup>

**<sup>1</sup>H NMR** (400 MHz, *d*<sub>6</sub>-DMSO) δ 2.10 (m, 1H, H<sub>4'</sub>/H<sub>5'</sub>), 2.62 (m, 1H, H<sub>4'</sub>/H<sub>5'</sub>), 2.75 (m, 1H, H<sub>4'</sub>/H<sub>5'</sub>), 3.02 (m, 1H, H<sub>4'</sub>/H<sub>5'</sub>), 5.06 (d, *J* = 7.5 Hz, 2H, CH<sub>2</sub>OH), 5.27 (dd, *J* = 13.2 and 5.4 Hz, 1H, H<sub>3'</sub>), 6.20 (t, *J* = 7.2 Hz, 1H, OH), 7.26-7.90 (m, 4H, Ar-CH).

**(*R,S*)-2-(1-(chloromethyl)-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (**4**)**



Following the procedure of Hess *et. al.*<sup>14</sup> Thionyl chloride (0.38 mL, 5.27 mmol) was added dropwise to a stirred solution of hydroxymethylthalidomide **3** (555 mg, 1.92 mmol) in DMF (4.80 mL) and stirred at 0°C for 1 h. The mixture was then poured over iced water, the precipitate collected and recrystallised from ethanol to afford the chloroalkyl compound **4** as a white crystalline solid (410 mg, 70%). The spectral data matched that reported in the literature.<sup>14</sup>

**m.p.** = 195-197°C

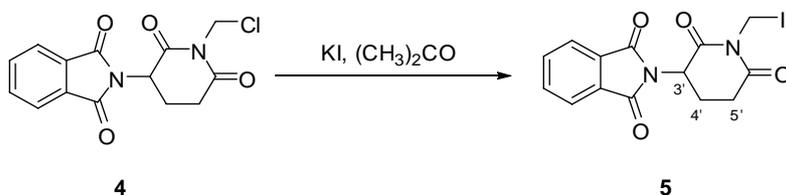
**<sup>1</sup>H NMR** (400 MHz, *d*<sub>6</sub>-DMSO): δ 2.12 (m, 1H, H<sub>4'</sub>/H<sub>5'</sub>), 2.57 (m, 1H, H<sub>4'</sub>/H<sub>5'</sub>), 2.88 (m, 1H, H<sub>4'</sub>/H<sub>5'</sub>), 3.06 (m, 1H, H<sub>4'</sub>/H<sub>5'</sub>), 5.36 (dd, *J* = 12.8 and 5.2 Hz, 1H, H<sub>3'</sub>), 5.52 (s, 2H, CH<sub>2</sub>Cl), 7.89-7.96 (m, 4H, Ar-H).

**<sup>13</sup>C NMR** (100.5 MHz, *d*<sub>6</sub>-DMSO): δ 20.7 (C<sub>4'</sub>/C<sub>5'</sub>), 31.0 (C<sub>4'</sub>/C<sub>5'</sub>), 47.2 (CH<sub>2</sub>Cl), 49.4 (C<sub>3'</sub>), 123.5 (2 x Ar-CH), 131.2 (2 x Ar-C), 135.0 (2 x Ar-CH), 167.0 (2 x C=O), 168.7 (C=O), 170.43 (C=O).

**MS EI, *m/z*** (%): 306 (12) [M]<sup>+</sup>, 271 [M-Cl]<sup>+</sup>, 172 (100), 104 (43).

**HRMS** calculated for C<sub>14</sub>H<sub>11</sub>ClN<sub>2</sub>O<sub>4</sub>: 306.0407; Found: 306.0408.

**(*R,S*)-2-(1-(iodomethyl)-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (**5**)**



Following the procedure of Hess *et. al.*<sup>14</sup> Potassium iodide (541 mg, 3.26 mmol) was added to stirred mixture containing chloroalkyl-thalidomide **4** (200 mg, 0.652 mmol) and acetone (6.52 mL) and stirred at reflux for 6 h. The solvent was then removed under reduced pressure and the residue dissolved in DCM (40 mL) and washed with sodium thiosulfate (2 x 2 mL).

