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Bioorganic & Medicinal Chemistry Letters 17 (2007) 5819–5824
Bioorganic & Medicinal Chemistry Letters

Synthesis and TNF expression inhibitory properties of new thalidomide analogues derived via Heck cross coupling

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Received 25 July 2007; revised 19 August 2007; accepted 21 August 2007
Available online 25 August 2007

Abstract—A library of new thalidomide analogues containing an olefin functionality were synthesised using a Heck cross coupling reaction from their aryl halogenated precursor. All analogues were tested for their ability to inhibit the synthesis of the proinflammatory cytokine Tumour Necrosis Factor (TNF). Compounds **22**, **29**, **33** and **37** were the most effective in this assay inhibiting TNF expression 50%, 69%, 52% and 50%, respectively.

Since its discovery, thalidomide [(*R*,*S*)-2-(2,6-Dioxo-3-piperidinyl)-1*H*-isoindole-1,3(2H)-dione (**1**) (Fig. 1)] has had a tumultuous history as a medicinal agent. Administered in the 1950s as a treatment for insomnia and as an antiemetic agent, the racemic compound **1** was assumed to be non-toxic compared to the other existing sedative drugs, the barbiturates. Later investigations found that while the *R*-isomer (at C3′) (*R*)-**1** was responsible for the sedative effect the *S*-isomer (*S*)-**1** had teratogenic properties.[1–3]

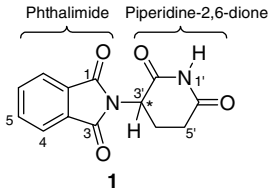

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Figure 1. Thalidomide **1**.

Alternative uses for thalidomide began in 1965, when Jacob Sheskin administered thalidomide (**1**) as a sedative to remarkably improve skin lesions of leprosy patients suffering from erythema nodosum leprosum (ENL).[4] In 1998, Celgene received FDA approval to use thalidomide (**1**) (Thalomid™) for the treatment of ENL.[5] More recently thalidomide (**1**) has been connected with the treatment of rheumatoid arthritis,[6,7] Behçet's disease,[7] Crohn's disease,[8] HIV/AIDS related illnesses,[8] mesothelioma,[9] and multiple myeloma.[3]

Thalidomide (**1**) appears to be a multi-target drug that impinges on a number of seemingly distinct cellular processes including peptidase inhibition, glucosidase inhibition, androgen receptor antagonism and more recently (cyclooxygenase) COX inhibition.[10] However, the precise mechanism of action of thalidomide (**1**) has been difficult to determine primarily due to its differing in vitro versus in vivo activities that are thought to result from the formation of numerous metabolic breakdown products in vivo. One of the most studied biological activities influenced by thalidomide (**1**), is the inhibition of the expression of the proinflammatory cytokine, tumour necrosis factor (TNF).[3] TNF is a central regulator of the inflammatory cascade controlling many effector pathways, the main being anti-angiogenic, anti-inflammatory and immunomodulatory. The molecular mode of action of thalidomide (**1**) on TNF expression is thought to involve the inflammatory NFκB signalling pathway, specifically inhibiting the activity of the IκB kinase, IKKα.[11] As part of our current study into small molecule inhibitors of TNF expression, we decided to screen new thalidomide analogues for enhanced ability to specifically inhibit the NFκB pathway.

Keywords: Thalidomide; Tumour necrosis factor (TNF); Heck reaction; Phthalimide; Cross coupling reaction; TNF reporter.
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0960-894X/$ - see front matter
doi:10.1016/j.bmcl.2007.08.042

A small number of thalidomide (**1**) analogues have been synthesised by attaching functional groups to the thalidomide (**1**) ring system. For example, the more water-soluble C5-carboxylic acid derivative of thalidomide displayed an improvement in (bFGF) growth factor inhibition. [12] In other assays, the C5-hydroxy-thalidomide caused a loss in activity when compared to the inhibition level of thalidomide (**1**) itself.[13] The two marketed drugs, Revimid™ and Actimid™,[5,14] containing amino groups bound to the left-hand aryl ring have shown excellent activity in several assays including TNF expression inhibition, and as such, have been marketed as immunomodulatory drugs (iMiDs).[3] Nevertheless, a literature search reveals few analogues which possess significant modifications of thalidomide's phthalimide ring system, other than by the addition of $-NH_2$, $-NO_2$, –OH and $-CO_2H$ functionalities at the C4 and C5 positions (**2**, Fig. 2). This paper illustrates the efficient production of a new series of thalidomide analogues, many containing olefin functionality, as well as the biological evaluation of these products using an assay involving the inhibition of expression of a TNF reporter gene.

