Supporting Information

Optimizing Natural Products by Biosynthetic Engineering: Discovery of Non-quinone Hsp90 Inhibitors

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Contents of Supporting Information

Experimental Details

Cloning and Sequencing of the Macbecin Biosynthetic Cluster. A cosmid library derived from *Actinosynnema pretiosum* subsp. *pretiosum* ATCC 31280 was isolated, screened and the region spanning the biosynthetic cluster for macbecin was sequenced using standard protocols[1]. Southern blot experiments were carried out using carbamoyltransferase-encoding gene *gdmN* (DIG-labelled) from the geldanamycin biosynthetic gene cluster of *Streptomyces hygroscopicus* NRRL 3602 (Accession number AY179507) as a heterologous probe to identify the initial cosmid 52. Further cosmids were then identified using probes generated from cosmid 52 by standard procedures. The sequenced region spans approx. 100 kbp and contains 23 open reading frames (Table S1). The sequence is deposited under the accession number EU827593.

Table S1. Location and Predicted Encoded Function of the Open Reading Frames within the Macbecin Biosynthetic Cluster

Coding region in cluster sequence	**Gene**	**Function of the encoded protein**
14925-17906*	*mbcRII*	Transcriptional regulator
18028-19074c	*mbcO*	Aminohydroquinate synthase
19266-20066c*	*mbcB*	unknown
20330-40654	*mbcAI*	Polyketide synthesis loading module + modules 1-3
40654-50856	*mbcAII*	Polyketide synthesis modules 4+5
50867-62488*	*mbcAIII*	Polyketide synthesis modules 6+7
62500-63273*	*mbcF*	Amide synthetase
63281-64849*	*mbcM*	Flavin dependant monooxygenase (C21)
64902-65696c*	*mbcC*	Phosphatase
65696-66853c*	*mbcD*	Oxidoreductase

Coding region in cluster sequence	**Gene**	**Function of the encoded protein**
66894-68057c*	*mbcE*	3-amino-5-hydroxybenzoic acid synthase
68301-68729*	*mbcL*	ADHQ dehydratase
68693-69661c*	*mbcQ*	AHBA kinase
70188-72194c*	*mbcN*	Carbamoyltransferase (C7 hydroxyl group)
72251-73339c	*mbcH*	Glycerol transferase/phosphatase
73339-74493c	*mbcI*	Acyl-ACP dehydrogenase
74493-74765c	*mbcJ*	Acyl carrier protein
74765-75628c*	*mbcK*	Glycerol-ACP dehydrogenase
75881-76534	*mbcG*	*O*-methyl transferase
76534-77799*	*mbcP*	Cytochrome P450 monooxygenase (4,5-desaturase)
77831-79051*	*mbcS*	Cytochrome P450 mononoxygenase (C15)
79119-79931*	*mbcT*	*O*-methyltransferase (C11 hydroxyl group)
79931-80713*	*mbcU*	*O*-methyltransferase (C15 hydroxyl group)

c indicates that the gene is encoded by the complement DNA strand;

* indicates more than one potential candidate start codon can been identified.

Genetic Engineering of *Actinosynnema pretiosum* subsp. *pretiosum*. Engineering of the macbecin biosynthetic cluster was carried out by standard methods[1]. For integration mutants, an internal fragment of the target gene was amplified by PCR, cloned into a pKC1132-based vector[2] and used to integrate into the strain giving a mutant strain which is selected by apramycin resistance. For deletion mutants, regions flanking the genes to be deleted were amplified by PCR, cloned together into pKC1132 and used to effect double recombination in *Actinosynnema pretiosum*. These engineering experiments are summarized in Table S2. Complementation of deletion mutants was achieved by amplifying the relevant gene, placing it under the *actI* promoter[3] and introducing it into

the ΦBT1 attachment site (Table S3).[4] Biot-3982 was generated by engineering the *mbcP,S,T&U* deletion into Biot-3970.