The organic layer was concentrated under reduced pressure and the white residue recrystallised from acetonitrile to afford the iodoalkyl product **5** as a white crystalline solid (146 mg, 56%). The spectral data matched that reported in the literature.<sup>11</sup>

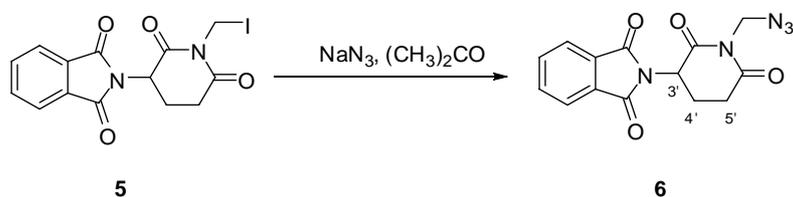
**m.p.** = 210-212°C.

**<sup>1</sup>H NMR** (300 MHz, CDCl<sub>3</sub>): δ 2.11-2.17 (m, 1H, H<sub>4'</sub>/H<sub>5'</sub>), 2.75-2.81 (m, 2H, H<sub>4'</sub>/H<sub>5'</sub>), 3.01-3.07 (m, 1H, H<sub>4'</sub>/H<sub>5'</sub>), 5.00-5.04 (dd, 12.6 and 5.4 Hz, 1H, H<sub>3'</sub>), 5.52 (s, 2H, CH<sub>2</sub>I), 7.76-7.79 (m, 2H, Ar-CH), 7.88-7.91 (m, 2H, Ar-CH).

**<sup>13</sup>C NMR** (75.5 MHz, *d*<sub>6</sub>-DMSO): δ 5.2 (CH<sub>2</sub>I), 21.7 (C<sub>4'</sub>/C<sub>5'</sub>), 32.1 (C<sub>4'</sub>/C<sub>5'</sub>), 50.3 (C<sub>3'</sub>), 124.0 (2 x Ar-CH), 131.8 (2 x Ar-C), 134.7 (2 x Ar-CH), 167.1 (2 x C=O), 167.3 (C=O), 169.6 (C=O).

**IR** (KBr, cm<sup>-1</sup>) *v*: 3474 (N-H), 3082, 1718 (C=O), 1388, 1139, 716.

**(*R,S*)-2-(1-(azidomethyl)-2,6-dioxopiperidin-3-yl)isoindoline-1,3-dione (**6**)**



Following the procedure of Hess *et al.*<sup>14</sup> Sodium azide (98 mg, 1.51 mmol) was added in one portion to a stirred solution of iodoalkylthalidomide **5** (300 mg, 0.753 mmol) in dry acetone (4.5 mL) and stirred at reflux for 24 h. The solution was then poured onto ice water and the white precipitate collected and recrystallised from ethanol to afford the azido compound **6** as a white crystalline solid (155 mg, 66%). The spectral data matched that reported in the literature.<sup>14</sup>

**m.p.** = 118-120°C.

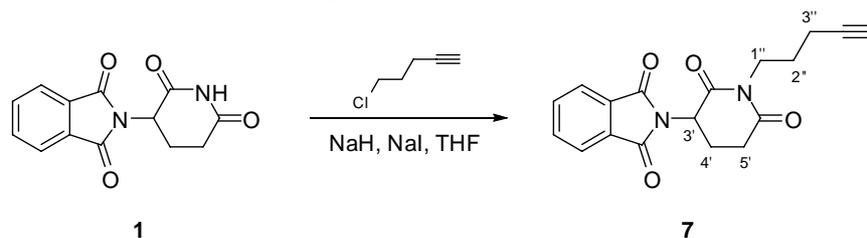
**<sup>1</sup>H NMR** (400 MHz, *d*<sub>6</sub>-DMSO): δ 2.13 (m, 1H, H<sub>4'</sub>/H<sub>5'</sub>), 2.60 (m, 1H, H<sub>4'</sub>/H<sub>5'</sub>), 2.83 (m, 1H, H<sub>4'</sub>/H<sub>5'</sub>), 3.03 (m, 1H, H<sub>4'</sub>/H<sub>5'</sub>), 5.08 (m, 2H, CH<sub>2</sub>), 5.36 (dd, *J* = 12.8 and 5.2 Hz), 7.85-7.96 (Ar-H).

