Absolute Stereochemical Assignment and Fluorescence Tuning of the Small Molecule Tool, (–)-Blebbistatin

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Keywords: Inhibitors / Asymmetric synthesis / Fluorescence

(–)-Blebbistatin (**1**), a recently discovered small molecule inhibitor of the ATPase activity of non-muscle myosin II has been prepared from methyl 5-methylanthranilate (**6**) in three steps. This flexible synthetic route has also been used to prepare a nitro group-containing analogue **12** that has modified fluorescence properties and improved stability under microscope illumination. The key step in the synthesis of **1** and its analogues was the asymmetric hydroxylation of the quinolone intermediate **3** using the Davis oxaziridine methodology. The absolute stereochemistry of (–)-blebbistatin (**1**) was shown to be *S* by X-ray crystal structure analysis of a heavy atom (bromine) containing analogue **11**, which was subsequently reduced and shown to be identical to **1**.

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Recent developments at the chemistry–biology interface have revitalised the search for specific small molecule modulators of protein function.[1] These molecules, once discovered and with their activity optimized, play an important role in improving our understanding of complex biological processes.[2] For example, a programme to identify selective inhibitors of the myosin protein subfamilies[3] has led to the discovery of several novel biological reagents including (–)-blebbistatin (**1**) (Figure 1).[4,5] In the two years since its discovery **1** has had a significant impact on biomedical research, with reports of its use in dissecting the mechanism of cancerous cell migration,[6] severing-induced axon retraction[7] and cell cycle control.[8] In a recent application,[4] **1** was used to study the role of non-muscle Myosin II (nmMII) in cytokinesis, the final and critical stage in the complex process that leads to the formation of two daughter cells from one mother cell. (+)-Blebbistatin (**2**) (the enantiomer of **1**) is also an important compound. As a result of its reduced activity against nmMII, it can function as a control compound in these types of studies.[4] To date, no experimental evidence that supports the assignment of the absolute stereochemistry of **1** has been reported despite its prominent role as a novel molecular tool. Unambiguous assignment of the absolute stereochemistry of (–)-blebbistatin (**1**) is also essential for the refinement of computational

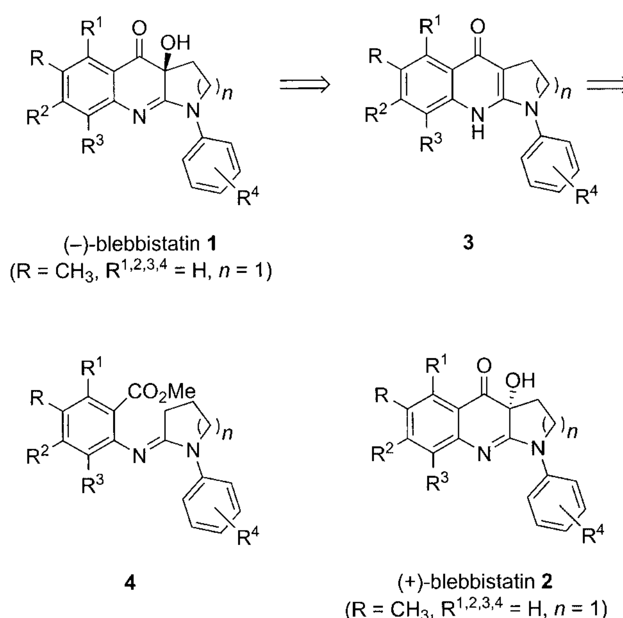

Figure 1. A flexible approach to (–)-blebbistatin (**1**) and its analogues.

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Supporting information for this article is available on the WWW under http://www.eurjoc.org or from the author.

 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim DOI: 10.1002/ejoc.200500103

models of the interaction of **1** and **2** with myosins.[9] These studies are designed to identify putative binding sites.

