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SUPPORTING INFORMATION

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

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Ref. No.: O200500103

Supporting Information

I. General Methods

Chemicals and solvents were purchased from Acros, Aldrich, Fisher Chemicals, Fluka and Lancaster and were used as received unless otherwise stated. Air and moisture sensitive reactions were carried out under an inert atmosphere of dried argon and glassware was oven dried (145 °C).

Tetrahydrofuran (THF) was dried by heating under reflux with sodium/benzophenone under a nitrogen atmosphere and collected by distillation. Dichloromethane (DCM) was dried by heating under reflux over calcium hydride and distilled under an atmosphere of nitrogen. Anhydrous methanol (MeOH) was purchased from Aldrich.

Analytical thin-layer chromatography (TLC) was performed on pre-coated TLC plates SIL G-25 UV_{254} (layer 0.25 mm silica gel with fluorescent indicator UV_{254}) (Aldrich). Developed plates were air dried and analysed under a UV lamp, Model UVGL-58 (Mineralight LAMP, Multiband $UV_{254/365}$ nm) and where necessary, stained with a solution of ceric ammonium molybdate or iodine on silica to aid identification. Flash column chromatography was performed using silica gel (40-63 μm, Fluorochem).

Melting points were determined using an Electrothermal 9100 capillary melting point apparatus. Values are quoted to the nearest 0.5 °C and are uncorrected.

^{1}H Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 300 (300.1 MHz), Bruker Avance 500 (499.9 MHz) or a Varian Gemini 2000 (300.0 MHz) spectrometer. ^{13}C NMR spectra using the PENDANT sequence were recorded on a Bruker Avance 300 (75.5 MHz) spectrometer. All other ^{13}C spectra were recorded on a Varian Gemini 2000 (75.5 MHz) spectrometer using composite pulse ^{1}H decoupling. All chemical shifts (δ) are recorded in ppm using the residual solvent as the internal reference in all cases ($CDCl_3$ δ_H 7.27 ppm, δ_C 77.0 ppm) ([D_8]THF δ_C 25.2 ppm, 67.2 ppm) ([D_6]DMSO δ_H 2.5 ppm, δ_C 39.5 ppm). Coupling constants (*J*) are quoted to the nearest 0.1 Hz. The following abbreviations are used:

s, singlet; d, doublet; dd, doublet of doublets; dt, doublet of triplets; t, triplet; q, quartet; p, pentet; m, multiplet and br, broad.

Fourier Transform Infrared (FT IR) spectra were recorded as Nujol mulls for solids and dichloromethane solutions for liquids using a Perkin-Elmer Paragon 1000 Fourier Transform spectrometer. Only selected absorptions are reported. Absorptions are reported in wavenumbers (cm^{-1}) and the following abbreviations are used: br, broad; m, medium; s, strong; w, weak.

Low resolution and high-resolution (HR) mass spectral analysis, (EI and CI) were recorded using a VG AUTOSPEC mass spectrometer or a Micromass GCT (Time-Of-Flight), high performance, orthogonal acceleration spectrometer coupled to an Agilent Technologies6890N GC system. Electrospray mass spectrometry (ESMS) was recorded on a high performance orthogonal acceleration reflecting TOF mass spectrometer, coupled to a Waters 2975 HPLC. Only major peaks are reported and intensities are quoted as percentages of the base peak.

Microanalysis for carbon, hydrogen and nitrogen were performed using EA 1110 CHNS CE INSTRUMENTS, Elemental Analyser.

The enantiomeric excess (*ee*) of the samples was measured using chiral high performance liquid chromatography (HPLC) and a polarimeter. The HPLC system includes a Waters 2996 photodiode array detector, Waters 2795 Alliance HT Separations Module, Micromass LCT, Thinkcenter IBM running MassLynx™ 4.0.Global. Separations were performed using a Daicel Chiralpak AD HPLC column.

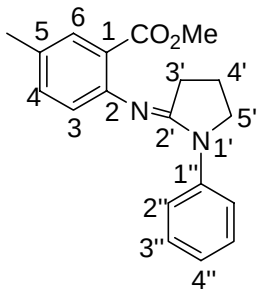
Optical Rotation measurements were performed with an Optical Activity AA-1000 polarimeter (Optical Activity Ltd. Polarimeter millidegree-autoranging) operating at 589 nm using a 2 mL solution cell with a 20 cm path length. Solvents, concentration and temperature are quoted in each case.

UV Absorption spectra were recorded on a Cary Varian model 300 absorption spectrophotometer, and time-integrated PL spectra were measured on a Jobin Yvon Fluoromax 2 fluorimeter. Measurements were recorded at a sample concentration of

10 mg L^{-1}, in HPLC grade methanol. The samples were excited at wavelengths of 420, 440, 460 and 488 nm, and the optical densities of the samples were similar and small (~0.2).

II. Synthetic Route to (*S*)-(-)-Blebbistatin (1)

1. Methyl 5-Methyl-2-(1-phenylpyrrolidin-2-ylideneamino)benzoate (4)



Phosphorus oxychloride (0.46 g, 0.30 mL, 3.0 mmol, 1.0 eq) was added dropwise to a solution of 1-phenyl-2-pyrrolidinone (**6**) (0.54 g, 3.4 mmol, 1.1 eq) in dry dichloromethane (3.0 mL) and the reaction was stirred for 3 hours at room temperature. A solution of anthranilate (**5**) (0.50 g, 3.0 mmol, 1.0 eq) in dry dichloromethane (12 mL) was then added and the reaction refluxed for 16 hours. The reaction mixture was cooled and concentrated *in vacuo*. The resulting solid was dissolved in aqueous hydrochloric acid (0.30 M, 100 mL) and extracted with dichloromethane (3 × 100 mL). The aqueous phase was then basified with aqueous sodium hydroxide solution (2.0 M, to pH 8) and extracted with ethyl acetate (3 × 100 mL). The first organic extracts were concentrated *in vacuo* and the resulting solid was carried through the above procedure three more times. All ethyl acetate extracts were combined, dried ($MgSO_4$) and concentrated *in vacuo* to give the desired compound (**4**) (0.38 g, 1.2 mmol, 41%) as a white crystalline solid. An analytical sample of **4** was prepared by recrystallisation from EtOAc/hexane; mp 119-120 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.98-2.09 (m (7 lines), 2 H; 4'-H), 2.32 (br s, 3 H; CH_3), 2.46 (t, ^3J(H,H) = 7.8 Hz, 2 H; 3'-H), 3.82 (s, 3 H, OCH_3), 3.87 (t, ^3J(H,H) = 6.9 Hz, 2 H; 5'-H), 6.72 (d, ^3J(H,H) = 8.1 Hz, 1 H; Ar-3-H), 7.01-7.10 (m, ^3J(H,H) = 7.4 Hz, 1 H; Ar-4"-H), 7.19 (ddq, ^3J(H,H) = 8.1 Hz, ^4J(H,H) = 2.2 Hz, ^4J(H,H) = 0.6 Hz, 1 H; Ar-4-H), 7.35 (dd, ^3J(H,H) = 8.7 Hz, ^3J(H,H) = 7.4 Hz, 2 H; Ar-3"-H), 7.65-7.68 (m, 1 H; Ar-6-H), 7.82 ppm (dd, ^3J(H,H) = 8.7 Hz, ^4J(H,H) = 0.9 Hz, 2 H; Ar-2"-H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ =19.7 (C4'), 20.5 (CH_3), 29.1 (C3'), 50.6 (C5'), 51.7 (OCH_3), 120.2 (C2"), 121.9 (C1), 122.9 (C4''), 123.0 (C3), 128.5 (C3"), 130.9 (C5), 131.0 (C6), 133.4 (C4), 141.4 (C1"), 150.5 (C2) , 159.7 (C2'), 167.8 ppm (C=O); IR

(CH_2Cl_2 thin film): $\tilde{\nu}_{max}$=1721 (s) (C=O), 1652 (s) (C=N), 756 (m) (Ar-H), 692 cm^{-1} (m); LRMS (EI): *m/z* (%): 307 (100) $[M]^+$, 308 (85) $[M]^+$, 249 (10) $[CO_2Me]$, 202 (45), 77 (14) $[C_6H_5]$; HRMS (EI): *m/z* calc'd for $C_{19}H_{20}N_2O_2$ $[M^+]$: 308.1524; found: 308.1519; Anal. calc'd for $C_{19}H_{20}N_2O_2$: C, 74.00; H, 6.54; N, 9.08; found: C, 74.02; H, 6.55; N, 9.05

X-RAY structure of Methyl 5-Methyl-2-(1-phenylpyrrolidin-2-ylideneamino)benzoate (4).

Summary of Data CCDC 243020

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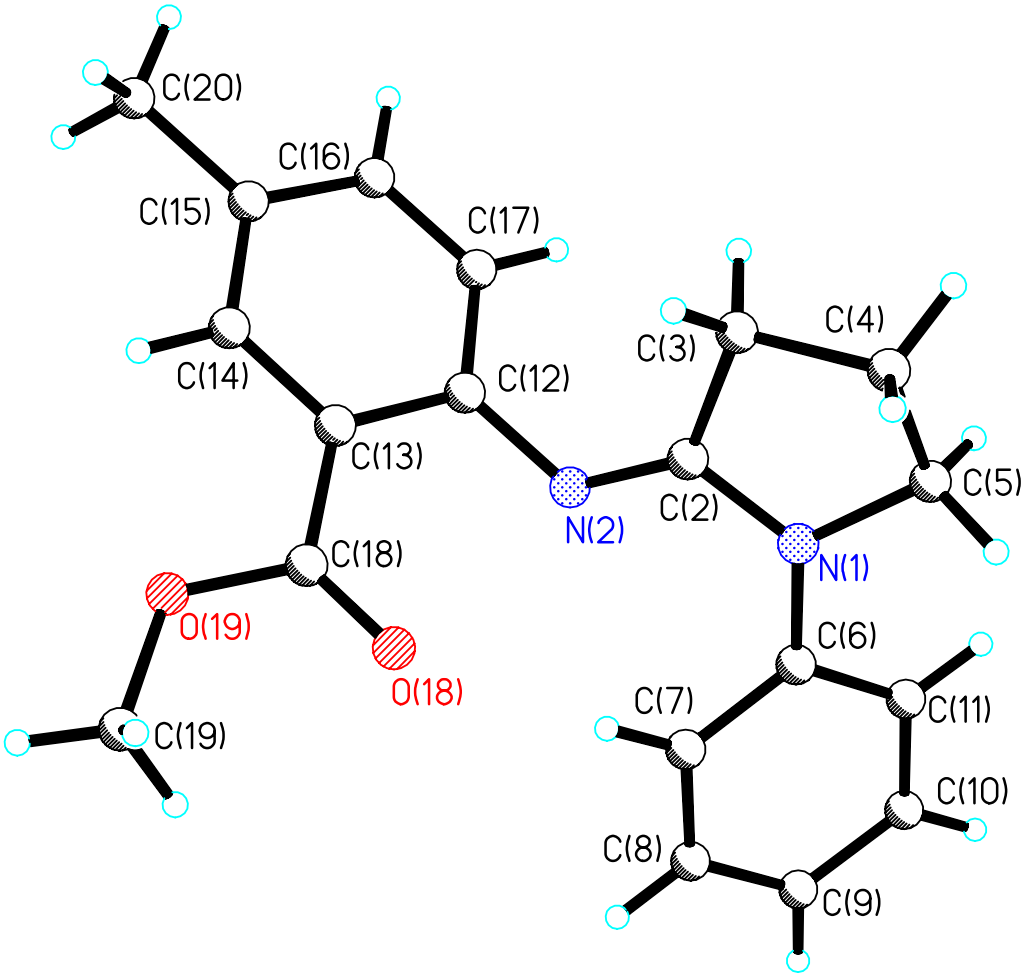
Journal: Angew.Chem.,Int.Ed.Engl. (0179)

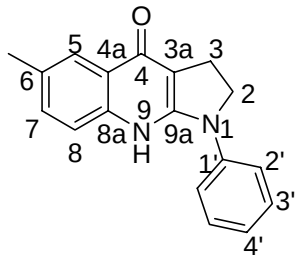
Formula: C19 H20 N2 O2

Unit cell parameters: a 10.4659(16) b 11.374(2) c 13.026(3)

Space group P212121

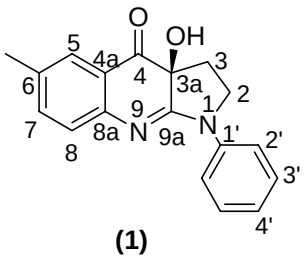


5. 6-Methyl-1-phenyl-1,2,3,9-tetrahydro-4*H*-pyrrolo[2,3-*b*]quinolin-4-one (3)

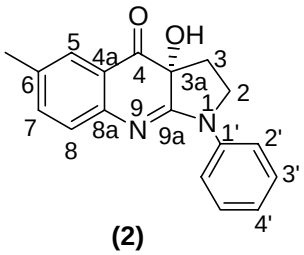


A solution of amidine (**3**) (0.20 g, 0.60 mmol, 1.0 eq) in anhydrous THF (30 mL) was cooled to –78 °C and stirred for 15 minutes. Lithium bis(trimethylsilyl)amide (1.0 M in THF, 1.9 mL, 1.9 mmol, 3.0 eq) was added dropwise to the reaction mixture. The reaction mixture was warmed to 0 °C over 3 hours and quenched at 0 °C with saturated ammonium chloride solution (5.0 mL). Further saturated ammonium chloride solution (150 mL) was added. The aqueous phase was extracted with dichloromethane (3 × 100 mL) and the combined organic extracts dried ($MgSO_4$) and concentrated *in vacuo* to give an orange solid. The solid was purified by flash column chromatography on silica gel (eluting with 100% ethyl acetate), to give the desired compound (**3**) (0.16 g, 0.60 mmol, 90%) as an off-white solid; mp 189-190 °C; ^{1}H NMR (300 MHz, [D_6]DMSO): δ=2.41 (s, 3 H; C$\underline{H}_3$), 3.17 (t, ^3J(H,H) = 8.0 Hz, 2 H; 3-$\underline{H}$), 4.06 (t, ^3J(H,H) = 8.0 Hz, 2 H; 2-$\underline{H}$), 6.96-7.02 (m, 1 H; Ar-4'-$\underline{H}$), 7.31 (dd, ^3J(H,H) = 8.3 Hz, ^4J(H,H) = 1.6 Hz, 1 H; Ar-7-$\underline{H}$), 7.34-7.41 (m, 2 H; Ar-3'-$\underline{H}$), 7.50 (d, ^3J(H,H) = 8.3 Hz, 1 H; Ar-8-$\underline{H}$), 7.76 (br s, 1 H; Ar-5-$\underline{H}$), 8.04-8.11 ppm (m, 2 H; Ar-2'-H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ=21.1 (C$\underline{H}_3$), 24.0 (C$\underline{3}$), 52.2 (C$\underline{2}$), 104.1 (C$\underline{3}$a), 119.6 (C$\underline{2}$'), 120.9 (C$\underline{4}$'), 123.3 (C$\underline{6}$), 123.6 (C$\underline{5}$), 124.2 (C$\underline{8}$) 129.4 (C$\underline{3}$'), 130.9 (C$\underline{7}$), 132.2 (C$\underline{1}$'), 138.6 (C$\underline{8}$a), 140.4 (C$\underline{4}$a),154.9 (C$\underline{9}$a), 163.0 ppm (C$\underline{4}$); IR (Nujol): $\tilde{\nu}_{max}$=1571 (s), 1490 (m), 1460 (s), 1309 (s), 1272 (w), 818 (w), 753 (m) (Ar-H), 188 cm^{-1}(m); LRMS (EI): *m/z* (%): 276 (100) $[M]^+$, 57 (13); HRMS (EI): *m/z* calc'd for $C_{18}H_{16}N_2O_1$ $[M^+]$: 276.1263; found: 276.1259.

