Efforts toward elucidating Thalidomide's molecular target: an expedient synthesis of the first Thalidomide biotin analogue†

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Herein we describe the synthesis of the first Thalidomide–biotin analogue in order to initiate investigations into the unknown molecular mode of action of Thalidomide. In this manner we describe the attachment of biotin tether through the Huisgen 1,3-dipolar cycloaddition or "click" synthetic methodology.

Introduction

The history behind the pharmaceutical development of Thalidomide, [(*R*,*S*)-2-(2,6-dioxo-3-piperidinyl)-1*H*-isoindole-1,3(2H)-dione (**1**) (Fig. 1)] is perhaps one of the most recognized accounts of a pharmaceutical for chemists and biochemists alike. This drug, as a racemic mixture, was administered in the 1950's to pregnant women as a treatment for insomnia and an antiemetic agent for morning sickness. It was later discovered however to have side effects in that the *R*-isomer (at C3′) (*R*)-**1** was responsible for the sedative response and the *S*-isomer (*S*)-**1** had teratogenic properties.[1,2,3] As a result of these findings, in 1962, this commercially popular drug was withdrawn, but not before 10,000 infants with various limb malformations were born. Currently, both academia and the pharmaceutical industry have highlighted new and exciting areas in Thalidomide (**1**) research with the most notable being the use of (**1**) as a treatment for multiple myeloma, an as yet incurable form of bone marrow cancer. To that end, the pharmaceutical company Celgene have received FDA approval to use Thalidomide (**1**), (Thalomid™), and analogues Revlimid and Actimid, (both containing an aromatic amino functionality), for the treatment of multiple myeloma and erythema nodosum leprosum (ENL).[4]

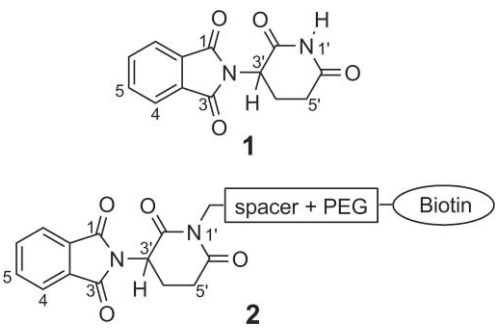

Fig. 1 Thalidomide and a generic target biotinylated Thalidomide analogue.

Despite being known for over 50 years, determining the precise mechanism of action of Thalidomide (**1**) has remained elusive.[5] There have been many reports of Thalidomide (**1**) and the influence of several cellular processes, for example the binding with α_1-acid glycoprotein (AGP), the interaction with g-rich proteins in FGF-2 gene, cyclooxygenase (COX-1 and 2) inhibition and possible protein kinase inhibition.[6] Another current hypothesis reported in the literature is that Thalidomide (**1**) inhibits in some manner the expression of the pro- inflammatory cytokine, tumour necrosis factor (TNF).[3,7] More specifically, the action of Thalidomide (**1**) is thought to involve the inflammatory NFκB signalling pathway, specifically inhibiting the activity of an IκB kinase, IKKα.[8] In an effort to elucidate the precise mode of action of Thalidomide (**1**), we envisaged the preparation of activity-based probe (Fig. 1), that if prepared, would allow us to gain insight and characterize Thalidomide's molecular target(s). This would also allow us to elaborate on our initial Thalidomide analogue studies[9,10] in being able to develop more potent compounds against a known biological target.

We felt that a biotinylated Thalidomide derivative based on **2** would suit the purpose of determining the precise mechanism of action of Thalidomide (**1**) as it contains the general design features that should be incorporated into molecules of this type. The Thalidomide fragment would act as a recognition element which would specifically bind to the molecular target(s) of interest. Biotin would act as a reporter that would permit rapid and sensitive detection of the cellular location of the probe and finally the polyethylene glycol (PEG) unit would act as a linker molecule between the reporter and the recognition element to increase solubility of the overall molecule in water and to ensure that the reporter does not interfere with binding to the target(s).

The placement of the PEG-linker was also chosen because of earlier analogue studies[3] by our group[9,10] and the pioneering work of Muller[11] and Hashimoto,[12] which suggest that functionalization of the left side phthalimide ring (phthaloyl) of Thalidomide results in compounds with improved biological activity.