**<sup>13</sup>C NMR** (75.5 MHz, *d*<sub>6</sub>-DMSO): δ 20.9 (C<sub>4'</sub>/C<sub>5'</sub>), 32.0 (C<sub>4'</sub>/C<sub>5'</sub>), 49.4 (C<sub>3'</sub>), 54.3 (CH<sub>2</sub>N<sub>3</sub>), 123.5 (2 x Ar-CH), 131.2 (2 x Ar-C), 134.9 (2 x Ar-CH), 167.0 (2 x C=O), 169.7 (C=O), 171.7 (C=O).

**IR** (KBr, cm<sup>-1</sup>) *v*: 3415 (N-H), 2102 (N<sub>3</sub>), 1691, 1392, 1193, 719.

**MS** EI, *m/z* (%): 314 (32) [M+H]<sup>+</sup>, 272 (13) [M+H-N<sub>3</sub>]<sup>+</sup>, 271 (100) [M-N<sub>3</sub>]<sup>+</sup>, 186 (38).

**(*R,S*)-2-(2,6-dioxo-1-(pent-4-ynyl)piperidin-3-yl)isoindoline-1,3-dione (7)**



Sodium hydride (57 mg, 1.41 mmol) was added to a stirred solution of (*R,S*)-thalidomide (**1**) (307 mg, 1.27 mmol) in DMF (2 mL) and stirred for 10 minutes. Sodium iodide (210 mg, 1.42 mmol) and 5-chloro-1-pentyne (150  $\mu$ L, 1.41 mmol) was then added simultaneously and stirred for 18 h at room temperature. As TLC analysis indicated unreacted starting material, an extra equivalent of sodium hydride and 5-chloro-1-pentyne was added and then stirred for a further 4 h at 40°C. The reaction mixture was then reduced *in vacuo*, dissolved in DCM and fused to silica before being subjected to column chromatography (2:3 ethyl acetate/hexane) to afford the title compound **7** as a white solid (138 mg, 36%). m.p. = 110-113°C.  $R_f$  = 0.27 (2:3 ethyl acetate/hexane).

**$^1\text{H NMR}$**  (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  1.78-1.80 (m, 2H,  $\text{H}_{2''}$ ), 1.98 (t,  $J$  = 2.4 Hz, 1H,  $\text{C}\equiv\text{CH}$ ), 2.10 (m, 1H,  $\text{H}_{4'}/\text{H}_{5'}$ ), 2.21-2.26 (m, 2H,  $\text{H}_{3''}$ ), 2.75 (m, 2H,  $\text{H}_{4'}/\text{H}_{5'}$ ), 2.98 (m, 1H,  $\text{H}_{4'}/\text{H}_{5'}$ ), 3.92 (m, 2H,  $\text{H}_{1''}$ ), 4.98 (dd,  $J$  = 12.8 and 5.2 Hz, 1H,  $\text{H}_{3''}$ ), 7.76 (m, 2H, Ar-H), 7.89 (m, 2H, Ar-H).

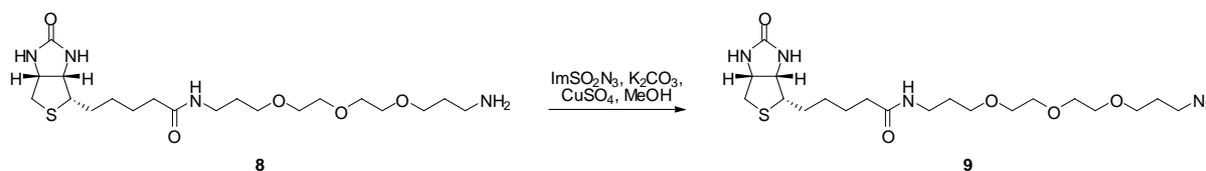
**$^{13}\text{C NMR}$**  (75.5 MHz,  $\text{CDCl}_3$ ):  $\delta$  16.4 ( $\text{C}_{2''}$ ), 22.1 ( $\text{C}_{4'}/\text{C}_{5'}$ ), 26.6 ( $\text{C}_{3''}$ ), 32.1 ( $\text{C}_{4'}/\text{C}_{5'}$ ), 39.9 ( $\text{C}_{1''}$ ), 50.2 ( $\text{C}_{3''}$ ), 68.8 ( $\text{C}\equiv\text{CH}$ ), 83.6 ( $\text{C}\equiv\text{CH}$ ), 123.8 (2 x Ar-CH), 131.8 (2 x Ar-C), 134.5 (2 x Ar-CH), 167.5 (2 x C=O), 168.7 (C=O), 170.9 (C=O).