6. (3a*S*)-3a-Hydroxy-6-methyl-1-phenyl-1,2,3,3a-tetrahydro-4H-pyrrolo[2,3-*b*]quinolin-4-one (1) ((*S*)-(-)-blebbistatin) + (3a*R*)-3a-Hydroxy-6-methyl-1-phenyl-1,2,3,3a-tetrahydro-4H-pyrrolo[2,3-*b*]quinolin-4-one (2) ((*R*)-(+)-blebbistatin)



(1)



(2)

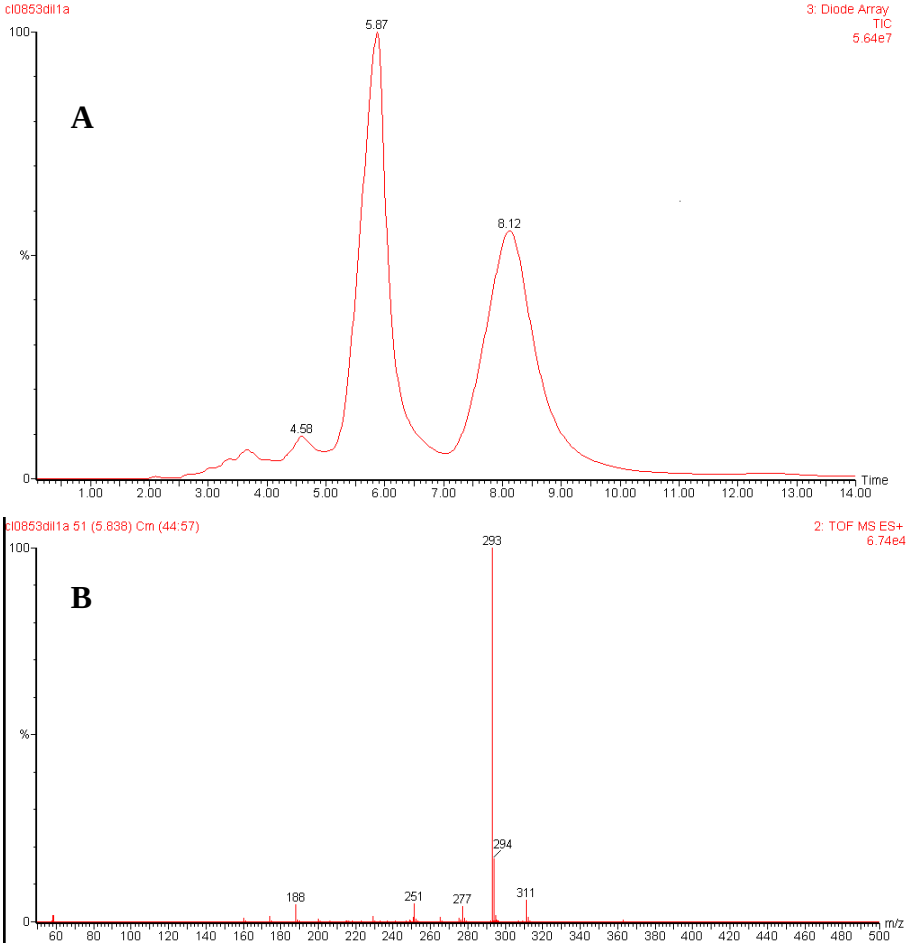
Method 1a: Photodegradation of quinolone (3) in DMSO

A solution of quinolone (**3**) (0.12 g, 0.42 mmol) in DMSO (5 mL) was allowed to stand open to the air in direct sunlight for 40 days. The reaction was concentrated *in vacuo* to give a dark yellow oil which was purified by flash column chromatography eluting with 20% ethyl acetate/hexane to give (±)-blebbistatin as a bright yellow solid (0.027 g, 0.092 mmol, 22%). Analytical data for (±)-blebbistatin prepared by this method was identical to that for (**1**) (see method 3) except for $[\alpha]_D^{26}$= 0 (c=0.1 in dichlorometahne).

Method 1b: Photodegradation of quinolone (3) in DMSO using a Mercury Lamp

A solution of quinolone (**3**) (0.028 g, 0.095 mmol) in DMSO (5.0 mL) was irradiated (mercury lamp (400 W) medium pressure, unfiltered) for 3 hours in a quartz flask in air. The mixture was poured into water (50 mL) and extracted with diethyl ether (3 x 50 mL). The combined organic extracts were washed with water (3 x 50 mL), dried ($MgSO_4$) and concentrated *in vacuo* to give a dark yellow oil. The crude oil was purified by flash column chromatography eluting with 20% ethyl acetate/hexane to give (±)-blebbistatin as a bright yellow solid (0.0080 g, 0.027 mmol, 29%). Analytical data for (±)-blebbistatin prepared by this method were identical to that for (**1**) (see method 3) except for $[\alpha]_D^{26}$= 0 (c=0.1 in dichloromethane). Chiral

hplc analysis indicated that the compound was prepared as a racemic mixture (two peaks of equal integration).



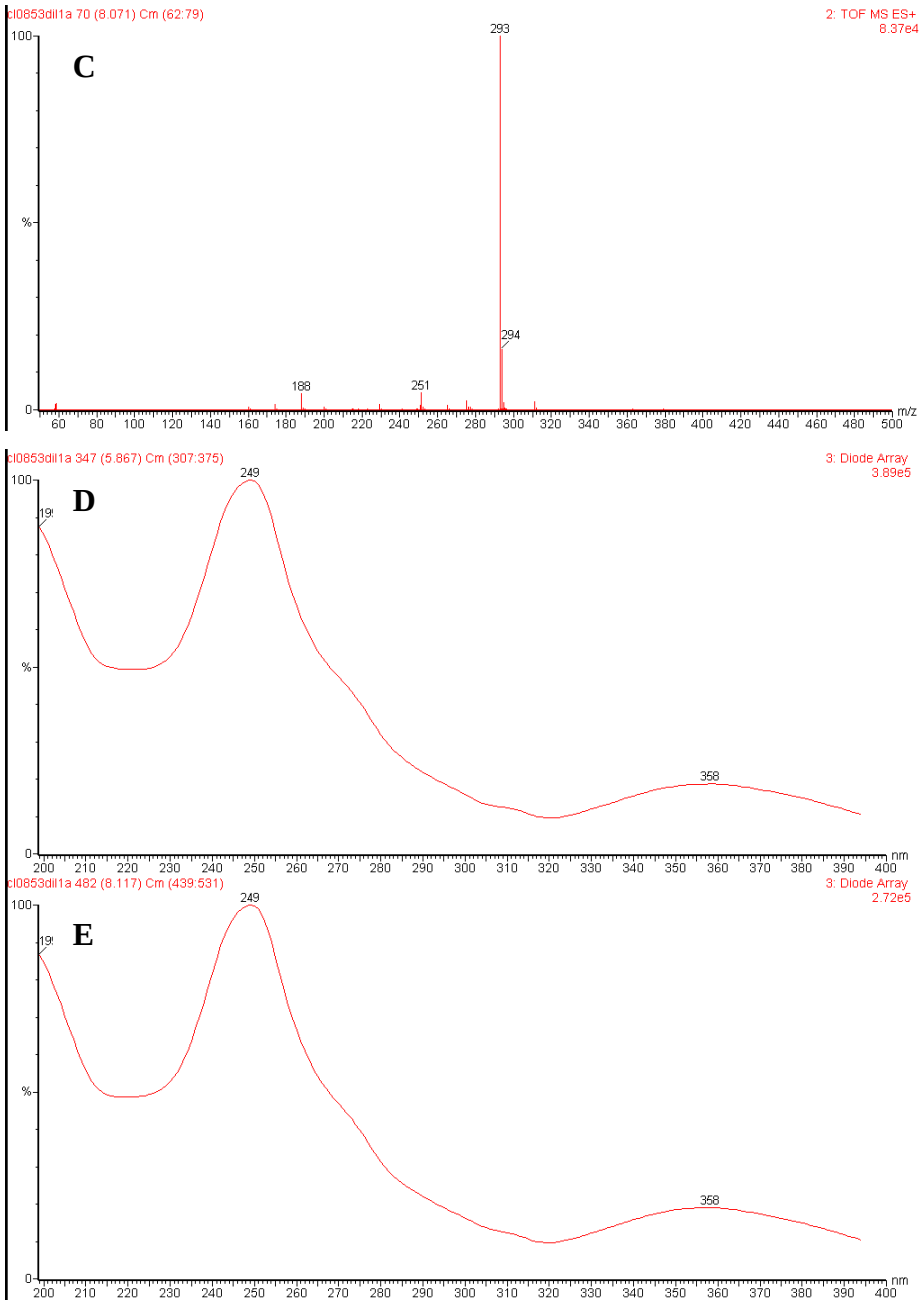


Figure S1: A), UV chromatogram of racemic blebbistatin. B), ESMS (+ve) of peak RT=5.87 min. $[M+H]^+$=293. C), ESMS (+ve) of peak RT=8.12 min. $[M+H]^+$=293. D), UV-vis spectra of peak RT=5.87 min. max=249 nm. E), UV-vis spectra of peak RT=8.12 min. max=249 nm.

Method 2: Photodegradation of quinolone (3) in THF using a Mercury Lamp

A solution of quinolone (**3**) (0.033 g, 0.12 mmol) in THF (5.0 mL) was irradiated (mercury lamp, 400 W, medium pressure, unfiltered) for 3 hours in a quartz flask under air. The mixture was poured into water (50 mL) and extracted with

diethyl ether (3 x 50 mL). The combined organic extracts were washed with water (3 x 50 mL), dried ($MgSO_4$) and concentrated *in vacuo* to give a dark yellow oil. The crude oil was purified by flash column chromatography eluting with 20% ethyl acetate/hexane to give (±)-blebbistatin as a bright yellow solid (0.009 g, 0.030 mmol, 26%). All the analytical data were identical to that for (**1**) (see method 3), except for $[\alpha]_D^{26}$= 0 (c=0.1 in dichloromethane) and chiral HPLC analysis (ee=0%).

Method 3: Figure 3 Entry 1:

A solution of quinolone (**3**) (0.040 g, 0.15 mmol, 1.0 eq) in dry THF (10 mL) was added dropwise to lithium bis(trimethylsilyl)amide (1.0 M in THF, 0.18 mL, 0.18 mmol, 1.2 eq) in dry THF (4.0 mL) at -78 °C under argon. The reaction was stirred for 30 minutes at -78 °C and a solution of the Davis reagent (**9**) (0.080 g, 0.35 mmol, 2.4 eq) in dry THF (5.0 mL) was added via cannula. After 16 hours at -10 °C saturated ammonium iodide solution (5.0 mL, 10 eq) and diethyl ether (10 mL) were added and the reaction warmed to room temperature. Saturated sodium thiosulfate solution (15 mL) was then added and the reaction extracted with diethyl ether (2 × 10 mL). The combined organic extracts were dried ($MgSO_4$) and concentrated *in vacuo* to give a yellow solid. The solid was partitioned between dichloromethane (100 mL) and aqueous hydrochloric acid solution (0.30 M, 100 mL). The aqueous phase was washed with dichloromethane (3 × 100 mL), basified with aqueous sodium hydroxide solution (2.0 M, to pH 8) and extracted with ethyl acetate (2 × 100 mL). The organic extracts were dried ($MgSO_4$) and concentrated *in vacuo* to give blebbistatin (0.031 g, 0.11 mmol, 70%) as a bright yellow solid. The enantiomeric excess of blebbistatin, prepared by this route, was 42% as determined by chiral-phase HPLC analysis; mp 192-193 °C; $[\alpha]_D^{26}$= -104 (c=0.2 in dichloromethane); ^{1}H NMR (300 MHz, $CDCl_3$): δ =2.13-2.20 (m, 1 H; 3-H), 2.30 (br s, 3 H; CH_3), 2.34 (dt, ^3J(H,H) = 15.6 Hz, ^2J(H,H) = 3.2 Hz, 1 H; 3-H), 3.69-3.76 (m, 2 H; 2-H), 6.9 (d, ^3J(H,H) = 8.1 Hz, 1 H; Ar-8-H), 7.10 (ddq, ^3J(H,H) = 8.1 Hz , ^4J(H,H) = 2.2 Hz, ^4J(H,H) = 0.6 Hz, 1H; Ar-7-H), 7.16-7.22 (m, 1 H; Ar-4'-H), 7.38-7.46 (m, 2 H; Ar-3'-H), 7.59-7.62 (m, 1 H; Ar-5-H), 7.79-7.84 ppm (m, 2 H; Ar-2'-H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ =20.6 (CH_3), 29.0 (C3), 48.3 (C2), 77.4 (C3a), 120.0 (C4a), 120.4 (C2'), 124.7 (C4'), 125.8 (C8), 127.1 (C5), 128.8 (C3'), 133.4 (C6), 137.2 (C7), 139.7 (C1'), 148.1 (C8a), 164.7 (C9a),

194.1 ppm (C4); IR (Nujol): $\tilde{\nu}_{max}$=1694 (s) (C=O), 1593 (s), 1299 (m), 1108 (m), 755 (m), 735 cm^{-1} (m); LRMS (CI^+): *m/z* (%): 293 (100) $[M+H]^+$, 277 (32); HRMS (EI): *m/z* calc'd for $C_{18}H_{16}O_2N_2$ $[M^+]$: 292.1213; found: 292.1216; Anal. calc'd for $C_{18}H_{16}O_2N_2$: C, 73.95; H, 5.52; N, 9.58: found: C, 74.04; H, 5.40; N, 9.69; HPLC (Daicel Chiralpak AD, Acetonitrile/Water 50:50, flow rate 0.8 ml min^{-1}, λ=254 nm): major enantiomer: t_R= 6.07 min: minor enantiomer: t_R= 8.57min.

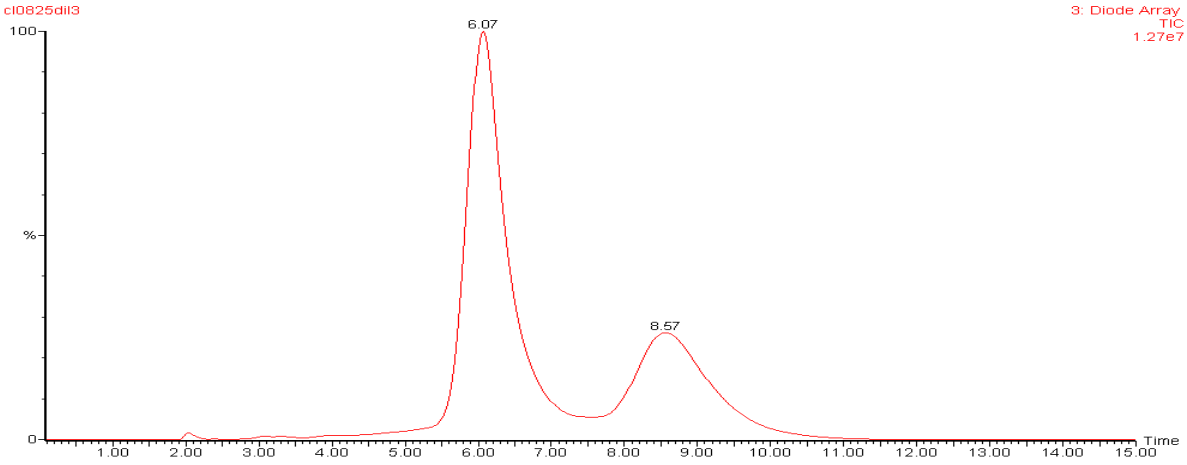


Figure S2; UV chromatogram of the two enantiomers of Blebbistatin, RT=6.07 min and RT=8.57 min.