As an accompanying part of this study, a second series of compounds were also targeted, those thalidomide derivatives containing a simplified aryl ring system instead of the piperidine-2,6-dione ring (**3**, Fig. 2). Such substituted *N*-phenylphthalimides are of high interest because they have been found to be inhibitors of TNF,[15] COX[10] and have tubulin binding properties.[16] At present none of the generic compounds **2** or **3** have been subjected to assays that specifically target NFκB signalling through the TNF promoter. This study also serves to obtain better knowledge of the as yet unknown mode of action of thalidomide (**1**) and related analogues.

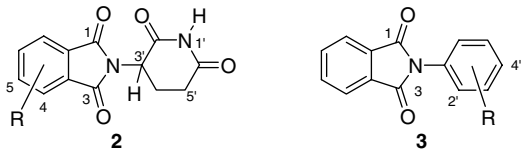
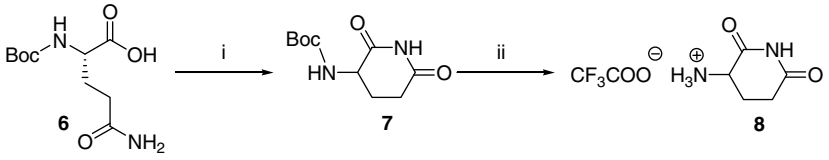


Figure 2. Target thalidomide analogues **2** and **3**.

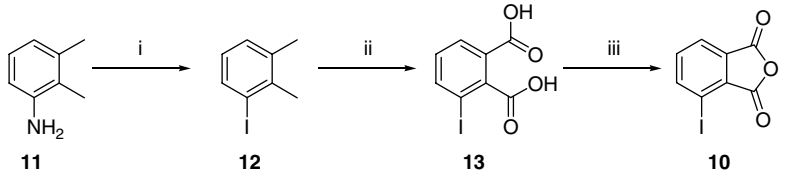
Two halogenated compounds **4** and **5** (Scheme 3) were targeted as starting materials in order to access a series of C4 and C5 analogues, which also contain the essential thalidomide (**1**) backbone. It was proposed that this aryl halogenation could serve as a handle for installation of other functional groups through utilisation of a Heck cross coupling reaction.[17] This modern transformation is not only mild, but highly selective, as it is compatible with a large range of functionalities as illustrated in its use in the total synthesis of a large number of more complex natural products.[18] Through the C–C cross coupling Heck reaction a myriad of commercially available olefins can be connected.

The synthesis of thalidomide (**1**) and related analogues began with the construction of the piperidine-2,6-dione ring. Thus, the reactive trifluoroacetic acid salt of aminoglutaramide **8** was synthesised in two steps from the commercially available *tert*-butoxycarbonyl-L-glutamide **6** in, ca. 72% yield, following the procedure of Muller or Brown (Scheme 1).[19] This ring system then served as a condensation partner for the phthalic anhydrides (**9**, **10** and**14**, Scheme 3). The iodinated phthalic anhydride **10** was synthesised from 2,3-dimethylaniline **11** (Scheme 2). Following a similar procedure to that of Kayser,[20] iodination of **11**, through a modified Sandmeyer protocol, provided iodobenzene **12** in reasonable yields (49%). A potassium permanganate mediated benzylic oxidation of **12** furnished the diacid **13** which upon treatment with acetic anhydride gave the C-4 iodophthalic anhydride **10**. The third condensation partner, 5-bromophthalic anhydride **9**, was purchased from Wako Chemicals.[21]

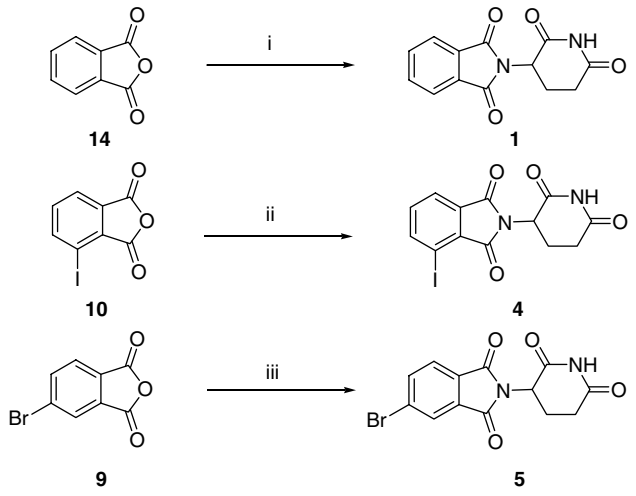
The condensation of phthalic anhydride (**14**) with the amine of the trifluoroacetic acid salt **8** provided thalidomide (**1**) in reasonable yields (55%) which was used as a standard in our TNF inhibition assay (Scheme 3). Sim-