Table S2. Primers used for Engineering Deletion and Integration Mutants of *A. pretiosum*

Experiment	Strain	Primers 5'→3'
mbcM deletion	Biot-3970	ATATACTAGTCACGTCACCGGCGCGGTGTCCGCGGACTTCGTCAACG ATATCCTAGGCTGGTGGCGGACCTGCGCGCGCGGTTGGGGTG ATATCCTAGGCACCACGTCGTGCTCGACCTCGCCCGCCACGC ATATTCTAGACGCTGTTCGACGCGGGCGCGGTCACCACGGGC
mbcT integrant	Biot-3820	TCTAGAACGAGCACACCTACGAGCAGTTCGAGAAGT TCTAGAGATCTCCAGGGTCTCCCGCCAAGTGCGTTC
mbcU integrant	Biot-3819	TCTAGACCGAGCTGGAGGGGGGCGCAGCCGACCCGA TCTAGACAGATCGGCCAGTTCGCGGAAGTTGCGTTG
mbcS integrant	Biot-3825	TTCGTGCAGCGGATCGTCGA ATCCCGGTGTGCGAGATCGT
mbcT&U deletion	Biot-3848	GGAAGCTTTCGGTAATGGGGAGACTCGACGCCGCCTGAC GGGATCCCCGAACACCCGTAACCACGCGGTGGCGTCCCCC GGGATCCGGGAACGGCCTTTCGGGGTCGGCTTGCGGGAGG GGGAATTCCCCCGGAGAGAAAGGCCGCCGCAGTGTTCAC
mbcP,S,T&U deletion	Biot-3827 Biot-3852	GGTCACTGGCCGAAGCGCACGGTGTCATGG CCTAGGCGACTACCCCGCACTACTACACCGAGCAGG CCTAGGAACGGGTAGGCGGGCAGGTCGGTG GTGTGCGGGCCAGCTCGCCCAGCACGCCCAC
mbcP integrant	Biot-3863	GCATGGTCGCCGGGCACTTC TCGACGGCGTTGGCGAAACC

Table S3. Primers for Amplification of Genes for Complementation of *A. pretiosum* Deletion Mutants

Gene	Strain		Primers 5'→3'
mbcT	Δ*T&U* + *mbcT*	Biot-3854	GGTCTAGAGGTCACGGGCGGTCTGCGGCGACCAGCAGG GGCATATGAGCGACACCACGCTGTCCGTGCCCGTCCC
mbcU	Δ*T&U* + *mbcU*	Biot-3856	GGTCTAGAGGTCACGGGTGTTCGGCTACTGCTAGGAAGCAGCC GGCATATGAGGCCCGTCCCCGAGGCCGTGGGCCGCC
mbcS	Δ*P,S,T&U* + *mbcS,T&U*	Biot-3879*	GGTCTAGAGGTCACCAGGTGACGGGCAGCTCGTAGACGCC GGCATATGACCCGCACCACCCCCACCCCCGACCTGGCCCC

*Biot-3879 contains a gene cassette of *mbcS,T&U*. The primers shown are for *mbcS*, those for *mbcT&U* are as given above.

General Microbiology. The production of macbecin has been described previously[5] and methods for production of the novel compounds are adapted from there. Using the published media, seed cultures were inoculated from a 3 day MAM plate (2 x 6 mm plugs for 30 mL in a 250 mL flask) and grown for 48 h at 28 °C. Production cultures were

grown in 2 litre flasks (375 mL per flask 5% inoculum) either for 24 h at 28 °C followed by 5 days at 26 °C, or for 6-7 days at 26 °C.

General Chemistry. NMR spectra were recorded on a Bruker Advance 500 spectrometer at 298 K operating at 500 MHz and 125 MHz for ^{1}H and ^{13}C respectively. Standard Bruker pulse sequences were used to acquire ^{1}H-^{1}H COSY, APT, HMBC and HMQC spectra. Coupling constants are given in hertz. Spectra were referenced to the residual proton or standard carbon resonances of the solvents in which they were run. LCMS was performed on an Agilent HP1100 HPLC system in combination with a Bruker Daltonics Esquire 3000+ mass spectrometer fitted with an electrospray source. High-resolution MS were measured on a Bruker BioApex II 4.7T FTICR fitted with an electrospray source and operating in positive ion mode. Production of macbecin analogues was determined by LCMS following extraction with an equal volume of ethyl acetate, removal of solvent under reduced pressure and dissolution of extract into methanol.