Method 4: Figure 3 Entry 2:

A solution of quinolone (**3**) (0.040 g, 0.15 mmol, 1.0eq) in dry THF (10 mL) was added dropwise to lithium diisopropylamine (1.0 M in THF, 0.50 mL, 0.20 mmol, 1.2eq) in dry THF (2.0 mL) at -78 °C under argon. The reaction was stirred for 30 minutes at -78 °C and a solution of the Davis reagent (**9**) (0.080 g, 0.40 mmol, 2.4eq) in dry THF (6.0 mL) was added via cannula. After 16 hours at 0 °C saturated ammonium iodide solution (5.0 mL, 10eq) and diethyl ether (5.0 mL) were added and the reaction warmed to room temperature. Saturated sodium thiosulfate solution (15 mL) was then added and the reaction extracted with diethyl ether (2 × 10 mL). The combined organic extracts were dried ($MgSO_4$) and concentrated *in vacuo* to give a yellow solid. The solid was partitioned between dichloromethane (100 mL) and aqueous hydrochloric acid solution (0.30 M, 100 mL). The aqueous phase was washed with dichloromethane (3 × 100 mL), neutralised with aqueous sodium

hydroxide solution (2.0 M) and extracted with ethyl acetate (2 × 100 mL). The organic extracts were dried ($MgSO_4$) and concentrated *in vacuo* to give blebbistatin (0.04 g, 0.1 mmol, 90%) as a bright yellow solid. All the analytical data for blebbistatin prepared by this route is identical to that described in method 3 except for the data on optical purity. The enantiomeric excess of blebbistatin was 31% as determined by chiral-phase HPLC analysis. HPLC (Daicel Chiralpak AD, Acetonitrile/Water 50:50, flow rate 0.8 ml min^{-1}, λ=254 nm): major enantiomer: t_R= 6.33 min: minor enantiomer: t_R= 8.77 min.

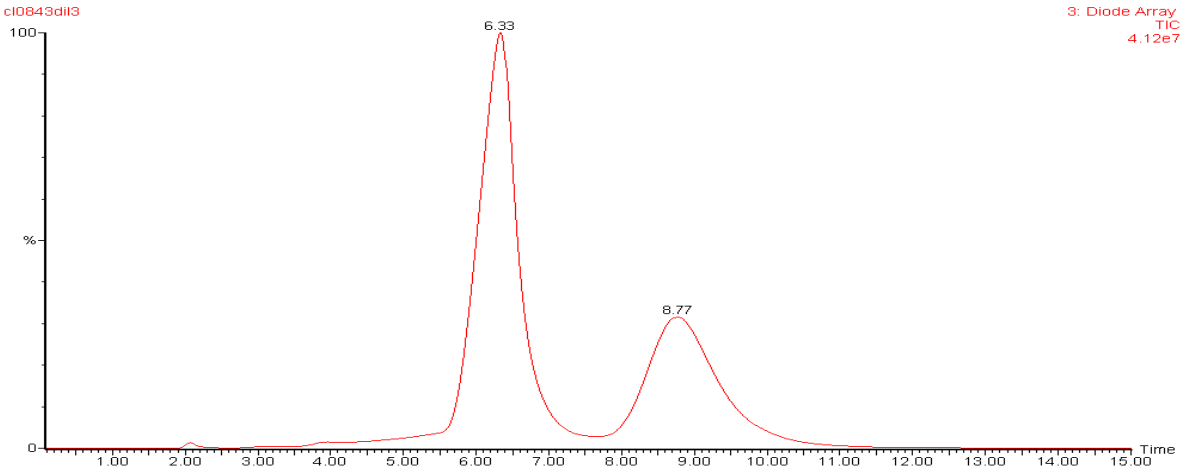


Figure S3; UV chromatogram of the two enantiomers of blebbistatin, RT=6.33 min and RT=8.77 min.

Method 5: Figure 3 Entry 3.

A solution of quinolone (**3**) (0.20 g, 0.70 mmol, 1.0eq) in dry THF (17 mL) was added dropwise to lithium bis(trimethylsilyl)amide (1.0 M in THF, 0.90 mL, 0.90 mmol, 1.2eq) in dry THF (4.0 mL) at -78 °C under argon. The reaction was stirred for 30 minutes at -78 °C and a solution of the Davis reagent (**10**) (0.50 g, 1.7 mmol, 2.4eq) in dry THF (10 mL) was added via cannula. After 16 hours at -10 °C saturated ammonium iodide solution (10 mL, 10eq) and diethyl ether (10 mL) were added and the reaction warmed to room temperature. Saturated sodium thiosulfate solution (15 mL) was then added and the reaction extracted with diethyl ether (2 × 10 mL). The combined organic extracts were dried ($MgSO_4$) and concentrated *in vacuo* to give a yellow solid. The solid was partitioned between dichloromethane (100 mL) and

aqueous hydrochloric acid solution (0.30 M, 100 mL). The aqueous phase was washed with dichloromethane (3 × 100 mL), basified with aqueous sodium hydroxide solution (2.0 M, to pH 8) and extracted with ethyl acetate (2 × 100 mL). The organic extracts were dried ($MgSO_4$) and concentrated *in vacuo* to give blebbistatin as a bright yellow solid (0.17 g, 0.6 mmol, 82%). The enantiomeric excess of blebbistatin was 86% as determined by chiral-phase HPLC analysis. HPLC (Daicel Chiralpak AD, Acetonitrile/Water 50:50, flow rate 0.8 mL min^{-1}, λ=254 nm): major enantiomer: t_R= 6.33 min: minor enantiomer: t_R= 8.60 min.

An analytical sample of blebbistatin was prepared by recrystallisation from MeCN; $[\alpha]_D^{26}$= -464 (c=0.2 in dichloromethane). Chiral HPLC analysis showed that after a single recrystallisation (*S*)-(-)-blebbistatin was prepared with an *ee* of >99%. HPLC (Daicel Chiralpak AD, Acetonitrile/Water 50:50, flow rate 0.8 mL min^{-1}, λ=254 nm): major enantiomer: t_R= 6.22 min.

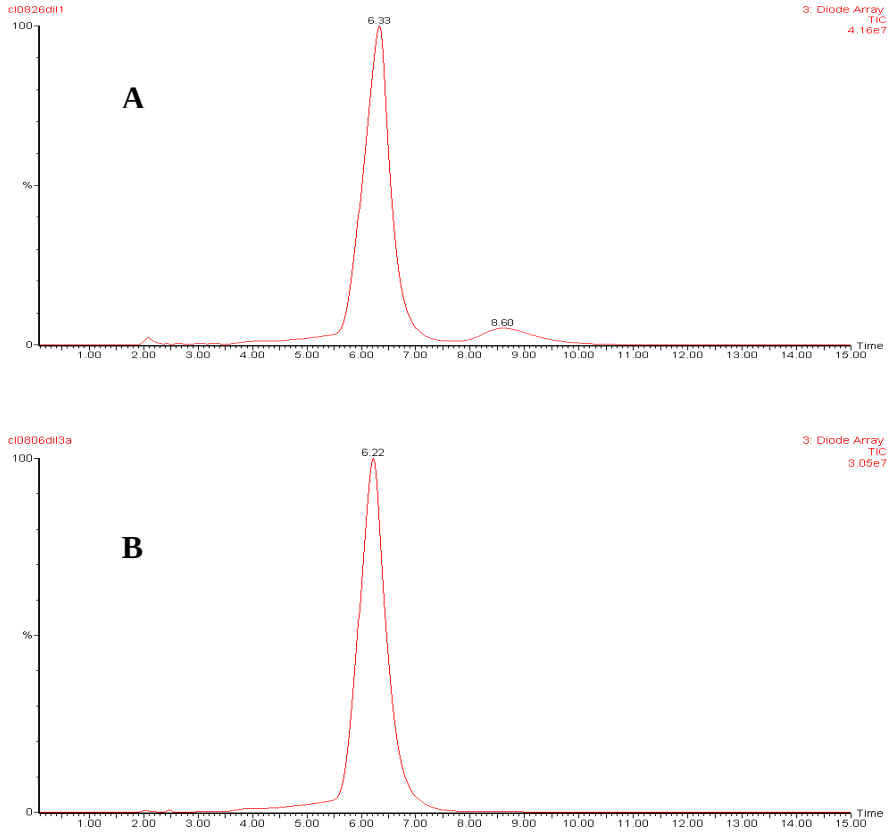
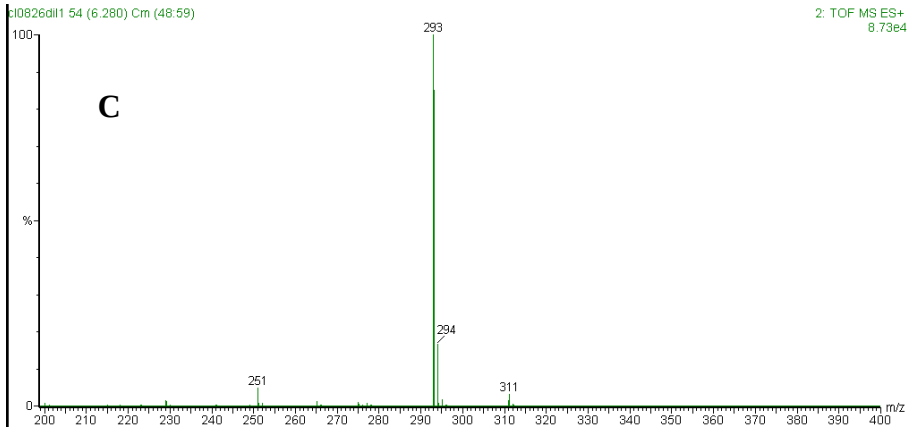


Figure S4: A), UV chromatogram of (**1**) blebbistatin RT=6.33 min, RT=8.60 min, B), UV chromatogram of cristallised (**1**) blebbistatin RT=6.22 min.



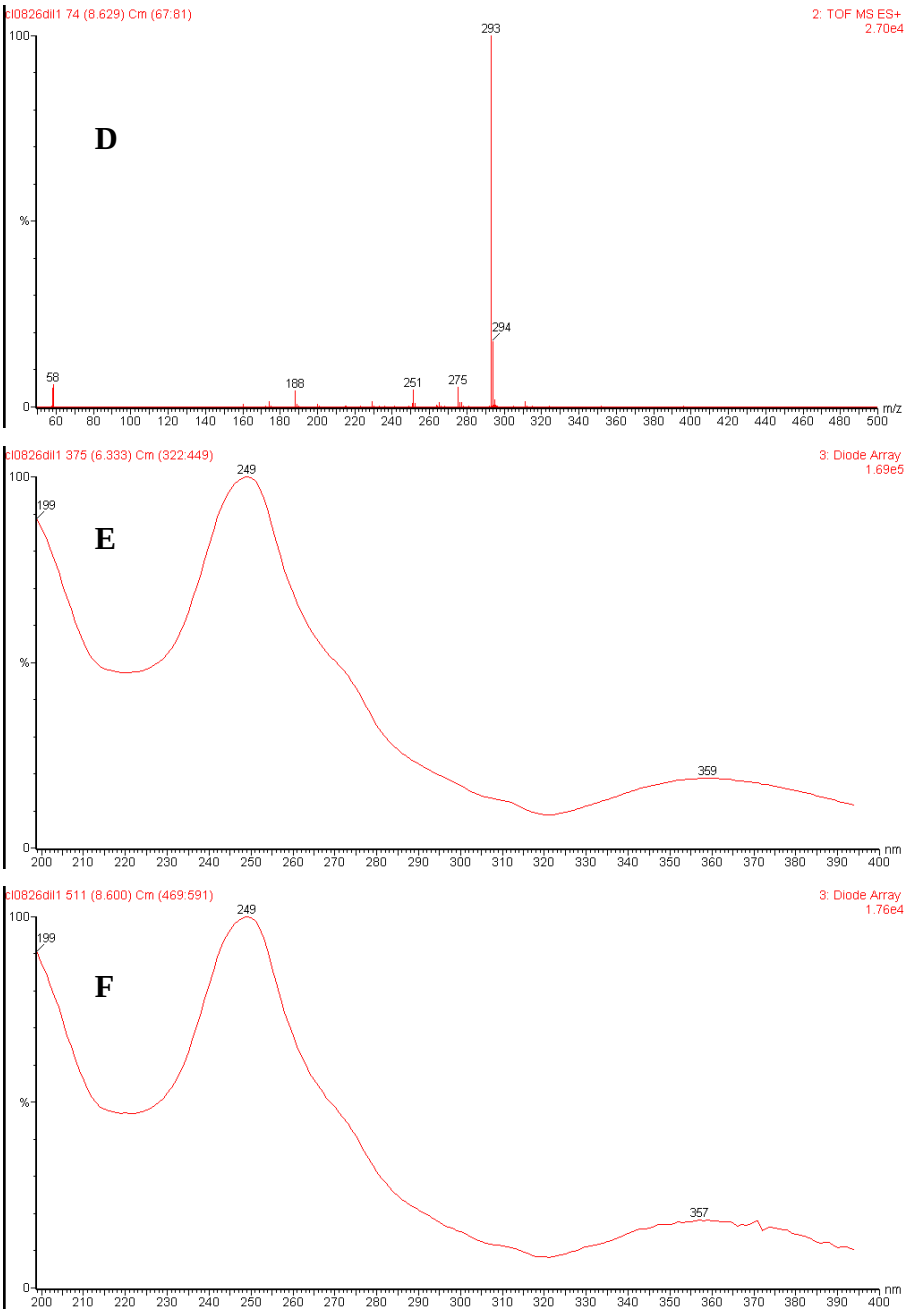


Figure S5: C), ESMS (+ve) of peak RT=6.33 min. $[M+H]^+$=293. D), ESMS (+ve) of peak RT=8.60 min. $[M+H]^+$=293. E), UV-vis spectra of peak RT=6.33 min. max=249 nm. F), UV-vis spectra of peak RT=8.60 min. max=249 nm.

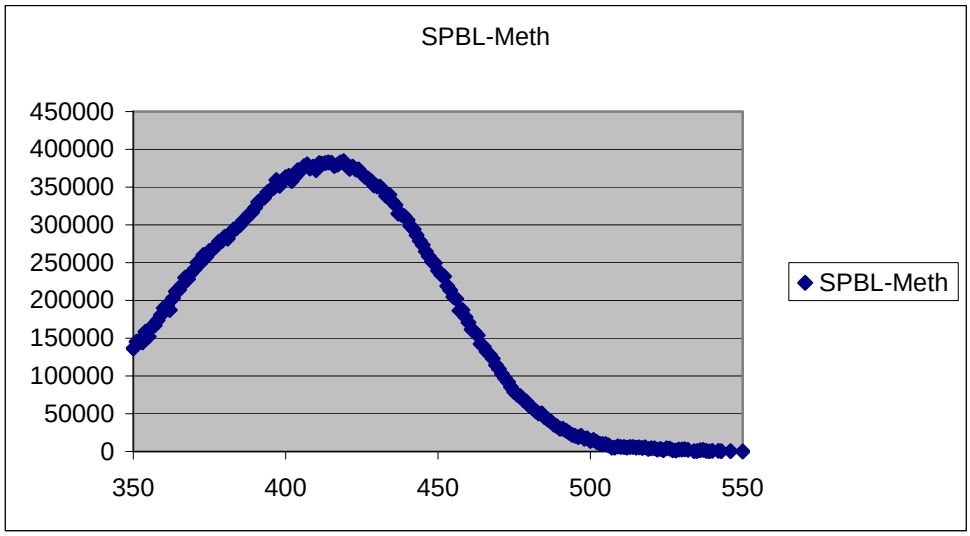


Figure S6: Excitation acquisition spectrum of (*S*)-(-)-blebbistatin (**1**), the detected emission wavelength is 600 nm.