Scheme 1. Synthesis of piperidine-2,6-dione **8**. Reagents and conditions: (i) CDI, 4-DMAP, THF, reflux, 24 h, 76%; (ii) 1:1 TFA/DCM, 22 °C, 1 h, 95% CDI, 1,1′-carbonyldiimidazole and TFA, Trifluoroacetic acid.



Scheme 2. Synthesis of 3-iodophthalic anhydride **10**. Reagents and conditions: (i) a—H_2O, HCl conc, $NaNO_2$, −15 °C; b—KI (aq), 49%; (ii) H_2O, $KMnO_4$, 80 °C, 4 days, 51%; (iii) Ac_2O, reflux, 3 h, 70%.

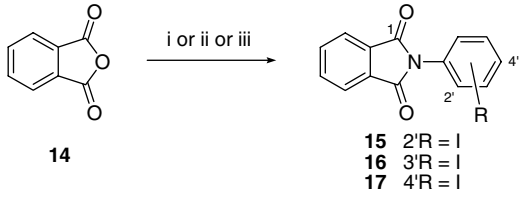


Scheme 3. Synthesis of thalidomide **1**, Iodo- **4** and bromothalidomide **5**. Reagents and conditions: (i, ii, iii) **8**, THF, Et_3N, 80 °C, 24 h, 49–61%.

ilarly, condensation of salt **8** and the corresponding iodinated phthalic anhydride **10** produced the phthalimide **4** in 61% yield. The final thalidomide (**1**) based Heck reaction precursor, 5-bromothalidomide **5**, was also produced through the same methodology in 49% yield.

The synthesis of the *N*-phenyl-phthalimides containing either the *ortho*-(**15**), *meta*-(**16**) or *para*-(**17**) iodide was carried out by condensation of phthalic anhydride and the respective anilines (Scheme 4). Generally, the yields of these condensations (60–88%) were higher than their amino piperidine-2,6-dione **8** counterparts.

The Heck reaction of the five precursors (**4**, **5**, **15**, **16** and **17**) and a range of olefins with various functionalities generally proceeded in an efficient manner to afford the alkenes **18** and **19** (Schemes 5 and 6).[22] The two catalytic conditions trailled were those developed by Fu,

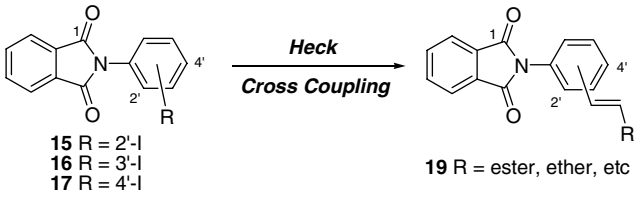


Scheme 4. Synthesis of the iodo-*N*-phenyl-phtalimides **15**–**17**. Reagents and conditions: (i) 2-Iodoaniline, toluene, 115 °C, 23 h, 60%; (ii) 3-Iodoaniline, toluene, 115 °C, 24 h, 88%; (iii) 4-Iodoaniline, toluene, 115 °C, 24 h, 53%.

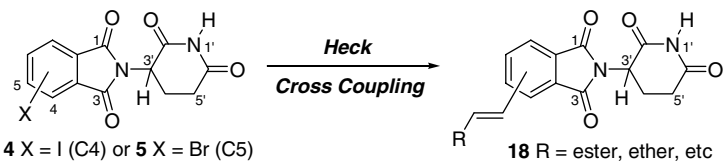
[$Pd_2(dba)_3$, $HP(t\text{-}Bu)_3BF_4$, Cy_2NMe] and Hermann/Beller [palladacycle, $(t\text{-}Bu)_4NOAc$].[23,17d,24] The olefins containing an electron-withdrawing group, as expected, produced larger quantities of the desired thalidomide analogues (ca. 70% yield). Advantageously, under these electron-rich phosphine catalytic conditions,[24] the electron-donating olefins also produced the desired analogues in reasonable yields (ca. 55%). Examination of general reactivity of all iodoaryl or bromoaryl substrates suggests that the most reactive substrate was the electron poor C-4 iodothalidomide **4**. Oppositely, the *ortho*-iodo-*N*-arylphthalic anhydride **15** is the least reactive, supposedly due to steric effects and slightly electron-rich nature of the iodoaryl ring.