Assessment of Compound Purity. Pure compound was prepared as a 0.1 mg/mL solution in methanol. This was examined by injection of 1 & 5 μL aliquots, and compared to an injection of 10 μL of the methanol dissolution solvent run immediately prior to the compounds. QC method 1: chromatography was achieved over a Phenomenex Hyperclone column (C_{18} BDS, 3 micron particle size, 150 x 4.6 mm) with a gradient elution process of: T=0, 10%B; T=2, 10%B; T=15, 100%B; T=17, 100%B; T=17.05, 10%B; T=20, 10%B. Elution was at 1 mL/min; mobile phase A=water + 0.1% formic acid; mobile phase B=acetonitrile + 0.1% formic acid. UV spectra were recorded between 190 and 400 nm, with extracted chromatograms taken at 210, 254 and 274 nm. Mass spectra were recorded between 100 and 1500 mu, and switching between positive

and negative modes. Purity of >95% was observed for each compound at all wavelengths (compared to sum of total peak areas). This was confirmed by inspection of the LCMS trace and ^{1}H and ^{13}C NMR spectra. Figure S1 shows the determination of purity of **5**.

Table S4. Minimum Purity of Compounds

Compound	Minimum purity
5	98%
6	99%
7	>99%
8	>99%
9	>99%
10	>99%
12	>99%

Extraction and Purification of Compounds. The relevant fermentation broth was extracted three times with equal volumes of ethyl acetate and the solvent removed from the combined extract. The residues were chromatographed over silica gel 60 eluting initially with $CHCl_3$:MeOH (95:5) followed by an increase in methanol concentration up to 10% as required, and collection of 250 mL fractions. The fractions were assayed by LCMS and those containing products combined. This material was further purified by reversed-phase HPLC over a Phenomenex-Luna C_{18}-BDS column (C_{18}-BDS, 5 micron particle size, 21.2 x 250 mm) eluting with a gradient of increasing acetonitrile versus water (gradient optimized for each compound) over a period of 25 min with a flow rate of 21 mL/min.

Isolation and Characterization of 5. The extract from 1 L of Biot-3970 fermentation broth gave an off white powder (86 mg). LCMS: Retention time (QC method 1) 9.9 min; *m/z* 525.7 $[M+Na]^+$, 1028.1 $[2M+Na]^+$, 501.6 $[M-H]^-$, 547.6 $[M+formate]^-$. High-resolution MS, $C_{28}H_{42}N_2O_6Na^+$: calculated, 525.2935; observed, 525.2924 (Δ 2.0 ppm).

NMR data for 5 (acetone-d_6)		
Position	**δ_H, mult. J (Hz)**	**δ_C**
1	-	171.8
2	-	135.5
2-CH_3	1.82, s	14.0
3	6.17, br. s	133.8
4a	2.40. m	28.5*
4b	2.19, m	-
5	1.46, m	33.6*
6	1.91, m	36.3
6-CH_3	0.87, d (7)	16.4
7	5.17 br. s	81.9
7-$CONH_2$	Not observed	159.0
8	-	134.0
8-CH_3	1.50, s	14.4
9	5.35, d (9.5)	131.4
10	2.45, m	36.0
10-CH_3	1.01, d (7)	18.6
11	3.60, dd (8.5, 3)	76.3
12	3.18, ddd (6, 3, 3)	84.1
12-OCH_3	3.30, s	57.6
13a	1.34, m	Not assigned
13b	1.55, m	-
14	1.63, m	36.7
14-CH_3	0.85, d (7)	21.3
15a	2.66, dd (12, 1.5)	43.9
15b	2.13, m	-
16	-	144.9
17	6.36, s	113.5
18	-	159.3
18-OH	8.22, br. s	-
19	7.34, bs	106.1
20	-	142.6
21	6.41, s	114.6

* Connectivity for these carbons could not be made and assignments given are based on similarity to related molecules.