15. X-RAY structure of Blebbistatin (1)

Summary of Data CCDC 238391

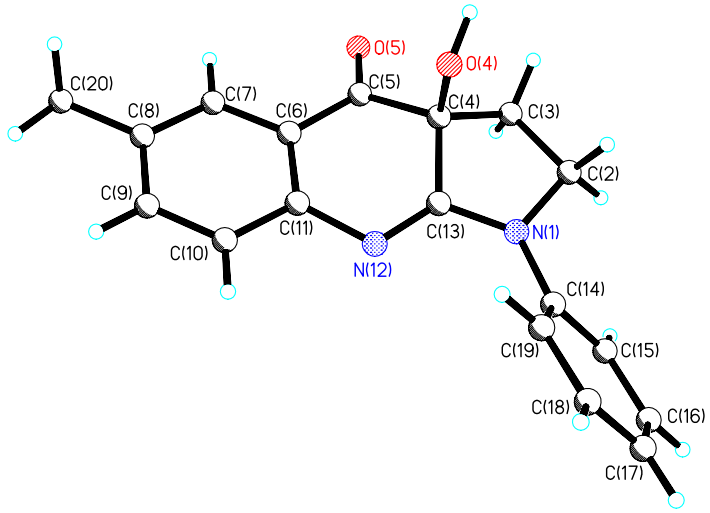
Authors: C. Lucas-Lopez, S. Patterson, T. Blum, A. F. Straight, J. Toth, A. M. Z. Slawin, T. J. Mitchison, J. R. Sellers, N. J. Westwood

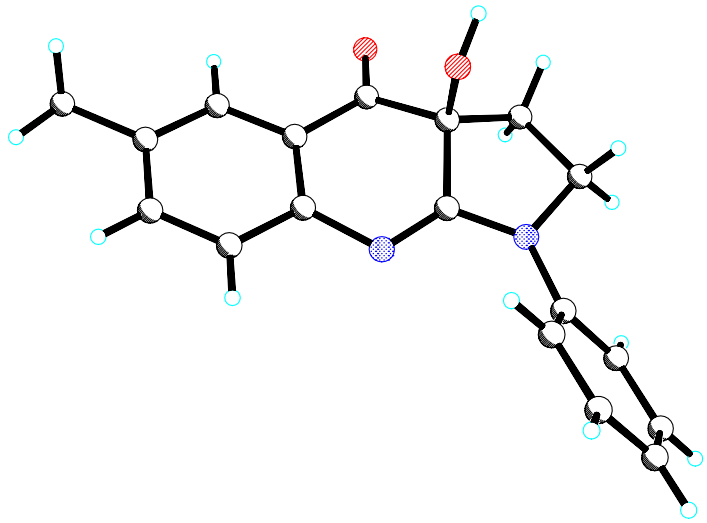
Journal: Angew.Chem.,Int.Ed.Engl. (0179)

Formula: C18 H16 N2 O2

Unit cell parameters: a 5.8412(5) b 19.9242(16) c 6.1298(5)

Space group P21





Method 7: Figure 3 Entry 4:

A solution of quinolone (**3**) (0.050 g, 0.18 mmol, 1.0eq) in dry THF (12 mL) was added dropwise to sodium bis(trimethylsilyl)amide (1.0 M in THF, 0.22 mL, 0.22 mmol, 1.2eq) in dry THF (4.0 mL) at -78°C under argon. The reaction was stirred for 30 minutes at -78°C and a solution of the Davis reagent (**10**) (0.13 g, 0.43 mmol, 2.4eq) in dry THF (6.0 mL) was added via cannular. After 72 hours at -78°C, saturated ammonium iodide solution (5.0 mL, 10eq) and diethyl ether (10 mL) were added and the reaction warmed to room temperature. Saturated sodium thiosulfate solution (15 mL) was then added and the reaction extracted with diethyl ether (2 × 10 mL). The combined organic extracts were dried ($MgSO_4$) and concentrated *in vacuo* to give a yellow solid. The solid was partitioned between dichloromethane (100 mL) and aqueous hydrochloric acid solution (0.30 M, 100 mL). The aqueous phase was washed with dichloromethane (3 × 100 mL), basified with aqueous sodium hydroxide solution (2.0 M, to pH 8) and extracted with ethyl acetate (2 × 100 mL). The organic extracts were dried ($MgSO_4$) and concentrated *in vacuo* to give blebbistatin (0.030 g, 0.10 mmol, 69%) as a bright yellow solid; $[\alpha]_D^{26}$= -403 (c=0.1 in dichloromethane). The enantiomeric excess of blebbistatin was 90% as determined by chiral-phase HPLC analysis. HPLC (Daicel Chiralpak AD, Acetonitrile/Water 50:50, flow rate 0.8 mL min^{-1}, λ=254 nm): major enantiomer: t_R= 6.17 min: minor enantiomer: t_R= 8.55 min.

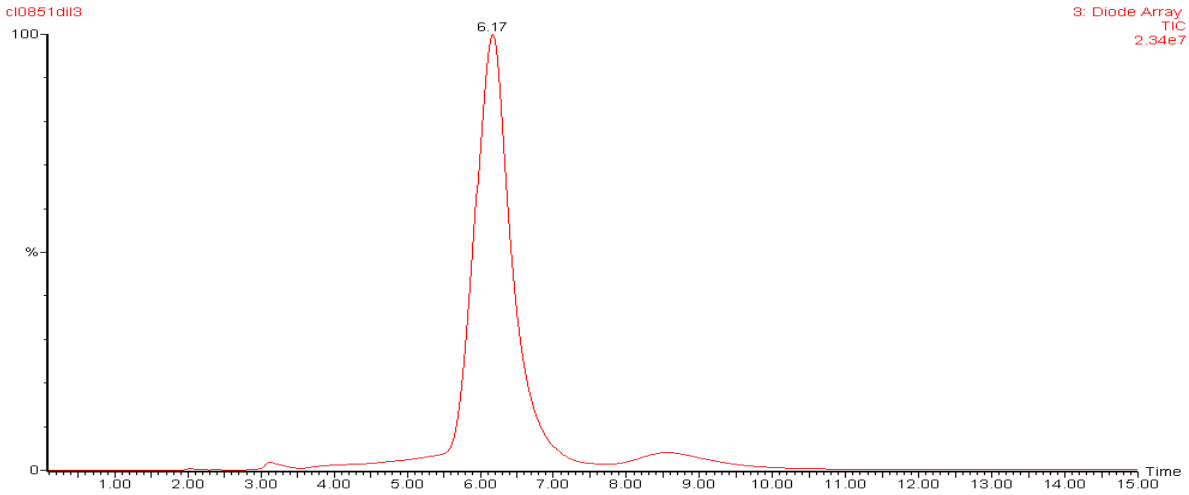


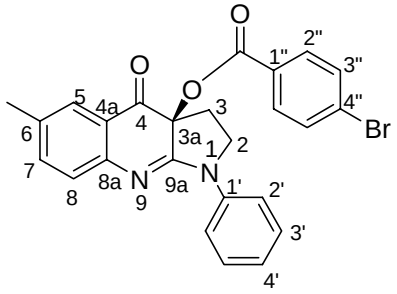
Figure S7: UV chromatogram of the two enantiomers of blebbistatin, RT=6.17 min and RT=8.55 min.

III. Proof of absolute stereochemistry of (*S*)-(-)-Blebbistatin (**1**)

1. Attempt to Brominate Blebbistatin.

A sample of optically enriched blebbistatin (0.030 g, 0.10 mmol, 1.0eq) was dissolved in dry DMF (3.0 mL). A solution of NBS (0.020 g, 0.10 mmol, 1.0eq) in dry DMF (3.0 mL) was added and the reaction stirred at room temperature for 24 hours. The reaction mixture was poured into water (50 mL) and extracted with diethyl ether (3 × 50 mL). The combined organic extracts were washed with water (3 × 50 mL), dried ($MgSO_4$) and concentrated *in vacuo* to afford a mixture of compounds from which it proved difficult to obtain a pure sample of a single compound.

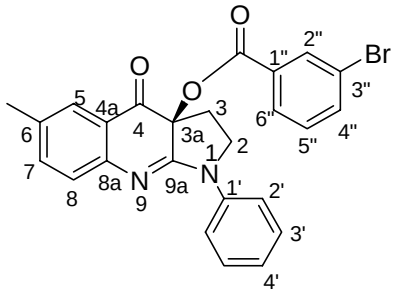
18 Synthesis of *S*-3a-(4-bromobenzoyloxy)-1-phenyl-2,3,3a,4-tetrahydro-1*H*-pyrrolo[2,3-*b*]quinolin-4-one



An optically enriched sample of (**1**) (0.090 g, 0.31 mmol, 1.0 eq) was dissolved in dichloromethane (28 mL) and a catalytic amount of DMAP (0.020 g, 0.16 mmol, 0.50 eq), dry pyridine (0.10 g, 1.2 mmol, 4.0 eq) and 4-bromo-benzoylchloride (0.70 g, 3.1 mmol, 10 eq) were added. The reaction mixture was stirred for 24 hours at room temperature, quenched with saturated ammonium chloride solution (5.0 mL) and then poured into water (50 mL, pH to 8) and extracted with dichloromethane (3 × 50 mL). The combined organic extracts were dried ($MgSO_4$) and concentrated *in vacuo*. The solid was purified by flash column chromatography on silica gel (eluting with 10% ethyl acetate/hexane), to give the title compound (0.15 g, 0.30 mmol, 100%(quant)) as a red solid. An analytical sample of this compound was prepared by recrystallisation from EtOAc/hexane; mp 161-162 °C; $[\alpha]_D^{26}$= -607

(c=0.1 in dichloromethane); ^{1}H NMR (300 MHz, $CDCl_3$): δ =2.30 (s, 3 H; C<u>H</u>$_3$), 2.69-2.89 (m, 2 H; 3-<u>H</u>), 4.01-4.09 (m (7 lines), 1 H; 2-<u>H</u>), 4.22 (dt, ^{3}J(H,H) = 9.5 Hz, ^{2}J(H,H) = 6.5 Hz, 1 H; 2-<u>H</u>), 7.16-7.23 (m, 2 H; Ar-4',8-<u>H</u>), 7.30 (ddq, ^{3}J(H,H) =8.1 Hz, ^{4}J(H,H) = 2.2 Hz, ^{4}J(H,H) = 0.6 Hz, 1 H; Ar-7-<u>H</u>), 7.40-7.50 (m, 4 H; Ar-3",3'-<u>H</u>), 7.55-7.58 (m, 1 H; Ar-5-<u>H</u>), 7.67-7.73 (m, 2 H; Ar-2"-<u>H</u>), 7.94-8.00 ppm (m, 2 H; Ar-2'-<u>H</u>); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ =20.6 (<u>C</u>H$_3$), 26.0 (<u>C</u>3), 48.1 (<u>C</u>2), 79.8 (<u>C</u>3a), 120.1 (<u>C</u>2'), 122.2 (<u>C</u>4"), 124.5 (<u>C</u>4'), 125.9 (<u>C</u>8), 127.1 (<u>C</u>5), 127.6 (<u>C</u>1"), 128.9 (<u>C</u>4a), 129.0 (<u>C</u>3'), 131.4 (<u>C</u>2"), 131.7(<u>C</u>3"), 133.6 (<u>C</u>6), 136.8 (<u>C</u>7), 140.0 (<u>C</u>1'), 148.3 (<u>C</u>8a), 160.3 (<u>C</u>9a), 164.1 (<u>C</u>=O), 192.3 ppm (<u>C</u>4); IR (Nujol): $\tilde{\nu}_{max}$=1734 (m) (C=O), 1591 (m), 1262 (m), 1089 (m), 1003 (m), 752 (m), 727 cm^{-1} (w) (Ar-H); LRMS (CI^{+}): *m/z* (%): 275 (100) [M-$C_7H_4O_2{}^{79}Br$]$^{+}$, 277 (66) [M-$C_7H_4O_2{}^{81}Br$]$^{+}$, 203 (40) [$C_7H_4O_2Br$]$^{+}$: HRMS (CI^{+}): *m/z* : calc'd for $C_{25}H_{20}N_2O_3{}^{79}Br$ [M+H]$^{+}$: 475.0657: found: 475.0661; Anal. calc'd for $C_{25}H_{19}BrN_2O_3$: C, 63.17; H, 4.03; N, 5.89; found: C, 63.37; H, 3.96; N, 5.59.

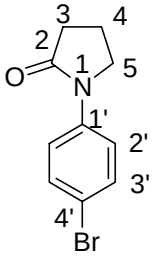
19. Synthesis of *S*-3a-(3-bromobenzoyloxy)-1-phenyl-2,3,3a,4-tetrahydro-1*H*-pyrrolo[2,3-*b*]quinolin-4-one



An optically enriched sample of (**1**) (0.090 g, 0.30 mmol, 1.0 eq) was dissolved in dichloromethane (28 mL) and a catalytic amount of DMAP (0.020 g, 0.20 mmol, 0.50 eq), dry pyridine (0.10 g, 1.2 mmol, 4.0 eq) and 3-bromo-benzoylchloride (0.70 g, 3.1 mmol, 10 eq) were added. The reaction mixture was stirred for 24 hours at room temperature, quenched with saturated ammonium chloride solution (5.0 mL) and then poured into water (50 mL, pH to 8) and extracted with dichloromethane (3 × 50 mL). The combined organic extracts were dried ($MgSO_4$) and concentrated *in vacuo.* The solid was purified by flash column chromatography on silica gel (eluting with 10% ethyl acetate/hexane), to give the title compound (0.14

g, 0.30 mmol, 97%) as a red solid. An analytical sample of this compound was prepared by recrystallisation from EtOAc/hexane; mp 128-129 °C; $[\alpha]_D^{26}$= -568 (c=0.1 in dichloromethane); ^{1}H NMR (300 MHz, $CDCl_3$): δ =2.32 (s, 3 H; C<u>H</u>$_3$), 2.70-2.91 (m, 2 H; 3-<u>H</u>), 4.01-4.11 (m (7 lines), 1 H; 2-<u>H</u>), 4.22 (dt, ^3J(H,H) = 9.5 Hz, ^2J(H,H) = 6.5 Hz, 1 H; 2-<u>H</u>), 7.17-7.24 (m, 3 H; Ar-8,4',5"-<u>H</u>), 7.32 (ddq, ^3J(H,H) = 8.1 Hz, ^4J(H,H) = 2.2 Hz, ^4J(H,H) = 0.6 Hz, 1 H; Ar-7-<u>H</u>), 7.42-7.49 (m, 2 H; Ar-3'-<u>H</u>), 7.57-7.60 (m, 1 H; Ar-5-<u>H</u>), 7.64 (ddd, ^3J(H,H) = 8.0 Hz, ^4J(H,H) = 2.0 Hz, ^4J(H,H) = 1.0 Hz, 1 H; Ar-4"-<u>H</u>), 7.79 (ddd, ^3J(H,H) = 8.0 Hz, ^4J(H,H) = 2.0 Hz, ^4J(H,H) = 1.0 Hz, 1 H; Ar-6"-<u>H</u>), 7.96-8.01 ppm (m, 3 H; Ar-2',2"-<u>H</u>); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ =20.6 (<u>C</u>H$_3$), 26.2 (<u>C</u>3), 48.1 (<u>C</u>2), 79.9 (<u>C</u>3a), 120.2 (<u>C</u>2'), 122.2 (<u>C</u>-Br), 122.4 (<u>C</u>1"), 124.6 (<u>C</u>4'), 125.9 (<u>C</u>8), 127.1 (<u>C</u>5), 128.5 (<u>C</u>6"), 129.0 (<u>C</u>3'), 130.0 (<u>C</u>5"), 130.6 (<u>C</u>4a), 132.8 (<u>C</u>2"), 133.6 (<u>C</u>6), 136.5 (<u>C</u>4"), 136.8 (<u>C</u>7), 139.9 (<u>C</u>1'), 148.2 (<u>C</u>8a), 160.2 (<u>C</u>9a), 163.5 (<u>C</u>=O), 192.2 ppm (<u>C</u>4); IR (Nujol): $\tilde{\nu}_{max}$=1734 (m) (C=O), 1593 (m), 1253 (m), 1054 (m), 740 (m), 710 (m) (Ar-H), 681 cm^{-1} (w); LRMS (EI): *m/z* (%): 474 (32), 476 (11), 274 (100) [M-$C_7H_4O_2{}^{79}Br$]$^+$, 276 (58) [M-$C_7H_4O_2{}^{81}Br$]$^+$, 200 (64), 202 (63), 187 (63), 185 (64), 156 (40), 149 (48), 154 (38); HRMS (EI): *m/z* : calc'd for $C_{25}H_{19}N_2O_3{}^{79}Br$ [M]$^+$: 474.0579: found: 474.0580 and calc'd for $C_{25}H_{19}N_2O_3{}^{81}Br$ [M]$^+$: 476.0559: found: 476.0564.