To effectively measure inhibition of NFκB pathway signalling by each analogue, a TNF transcriptional reporter cell line was used. The green fluorescent protein (GFP) reporter gene, under the control of the NFκB-responsive human TNF promoter, was inserted into the genome of the human T cell line, Jurkat to generate a transcriptional reporter line, FRT-Jurkat TNF, as previously described.[25,26] GFP expression, as a measure of TNF promoter activity, was quantitated by flow cytometry by measuring the fluorescence intensity of individual cells. This method had the added advantage of being able to easily assess cellular toxicity of each compound, by comparing forward and side scatter (as a measure of cellular size and granularity) during flow cytometry. The cell population in each assay that exhibited low granularity was considered to be dead. This was confirmed by staining with propidium iodide. All thalidomide and *N*-phenylphthalimide derivatives were assayed in triplicate at concentrations of 100 μM and the percentage inhibition of TNF expression (relative to TNF expression from solvent treated control cells) for each compound measured.

The results of the biological screening illustrate many analogues which exhibited a higher inhibition of TNF expression than thalidomide (**1**). Compounds containing an olefinic attachment on the aryl ring of thalidomide as well as halogenated thalidomide precursors are shown in



Scheme 6. Heck reaction of *N*-phenylphthalimides **15**–**17**.



Scheme 5. Heck reaction of iodo and bromo thalidomides **4** and **5**.

Table 1. The data imply that an oxygen atom directly attached to the C4 or C5 olefin provides an enhanced inhibitory quality. When assayed at 100 μM concentrations substrate **22** containing a furan attachment at C4 displays a 50% TNF production inhibition while the *O*-Butyl analogue **23** shows a 47% inhibition. In comparison, thalidomide (**1**) at the same concentration has a 38% inhibition. Remarkably derivative **29**, containing an *O*-Butyl group at C5, shows astonishing TNF expression inhibition at 69%, however this compound, unlike all of the other derivatives, displays high cytotoxicity at 100 μM (Fig. 3).

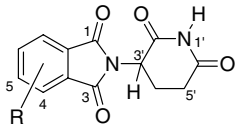
As a general rule, compounds containing an electron-withdrawing substituent at C4 or C5 in the form of an ester **20**, **26** or cyclic ketone **25** and **31** inhibited TNF expression to a similar to or slightly lesser extent than thalidomide (**1**) itself. Increasing the size of the C4 or C5 conjugated ester from CO_2Me to CO_2Bu (**20** → **21** or **26** → **27**) slightly improved the inhibitory activity. Interestingly, the halogenated analogues **4** and **5** (which could block carbon sites of further hydroxylation) failed to have any influence on the inhibition of TNF expression in comparison to thalidomide (**1**) itself. This result coupled with the other entries suggests that additional functionalities at C4 and C5 either enhance the inhibition of TNF expression or have the similar inhibitory qualities as thalidomide (**1**) itself.

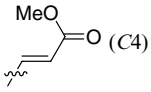
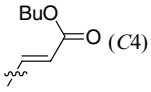
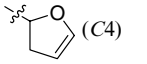
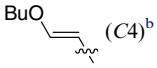
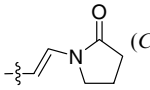
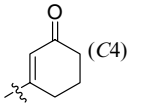
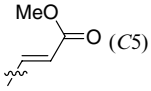
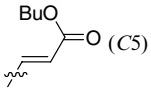
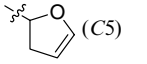
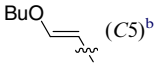
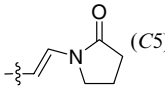
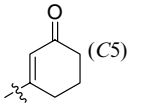
Derivatives of the *N*-arylphthalimide (Table 2) also provide valuable insight into the type of substitution require for enhanced inhibition of TNF expression. Specifically, *ortho* substitution (or C2′) provided enhanced inhibitory qualities when compared to thalidomide (**1**) at 100 μM. Iododerivative **15** and methyl or butyl esters (**32** and **33**) displayed a 40%, 48% and 52% inhibition respectively, compared to thalidomide (**1**) at 38% (Table 2). With these three groups the C2′ substitution provides a slightly better inhibitory activity as opposed to C3′ and C4′. This improvement mirrors results by Hashimoto[27] and Barreiro[15b] which have seen TNF expression reduction when examining the methyl and isopropyl derivatives of thalidomide (**1**).