Here we clarify several chemical issues relating to (–)-blebbistatin (**1**) and its analogues. We describe an efficient and flexible synthetic approach to highly optically enriched (–)-blebbistatin (**1**) and prove, for the first time, that the absolute stereochemistry of (–)-blebbistatin (**1**) is *S* (Figure 1). In addition, we report that incorporation of a nitro functional group into the blebbistatin core structure improves the physical properties of blebbistatin, extending the utility of these reagents. The nitro analogue of **1** is not fluorescent (Figure 5) and is also stable for extended periods of time at wavelengths typically used for the fluorescence-based imaging of live cells, unlike (–)-blebbistatin **1** (Figure 6).[10]

Our approach to (–)-blebbistatin (**1**) relies on late stage asymmetric oxidation of the quinolone **3** to the previously unknown heterocyclic core structure in **1** (Figure 1). It was envisaged that conversion of **3** to **1** could be carried out using Davis oxaziridine methodology.[11] **3** can be accessed rapidly from commercially available starting materials via the corresponding amidine **4**.

Reaction of the pyrrolidinone **5** with the anthranilate **6** in the presence of phosphorus oxychloride gave the desired amidine **4** in 41% yield (Figure 2). ^{1}H NMR analysis of the reaction between **5** and $POCl_3$ did not support the efficient formation of an imonium intermediate.[12] Reaction of **5** and **6** under microwave irradiation in the absence of $POCl_3$ did not produce the desired amidine **4** even in the presence of a dehydrating agent. Attempts to optimize this reaction by addition of base[12] or by the use of alternative reagents ($SOCl_2$, MeOTf, PCl_5) proved unsuccessful. The pyrrolidinone dimer **7** was also isolated on a number of occasions.[13] Cyclisation of **4** to the desired quinolone **3** was carried out using excess lithium bis(trimethylsilyl)amide (LiHMDS) in high yield. The quinolone **3**, an off-white solid, was found to be stable in the absence of air and light, and was readily prepared and stored for extended periods of time in multigramme quantities. However, it was observed that a DMSO solution of **3** slowly decomposed in air in the presence of light to give (±)-blebbistatin. The rate and yield of this process was increased by irradiation of **3** in DMSO or THF using a medium-pressure mercury lamp (400 W, unfiltered) or upon irradiation (365 nm) of **3** supported on silica gel. Interestingly, (±)-blebbistatin was initially discovered following biological assays carried out with an "aged" DMSO solution of **8** (Figure 2). The azatacrine[14] analogue **8** is not an inhibitor of nmMII and the observed activity of the "aged" sample results from degradation of **8** to (±)-blebbistatin.

As a result of the above observations care was taken to exclude any oxygen from the subsequent oxidation reactions. In initial experiments, treatment of the anion generated from **3** using LiHMDS or LDA with oxaziridine **9** or **10** at –10 °C or 0 °C, respectively, gave high yields of optically enriched (–)-blebbistatin (**1**) (Figure 3, Entries 1–3).[11] However, little or no reaction was observed when these transformations were carried out at temperatures of –40 °C or below. Generation of the sodium enolate of **3** using NaHMDS resulted in a more reactive enolate that reacted with **10** at –78 °C to give highly optically enriched (–)-blebbistatin (**1**) in 69% yield and 90% *ee* as judged by chiral HPLC analysis of the crude reaction mixture (Figure 3, Entry 4). A single recrystallisation from acetonitrile of the crude reaction mixture prepared according to Entry 3 (Figure 3) provided a route to **1** in >99% *ee* ($[\alpha]_D^{26}$ = –464 (c = 0.2 in dichloromethane).[15,16] (*S*)-(–)-Blebbistatin (**1**) inhibits nmMIIa with an IC_{50} of 7.1 μM (see supporting information; for supporting information see also the footnote on the first page of this article), whilst **2** is significantly less active than **1**. This result clarifies the conflicting literature reports.[4,9]