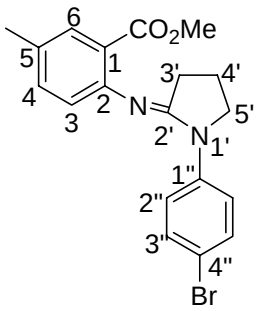
20. Synthesis of 1-(4'-Bromo-phenyl)-pyrrolidin-2-one[1]



1-Phenyl-2-pyrrolidinone (**6**) (5.0 g, 31 mmol, 1.0 eq) and *N* bromosuccinimide (5.3 g, 31 mmol, 1.0 eq) were dissolved in anhydrous dimethylformamide (40 mL). The reaction mixture was left to stir at room temperature for two days. The mixture was poured into water (50 mL) and extracted with diethyl ether (3 × 50 mL). The organic extracts were washed with water (3 × 50 mL), dried ($MgSO_4$) and concentrated *in vacuo* to give a white solid (6.95 g). The crude solid was purified by recrystallisation from hexane/ethyl acetate to afford 1-(4'-

Bromo-phenyl)-pyrrolidin-2-one (3.64 g, 15.2 mmol, 50%) as white needles; mp 99-100 °C (lit 97.5 °C)[1]; ^{1}H NMR (300 MHz, $CDCl_3$): δ =2.11-2.23 (m, 2 H; 4-H), 2.58-2.65 (m, 2 H; 3-H), 3.81-3.87 (m, 2 H; 5-H), 7.44-7.49 (m, 2 H; Ar-3'-H), 7.51-7.56 ppm (m, 2 H; Ar-2'-H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ =17.8 (C4), 32.6 (C3), 48.5 (C5), 117.1 (C4'), 121.2 (C3'), 131.7 (C2'), 138.4 (C1'), 174.2 ppm (C2); IR (Nujol): $\tilde{\nu}_{max}$=1684 (s) (C=O), 1582 (m), 1309 (m), 1224 (m), 1065 (w), 828 cm^{-1} (s); LRMS (ES^+): *m/z* (%): 296 (28), 262 (100) [^{79}M+23]$^+$, 264 (96) [^{81}M+23]$^+$; HRMS (ES^+): *m/z* calc'd for $C_{10}H_{10}NONa^{79}Br$ [M+23]$^+$: 261.9843; found: 261.9850 and calc'd for $C_{10}H_{10}NONa^{81}Br$ [M+23]$^+$: 263.9823; found: 263.9835; Anal. calc'd for $C_{10}H_{10}NOBr$: C, 50.02; H, 4.20; N, 5.83; found: C, 49.72; H, 3.98; N, 5.66.

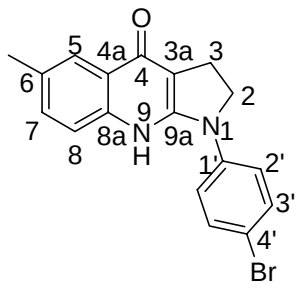
21. Synthesis of 2-[1-(4-Bromo-phenyl)-pyrrolidin-2-ylideneamino-5-methyl-benzoic acid methyl ester



Phosphorus oxychloride (0.81 g, 5.3 mmol, 0.90 eq) was added dropwise to a solution of 1-(4-bromo-phenyl)-2-pyrrolidinone (1.3 g, 5.3 mmol, 0.90 eq) in dry dichloromethane (8.0 mL) and the reaction was stirred for 3 hours at room temperature. A solution of anthranilate (**5**) (0.98 g, 5.9 mmol, 1.0 eq) in dry dichloromethane (15 mL) was then added and the reaction refluxed for 16 hours. The reaction mixture was cooled and concentrated *in vacuo*. The resulting solid was dissolved in aqueous hydrochloric acid (0.30 M, 100 mL) and extracted with dichlroromethane (3 × 100 mL). The aqueous phase was then basified with aqueous sodium hydroxide solution (2.0 M, to pH 8) and extracted with ethyl acetate (3 × 100 mL). The first organic extracts were concentrated *in vacuo* and the resulting solid was carried through the above procedure three more times. All ethyl acetate extracts were combined, dried ($MgSO_4$) and concentrated *in vacuo* to give the desired compound 2-

[1-(4-Bromo-phenyl)-pyrrolidin-2-ylideneamino-5-methyl-benzoic acid methyl ester (0.54 g, 1.4 mmol, 26%) as a pale-yellow oil; ^{1}H NMR (300 MHz, $CDCl_3$): δ=1.98-2.09 (m (6 lines), 2 H; 4'-H), 2.32 (s, 3 H; CH_3), 2.45 (t, ^{3}J(H,H) = 7.8 Hz, 2 H; 3'-H), 3.80 (s, 3 H; OCH_3), 3.81-3.86 (m, 2 H; 5'-H), 6.70 (d, ^{3}J(H,H) = 8.1 Hz, 1 H; Ar-3-H), 7.19 (ddd, ^{3}J(H,H) =8.1 Hz, ^{4}J(H,H) =2.1 Hz, ^{4}J(H,H) = 0.6 Hz, 1 H; Ar-4-H), 7.41-7.46 (m, 2 H; Ar-3"-H), 7.67 (d, ^{3}J(H,H) = 2.0 Hz, 1 H; Ar-6-H), 7.71-7.77 ppm (m, 2 H; Ar-2"-H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ =20.0 (C4'), 21.0 (CH_3), 29.5 (C3'), 50.6 (C5'), 52.1 (OCH_3), 115.9 (C4"), 121.6 (C1), 122.1 (C2"), 123.3 (C3), 131.5 (C5), 131.7 (C3"), 131.8 (C6), 134.0 (C4), 140.9 (C1"), 150.6 (C2), 160.2 (C2') 168.0 ppm (C=O); IR (Nujol): $\tilde{\nu}_{max}$= 1719 (m) (C=O), 1668 (m) (C=N), 1582 (m), 1197 (m), 1078 (m), 1003 (m), 826 cm^{-1} (m); LRMS (CI^{+}): *m/z* (%): 389 (82) [^{81}M+H]$^{+}$, 387 (100) [^{79}M+H]$^{+}$, 308 (15) [M-Br]$^{+}$; HRMS (CI^{+}): *m/z* calc'd $C_{19}H_{20}N_2O_2{}^{79}Br$ [M + H]$^{+}$: 387.0708; found: 387.0697 and calc'd $C_{19}H_{20}N_2O_2{}^{81}Br$ [M + H]$^{+}$: 389.0688; found: 389.0703.

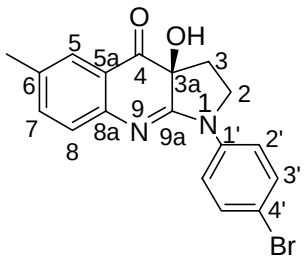
22. Synthesis of 1-(4-Bromo-phenyl)-6-methyl-1,2,3,9-tetrahydro-pyrrolo[2,3-*b*]quinolin-4-one



A solution of 2-[1-(4-Bromo-phenyl)-pyrrolidin-2-ylideneamino-5-methyl-benzoic acid methyl ester (0.47 g, 1.2 mmol, 1.0 eq) in anhydrous THF (45 mL) was cooled to –78 °C and stirred for 15 minutes. Lithium bis(trimethylsilyl)amide (1.0 M in THF, 3.7 mL, 3.7 mmol, 3.0 eq) was added dropwise to the reaction mixture. The reaction mixture was warmed to 0 °C over three hours. The reaction mixture was quenched at 0 °C with saturated ammonium chloride solution (5.0 mL). Further saturated ammonium chloride solution (150 mL) was added. The aqueous phase was extracted with dichloromethane (3 × 100 mL) and the combined organic extracts dried ($MgSO_4$), and concentrated *in vacuo.* The crude product was purified by flash column

chromatography on silica gel (eluting with 100% ethyl acetate), to give the desired compound 1-(4-Bromo-phenyl)-6-methyl-1,2,3,9-tetrahydro-pyrrolo[2,3-*b*]quinolin-4-one (0.26 g, 0.70 mmol, 60%) as a brown solid; mp 187-188 °C; ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ=2.41 (s, 3 H; C<u>H</u>$_3$), 3.16 (t, ^{3}J(H,H) = 8.1 Hz, 2 H; 3-<u>H</u>), 4.02 (t, ^{3}J(H,H) = 8.1 Hz, 2 H; 2-<u>H</u>), 7.32 (dd, ^{3}J(H,H) = 8.4 Hz, ^{4}J(H,H) = 2.0 Hz, 1 H; Ar-7-<u>H</u>), 7.49-7.54 (m, 3 H; Ar-3',8-<u>H</u>), 7.77 (br s, 1 H; Ar-5-<u>H</u>), 8.04-8.09 ppm (m, 2 H; Ar-2'-<u>H</u>); ^{13}C NMR (75.5 MHz, [D$_6$]DMSO): δ=21.0 (<u>C</u>H$_3$), 22.0 (<u>C</u>2'), 48.0 (<u>C</u>3'), 106.3 (<u>C</u>3a), 112.0 (<u>C</u>4'), 118.7 (<u>C</u>6), 119.0 (<u>C</u>2'), 120.5 (<u>C</u>5), 126.2 (<u>C</u>3'), 130.4 (<u>C</u>7), 131.0 (<u>C</u>4a), 131.1 (<u>C</u>8), 141.4 (<u>C</u>1'), 146.2 (<u>C</u>8a), 153.9 (<u>C</u>9a), 159.1 ppm (<u>C</u>4): IR (Nujol): $\tilde{\nu}_{max}$=1628 (m) (C=O), 1572 (m), 1312 (m), 1054 (w), 1002 (w), 818 (w), 727 cm^{-1} (w) (Ar-H); LRMS (EI): *m/z* (%):354 (100) [^{79}M]$^{+}$, 356 (85) [^{81}M]$^{+}$, 273 (12); HRMS (EI): *m/z* calc'd for $C_{18}H_{15}N_2O^{79}Br$ [M]$^{+}$: 354.0368; found: 354.0364.

23. Synthesis of 1-(4-Bromo-phenyl)-3a-hydroxy-6-methyl-1,2,3,3a-tetrahydro-pyrrolo[2,3-*b*]quinolin-4-one (11)



A solution of 1-(4-Bromo-phenyl)-6-methyl-1,2,3,9-tetrahydro-pyrrolo[2,3-*b*]quinolin-4-one (0.22 g, 0.60 mmol, 1.0 eq) in dry THF (15 mL) was added dropwise to lithium bis(trimethylsilyl)amide (1.0 M in THF, 0.74 mL, 0.74 mmol, 1.2 eq) in dry THF (4.0 mL) at -78 °C under argon. The reaction was stirred for 30 minutes at -78 °C and a solution of the Davis reagent (**10**) (0.45 g, 1.5 mmol, 2.4 eq) in dry THF (10 mL) was added via cannula. After 16 hours at -10 °C saturated ammonium iodide solution (10 mL, 10 eq) and diethyl ether (10 mL) were added and the reaction warmed to room temperature. Saturated sodium thiosulfate solution (15 mL) was then added and the reaction extracted with diethyl ether (2 × 10 mL). The combined organic extracts were dried ($MgSO_4$) and concentrated *in vacuo* to give a yellow solid.

The solid was partitioned between dichloromethane (100 mL) and aqueous hydrochloric acid solution (0.30 M, 100 mL). The aqueous phase was washed with dichloromethane (3 × 100 mL), basified with aqueous sodium hydroxide solution (2.0 M, to pH 8) and extracted with ethyl acetate (2 × 100 mL). The ethyl acetate extracts were dried ($MgSO_4$) and concentrated *in vacuo* to give the desired compound (**11**) (0.16 g, 0.40 mmol, 68%) as a red solid. The enantiomeric excess of (**11**) was 88% as determined by chiral-phase HPLC analysis; mp 183-184 °C; $[\alpha]_D^{26}$= -281 (c=0.1 in dichloromethane); ^{1}H NMR (300 MHz, $CDCl_3$): δ =2.22-2.29 (m, 1 H; 3-H), 2.32 (s, 3 H; CH_3), 2.41-2.49 (m, 1 H; 3-H), 3.78-3.86 (m, 1 H; 2-H), 3.95-4.06 (m, 1 H; 2-H), 7.10 (d, ^3J(H,H) = 8.1 Hz, 1 H; Ar-8-H), 7.25 (ddd, ^3J(H,H) =8.2 Hz, ^4J(H,H) =2.2 Hz, ^4J(H,H) = 0.6 Hz, 1 H; Ar-7-H), 7.47-7.54 (m, 2 H; Ar-3'-H), 7.62-7.65 (m, 1 H; Ar-5-H), 7.77-7.82 ppm (m, 2 H; Ar-2'-H); ^{13}C NMR (75.5 MHz, [D_8]THF): δ =20.6 (CH_3), 29.5 (C3), 48.2 (C2), 73.8 (C3a), 116.3 (C4'), 121.8 (C2'), 122.2 (C4a), 126.9 (C8), 127.3 (C5), 132.1 (C3'), 133.6 (C6), 136.6 (C7), 141.3 (C1'), 149.8 (C8a), 166.3 (C9a), 194.8 ppm (C4); IR (Nujol): $\tilde{\nu}_{max}$=3304 (br) (O-H), 1673 (s) (C=O), 1615 (s) (C=N), 1588 (w), 1490 (w), 1297 (m), 1258 (m), 1102 (w), 835 (w), 801 cm^{-1} (w); LRMS (CI^+): 373 (90) $[^{81}M+H]^+$, 371 (100) $[^{79}M+H]^+$, 355 (45), 344 (14), 292 (50) $[M-Br]^+$, 274 (9): HRMS (CI^+): *m/z* calc'd $C_{18}H_{16}N_2O_2{}^{79}Br$ $[M + H]^+$: 371.0395; found: 371.0395; Anal. calc'd for $C_{18}H_{15}BrN_2O_2$: C, 58.24; H, 4.07; N, 7.55; found: C, 58.21; H, 3.87; N, 7.63; HPLC (Daicel Chiralpak AD, Acetonitrile/Water 50:50, flow rate 0.8 ml min^{-1}, λ=254 nm): major enantiomer: t_R= 29.12 min: minor enantiomer: t_R= 59.25 min.

An analytical sample of (**11**) was prepared by recrystallisation from MeCN; $[\alpha]_D^{26}$= -526 (c=0.1 in dichloromethane). Chiral HPLC analysis showed that after a single recrystallisation (**11**) was prepared with an *ee* of >99%. HPLC (Daicel Chiralpak AD, Acetonitrile/Water 50:50, flow rate 0.8 ml min^{-1}, λ=254 nm): major enantiomer: t_R= 29.18 min.

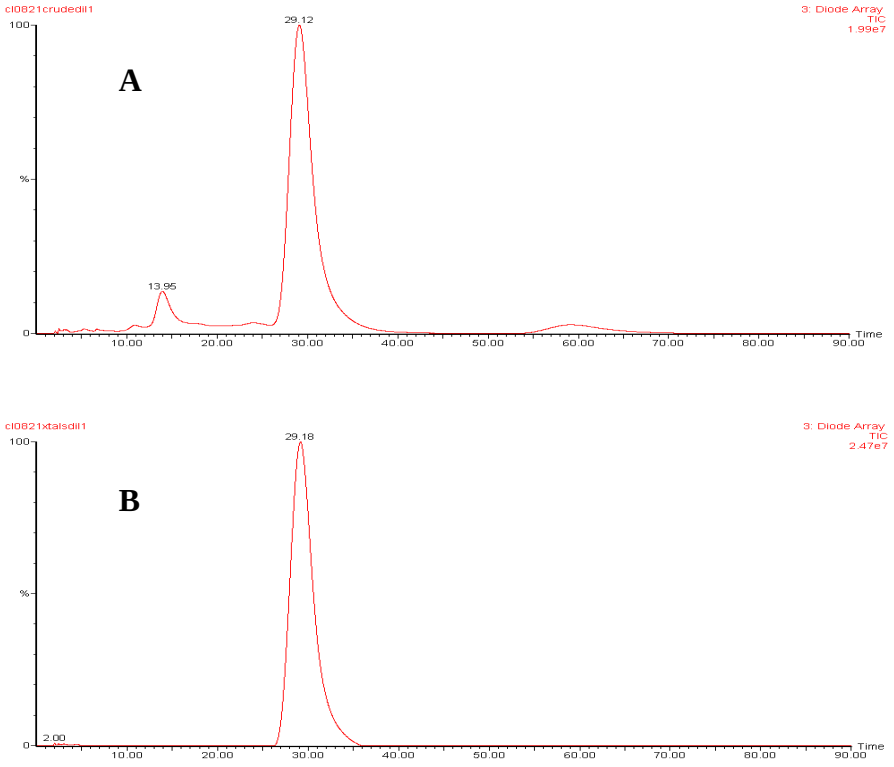
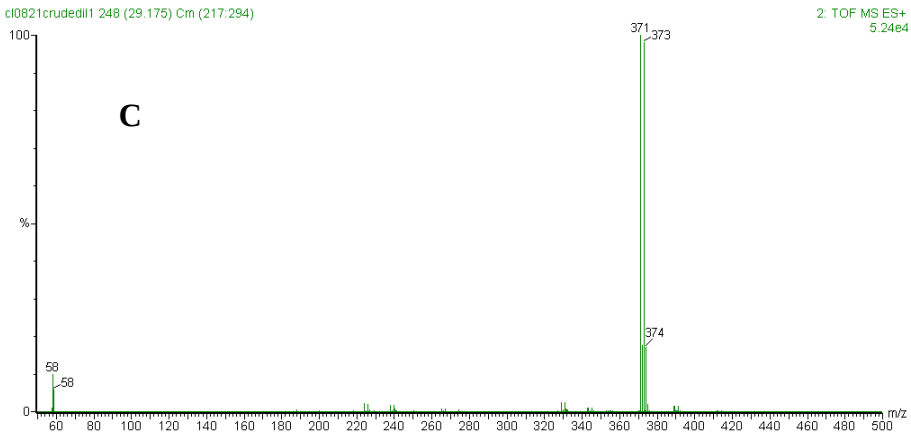


Figure S8: A) UV chromatogram of the two enantiomers of br-blebbistatin, B) UV chromatogram of the cristallised br-blebbistatin.



