

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

Cristina Lucas-Lopez,^{[a],‡} Stephen Patterson,^{[a],‡} Till Blum,^[a] Aaron F. Straight,^[b],c] Judit Toth,^[d] Alexandra M. Z. Slawin,^[a] Timothy J. Mitchison,^[b] James R. Sellers,^[d] and Nicholas J. Westwood^{*,[a]}

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(–)-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 quino-

lone 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

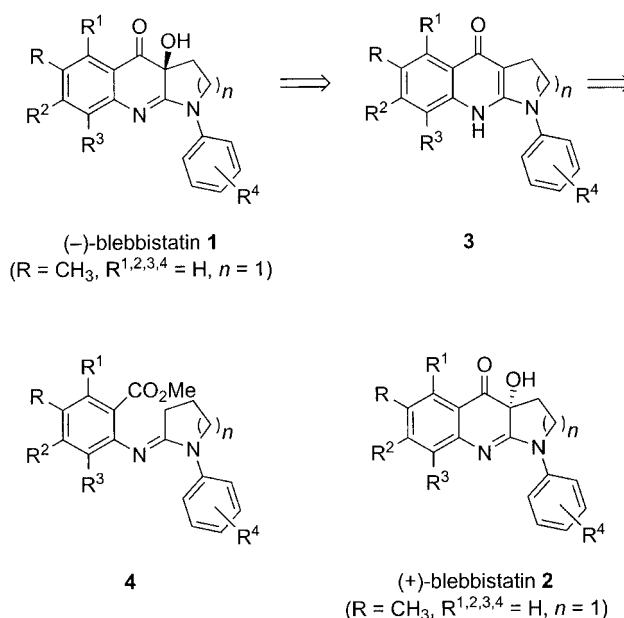


Figure 1. A flexible approach to (–)-blebbistatin (**1**) and its analogues.

[a] School of Chemistry and Centre for Biomolecular Sciences, University of St. Andrews, North Haugh, St. Andrews, Fife, KY16 9ST, UK
Fax: +44-1334-462595
E-mail: njw3@st-andrews.ac.uk

[b] Institute of Chemistry and Cell Biology and the Department of Systems Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA

[c] Current address: Department of Biochemistry, Stanford University, School of Medicine, 297 Campus Dr., B400, Stanford, CA 94305, USA

[d] Laboratory of Molecular Cardiology, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892-1762, USA

‡ These authors contributed equally to this work.

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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). ¹H NMR analysis of the reaction between **5** and POCl₃ did not support the efficient formation of an imonium intermediate.^[12] Reaction of **5** and **6** under microwave irradiation in the absence of POCl₃ 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 (SOCl₂, MeOTf, PCl₅) 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 multi-gramme 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 IC₅₀ 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]