NMR data for 5 (dmso-d_6)		
Position	**δ_H, mult. J (Hz)**	**δ_C**
NH	9.28	-
1	-	170.6
2	-	132.0
2-CH_3	17.1, s	13.2
3	5.81, br	134.2
4a	2.02. m	25.2
4b	2.20, m	-
5a	1.02, m	30.9
5b	1.18, m	-
6	1.66, m	33.6
6-CH_3	0.81, d (6.5)	15.2
7	4.70, d (6.1)	81.7
7-$CONH_2$	Not observed	156.7
8	-	131.1
8-CH_3	1.34, s	11.9
9	5.21, d (9.9)	131.8
10	2.32, m	34.1
10-CH_3	0.91, d (6.8)	17.1
11	3.38, m	73.6
11-OH	4.37, d (5.1)	-
12	2.99, m	81.1
12-OCH_3	3.20, s	56.3
13a	1.41, m	34.0
13b	1.15, m	-
14	1.68, m	31.8
14-CH_3	0.75, d (6.5)	19.0
15a	2.22, m	42.4
15b	2.51, m	-
16	-	141.5
17	6.23, s	112.4
18	-	157.4
18-OH	9.22, s	-
19	6.78, s	105.2
20	-	140.1
21	6.23, s	114.2

Isolation and Characterization of 6. The extract from 1.3 L of Biot-3970 fermentation broth gave an off white powder (246 mg). LCMS: Retention time (QC method 1) 8.6

min; *m/z* 541.5 $[M+Na]^+$, 1059.4 $[2M+Na]^+$, 517.4 $[M-H]^-$, 563.5 [M+formate]$^-$. High-resolution MS, $C_{28}H_{42}N_2O_7Na^+$: calculated, 541.2884; observed, 541.2866 (Δ 3.3 ppm).

NMR data for 6 (in acetone-d_6)		
Position	**δ_H, mult. J (Hz)**	**δ_C**
1	-	172.5
2	-	135.5
2-CH_3	1.81, s	14.3
3	6.02, s	133.7*
4a	2.34, m	27.7*
4b	2.12, m	-
5	1.32, m	36.1*
5	1.21, m	
6	1.84, m	35.7
6-CH_3	0.86, d (7)	16.4
7	5.01 br. s	82.1
7-$CONH_2$	-	159.5
8	-	134.5
8-CH_3	1.44, s	13.9
9	5.29, d (9.5)	132.7
10	2.42, m	35.7
10-CH_3	1.00, d (7)	18.8
11	3.62, dd (8.5, 3)	75.9
12	3.15, ddd (6,3,3)	83.4
12-OCH_3	3.30, s	57.5
13	1.84, m	32.9
14	1.84, m	40.5
14-CH_3	0.75, d (6.5)	15.9
15	4.62, d (1.5)	76.5
16	-	141.9
17	6.32, s	111.8
18	-	158.5
18-OH	8.38, br. s	-
19	7.16, s	107.2
20	-	148.1
21	6.76, s	110.6

* Connectivity for these carbons could not be made and assignments given are based on similarity to related molecules.

Isolation and Characterization of 7. The extract from 1 L of Biot-3820 fermentation broth gave an off white powder (80 mg). LCMS: Retention time (QC method 1) 10.4 min; *m/z* 553.2 $[M+Na]^+$, 1083.0 $[2M+Na]^+$, 529.2 $[M-H]^-$. Compound **10** (45 mg) was

additionally isolated from this experiment. High-resolution MS, $C_{28}H_{38}N_2O_8Na^+$: calculated, 553.2520; observed, 553.2537 (Δ 3.1 ppm).

NMR data for 7 (in acetone-d_6)		
Position	**δ_H, mult. J (Hz)**	**δ_C**
1	-	170.7
2	-	134.2
2-CH_3	1.95, s	14.8
3	7.24, d (12)	130.9
4	6.33, dd (12, 11)	124.4
5	5.78, dd, (11, 7)	144.1
6	3.09, m	39.9
6-CH_3	1.02, d (7)	13.5
7	5.32, d (4.5)	81.2
7-$CONH_2$	-	158.0
8	-	135.2
8-CH_3	1.65, s	15.3
9	5.58, d (9.5)	132.4
10	2.67, m	34.9
10-CH_3	0.97, d (6.5)	15.7
11	3.56, dd (8.5, 2.5)	74.5
12	3.42, ddd (2.5,3,6)	83.2
12-OCH_3	3.29, s	57.4
13a	1.85, m	35.3
13b	1.82, m	-
14	1.68, m	34.9
14-CH_3	0.87, d (7)	16.2
15	4.27, d (1.5)	73.5
16	-	150.0
17	6.62, s	134.1
18	-	189.5
19	7.22, s	113.5
20	-	141.5
21	-	185.3