Similarly, as with the first set of derivatives (Table 1), the vinyl *O*-butyl ethers (**34**, **38** and **44**) all have an improved activity when compared to thalidomide (**1**) itself. The *meta*-substituted 2-cyclohexen-1-one **40** also shows promising TNF inhibitory activity at 47%.[28]

Through our initial studies, the greatest inhibitor of TNF expression, vinyl butyl ether **29**, was selected for additional studies due to the large degree of cell death that was apparent when analysing the flow cytometry data (Fig. 3a). It should be noted that compound **29** was the only such compound in this study which displayed a high degree of cytotoxicity at 100 μM. To determine whether lower concentrations of compound **29** showed similar levels of TNF inhibition without the cytotoxic effects, the consequences of different doses over time were assessed. At the four concentrations tested (1, 10, 50 and 100 μM) it was clear that the inhibition of gene expression increased over time. At 30-h

Table 1. C4 and C5 thalidomide analogues and their inhibition of tumour necrosis factor (TNF) expression



Compound	R (carbon substitution)	% Inhibition[a,c]	*p* value[d]
Thalidomide **1**	H	38	—
4	I (*C4*)	37	0.1587
20	 (*C4*)	40	0.4961
21	 (*C4*)	44	*0.0068*
22	 (*C4*)	50	*0.0207*
23	 (*C4*)[b]	47	*0.0023*
24	 (*C4*)	38	0.2519
25	 (*C4*)	38	0.462
5	Br (*C5*)	35	0.1334
26	 (*C5*)	39	0.8013
27	 (*C5*)	42	0.2318
28	 (*C5*)	40	0.0542
29	 (*C5*)[b]	69	*0.0169*
30	 (*C5*)	36	*0.0441*
31	 (*C5*)	35	*0.0472*

[a] The assay was carried out at a concentration of 100 μM using the TNF promoter reporter line.

[b] Due to stereoselectivity of the Heck reaction as well as difficulties associated with isomer separation these compounds were tested as a mixture of 1,2-*E* and Z isomers, compound **23** *E*:*Z* – 2.6: 1; compound **29** *E*:*Z* – 2.9:1.

[c] All compounds were assayed in triplicate and as a racemic mixture.

[d] The *p* values which have a significant difference compared to thalidomide **1** ($p < 0.05$) are shown in italics. Significance was estimated using the unpaired Student's *t*-test.

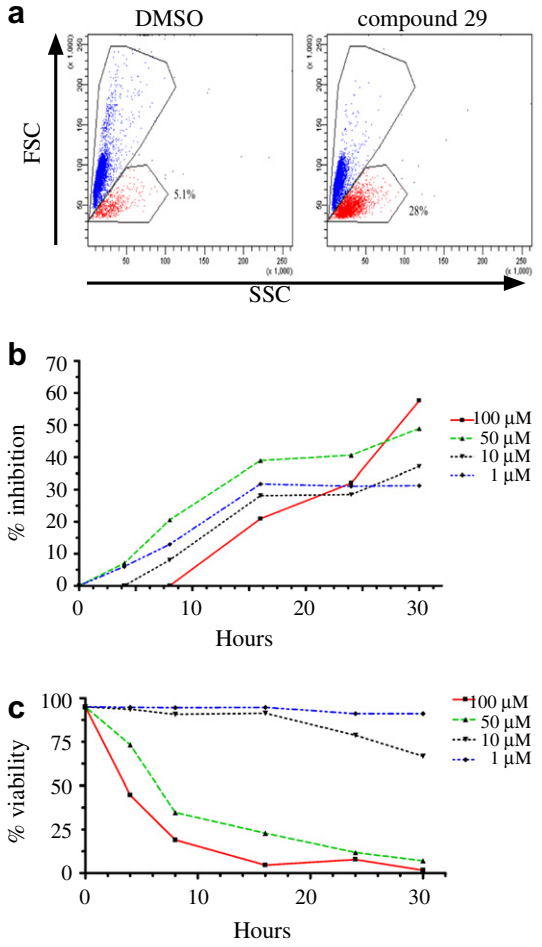
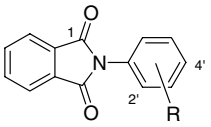