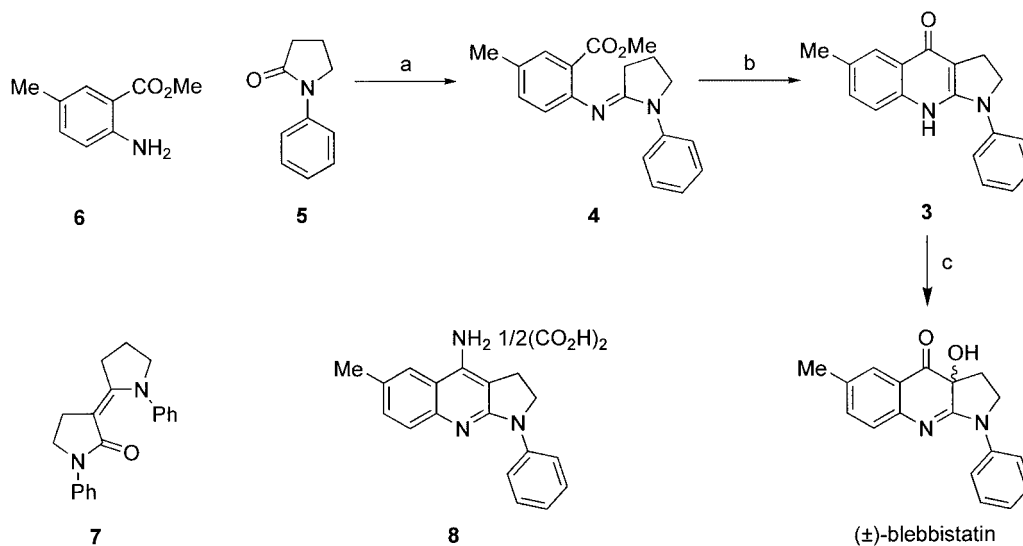
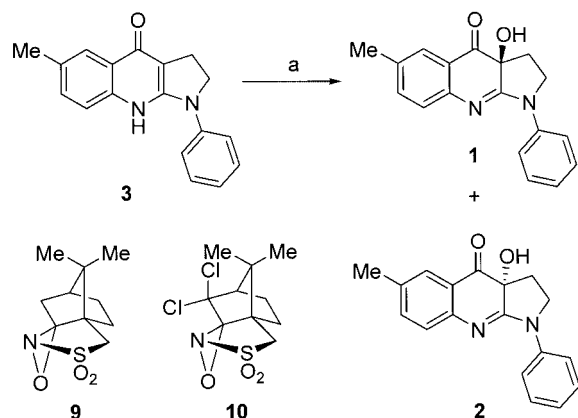


Figure 2. Preparation of (±)-blebbistatin. Reagents and conditions: a) i) POCl₃, CH₂Cl₂, 25 °C, 3 h; ii) 40 °C, 16 h, 41%; b) LiHMDS (3 equiv.), –78 °C, 3 h, 90%; c) O₂, hv, DMSO, 25 °C, 3 h, 29%; or O₂, hv, 25 °C, 3 h, THF, 26%. LiHMDS = lithium bis(trimethylsilyl)amide.



	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 ¹H 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 radio-labelled 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-

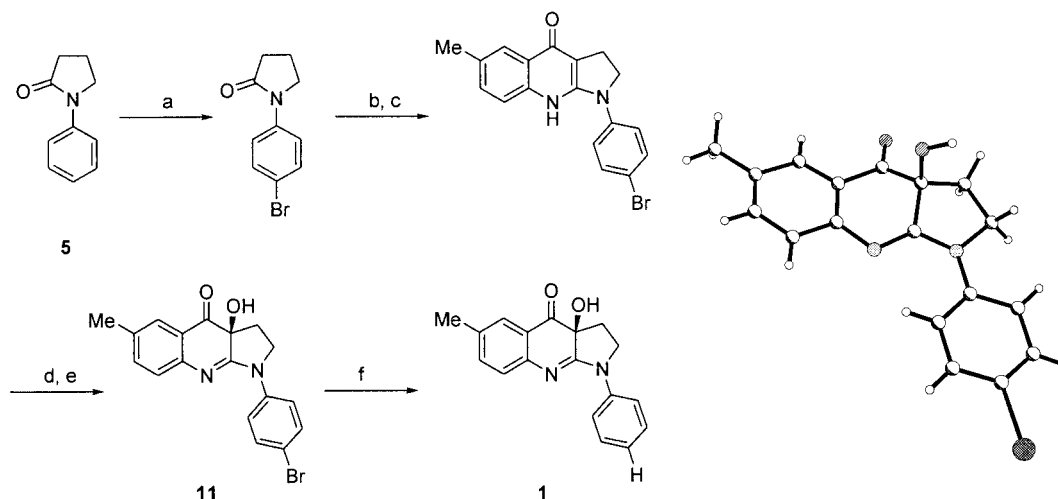


Figure 4. Assignment of the absolute stereochemistry of (-)-blebbistatin (**1**). Reagents and conditions: a) NBS, DMF, 25 °C, 2 days, 50%; b) i) POCl₃, CH₂Cl₂, 25 °C, 3 h; ii) **6**, 40 °C, 16 h, 26%; c) LiHMDS (3 equiv.), -78 °C to 0 °C, 3 h, 60%; d) i) LiHMDS (1.2 equiv.), THF, -78 °C, 30 min; ii) **10** (2.4 equiv.), -10 °C, 16 h, 68%, 88% *ee*; e) recrystallisation from acetonitrile >99% *ee*. [α]_D²⁵ = -526 (*c* = 0.1, CH₂Cl₂); f) H₂, 1% Pd/C, Et₃N, DMF/MeOH, 25 °C, 24 h, 99%. NBS = *N*-bromosuccinimide, DMF = *N,N*-dimethylformamide.