**IR** (KBr,  $\text{cm}^{-1}$ )  $\nu$ : 3415 (N-H), 3275 ( $\text{C}\equiv\text{H}$ ), 2115 ( $\text{C}\equiv\text{C}$ ), 1710 (C=O), 1349, 1156.

**MS** EI,  $m/z$  (%): 324 (19)  $[\text{M}]^+$ , 296 (14), 173 (100).

**HRMS** calculated for  $\text{C}_{18}\text{H}_{16}\text{N}_2\text{O}_4$ : 324.1101; found: 324.1108.

***N*-(13-Amino-4,7,10-trioxatridecanyl)biotinamide (9)**



Imidazole-1-sulfonyl azide hydrochloride (28 mg, 0.13 mmol) was added to amine (**8**) (50 mg, 0.11 mmol),  $\text{K}_2\text{CO}_3$  (23 mg, 0.17 mmol) and  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (1 mg, 4  $\mu$ mol) in MeOH (2 mL) and the mixture stirred at room temperature (12 h). The mixture was concentrated and co-evaporated with PhMe ( $2 \times 5$  mL), followed by flash chromatography (MeOH/EtOAc, 3:17) to give (**9**) as a colourless solid (44 mg, 83%).

m.p. = 120-124 °C.

**$^1\text{H NMR}$**  (500 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  1.40-1.47, 1.55-1.85 (2m, 10H, H-1 $\square$ , H-1 $\square$ , H-2 $\square$ , H-2 $\square$ , H-3 $\square$ , H-3 $\square$ , H-7 $\square$ , H-7 $\square$ , H-14 $\square$ , H-14 $\square$ ), 2.20 (t, 1H, 7.2 Hz, H-4 $\square$ , H-4 $\square$ ), 2.70 (d, 1H,  $J$  = 12.7 Hz, H-1), 2.92 (dd, 1H,  $J$  = 5.0, 12.7 Hz, H-1), 3.19-3.22 (m, 1H, H-4), 3.25, 3.39 (2

x dd, 4H, H-6□, H-6□, H-15□, H-15□), 3.50-3.63 (4m, 12H, H-8□, H-8□, H-9□, H-9□, H-10□, H-10□, H-11□, H-11□, H-12□, H-12□, H-13□, H-13□), 4.30 (dd, 1H,  $J = 4.6, 7.7$  Hz, H-3), 4.49 (ddd, 1H,  $J = 0.7, 4.9, 7.7$  Hz, H-2).