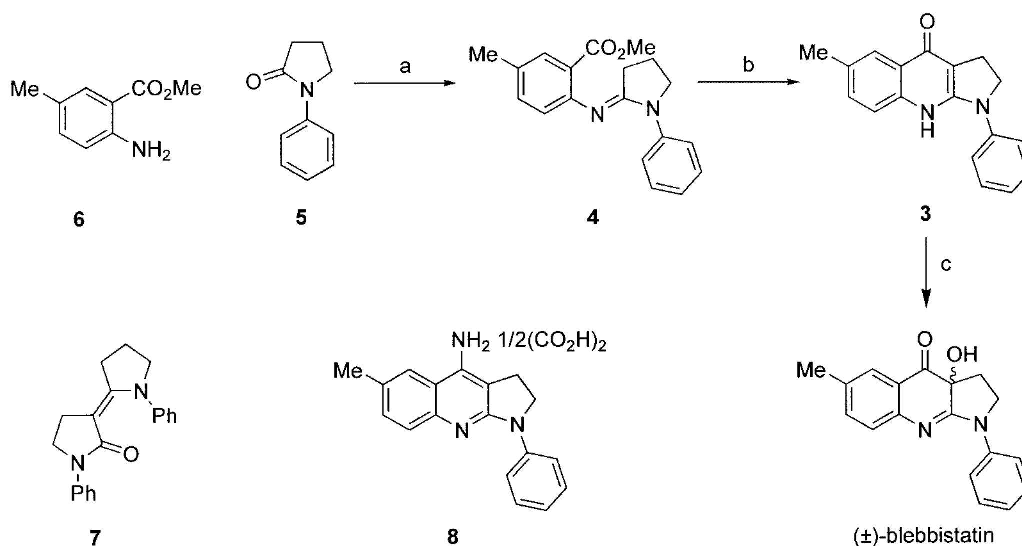

Figure 2. Preparation of (±)-blebbistatin. Reagents and conditions: a) i) $POCl_3$, CH_2Cl_2, 25 °C, 3 h; ii) 40 °C, 16 h, 41%; b) LiHMDS (3 equiv.), –78 °C to 0 °C, 3 h, 90%; c) O_2, *hv*, DMSO, 25 °C, 3 h, 29%; or O_2, *hv*, 25 °C, 3 h, THF, 26%. LiHMDS = lithium bis(trimethylsilyl)amide.

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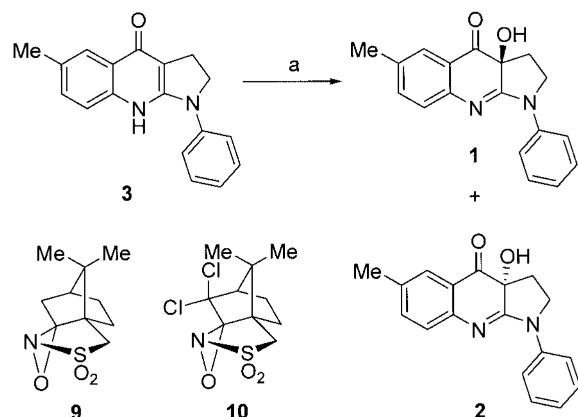

	Base	Oxaziridine	*T* [°C]	%Yield	%*ee*
1	LiHMDS	**9**	–10	70	42
2	LDA	**9**	0	90	31
3	LiHMDS	**10**	–10	82	86
4	NaHMDS	**10**	–78	69	90

Figure 3. Preparation of enantiomerically enriched blebbistatin using the Davis oxaziridine methodology. Reagents and conditions: a) LiHMDS or LDA or NaHMDS (1.2 equiv.), THF, oxaziridine **9** or **10** (2.4 equiv.). The Table summarizes the yield and enantiomeric excess (*ee*) obtained as a function of base, temperature and reagent. All *ee* values were determined using chiral HPLC analysis of the crude reaction mixture (see supporting information). LDA = Lithium diisopropylamide, NaHMDS = sodium bis(trimethylsilyl)-amide.