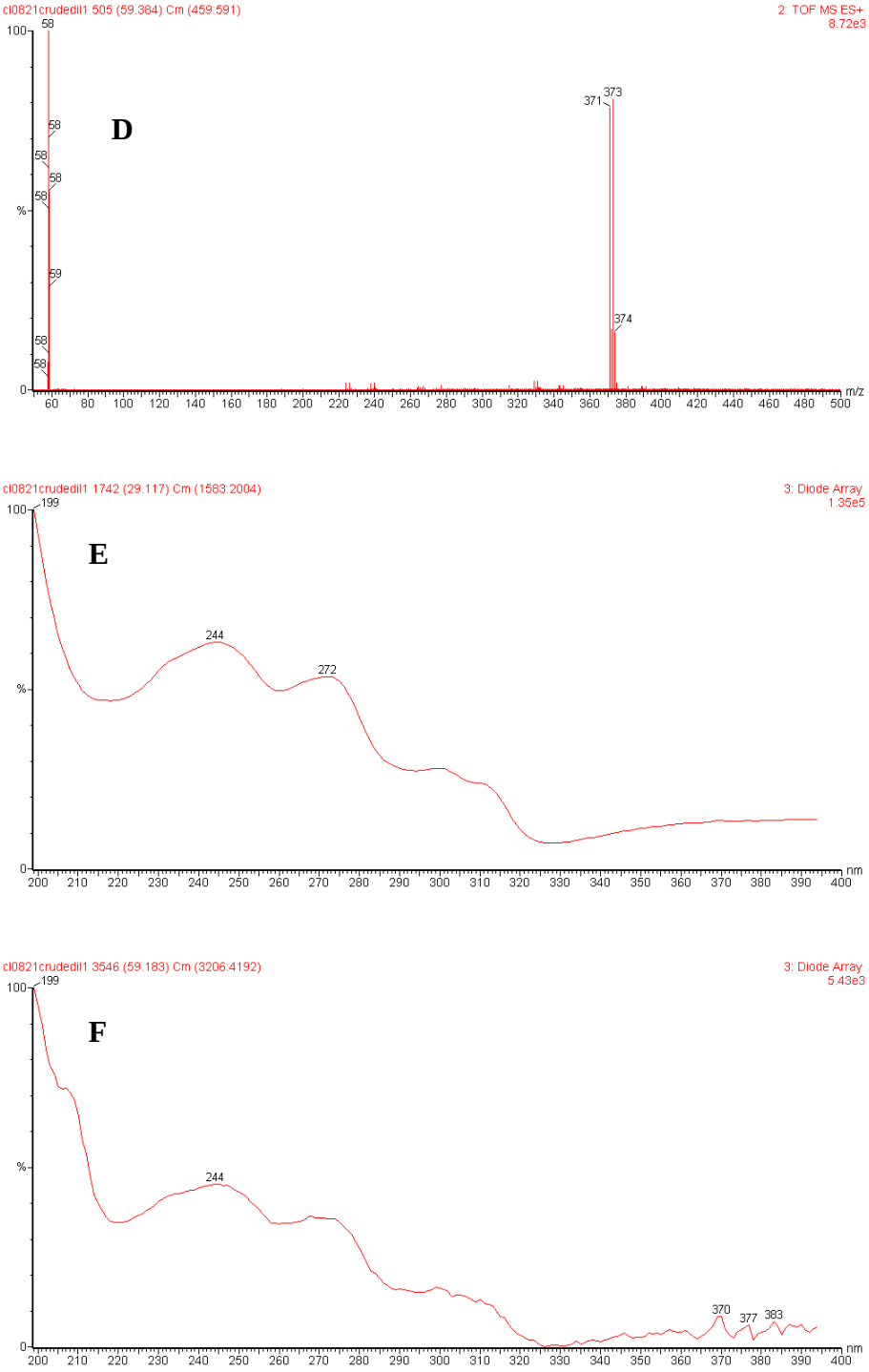


Figure S9: C), ESMS (+ve) of peak RT=29.12 min. $[M+H]^+$=371, $[M+H]^+$=373. D), ESMS (+ve) of peak RT=59.25 min. $[M+H]^+$=371, $[M+H]^+$=373. E), UV-vis spectra of peak RT=29.12 min. max=244 nm. F), UV-vis spectra of peak RT=59.25 min. max=244 nm.

X-RAY structure of 1-(4-Bromo-phenyl)-3a-hydroxy-6-methyl-1,2,3,3a-tetrahydro-pyrrolo[2,3-*b*]quinolin-4-one (11)

Summary of Data CCDC 238392

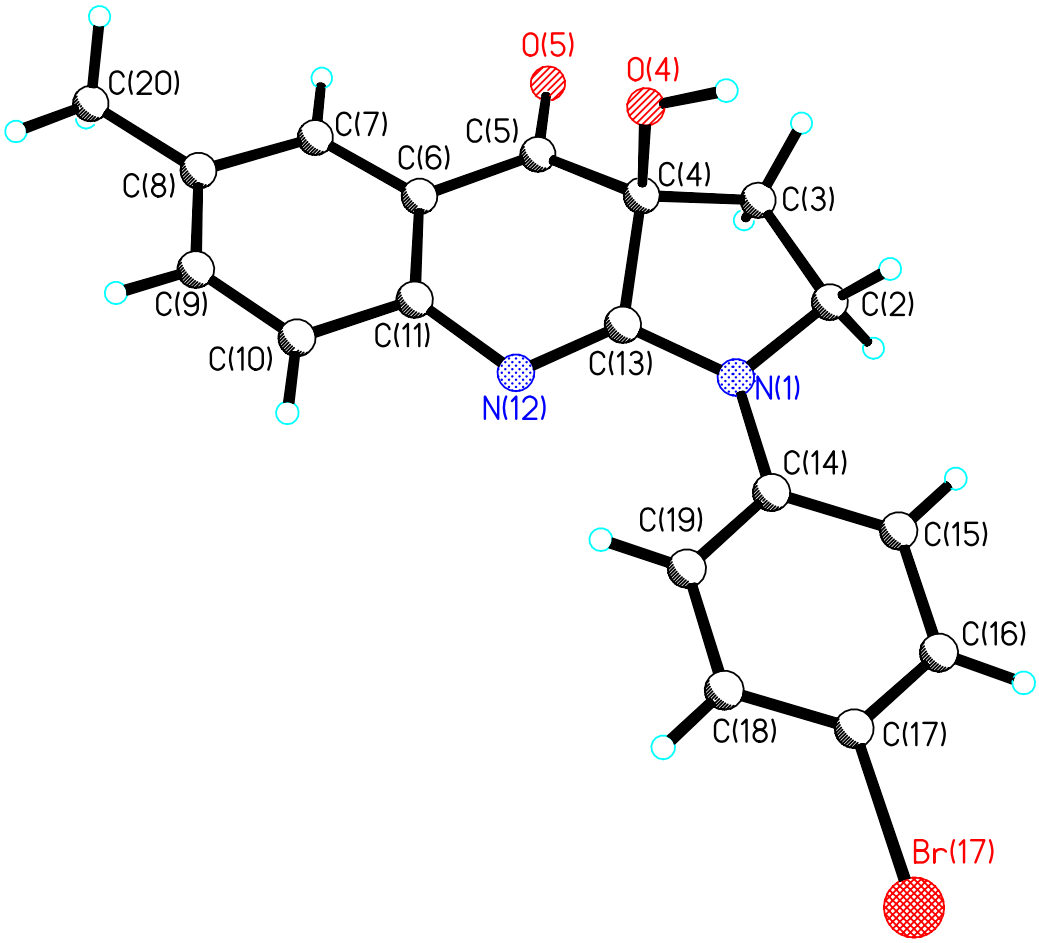
Authors: C. Lucas-Lopez, S. Patterson, T. Blum, A. F. Straight, J. Toth, A. M. Z. Slawin, T. J. Mitchison, J. R. Sellers, N. J. Westwood

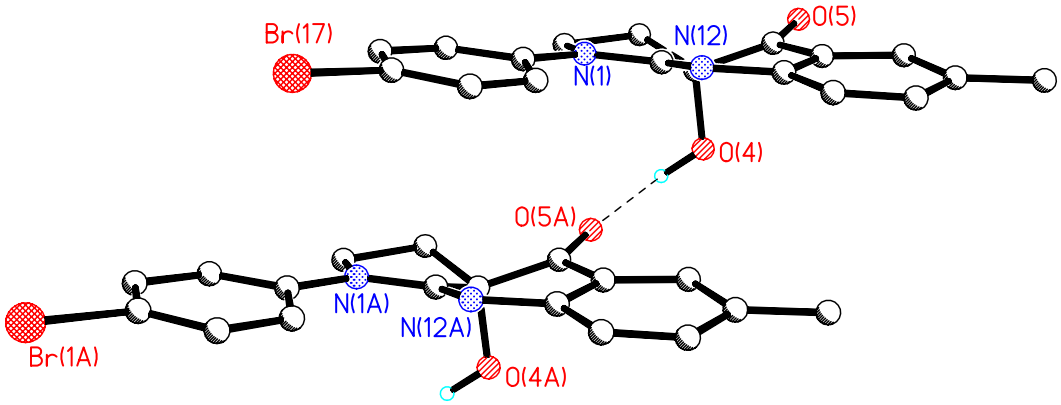
Journal: Angew.Chem.,Int.Ed.Engl. (0179)

Formula: C18 H15 Br N2 O2

Unit cell parameters: a 5.6627(11) b 11.588(2) c 23.364(5)

Space group P212121




Br(17)
N(1)
N(12)
O(5)
O(4)
O(5A)
N(1A)
N(12A)
Br(1A)
O(4A)

24. Reduction of 1-(4-Bromo-phenyl)-3a-hydroxy-6-methyl-1,2,3,3a-tetrahydro-pyrrolo[2,3-*b*]quinolin-4-one

To a solution of 1-(4-Bromo-phenyl)-3a-hydroxy-6-methyl-1,2,3,3a-tetrahydro-pyrrolo[2,3-*b*]quinolin-4-one (0.015 g, 0.040 mmol, 1.0eq) in a 1:1 mixture of methanol and dimethyl formamide (6.0 mL) were added triethylamine (0.015 g) and 1% Pd/C (0.015 g) under argon. This suspension was then degassed under vacuum. Hydrogen was then introduced and the suspension was stirred at room temperature under hydrogen for 24 hours. The solid material was filtered off and washed with methanol (2.0 mL). The filtrate was concentrated *in vacuo* and the residue was purified by flash column chromatography on silica gel (eluting with 20% ethyl acetate/hexane), to give the desired compound (**1**) (0.012 g, 0.040 mmol, 99%(quant)) as bright yellow solid. ^{1}H NMR and MS agrees with the authentic material, Blebbistatin (**1**) prepared by method 4, entry 1.

25. Chiral HPLC to compare reduced br-blebbistatin with Blebbistatin.

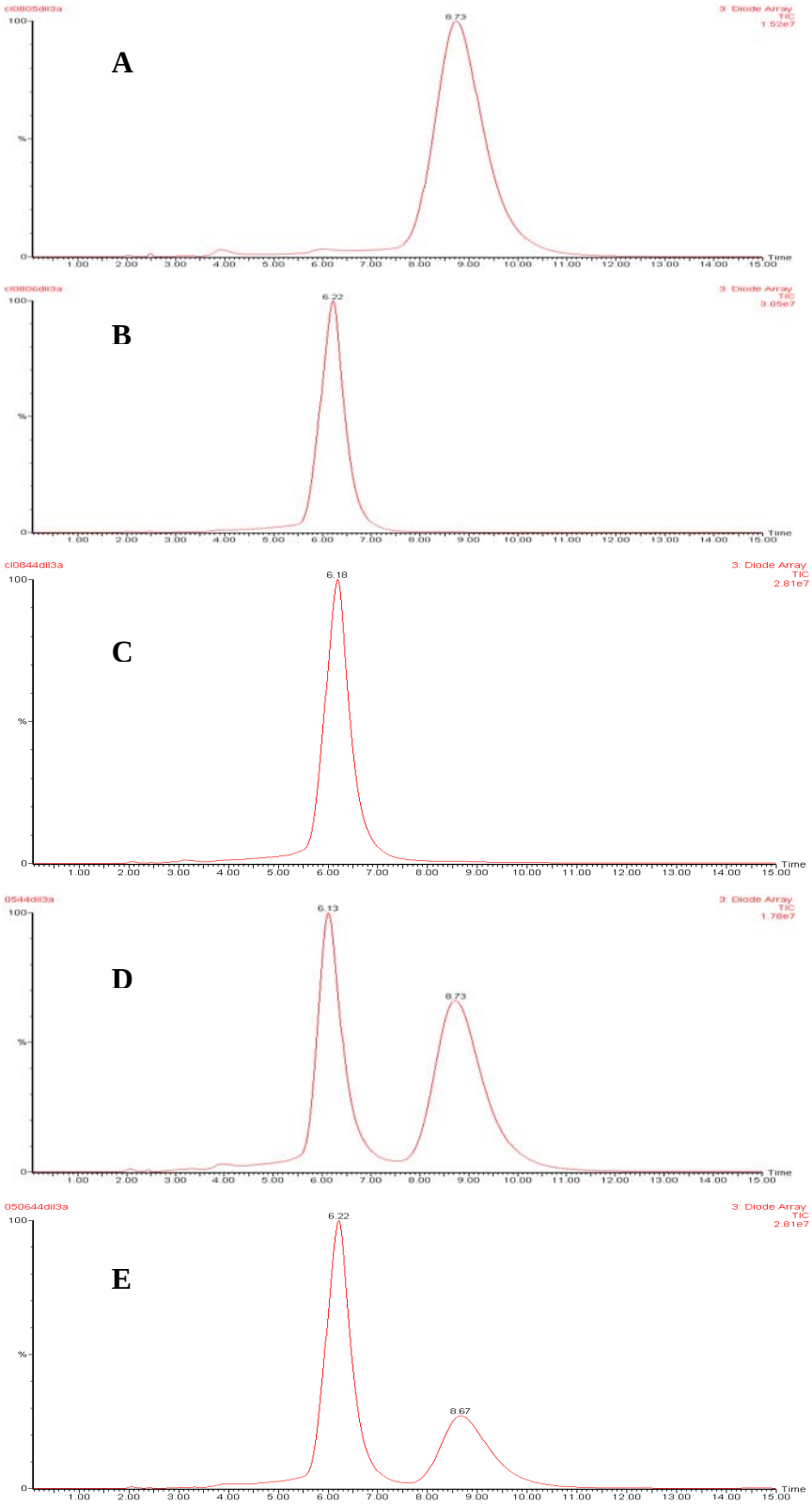


Figure S9: UV chromatograms of A) cristallised material (**2**), RT=8.73 min, B) cristallised material (**1**), RT=6.22 min, C) reduced br-blebbistatin RT=6.18 min, D) A+C, RT=6.13 min, RT=8.73 min. E) B+C, RT=6.22 min, RT=8.67 min.