Isolation and Characterization of 8. The extract from 1.5 L of Biot-3825 fermentation broth gave an off white powder (270 mg). LCMS: Retention time (QC method 1) 13.5 min; *m/z* 551.4 $[M+Na]^+$, 1079.2 $[2M+Na]^+$, 527.4 $[M-H]^-$. Compound **10** (680 mg) was

additionally isolated from this experiment (see below). High-resolution MS, $C_{29}H_{40}N_2O_7Na^+$: calculated, 551.2728; observed, 551.2718 (Δ 1.7 ppm).

NMR data for 8 (in acetone-d_6)		
Position	**δ_H, mult. J (Hz)**	**δ_C**
1	-	171.2
2	-	134.4
2-CH_3	1.94, s	13.2
3	7.25, d (12)	131.1
4	6.33, dd (12, 11)	125.6
5	5.68, dd, (11, 7)	143.5
6	3.19, m	35.4
6-CH_3	1.01, d (7)	15.2
7	5.76, d (4.5)	80.2
7-$CONH_2$	-	158.0
8	-	135.8
8-CH_3	1.57, s	16.0
9	5.32, d (9.5)	129.8
10	2.47, m	38.2
10-CH_3	1.02, d (6.5)	18.4
11	3.21, dd (8.5, 2.5)	85.5
11-OCH_3	3.44, s	61.1
12	3.68, ddd (2.5,3,6)	84.7
12-OCH_3	3.30, s	56.9
13	1.33, m	35.2
14	1.74, m	37.3
14-CH_3	0.92, d (7)	22.3
15	3.17, m	36.2
16	-	147.7
17	6.54, d (1.5)	134.6
18	-	189.4
19	7.13, d (1.5)	113.7
20	-	141.1
21	-	185.1

Isolation and Characterization of 9. The extract from 2.5 L of Biot-3819 fermentation broth gave an amorphous white powder (292 mg). LCMS: Retention time (QC method 1) 12.3 min; *m/z* 567.4 $[M+Na]^+$, 1111.4 $[2M+Na]^+$, 543.3 $[M-H]^-$. High-resolution MS, $C_{29}H_{40}N_2O_8Na^+$: calculated, 567.2677; observed, 567.2675 (Δ 3.1 ppm).

NMR data for 9 (in acetone-d_6)		
Position	**δ_H, mult. J (Hz)**	**δ_C**
1	-	171.2
2	-	134.4
2-CH_3	1.94, s	13.4
3	7.24, d (12)	131.2
4	6.36, dd (12, 11)	125.7
5	5.67, dd, (11, 7)	143.5
6	3.18, m	35.4
6-CH_3	1.00, d (7)	15.1
7	5.77, d (4.5)	80.2
7-$CONH_2$	-	158.1
8	-	133.4
8-CH_3	1.58, s	16.1
9	5.33, d (9.5)	130.1
10	2.50, m	39.5
10-CH_3	1.02, d (6.5)	18.5
11	3.26, dd (8.5, 2.5)	85.5
11-OCH_3	3.46, s	61.1
12	3.66, ddd (2.5,3,6)	85.1
12-OCH_3	3.29, s	56.9
13	1.33, m	36.2
14	1.74, m	42.3
14-CH_3	0.80, d (7)	14.7
15	5.14, m	68.8
16	-	150.8
17	6.65, d (1.5)	134.6
18	-	189.5
19	7.22, d (1.5)	113.4
20	-	141.1
21	-	185.0

Isolation and Characterization of 10. The extract from 1.5 L of Biot-3825 fermentation broth gave an off white powder (680 mg). LCMS: Retention time (QC method 1) 12.6 min; *m/z* 537.1 $[M+Na]^+$, 1051.0 $[2M+Na]^+$, 513.1 $[M-H]^-$. Compound **8** (270 mg) was additionally isolated from this experiment (see above). High-resolution MS, $C_{28}H_{38}N_2O_7Na^+$: calculated, 537.2571; observed, 537.2553 (Δ 3.3 ppm).