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).

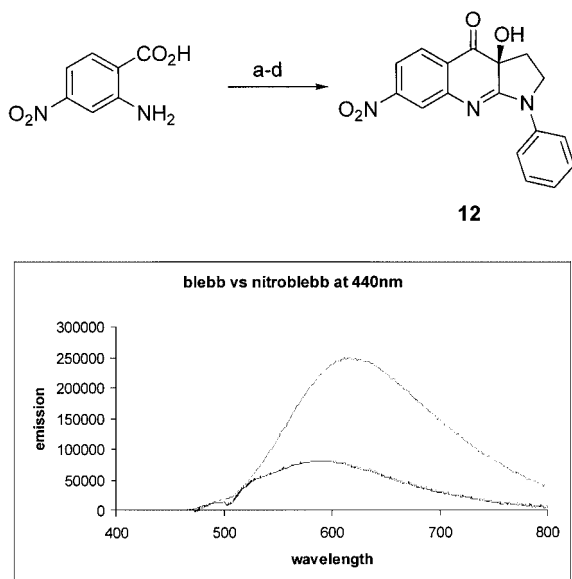


Figure 5. Preparation of a blebbistatin analogue containing a nitro functionality resulting in improved optical properties. Reagents and conditions: a) MeOH, H₂SO₄, 65 °C, 96 h, 81%; b) i) POCl₃, **5**, CH₂Cl₂, 25 °C, 3 h; ii) 40 °C, 72 h, 22%; c) LiHMDS (2.5 equiv.), –78 °C to 0 °C, 96 h, 44%; d) i) 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.

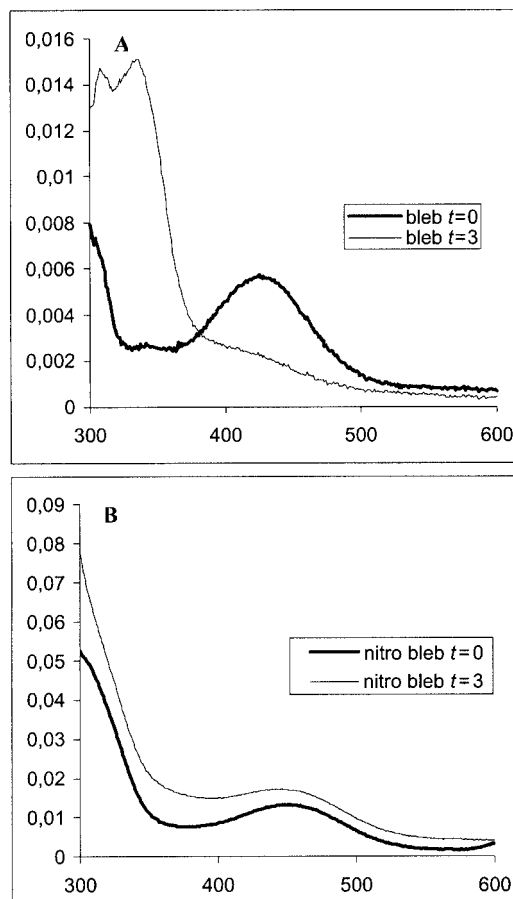


Figure 6. A) UV spectra of (*S*)-(–)-blebbistatin (**1**) in PBS (solid line). The UV spectra of **1** after exposure to filtered light (436 and 510 nm) for 3 hours (dashed line), showing that complete degradation of **1** occurs under these conditions consistent with literature reports.^[10] B) UV spectra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

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