Results and Discussion

In the retrosynthetic analysis of our biotin–Thalidomide analogue **2** we could foresee two methods to attach the PEG-biotin fragment, the first through a simple nucleophilic displacement and the second through "click" chemistry.[13] The synthesis of the three

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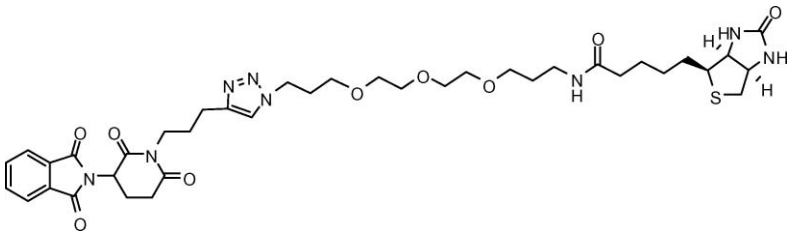
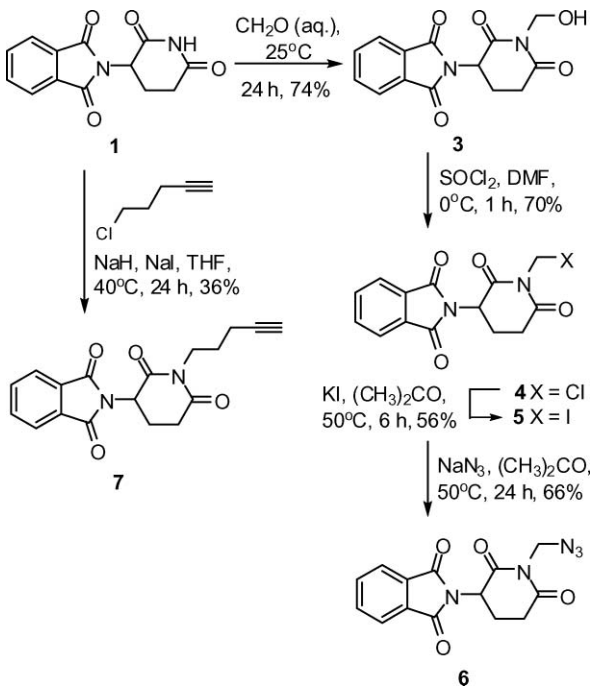
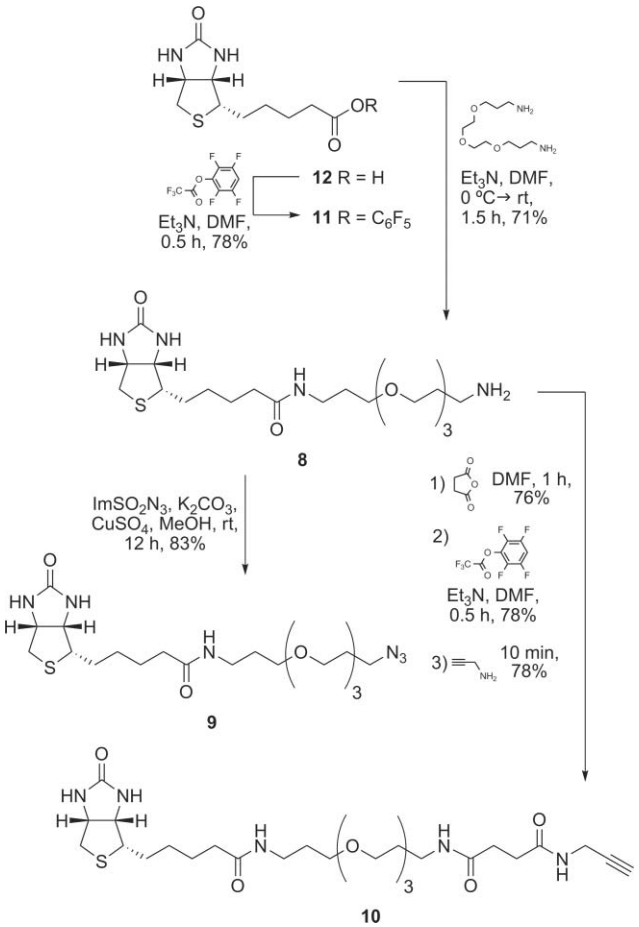


different Thalidomide analogues that could be putatively used to construct **2** are illustrated in Scheme 1. Following the procedure of Hess,[14] Thalidomide (**1**) was treated with an aqueous solution of formaldehyde to afford **3** in 74% yield and thus introducing a new hydroxymethylene group to the piperidine-2,6-dione ring.