NMR data for 10 (in acetone-d_6)		
Position	**δ_H, mult. J (Hz)**	**δ_C**

1	-	170.6
2	-	134.2
2-CH_3	1.97, s	14.1
3	7.19, d (12)	130.7
4	6.37, dd (12, 11)	124.4
5	5.80, dd, (11, 7)	145.1
6	3.06, m	41.5
6-CH_3	1.13, d (7)	13.5
7	5.11, d (4.5)	83.2
7-$CONH_2$	-	158.0
8	-	135.9
8-CH_3	1.67, s	13.9
9	5.57, d (9.5)	132.7
10	2.49, m	34.1
10-CH_3	1.05, d (6.5)	15.4
11	3.26, dd (8.5, 2.5)	74.6
11-OCH_3	3.46, s	61.1
12	3.30, ddd (2.5,3,6)	82.6
12-OCH_3	3.30, s	57.6
13a	1.32, m	33.0
13b	1.59, m	-
14	1.61, m	28.5
14-CH_3	0.86, d (7)	24.8
15a	2.22, m	41.6
15b	2.10, m	-
16	-	147.4
17	6.46, d (1.5)	136.6
18	-	189.7
19	7.24, d (1.5)	113.7
20	-	141.9
21	-	184.9

Isolation and Characterization of 12. The extract from 2 L of Biot-3827 fermentation broth gave an amorphous white solid (62 mg). LCMS: Retention time (QC method 1) 12.4 min; *m/z* 539.2 $[M+Na]^+$, 1055.4 $[2M+Na]^+$, 515.3 $[M-H]^-$, 1031.6 $[2M-H]^-$. High-resolution MS, $C_{28}H_{40}N_2O_7Na^+$: calculated, 539.2728; observed, 539.2734 (Δ 1.2 ppm).

NMR data for 12 (in acetone-d_6)		
Position	**δ_H, mult. J (Hz)**	**δ_C**
1	-	170.8
2	-	134.8

2-CH_3	1.86, s	13.6
3	6.39, dd (12, 4)	139.2
4	2.30, m	27.3
5	2.46, m	33.7
6	2.69, m	35.8
6-CH_3	0.96, d (7)	14.1
7	5.08, d (4.5)	81.6
7-$CONH_2$	-	158.5
8	-	133.5
8-CH_3	1.58, s	16.4
9	5.50, d (9.5)	136.2
10	2.36, m	38.3
10-CH_3	1.02, d (6.5)	14.7
11	2.84, dd (8.5, 2.5)	75.9
12	3.41, ddd (2.5,3,6)	82.8
12-OCH_3	3.31, s	58.1
13	1.41, m	34.6
14	1.62, m	40.3
14-CH_3	0.89, d (7)	23.7
15	1.96, m	38.3
16	-	147.8
17	6.49, d (1.5)	134.6
18	-	189.6
19	7.16, d (1.5)	113.6
20	-	142.2
21	-	185.4

Assessment of Solubility. Method 1: A 10 mM stock solution of compound was prepared in 100% DMSO at room temperature. Triplicate 0.01 mL aliquots are made up to 0.5 mL with either 0.1 M PBS (pH 7.3) or DMSO in amber vials. The resulting 0.2 mM solutions were shaken in the dark, at room temperature for 6 h. The suspension was filtered through a glass fibre C filter into a 96 well plate. Method 2: A known weight of compound was ddede into 0.25 mL pH 7.3 phosphate buffered saline (Oxoid BR0014G Dulbecco A PBS). The suspension was equilibrated with shaking at 25 °C for 24 hours then the pH was measured. The suspension was filtered through a glass fibre C filter into a 96 well plate.

In each case the solutions were diluted by appropriate amounts and quantitation was by HPLC with reference to a standard solution of approximately 0.1 mg mL^{-1} in DMSO. Different volumes of the standard, diluted and undiluted sample solutions were injected. Assays were run at Pharmorphix Ltd (Cambridge, UK).