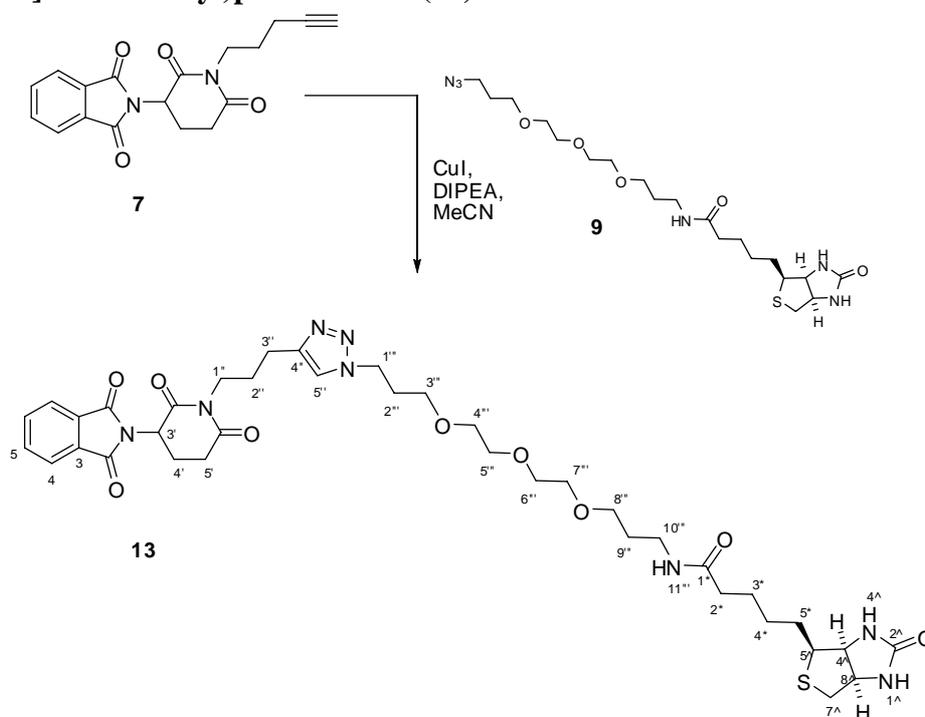
$^{13}\text{C}$  NMR (125.8 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  26.86, 29.50, 29.79, 30.17, 30.42 (C-1□, C-2□, C-3□, C-7□, C-14□), 36.86 (C-4□), 41.03 (C-1), 37.85, 49.11 (C-6□, C-15□), 56.99 (C-4), 61.63 (C-2), 63.39 (C-3), 68.94, 69.98, 71.26, 71.29, 71.55, 71.56 (6C, C-8□, C-9□, C-10□, C-11□, C-12□, C-13□), 166.09 (C-5), 175.96 (C-5□).

IR (film,  $\text{cm}^{-1}$ )  $\nu$ : 2111 ( $\text{N}_3$ ).

HRMS calculated for  $\text{C}_{20}\text{H}_{37}\text{N}_6\text{O}_5\text{S}$   $[\text{M}+\text{H}]^+ = 473.2546$ ; found  $[\text{M}+\text{H}]^+ = 473.2555$ .

ANALYSIS calculated for  $\text{C}_{20}\text{H}_{36}\text{N}_6\text{O}_5\text{S}$ : C, 50.83; H, 7.68. Found: C, 50.74; H, 7.71%.

**(*R,S*)-*N*-(2-(2-(2-(2-(4-(3-(3-(1,3-dioxisoindolin-2-yl)-2,6-dioxopiperidin-1-yl)propyl)-1*H*-1,2,3-triazol-1-yl)ethoxy)ethoxy)ethoxy)ethyl)-5-((3*aS*,4*S*,6*aR*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamide (13)**



Azido-PEG-biotin (**9**) (15 mg, 0.0360 mmol) was added in one portion to stirred solution of pentynyl-thalidomide **7** (12 mg, 0.0396 mmol), CuI (1 mg, 0.00396 mmol), diisopropylethylamine (7  $\mu\text{L}$ , 0.0396 mmol) in acetonitrile (2 mL) and the ensuing mixture stirred at room temperature for 18 h. TLC analysis indicated starting material remaining, thus the reaction mixture was heated to 40°C and stirred for a further 48 h. The resulting mixture was then fused to reverse phase silica and purified using gravity reverse-phase column chromatography (7:3 methanol/water) to afford the 1,2,3-triazole product (**13**) as a waxy white solid (27 mg, 99%).

$R_f = 0.31$  (7:3 methanol/water).

$^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ ): 1.41 (m, 2H,  $\text{H}_{4^*}$ ), 1.56-1.78 (m, 6H,  $\text{H}_{9^{***}}/\text{H}_{2^*}/\text{H}_{5^*}$ ), 1.95 (m, 2H,  $\text{H}_{2^{**}}$ ), 2.08-2.17 (m, 5H, ( $\text{H}_{4^*}/\text{H}_{5^*}/\text{H}_{2^{***}}/\text{H}_{3^*}$ )), 2.68-2.89 (m, 5H,  $\text{H}_{7^A}/\text{H}_{4^*}/\text{H}_{5^*}/\text{H}_{3^{**}}$ ), 2.87 (m, 1H,  $\text{H}_{7^A}$ ), 2.95 (m, 1H,  $\text{H}_{4^*}/\text{H}_{5^*}$ ), 3.12 (m, 1H,  $\text{H}_{5^A}$ ), 3.33 (m, 2H,  $\text{H}_{10^{***}}$ ), 3.41 (td,  $J = 6$  and 2.4