Scheme 1 Synthesis of Thalidomide fragments



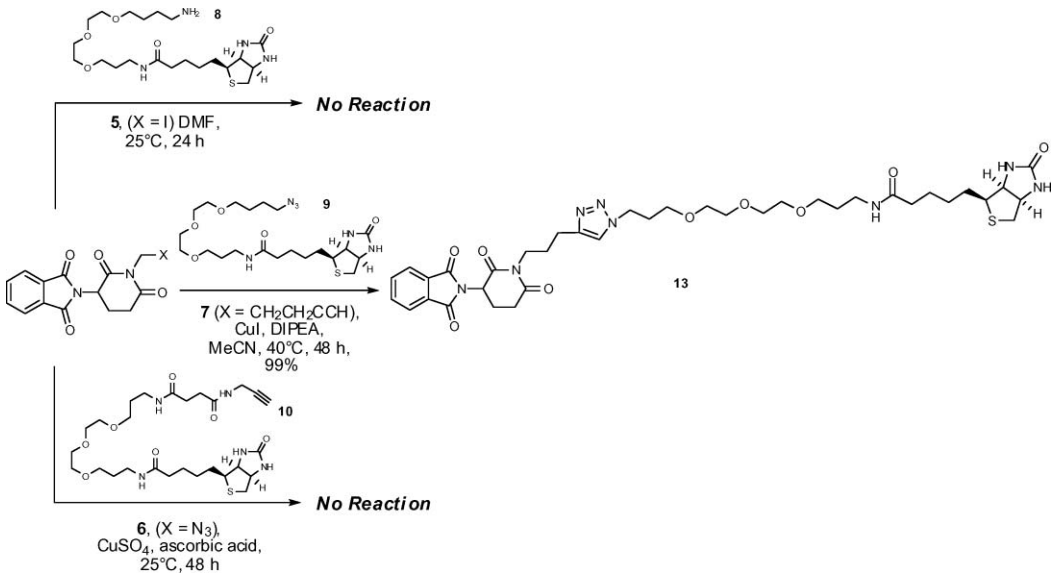
Scheme 2 Synthesis of biotin precursors

Treatment of alcohol **3** with thionyl chloride furnished the alkylchloride **4** in 70% yield. A Finkelstein reaction of this latter compound then produced the iodoalkyl compound **5** (56%).[14] Thalidomide derivative **5** served as one of the substrates for our key biotin–PEG attachment sequence.

Alternatively, one of the Huisgen cycloaddition precursors, alkyl azide **6**, was also synthesised following the procedure of Hess (see ESI†).[14] The last of our key Thalidomide fragments, compound **7**, was prepared following treatment of Thalidomide (**1**) with NaH, NaI and 5-chloro-1-pentyne. This secondary compound was devised to provide an alternative for the Huisgen 1,3-dipolar cycloaddition dienophile, where the point of attachment was considered more remote from the Thalidomide ring system.

The synthesis of the biotin fragments **8**,[15] **9** and **10**[16,17,18] (Scheme 2) were achieved using a divergent synthesis utilizing a known activated biotin ester **11**, which is readily prepared from biotin **12**.[16] Following this process the amine derivatized PEG linker **8** was prepared through amidation of **11** with the symmetrical amine 4,7,10-trioxa-1,13-tridecanediamine (71%).[19] Formation of the azide **9** from **8** proceeded in excellent yield (83%) using the novel diazotransfer reagent imidazole-1-sulfonyl azide.[20] The final biotin analogue, **10** was prepared according to the literature.[17]

The attachment of the two fragments (Scheme 3) through a simple S_N2 process proved problematic. When alkyl iodide **5** was treated with the biotin fragment **8** no reaction was observed even over long periods. Subjecting a similar substrate, compound **6**, to standard Huisgen 1,3-dipolar cycloaddition conditions ($CuSO_4$, ascorbic acid and alkyne **10**), also failed to provide any of the desired biotinylated product. It is assumed that the azide was not remote enough for the alkyne to approach in this instance. The final Thalidomide substrate **7**, containing a longer alkyne tether, was subjected to reaction with azido-PEG-biotin **9**. Under the "click" reaction conditions, CuI, diisopropylethyl amine, the



Scheme 3 Synthesis of Thalidomide biotin analogue

desired 1,4-triazole **13** was produced with complete regioselectivity and excellent yields (99%) as a mixture of diastereoisomers.[13f]

In many instances in the literature we found that many synthetically prepared biotinylated analogues were not rigorously identified spectroscopically. This may be in part due to their formation being carried out on a small scale or only in a reaction *in vivo*. In this described procedure we sought full characterisation prior to any further biological studies.