Table S5. Solubility Measurements of **4** and **5**

Compound #	Method	Solubility μM
4	1	81
5	1	>200
5	2	716

Binding Affinity to Hsp90. Isothermal titration calorimetry was used to determine binding affinity to Hsp90 for **5** and **6** (Figure S2). Yeast Hsp90 was dialysed against 20 mM Tris pH 7.5 containing 1 mM EDTA and 5 mM NaCl and then diluted to 8 μM in the same buffer, but containing 2% DMSO. The macbecin analogues were dissolved in 100% DMSO at a concentration of 50 mM and subsequently diluted to 100 μM in the same buffer as for Hsp90 with 2% DMSO. Heats of interaction were measured at 30^{o}C on a MSC system (Microcal), with a cell volume of 1.458 mL. 10 aliquots of 27 μL of 100 μM of each analogue were injected into 8 μM yeast Hsp90. Heats of dilution were determined in a separate experiment by injecting the macbecin analogues into buffer containing 2% DMSO, and the corrected data fitted using a nonlinear least square curve-fitting algorithm (Microcal Origin) with three floating variables: stoichiometry, binding constant and change in enthalpy of interaction.

X-ray Crystal Structure of 5-Hsp90 Complex. 50 μL of 1.6 mM N-terminal domain of Hsp90 was mixed with 1 mL of 20 mM Tris pH 7.5 and 1 mM EDTA. **5** (2 μL of 50

mM) in 100% DMSO was then added and mixed with the protein. After incubation at 4°C the complex was concentrated to 2 mg mL^{-1} (80 μM N-terminal domain). Crystals were grown by the hanging drop vapour diffusion method by mixing 1 μL of the drug complex with 1 μL of precipitant. The precipitant was prepared by diluting 0.25 volumes of stock precipitant (100 mM sodium succinate pH 5.0, 200 mM $(NH_4)_2SO_4$ and 30% PEGME 550) with 0.75 volumes of protein buffer (20 mM TRIS pH 7.5 and 1 mM EDTA). The final concentration of the precipitant in the crystallization was 25 mM sodium succinate pH 5.0, 50 mM $(NH_4)_2SO_4$ and 7.5% PEGME 550 and the protein was at 1 mg mL^{-1}. Single crystals appeared overnight of approximate dimensions 0.3 x 0.2 x 0.2 mm. These were flash frozen after stepwise addition of glycerol to 30% and data collected on station ID23.1 at the ESRF. The data were integrated using MOSFLM and scaled and merged using SCALA in CCP4.

The complex was initially solved by isomorphous replacement using a previously determined N-terminal structure (PDB code 1AH6) in the usual space group, $P4_322$. The model was refined in REFMAC5 in CCP4 and rebuilt using COOT. Refinement proceeded satisfactorily in C2, with four molecules in the asymmetric unit. The inhibitor library was built using SKETCHER. The inhibitor molecule and the waters were added in the final stages.

Table S6: Hydrogen Bond Interactions Seen in the **5**-Hsp90 Crystal Structure, between the Ligand and the Receptor or between the Ligand and Solvent.

Ligand	Receptor	Residue	Type	Distance
carbamate NH	O	HOH 10	H-don	2.27
phenol OH	O	Asp 40	H-don	1.90
amide NH	O	HOH 59	H-don	1.86
carbamate NH	O	Asp 79	H-don	1.75
carbamate O (C=O)	O	HOH 8	H-acc	2.81

C11 hydroxy O	N	Lys 44	H-acc	2.11
amide O (C=O)	N	Phe 124	H-acc	1.99

Solution Structure Determination for 5. The NOESY spectrum for **5** was obtained at 298 K in DMSO-d_6. The key NOE correlations are shown in Figure S3. These are consistent with the folded C-conformation as shown and with the data reported for reblastin.[6]