Hz, 2H, H<sub>3''</sub>), 3.52-3.63 (m, 10H, H<sub>4''</sub>/H<sub>5''</sub>/H<sub>6''</sub>/H<sub>7''</sub>/H<sub>8''</sub>), 3.86 (m, 2H, H<sub>1''</sub>), 4.29 (m, 1H, H<sub>1^</sub>), 4.42 (t, *J* = 7.2 Hz, 2H, H<sub>1''</sub>), 4.47 (m, 1H, H<sub>4^</sub>), 5.02 (m, 1H, C<sub>3'</sub>), 5.37 (s, 1H, H<sub>1^</sub>), 6.11 (s, 1H, H<sub>3^</sub>), 6.61 (m, 1H, NHCO), 7.44 (d, *J* = 3 Hz, 1H, H<sub>5''</sub>), 7.76 (m, 2H, Ar-CH), 7.87 (m, 2H, Ar-CH).

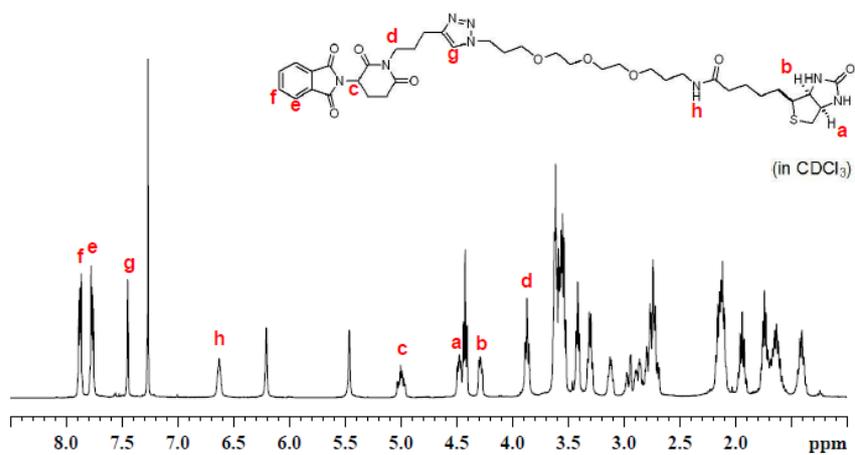
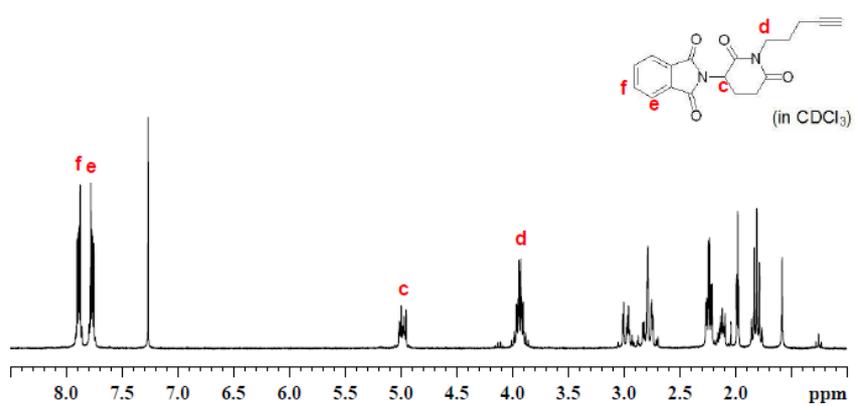
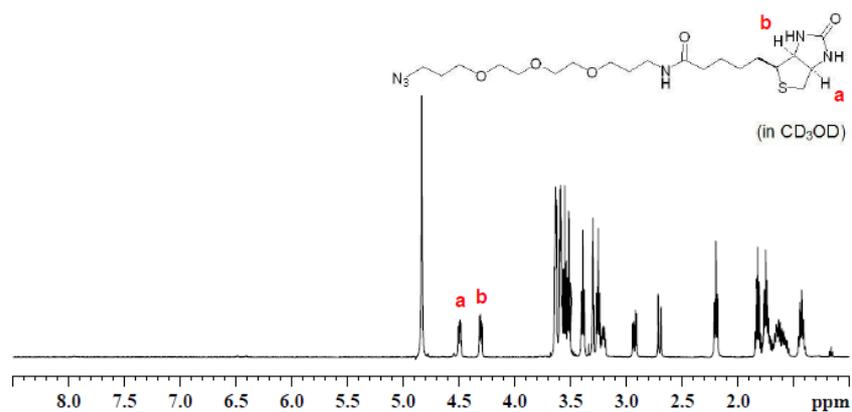
**<sup>13</sup>C NMR** (150.9 MHz, CDCl<sub>3</sub>): 22.12 (C<sub>4</sub>/C<sub>5</sub>), 23.2 (C<sub>2''</sub>/C<sub>3\*</sub>), 25.7 (C<sub>9''</sub>/C<sub>2\*</sub>/C<sub>5\*</sub>), 27.3 (C<sub>2''</sub>), 28.2 and 28.3 (C<sub>3''</sub>/C<sub>4\*</sub>/C<sub>9''</sub>/C<sub>2\*</sub>/C<sub>5\*</sub>), 29.1 (C<sub>9''</sub>/C<sub>2\*</sub>/C<sub>5\*</sub>), 30.4 (C<sub>9''</sub>/C<sub>2\*</sub>/C<sub>5\*</sub>), 32.1 (C<sub>4</sub>/C<sub>5</sub>), 36.1 (C<sub>9''</sub>/C<sub>2\*</sub>/C<sub>5\*</sub>), 37.8 (C<sub>10''</sub>), 40.2 (C<sub>1''</sub>), 40.7 (C<sub>7^</sub>), 47.1 (C<sub>1''</sub>), 50.3 (C<sub>3'</sub>), 55.7 (C<sub>5^</sub>), 60.2 (C<sub>8^</sub>), 61.9 (C<sub>4^</sub>), 67.4 (C<sub>3''</sub>), 70.6, 70.5, 70.3, 70.2, 70.11 (C<sub>4''</sub>/C<sub>5''</sub>/C<sub>6''</sub>/C<sub>7''</sub>/C<sub>8''</sub>), 121.6 (C<sub>5''</sub>), 123.8 (2 x Ar-CH), 131.0 (2 x Ar-C), 134.6 (2 x Ar-CH), 147.2 (C<sub>4''</sub>), 163.6 (C<sub>2^</sub>), 167.5 (C<sub>1</sub> and C<sub>3</sub>), 168.8 (C<sub>2'</sub>), 171.1 (C<sub>1'</sub>), 173.1 (NHCO, C<sub>1\*</sub>).