In order to assign by crystallographic analysis the absolute stereochemistry of (–)-blebbistatin (**1**), we decided to prepare an analogue that contained a heavy atom (bromine). Purification of the products resulting from direct bromination of **1** (or intermediates en route to **1**) with NBS proved difficult. Additionally, reactions to acylate the tertiary alcohol functionality in **1** with various bromobenzoyl chlorides gave the desired products, but did not yield sufficiently high quality crystals for X-ray analysis. Attempts to prepare an analogue of **1** with a bromine atom in place of the methyl substituent also proved unsuccessful due to the low yield of the reaction between methyl 5-bromoanthranilate and pyrrolidinone **5**. X-ray quality crystals of the bromine-containing analogue **11** were successfully prepared using similar methods to those described previously (see Figure 4 legend for further details). A highly enantiomerically enriched sample of **11** (>99% *ee* as judged by chiral HPLC analysis) was then reduced using hydrogen and 1% palladium on carbon in the presence of triethylamine.[17] The resulting sample of (–)-blebbistatin (**1**) was shown by chiral HPLC and ^{1}H NMR analysis to be identical to a sample of **1** prepared as described in Figure 3, hence confirming that the absolute stereochemistry of (–)-blebbistatin (**1**) is *S* (as drawn).[15,16] Formation of **1** presumably proceeds through an analogous transition state to the one proposed by Davis.[11,16] Blebbistatin analogue **11** can also function as a useful precursor for the synthesis of a radiolabelled derivative of **1** (by palladium-catalysed tritiation) and for the preparation of (*S*)-(–)-blebbistatin (**1**) analogues functionalised at C4′. Preliminary data on the activity of compounds substituted at C4′ shows that they retain nmMII ATPase inhibitory activity.[4]

An informative method of studying protein function in a cell uses fluorescence microscopy techniques on live cells that are expressing a green fluorescent protein (GFP)-labeled version of the protein of interest.[18] Unfortunately, the fluorescence of **1** is one factor that limits its application in this type of experiment.[19] In order to observe GFP-labeled proteins it is necessary to irradiate live cells with light at a wavelength between 420 and 490 nm (488 nm in confocal microscopy applications). The light that is subsequently emitted is collected using pass filters with a typical wavelength range of between 520 and 570 nm. As shown in Fig-