A comparison of the ^{1}H NMR of each of the cycloaddition substrates and the biotinylated Thalidomide indicates a signal attributed to the triazolic proton was calculated to be equal to the integral of the signal assigned to the H3′ proton of the Thalidomide backbone. Other key ^{1}H NMR resonances can be clearly associated with this new biotin Thalidomide derivative **13**, while the 1,5-triazole regioisomer is not observed. Additionally, a $[M+H]^+$ peak was observed at *m/z* 797 in the Fast Atom Bombardment (FAB) mass spectrum of compound **13**, whilst a lack of an absorption peak at 2115 cm^{-1} in the IR spectrum indicated the absence of any staring material (see supporting information and illustrated ^{1}H NMR spectra comparison, ESI†).

In this procedure, 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 µL, 0.0396 mmol) in acetonitrile (2 mL) and the ensuing mixture stirred at room temperature for 18 h. Following 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 (elution with 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}H NMR (600 MHz, $CDCl_3$): 1.41 (m, 2H, H_{4*}) 1.56–1.78 (m, 6H, $H_{9‴}/H_{2*}/H_{5*}$), 1.95 (m, 2H, $H_{2″}$), 2.08–2.17 (m, 5H, $(H_{4′}/H_{5′}/H_{2‴}/H_{3*})$, 2.68–2.89 (m, 5H, $H_{7^\wedge}/H_{4′}/H_{5′}/H_{3″}$), 2.87 (m, 1H, $H_{7^\wedge}$), 2.95 (m, 1H, $H_{4′}/H_{5′}$), 3.12 (m, 1H, $H_{5^\wedge}$), 3.33 (m, 2H, $H_{10‴}$), 3.41 (td, J = 6 and 2.4 Hz, 2H, $H_{3‴}$), 3.52–3.63 (m, 10H, $H_{4‴}/H_{5‴}/H_{6‴}/H_{7‴}/H_{8‴}$), 3.86 (m, 2H, $H_{1″}$), 4.29 (m, 1H, $H_{1^\wedge}$), 4.42 (t, J = 7.2 Hz, 2H, $H_{1‴}$), 4.47 (m, 1H, $H_{4^\wedge}$), 5.02 (m, 1H, $C_{3′}$), 5.37 (s, 1H, $H_{1^\wedge}$), 6.11 (s, 1H, $H_{3^\wedge}$), 6.61 (m, 1H, NHCO), 7.44 (d, J = 3 Hz, 1H, $H_{5″}$), 7.76 (m, 2H, Ar–CH), 7.87 (m, 2H, Ar–CH); ^{13}C NMR (150.9 MHz, $CDCl_3$): 22.12 ($C_{4′}/C_{5′}$), 23.2 ($C_{2‴}/C_{3*}$), 25.7 ($C_{9‴}/C_{2*}/C_{5*}$), 27.3 ($C_{2″}$), 28.2 and 28.3 ($C_{3″}/C_{4*}/C_{9‴}/C_{2*}/C_{5*}$), 29.1 ($C_{9‴}/C_{2*}/C_{5*}$), 30.4 ($C_{9‴}/C_{2*}/C_{5*}$), 32.1 ($C_{4′}/C_{5′}$), 36.1 ($C_{9‴}/C_{2*}/C_{5*}$), 37.8 ($C_{10‴}$), 40.2 ($C_{1″}$), 40.7 ($C_{7^\wedge}$), 47.1 ($C_{1‴}$), 50.3 ($C_{3′}$), 55.7 ($C_{5^\wedge}$), 60.2 ($C_{8^\wedge}$), 61.9 ($C_{4^\wedge}$), 67.4 ($C_{3‴}$), 70.6, 70.5, 70.3, 70.2, 70.11 ($C_{4‴}/C_{5‴}/C_{6‴}/C_{7‴}/C_{8‴}$), 121.6 ($C_{5″}$), 123.8 (2 x Ar–CH), 131.0 (2 x Ar–C), 134.6 (2 x Ar–CH), 147.2 ($C_{4″}$), 163.6 ($C_{2^\wedge}$), 167.5 (C_1 and C_3), 168.8 ($C_{2′}$), 171.1 ($C_{1′}$), 173.1 (NHCO, C_{1*}); IR (NaCl, cm^{-1}) υ: 3285 (N–H), 2925, 1716 (C═O), 1680 (C═O), 1391, 1151 (C–O), 722; MS FAB, *m/z* (%): 797 (100) $[M+H]^{\bullet+}$, 796 (9) $[M]^{\bullet+}$, 767 (12), 514 (45); FAB HRMS calculated for $C_{38}H_{52}N_8O_9S$ $[M+H]^{\bullet+}$ = 797.3578; found $[M+H]^{\bullet+}$ = 797.3633.