26. Prediction of the stereochemistry of (*S*)-(-)-Blebbistatin (1).

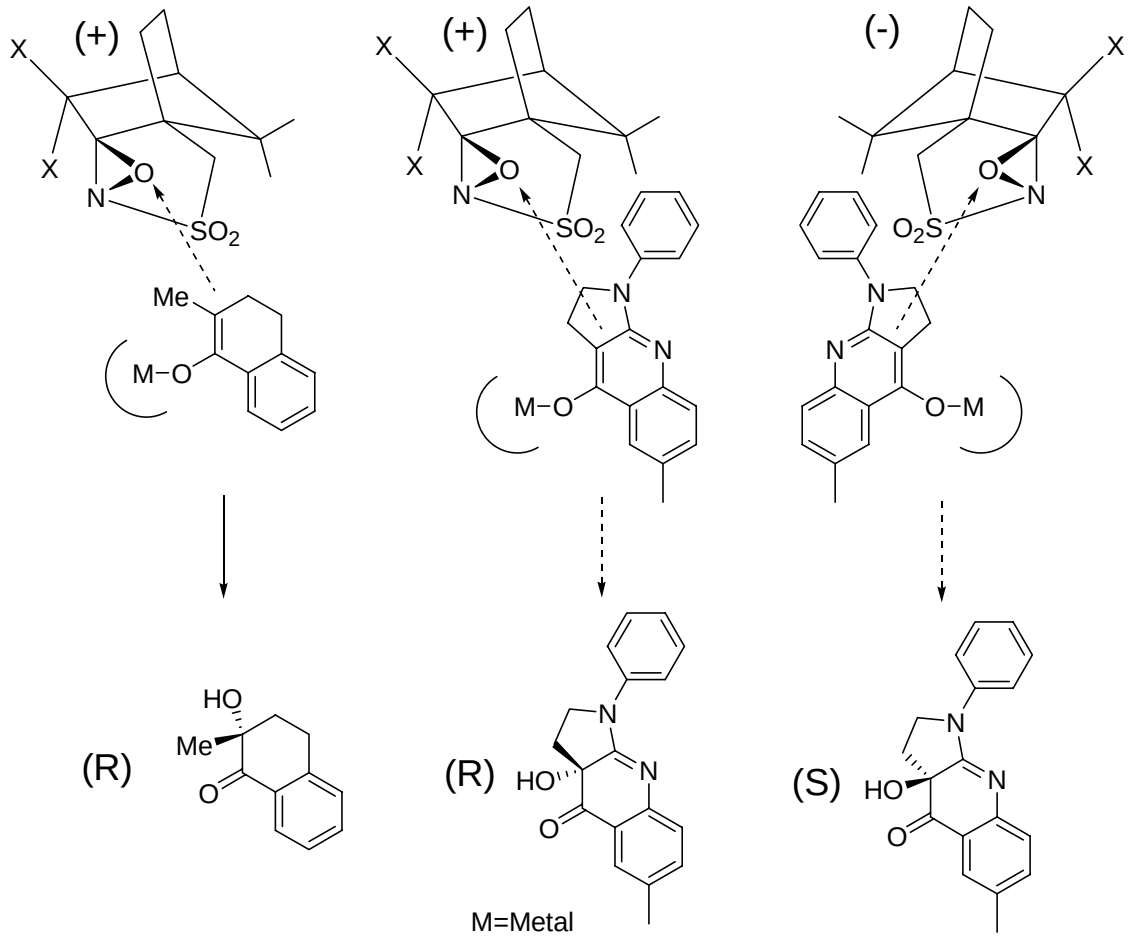
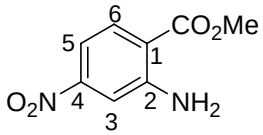


Figure S10: A schematic representation of Davis' rationalisation of the sense of asymmetric induction[2] and its application to (*S*)-(-)-Blebbistatin.

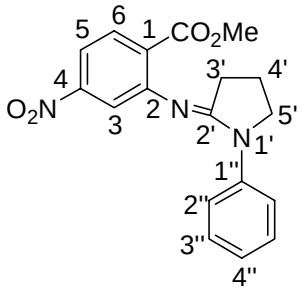
VII. Synthetic route to a nitro-containing analogue of (*S*)-(-)-Blebbistatin (1)

27. 2-Amino-4-nitro-benzoic acid methyl ester



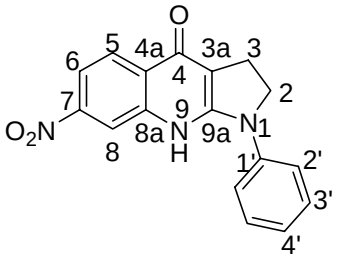
4-Nitro-2-amino benzoic acid (0.45 g, 2.5 mmol, 1.0 eq) was dissolved in methanol (50 mL) and H_2SO_4 (conc., 2.0 mL) was added. The reaction mixture was refluxed for four days, cooled and concentrated *in vacuo*. The resulting solid was dissolved in aqueous sodium hydroxide solution (2.0 M, 100 mL) and extracted with ethyl acetate (3 × 100 mL). The organic extracts were combined, dried ($MgSO_4$), and concentrated *in vacuo* to give a solid. The solid was purified by flash column chromatography on silica gel (eluting with 10% ethyl acetate/hexane), to give the desired compound 2-Amino-4-nitro-benzoic acid methyl ester (0.39 g, 2.0 mmol, 81%) as a red solid; mp 153-154 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ=3.91 (s, 3 H; OC<u>H</u>$_3$), 7.39 (dd, ^3J(H,H) = 8.8 Hz, ^4J(H,H) = 2.3 Hz, 1 H; Ar-5-<u>H</u>), 7.50 (dd, ^4J(H,H) = 2.3 Hz, ^4J(H,H) = 0.4, 1 H; Ar-3-H), 8.00 ppm (dd, ^3J(H,H) = 8.8 Hz, ^4J(H,H) = 0.4 Hz, 1 H; Ar-6-<u>H</u>); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ =52.2 (<u>C</u>H$_3$), 110.0 (<u>C</u>5), 111.1 (<u>C</u>3), 114.9 (<u>C</u>1), 132.8 (<u>C</u>6), 132.9 (<u>C</u>2), 150.6 (<u>C</u>-NO$_2$), 167.3 ppm (<u>C</u>=O); IR (Nujol): $\tilde{\nu}_{max}$=3487 (m) (N-H), 3378 (m) (N-H), 1701 (m) (C=O), 1519 (m), 1251 (m), 730 cm^{-1} (m) (Ar-H); LRMS (CI$^+$): *m/z* (%): 197 (100) [M+H]$^+$, 165 (14), 135 (17); HRMS (CI$^+$): *m/z* calc'd for $C_8H_9N_2O_4$ [M+H]$^+$: 197.0562; found: 197.0568.

28. Synthesis of methyl 4-nitro-2-(1-phenylpyrrolidin-2-ylideneamino)benzoate



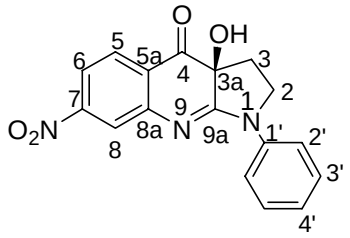
Phosphorus oxychloride (0.62 g, 0.38 mL, 4.1 mmol, 1.0 eq) was added dropwise to a solution of 1-phenyl-2-pyrrolidinone (**6**) (0.72 g, 4.5 mmol, 1.1 eq) in dry dichloromethane (10 mL) and the reaction was stirred for 3 hours at room temperature. A solution of 2-Amino-4-nitro-benzoic acid methyl ester (0.80 g, 4.1 mmol, 1.0 eq) in dry dichloromethane (20 mL) was then added and the reaction refluxed for 72 hours. The reaction mixture was cooled and concentrated *in vacuo*. The resulting solid was dissolved in aqueous hydrochloric acid (0.30 M, 100 mL) and extracted with dichloromethane (3 × 100 mL). The aqueous phase was then basified with aqueous sodium hydroxide solution (2.0 M, to pH 8) and extracted with ethyl acetate (3 × 100 mL). The first organic extracts were concentrated *in vacuo* and the resulting solid was carried through the above procedure three more times. All ethyl acetate extracts were combined, dried ($MgSO_4$) and concentrated *in vacuo* to give the desired compound methyl 4-nitro-2-(1-phenylpyrrolidin-2-ylideneamino)benzoate (0.30 g, 0.90 mmol, 22%) as a crystalline yellow solid; mp 111-112 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ =2.03-2.15 (m, 2 H; 4'-H), 2.52 (t, ^3J(H,H) = 7.8 Hz, 2 H; 3'-H), 3.86 (s, 3 H; OCH_3), 3.91 (t, ^3J(H,H) = 6.9 Hz, 2 H; 5'-H), 7.06-7.13 (m, 1 H; Ar-4"-H), 7.32-7.39 (m, 2 H; Ar-3"-H), 7.66 (d, ^3J(H,H) = 2.3 Hz, 1 H; Ar-3-H), 7.71-7.81 (m, 3 H; Ar-2",5-H), 7.92 ppm (d, ^3J(H,H) = 8.6 Hz, 1 H; Ar-6-H); ^{13}C NMR (75.5 MHz, $CDCl_3$): δ =19.6 (C4'), 29.5 (C3'), 50.9 (C5'), 52.2 (OCH_3), 115.7 (C5), 118.0 (C3), 120.8 (C2"), 123.7 (C4"), 128.2 (C1), 128.5 (C3"), 131.5 (C6), 140.6 (C1'), 150.0 (C4), 153.9 (C2), 160.2 (C2'), 166.1 ppm (C=O); IR (Nujol): $\tilde{\nu}_{max}$=1726 (s) (C=O), 1653 (s) (C=N), 1588 (m), 1306 (w), 1238 (w), 1198 (w), 762 cm^{-1} (w) (Ar-H); LRMS (CI$^+$): *m/z* (%): 368 (10), 340 (100) $[M+H]^+$, 310 (9): HRMS (CI$^+$): *m/z* calc'd for $C_{18}H_{18}N_3O_4$ $[M+H]^+$: 340.1297; found: 340.1294.

28. Synthesis of 7-Nitro-1-phenyl-1,2,3,9-tetrahydro-pyrrolo[2,3-*b*]quinolin-4-one



A solution of 4-nitro-2-(1-phenylpyrrolidin-2-ylideneamino)benzoate (0.25 g, 0.74 mmol, 1.0 eq) in anhydrous THF (15 mL) was cooled to –78 °C and stirred for 15 minutes. Lithium bis(trimethylsilyl)amide (1.0 M in THF, 0.74 mL, 0.74 mmol, 1.0 eq) was added dropwise to the reaction mixture. The reaction mixture was warmed to 0 ºC and stirred for 16 hours. Additional base (0.39 mL, 0.5 eq) was added every 24 hours. After 4 days the reaction was quenched by addition of saturated ammonium chloride solution (5.0 mL) and the reaction allowed to warm to room temperature. Further saturated ammonium chloride solution (150 mL) was added, the aqueous phase was extracted with dichloromethane (3 × 100 mL) and the combined organic extracts dried ($MgSO_4$) and concentrated *in vacuo*. The solid was purified by flash column chromatography on silica gel (eluting with 100% ethyl acetate), to give the desired compound 7-Nitro-1-phenyl-1,2,3,9-tetrahydro-pyrrolo[2,3-*b*]quinolin-4-one (0.099 g, 0.32 mmol, 44%) as an orange solid; mp 270-271 °C; ^{1}H NMR (300 MHz, [D_4]MeOD): δ =4.15 (t, ^3J(H,H) = 8.3 Hz, 2 H; 3-H), 5.14 (t, ^3J(H,H) = 8.3 Hz, 2 H; 2-H), 8.21 (t, ^3J(H,H) = 7.5 Hz, 1 H; Ar-4”-H), 8.40-8.47 (m, 2 H; Ar-3”-H), 8.59-8.66 (m, 2 H; Ar-2”-H), 8.96 (dd, ^3J(H,H) = 8.9 Hz, ^4J(H,H) = 2.3 Hz, 1 H; Ar-6-H), 9.18 (d, ^3J(H,H) = 8.9 Hz, 1 H; Ar-5-H), 9.33 ppm (d, ^4J(H,H) = 2.3 Hz, 1 H; Ar-8-H): ^{13}C NMR: (75.5 MHz, [D_8]THF): δ =22.5 (C3), 49.4 (C2), 110.4 (C3a), 115.7 (C6), 118.7 (C2’), 122.1 (C8), 122.4 (C4’), 123.2 (C5), 124.0 (C4a), 129.0 (C3’), 142.6 (C1’), 148.8 (C8a), 149.2 (C7), 154.6 (C9a), 162.1 (C4): IR (Nujol): $\tilde{\nu}_{max}$=1628 (m) (C=O), 1575 (m), 1542 (m), 1335 (m), 1295 (m), 750 (w) (Ar-H), 727 cm^{-1} (m) (Ar-H); LRMS (ES$^+$): *m/z* (%): 308 (100) [M+H]$^+$; HRMS (ES$^+$): *m/z* calc’d for $C_{17}H_{14}N_3O_3$ [M+H]$^+$: 308.1035; found: 308.1036.

29. Synthesis of 3a-Hydroxy-7-nitro-1-phenyl-1,2,3,3a-tetrahydro-pyrrolo[2,3-*b*]quinolin-4-one



A solution of 7-Nitro-1-phenyl-1,2,3,9-tetrahydro-pyrrolo[2,3-*b*]quinolin-4-one (0.032 g, 0.10 mmol, 1.0 eq) in dry THF (6.0 mL) was added dropwise to lithium bis(trimethylsilyl)amide (1.0 M in THF, 0.12 mL, 0.12 mmol, 1.2 eq) in dry THF (2.0 mL) at -78 °C under argon. The reaction was stirred for 30 minutes at -78 °C and a solution of the Davis reagent (**9**) (0.093 g, 0.31 mmol, 3.1 eq) in dry THF (6.0 mL) was added via cannula. After 32 hours at -10 °C saturated ammonium iodide solution (5.0 mL, 10 eq) and diethyl ether (10 mL) were added and the reaction warmed to room temperature. Saturated sodium thiosulfate solution (15 mL) was then added and the reaction extracted with diethyl ether (2 × 10 mL). The combined organic extracts were dried ($MgSO_4$) and concentrated *in vacuo* to give a orange solid. The solid was partitioned between dichloromethane (100 mL) and aqueous hydrochloric acid solution (0.30 M, 100 mL). The aqueous phase was washed with dichloromethane (3 × 100 mL), basified with aqueous sodium hydroxide solution (2.0 M, to pH 8) and extracted with ethyl acetate (2 × 100 mL). The organic extracts were dried ($MgSO_4$) and concentrated *in vacuo* to give a red solid. The solid was purified by flash column chromatography on silica gel (eluting with 20% ethyl acetate/hexane), to give the desired product 3a-Hydroxy-7-nitro-1-phenyl-1,2,3,3a-tetrahydro-pyrrolo[2,3-*b*]quinolin-4-one (0.010 g, 0.032mmol, 31%) as a red solid. The enantiomeric excess of 3a-Hydroxy-7-nitro-1-phenyl-1,2,3,3a-tetrahydro-pyrrolo[2,3-*b*]quinolin-4-one, prepared by this route was 76% as determined by chiral-phase HPLC analysis; mp 187-188 °C. $[\alpha]_D^{26}$= -418 (c=0.05 in dichloromethane); ^{1}H NMR (300 MHz, [D_8]THF): δ =2.29-2.42 (m, 2 H; 3-H), 3.98-4.07 (m, 1 H; 2-H), 4.17-4.27 (m, 1 H; 2-H), 7.10-7.18 (m, 1 H; Ar-4'-H), 7.35-7.44 (m, 2 H; Ar-3'-H), 7.80 (dd, ^3J(H,H) = 8.4 Hz, ^4J(H,H) = 2.2 Hz, 1 H; Ar-6-H), 7.94 (dd, ^3J(H,H) = 8.4 Hz, ^5J(H,H) = 0.3 Hz, 1 H; Ar-5-H), 7.99 (dd, ^4J(H,H) = 2.2 Hz, ^5J(H,H) = 0.3 Hz, 1 H; Ar-8-H), 8.09-8.14

ppm (m, 2 H; Ar-2'-H); ^{13}C NMR (75.5 MHz, [D_8]THF): δ =29.31 (C3), 49.0 (C2), 75.0 (C3a), 117.3 (C6), 121.1 (C2'), 121.3 (C8), 125.0 (C4'), 126.7 (C4a), 128.3 (C5), 129.3 (C3'), 141.4 (C1'), 153.0 (C8a), 154.2 (C7), 167.8 (C9a), 193.0 ppm (C4); IR (Nujol): $\tilde{\nu}_{max}$= 1707 (s) (C=O), 1625 (m) (C=N), 1578 (m), 1256 (m), 739 (m) (Ar-H), 721 cm^{-1} (w) (Ar-H); LRMS (ES$^+$): *m/z* (%): 324 (100) [M+H]$^+$,356 (95) [M+MeOH]$^+$, 413 (10); HRMS (ES$^+$): *m/z* calc'd for $C_{17}H_{14}N_3O_4$ [M+H]$^+$: 324.0984 ; found: 324.0987.