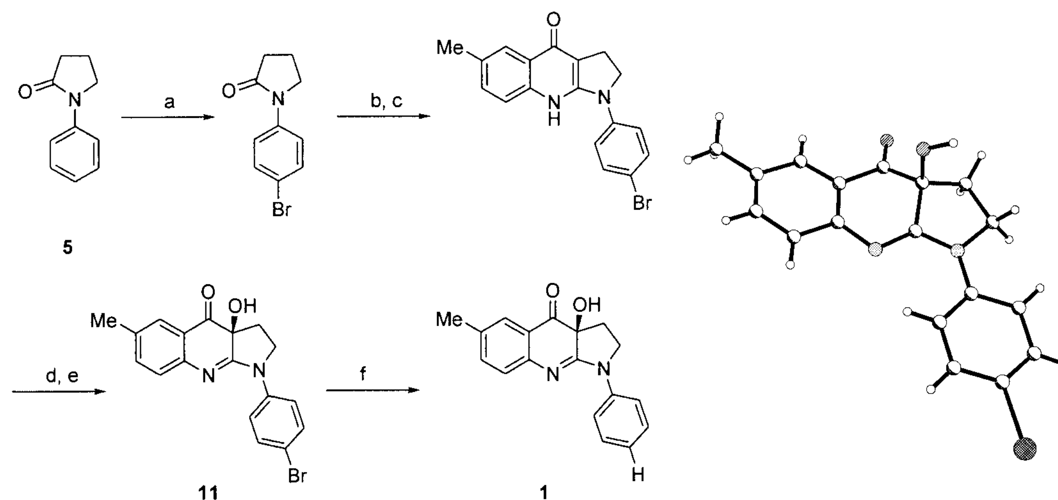

Figure 4. Assignment of the absolute stereochemistry of (–)-blebbistatin (**1**). Reagents and conditions: a) NBS, DMF, 25 °C, 2 days, 50%; b) i) $POCl_3$, CH_2Cl_2, 25 °C, 3 h; ii) **6**, 40 °C, 16 h, 26%; c) LiHMDS (3 equiv.), –78 °C to 0 °C, 3 h, 60%; d) i) LiHMDS (1.2 equiv.), THF, –78 °C, 30 min; ii) **10** (2.4 equiv.), –10 °C, 16 h, 68%, 88% *ee*; e) recrystallisation from acetonitrile >99% *ee*. $[\alpha]_D^{26}$ = –526 (*c* = 0.1, CH_2Cl_2); f) H_2, 1% Pd/C, Et_3N, DMF/MeOH, 25 °C, 24 h, 99%. NBS = *N*-bromosuccinimide, DMF = *N*,*N*-dimethylformamide.

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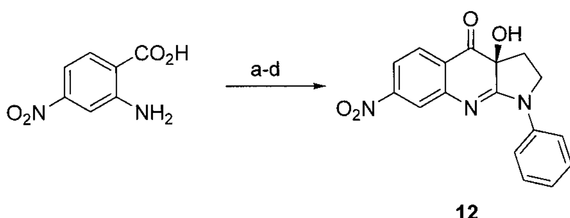
ure 5 (solid line), **1** exhibits significant fluorescence emission in the GFP emission wavelength range with an emission maximum at 601 nm. It was proposed that addition of a nitro functional group to the chromophore within (*S*)-(–)-blebbistatin (**1**) would modify the fluorescence properties without having a major effect on the biological activity, hence overcoming this limitation.[20] Optically enriched **12** was prepared (see Figure 5 legend for details[16]) and subsequent analysis of the fluorescence properties of **12** showed that there was a significant reduction in fluorescence emission in the required wavelength range following excitation at 440 nm. Biochemical and cell-based assays using **12** showed that it retained the desired biological activity (see supporting information).



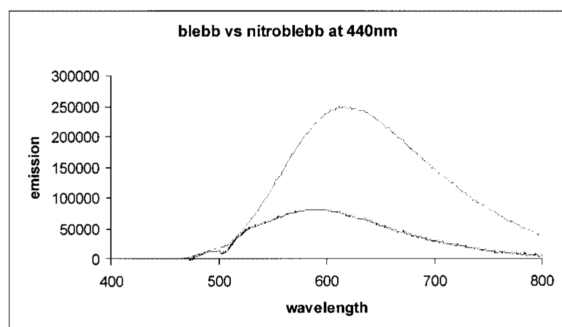


Figure 5. Preparation of a blebbistatin analogue containing a nitro functionality resulting in improved optical properties. Reagents and conditions: a) MeOH, H_2SO_4, 65 °C, 96 h, 81%; b) i) $POCl_3$, **5**, CH_2Cl_2, 25 °C, 3 h; ii) 40 °C, 72 h, 22%; c) LiHMDS (2.5 equiv.), –78 °C to 0 °C, 96 h, 44%; d) i) LiHMDS (1.2 equiv.), THF, –78 °C, 30 min; ii) **10** (3.1 equiv.), –10 °C, 32 h, 31%, 76% *ee*. Fluorescence emission spectrum of (–)-blebbistatin (**1**) (solid line) and its nitro analogue **12** (dashed line) in the wavelength range 450–800 nm after excitation at 440 nm.

A recent microscopy-based study has identified a further limitation of (*S*)-(–)-blebbistatin (**1**) that limits its use in live-cell imaging experiments. It was shown that prolonged exposure to filtered light (450–490 nm) results in degradation of (±)-blebbistatin to an unidentified non-inhibitory product via cytotoxic intermediates.[10] In analogous experiments performed by us the blebbistatin analogue **12** was shown to be stable to prolonged irradiation at the same wavelengths (see B in Figure 6), whereas **1** decomposed (see A in Figure 6). This coupled with its reduced fluorescence and retained biological activity suggests that analogue **12** will be an excellent reagent for imaging experiments with live cells, a situation where (*S*)-(–)-blebbistatin (**1**) itself is sub-optimal.