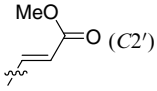
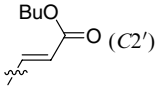
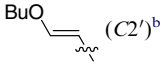
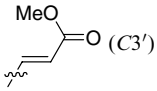
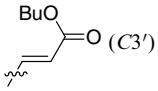
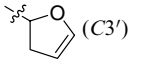
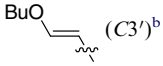
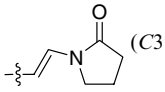
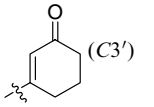
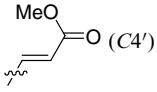
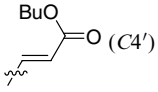
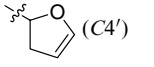
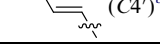
Figure 3. Biological effects of compound **29**. The effect of compound **29** on inhibition of TNF expression and cellular viability was assessed using the FRT-Jurkat TNF reporter cell line. (a) Viable and non-viable cells present following either the solvent dimethylsulfoxide alone (DMSO) or compound **29** treatment, were counted by gating on each cell population using a forward scatter (FSC) versus side scatter (SSC) plot. Cells were analysed using an EPICS XL™ flow cytometer (Beckman Coulter, Fullerton, CA, USA). Data analysis was performed using FlowJo software (TreeStar, Ashland, OR, USA). (b) Percentage inhibition of TNF expression over a 30-h time period was assessed following addition of compound **29** at different concentrations. Inhibition was determined as a change in GFP fluorescence which was detected at a wavelength of 515 nm on the FL3 channel of the instrument. (c) The percentage of viable cells was determined (as outlined in **A**) at the concentrations shown, over the 30 h compound **29** treatment period.

incubation, there was a dose-dependent increase in inhibition, although the percent inhibition at the lower two concentrations was not significantly different to thalidomide (**I**) itself (Fig. 3b). The effects of concentration of compound **29** on the levels of cellular viability also were investigated in the same experiment (Fig. 3c). The results indicated that at lower doses of compound **29** (1 μM and 10 μM), cellular viability was very high and was close to 100% at 1 μM. Investigations are underway to determine the mode of action of this unique derivative.

In summary, this study provides an excellent platform for constructing and testing new thalidomide analogues.

Table 2. C2′, C3′ and C4′ *N*-aryl phthalimide analogues and their inhibition of tumour necrosis factor (TNF) expression



Compound	R (carbon substitution)	% Inhibition[a,c]	*p* value[d]
1	Thalidomide	38	—
15	I (C2′)	40	0.3992
32	 (C2′)	48	*0.0101*
33	 (C2′)	52	*0.0013*
34	 (C2′)[b]	47	*0.0019*
16	I (C3′)	37	0.3000
35	 (C3′)	44	*0.0214*
36	 (C3′)	35	0.1018
37	 (C3′)	50	*0.0121*
38	 (C3′)[b]	49	*0.0052*
39	 (C3′)	36	0.4506
40	 (C3′)	47	*0.0041*
17	I (C4′)	36	0.2062
43	 (C4′)	41	0.1332
44	 (C4′)	35	0.1853
45	 (C4′)	38	0.4506
46	 (C4′)[b]	48	0.3198

[a] The assay was carried out at a concentration of 100 μM using the TNF promoter reporter line.

[b] Due to stereoselectivity of the Heck reaction as well as difficulties associated with isomer separation these compounds were tested as a mixture of 1,2-*E* and Z isomers, compound **34** *E*:*Z* – 5.2:1; compound **38** *E*:*Z* 2.1:1; compound **46** *E*: *Z* 1.5:1.

[c] All compounds were assayed in triplicate and as a racemic mixture.

[d] The *p* values which have a significant difference compared to thalidomide **1** ($p < 0.05$) are shown in italics. Significance was estimated using the unpaired Student's *t*-test.