V. Fluorescence Data and Light stability experiments.

1. Procedure for fluorescence

All emission acquisition spectra were corrected for the Raman lines of methanol by running the emission acquisition experiments separately with methanol and subtracting the resulting methanol spectra from the spectra obtained from the compounds dissolved in methanol.

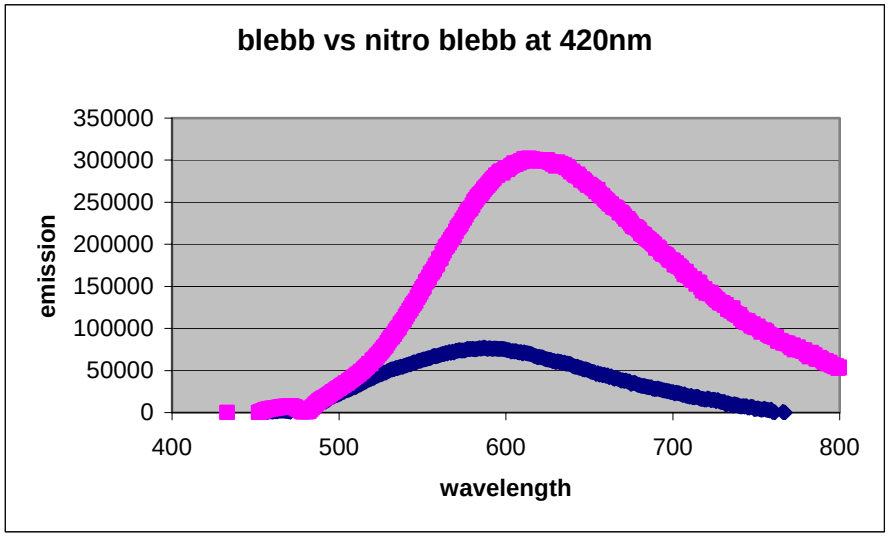


Figure S11: Emission acquisition spectra of (-)-blebbistatin (**1**) (pink) and nitroblebbistatin (**12**) (blue) when excited at a wavelength of 420nm.

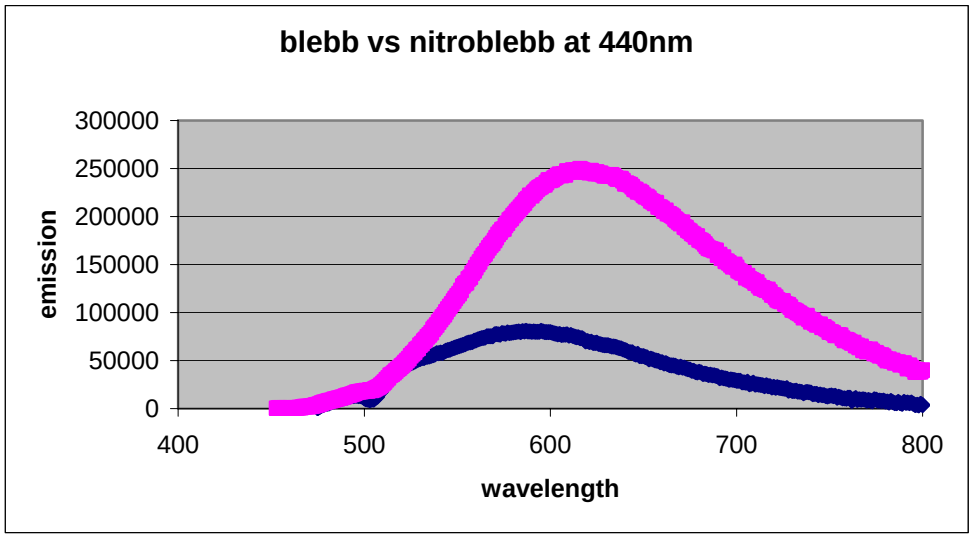


Figure S12: Emission acquisition spectra of (-)-blebbistatin (**1**) (pink) and nitroblebbistatin (**12**) (blue) when excited at a wavelength of 440nm.

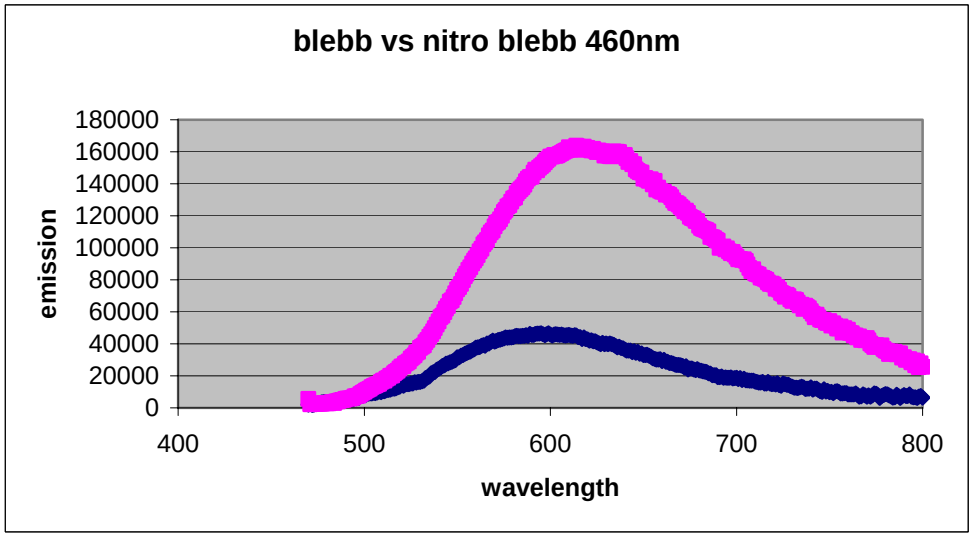


Figure S13: Emission acquisition spectra of (-)-blebbistatin (**1**) (pink) and nitroblebbistatin (**12**) (blue) when excited at a wavelength of 460nm.

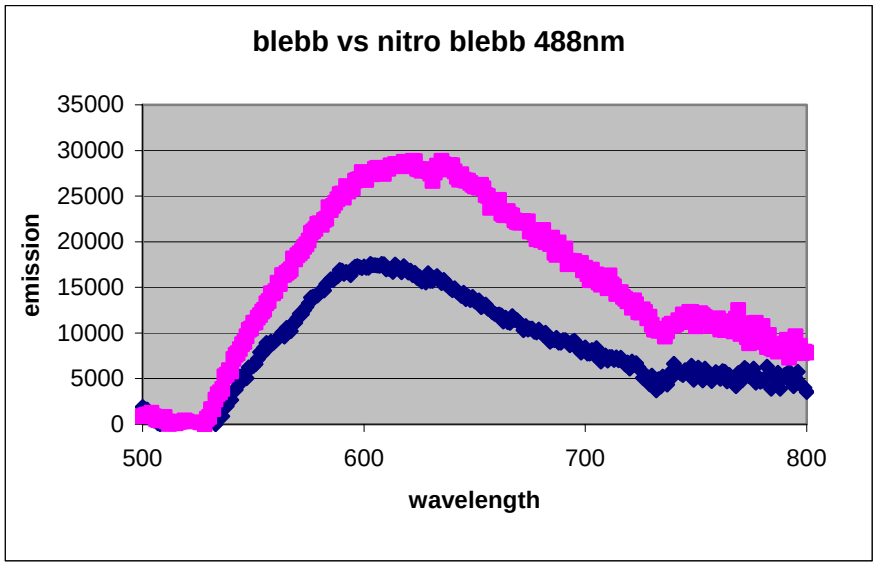


Figure S14: Emission acquisition spectra of (-)-blebbistatin (**1**) (pink) and nitroblebbistatin (**12**) (blue) when excited at a wavelength of 488nm.

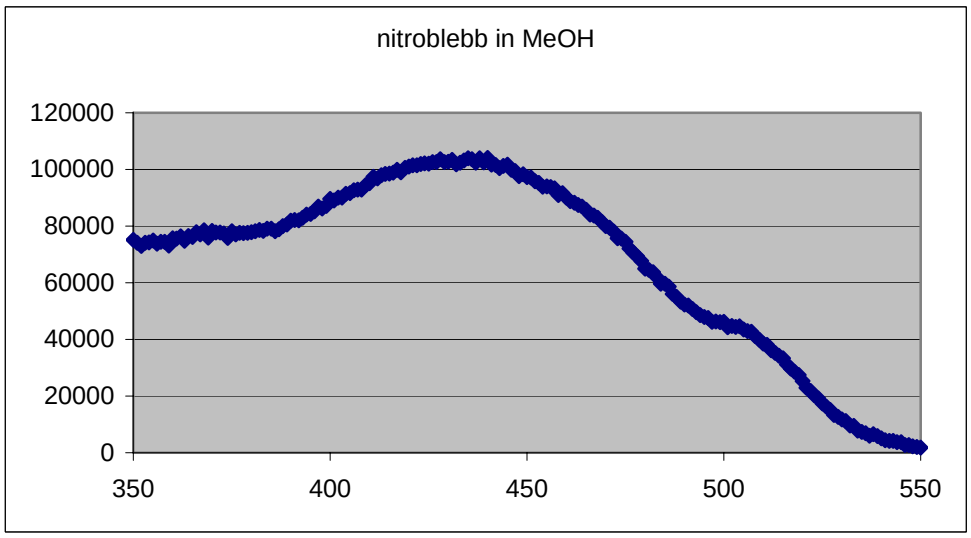


Figure S15: Excitation acquisition spectrum of nitroblebbistatin (**12**), the detected emission wavelength is 600 nm.

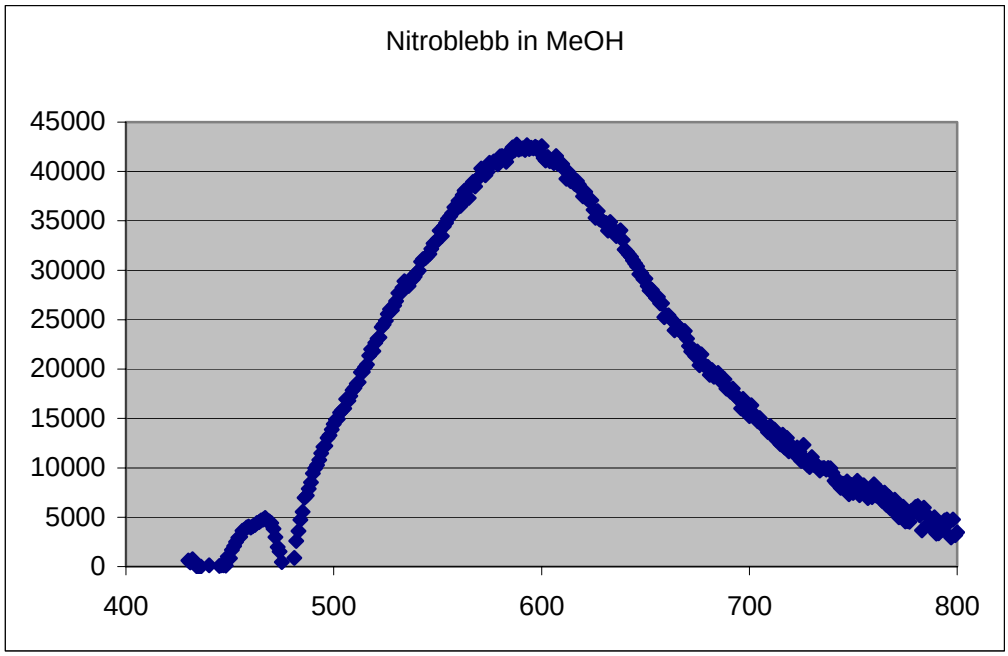
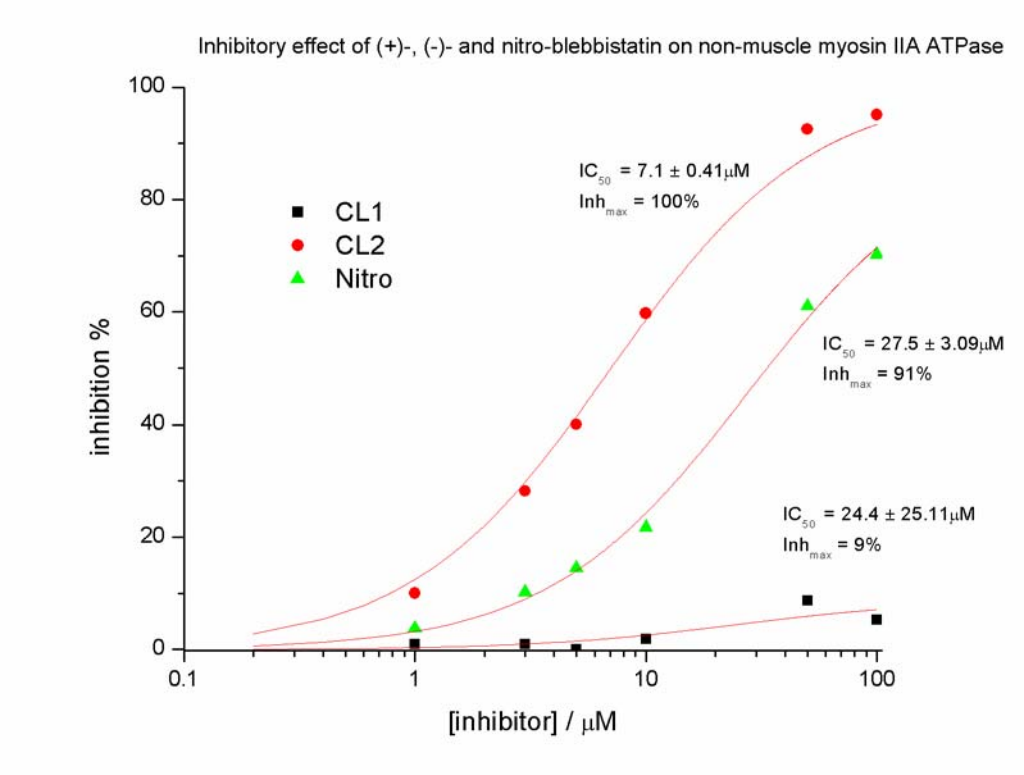


Figure S16: UV-vis spectra of nitroblebbistatin (**12**).

2. Procedure for determining light stability.

Stock solutions (DMSO) of (*S*)-(-)-Blebbistatin (**1**) and nitroblebbistatin (**12**) diluted to 20 μM in PBS were irradiated with filtered light (excitation maximas 436 and 510 nm) for 3 hours in a quartz cuvette using a Nikon Microphot-FXA microscope equipped with a XF135 filter set (Omega Optical inc.). The ×20 objective was used during the irradiation. After 3 hours the solutions were diluted to 1 μM in PBS and spectra recorded using a Perkin Elmer Lambda 35 UV/vis spectrometer.

VI. Biological Experiments



The biological activity of (**1**), (**2**) and (**12**) were assessed as described by Mitchison *et al.*[3]. For further details contact JRS.

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