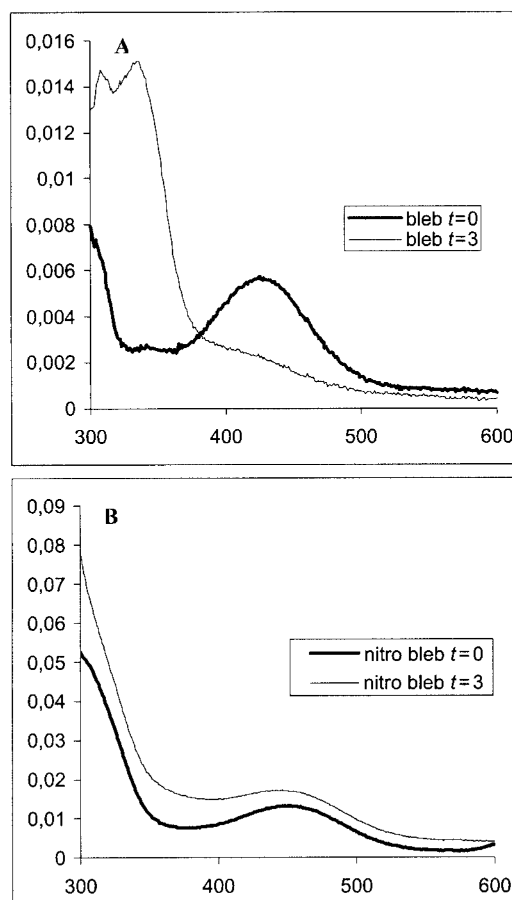


Figure 6. A) UV spectra of (*S*)-(–)-blebbistatin (**1**) in PBS (solid line). The UV spectra of **1** after exposure to filtered light (436 and 510 nm) for 3 hours (dashed line), showing that complete degradation of **1** occurs under these conditions consistent with literature reports.[10] B) UV spectra of **12** in PBS (solid line) and **12** after exposure to filtered light (436 and 510 nm) for 3 hours (dashed line) indicating that **12** is stable under these conditions. PBS = phosphate-buffered saline.

In summary, we report that the absolute stereochemistry of the selective small molecule myosin inhibitor (–)-blebbistatin (**1**) is *S*. In addition, we describe a flexible and efficient route to highly optically enriched **1** (>99% *ee*) using the Davis oxaziridine methodology. (*S*)-(–)-Blebbistatin (**1**) has already been used as a small molecule tool in a number of biological studies.[6–8,21] The synthetic approach we report enables rapid access to both enantiomers of blebbistatin (**1** and **2**) and its analogues in large quantities. We have also demonstrated that incorporation of a nitro functionality into the chromophore of **1** tunes its fluorescent properties and improves its stability, extending the scope of biological experiments in which analogues of (*S*)-(–)-blebbistatin (**1**) can be used.

Acknowledgments

The authors would like to thank the following for funding: EPSRC (N. J. W., C. L.-L.), The Royal Society (N. J. W. Fellowship), The

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Wellcome Trust (S. P.), Deutscher Akademischer Austauschdienst (T. B. Fellowship), NIH GM062566 (T. J. M.). For analytical and microscopy support: Melanja Smith (^{1}H NMR), Carolyn Horesburgh (mass spectrometry), Dr. Graham Turnbull, Prof. Ifor Samuel and Mark Goossens (fluorescence measurements), Dr. Tony Vaughan (microscopy). For helpful discussions during preparation of the manuscript: Dr. Stuart Conway, Dr. David Smith and Prof. Gary Ward.

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Received: February 08, 2005

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