This method for the analysis of thalidomide analogues provides an accurate, simple and more targeted method for the determination of the effects on signalling through the NFκB inflammatory pathway as measured by inhibition of TNF transcriptional activity, with the added advantage of concurrently assessing cytotoxicity of each derivative. The Heck cross coupling was proven to be an excellent method for the attachment of olefins to the thalidomide and phthalimide ring frameworks. Of the newly produced analogues the compounds **22**, **29**, **33** and **37** which had a TNF expression inhibitory activity 50%, 69%, 52% and 50%, respectively, showed the greatest potential.

At varying concentrations the *O*-Bu derivative **29** remains a better TNF expression inhibitor than thalidomide itself. At 10 μM the inhibition drops to 52% while thalidomide (**1**) is at 35%. At 1 μM, inhibition due to derivative **29** and thalidomide (**1**) is closer, at 32% and 31%, respectively.

Acknowledgments

The authors thank the University of Western Australia for the award of a UWA research grant. A scholarship for M.S. from Temasek Polytechnic is also gratefully acknowledged.

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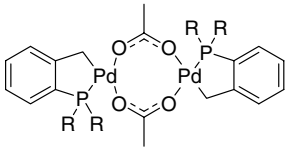
22. General Heck cross coupling procedure: (*E*)-methyl 3-(2-(2,6-dioxopiperidin-3-yl)-1,3-dioxoisoindolin-4-yl)acrylate (**20**). $Pd_2(dba)_3$ (11.9 mg, 0.013 mmol) was added to a solution of $HP(t\text{-}Bu)_3BF_4$ (7.5 mg, 0.026 mmol) and Cy_2NMe (32.5 μL, 0.15 mmol) in dioxane (0.5 mL), the solution was degassed and stirred at room temperature for 1 h. The resulting mixture was treated with phthalimide **4** (50 mg, 0.13 mmol), methyl acrylate (27.5 μL, 0.30 mmol) and heated to 50 °C for 3 h. The reaction mixture was cooled to room temperature, dissolved in $CHCl_3$ (60 mL), washed with saturated NH_4Cl (5× 1 mL), dried ($MgSO_4$), filtrated and concentrated under reduced pressure to afford dark brown solid. Subjection of this crude product to gradient flash chromatography (1:1 → 3:7 Hexane/EtOAc) and concentration of appropriate fractions produced desired ester **20** (33 mg, 73%) as a colourless solid. m.p. = 244–248 °C. $R_f = 0.16$ (1:1 hexane/EtOAc) IR (KBr, cm^{-1}) *v*: 1719.1 (C=O), 1642.5 (C=C), 1397.3, 1199.0 (O-CH_3), 748.6. ^1H NMR (500 MHz, DMSO-d_6): δ 2.03–2.10 (m, 1H, $H_{4'}/H_{5'}$), 2.50–2.64 (m, 2H, $H_{4'}/H_{5'}$), 2.85–2.94 (m, 1H, $H_{4'}/H_{5'}$), 3.77 (s, 3H, CH_3), 5.17 (dd, J = 12.8, 5.5 Hz, 1H, $H_{3'}$), 6.99 (d, J = 16.3 Hz, 1H, H_A), 7.87–7.98 (m, 2H, Ar-H), 8.36 (d, J = 7.6 Hz, 1H, Ar-H), 8.53 (d, J = 16.3 Hz, 1H, H_B), 11.14 (s, 1H, N-H). ^{13}C NMR (125.8 MHz, DMSO-d_6): δ 21.9, 30.9, 49.0, 51.9, 123.3, 124.7, 127.8, 132.0, 132.1, 132.3, 134.9, 136.8, 166.1, 166.5, 167.3, 169.8, 172.8. MS-FAB (*m*/*z*) (100): 343 (53) $[M + H]^+$, 311 (93) (M-CH_3O), 282 (28), 179 (27), 149 (100). $C_{17}H_{15}N_2O_6$ (342.31); HRMS-FAB-MS requires: $[C_{17}H_{15}N_2O_6 + H]^+$, 343.0930. found 343.0955.
23. Palladacycle in this case refers to *trans*–{di(μ-acetato)-bis[*ortho*-(di-*ortho*-tolylphosphanyl)benzyl]dipalladium (II) (Below) developed by Herrmann, W. A.; Broßmer, C. B.; Öfele, K.; Reisinger, C-P.; Priermeier, T.; Beller, M.; Fisher, H. *Angew. Chem.* **1995**, *107*, 1989; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 1844



R = *o*-Tol
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28. Unfortunately the Heck reaction involving 2-cyclohexen-1-one at the C2′ and C4′ positions only resulted in trace amounts of the desired olefins.