**IR** (NaCl, cm<sup>-1</sup>) *v*: 3285 (N-H), 2925, 1716 (C=O), 1680 (C=O), 1391, 1151 (C-O), 722.

**MS FAB**, *m/z* (%): 797 (100) [M+H]<sup>+</sup>, 796 (9) [M]<sup>+</sup>, 767 (12), 514 (45).

**FAB HRMS** calculated for C<sub>38</sub>H<sub>52</sub>N<sub>8</sub>O<sub>9</sub>S [M+H]<sup>+</sup> = 797.3578; found [M+H]<sup>+</sup> = 797.3633.

## $^1\text{H}$ NMR Comparison Study



## TNF Reporter Gene Assay

The effect of compound **13** on the inhibition of TNF expression and cellular viability was assessed using the FRT-Jurkat TNF reporter cell line. Analysis was done using a FACSCalibur 4-colour Flow Cytometer (Becton, Dickinson and Company, New Jersey, USA). Data analysis was performed using FlowJo software (TreeStar, Ashland, OR, USA). Viable and non-viable cells present following either solvent (DMSO) alone or compound **13** treatment for 24 hours, were assayed using flow cytometry. Cells were counted by gating on each cell population using a forward scatter (FSC) versus side scatter (SSC) plot. Inhibition was determined as a decrease in GFP fluorescence which was detected at a wavelength of 515 nm on the FL3 channel of the instrument. The percentage of viable cells was determined as the percentage of cells inside the FSC/SSC gate that encompassed the major population in solvent-only treated cells. Viability was confirmed following propidium iodide staining and detection of fluorescence at 570 nm on the FL2 channel.<sup>9, 10</sup>