TNF Reporter Gene Biological Assay

The biotin-linked Thalidomide analogue **13** was initially assayed in the Jurkat T-cell line containing the GFP reporter construct (see ESI†) to determine whether the biotinylation has affected the parent drug's activity. At 10 and 100 µM concentrations, the biotin-linked Thalidomide analogue (**13**) showed no significant difference in TNF inhibition levels to that of (*R*,*S*)-Thalidomide (**1**) (Fig. 2). At 100 µM concentration, the biotin-linked analogue **13** displayed a 65% inhibition of TNF expression, comparable to 67% inhibition by that of (*R*,*S*)-Thalidomide (**1**). Despite these promising results, the observed DMSO-induced TNF inhibition is higher than previously observed. Some of the increased solvent-based inhibition is due to a 1% cell volume of DMSO being used in this assay. Increasing volumes of DMSO have been shown to cause inhibition of NFκB signalling and TNF formation in rat liver studies, which may explain why DMSO-induced TNF inhibition is observed.[21] Despite these DMSO-based discrepancies, the comparison of activity between the biotin-linked Thalidomide analogue **13** and (*R*,*S*)-Thalidomide (**1**) remains relatively unaffected. Thus, these results show that attachment of

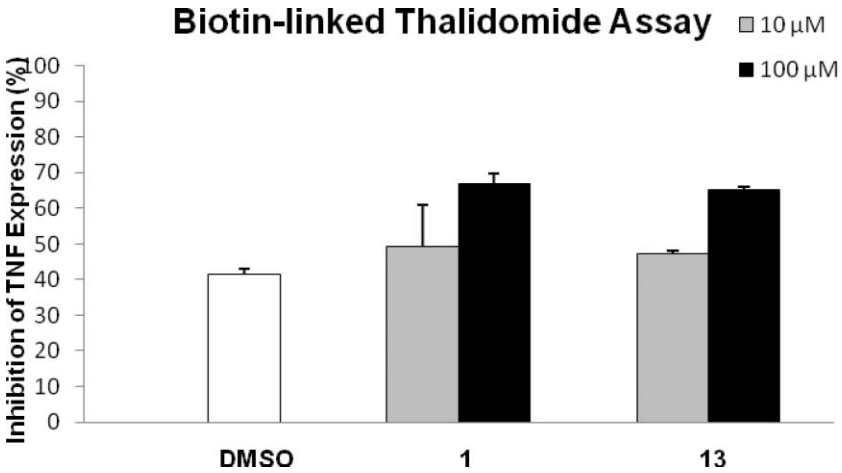


Fig. 2 Biological activity of the biotin-linked Thalidomide analogue (**13**) compared to (*R*,*S*)-Thalidomide (**1**).

a biotin fragment (**13**) to the piperidine ring does not significantly affect the biological activity of Thalidomide (**1**).

In conclusion, we have devised a short elegant route to a biotin derived Thalidomide analogue **13**. In this four step process we have used the a novel diazotransfer reagent to generate the biotin-azide fragment and a Huisgen 1,3-dipolar cycloaddition or click chemistry as the key reaction. Given that the production of **13** as a putative activity-based probe is viable it gives us the opportunity to investigate the localisation of Thalidomide and thus identify, in principle, this molecules cellular targets. Using fluorescein isothiocyanate (FITC)-conjugated streptavidin and confocal microscopy we envisage that we will visualise the cellular location of **13** and thus give insight into Thalidomide's (**1**) mode of action. What is also of interest to us, and currently under development, is an analogue of **13** incorporating a photoaffinity label.[22] Molecules such as these[6a,23] could be used in conjunction with streptavidin affinity-based chromatography[24] to elucidate whether Thalidomide (**1**) has multiple cellular targets.

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