Investigation of the Reactivity of Ansamycin Hsp90 Inhibitors with Glutathione in vitro. The procedure was adapted from that published.[7] Test compounds (**5**, **6**, and 17-DMAG, all at 50 μM) were dissolved in 0.1 M potassium phosphate buffer at pH 7.0. Analysis was performed by LCMS, with chromatography achieved over a Phenomenex Hyperclone column (C_{18} BDS, 3 micron particle size, 150 x 4.6 mm) with a gradient elution process of: T = 0, 10%B; T = 11 min, 100%B. Elution was at 1 mL/min; mobile phase A = water + 0.1% formic acid; mobile phase B=acetonitrile + 0.1% formic acid, and the following retention times were observed (**5**, R_t = 7.3 min; **6**, R_t = 6.3 min; 17-DMAG, R_t = 7.5 min). Samples were analyzed immediately after dissolution and after 24 hours at 37°C in the presence or absence of glutathione (10 mM). The amount of compound remaining after 24 h was determined by the absorbance at λ = 255 nm compared with that in the zero time samples. No change in the T = 0 or T = 24 h samples were observed for compounds **5** and **6**. 17-DMAG was completely absent from the T = 24 h (+ glutathione) sample. A new peak (R_t = 5.1 min) appeared with an m/z = 922.2 (positive mode) and m/z = 920.2 (negative mode). This is consistent with a GSH-DMAG adduct observed as published.[7]

In vitro Inhibition of Cancer Cell Growth. In vitro evaluation of compounds for anticancer activity in a panel of human tumor cell lines in a monolayer proliferation assay was carried out at Oncotest GmbH, Freiburg. Compounds were tested at 5 concentrations (0.001, 0.01, 0.1, 1, 10 μM) in a panel of 6 cell lines in duplicate (CNXF-498NL, HT29, LXF-1121L, MCF7, MEXF-394NL, DU145).[8-10] Cell lines, are grown at 37°C in a humidified atmosphere (95% air, 5% CO_2) in a 'ready-mix' medium containing RPMI 1640 medium, 10% fetal calf serum, and 0.1 mg/mL gentamicin (PAA, Cölbe, Germany). A modified propidium iodide assay was used to assess the effects of compounds on the growth of tumor cell lines.[11] IC_{50} values were estimated by plotting compound concentration versus cell viability.

The relative expression of NAD(P)H/quinone oxidoreductase (NQO1) in these cell lines was assessed using Human Genome U95 Affymetrix arrays at Oncotest GmbH, Freiburg.[12] The removal of NQO1 dependency of activity is shown in Figure S4.

Estimation of Maximum Tolerated Therapeutic Dose (MTTD) and In vivo Inhibition of MAXF 401 Tumor Xenograft Growth. These experiments were carried out at Oncotest GmbH, Freiburg, Germany and were conducted according to the guidelines of the German Animal Health and Welfare Act (Tierschutzgesetz).

MTTD was estimated by dose escalation in non-tumor bearing NMRI nu/nu mice. **5** was formulated in the vehicle: 5% DMSO, 10% Cyclodextrin (HP-ß-CD) in 0.15M saline and was dosed i.p.. At a dose level of 250 mg/kg/d of **5** given daily for 5 days caused only moderate median body weight losses (3.8%).

The antitumor activity and the side effects of **5** were investigated nude mice bearing patient derived xenografts of the human mammary cancer MAXF 401 (mean

tumor diameter: 6-8 mm at day 0 - the first day of dosing). One Group of 6 mice bearing 2 tumors was treated with **5** at 85 mg/kg/day i.p. on days 0-4, 7-11, 14-18, 21-25, 28-32. A second group was dosed with 17-AAG formulated in 10% DMSO, 0.05% Tween80 in PBS dosed i.p. at the same schedule, alongside a vehicle control group dosed at 10 mL/kg (10% DMSO, 0.05% Tween80 in PBS). 17-AAG is not soluble n the vehicle used for compound **5**. The MTTD for 17-AAG in this vehicle is 50 mg/kg/d. Body weights were determined twice a week and tumor volume was determined on Day 0 and then twice weekly. Antitumor activity was evaluated as maximum tumor volume inhibition versus the vehicle control group. Relative tumor volumes (RTVs) were calculated as a percentage of the individual tumor volume on Day 0. Group tumor volumes were expressed as the median RTV volumes of individual tumors in a group (group median RTV). The experiment was terminated on Day 35.

When administered i.p. at 85 mg/kg/d on days 0-4, 7-11, 14-18, 21-25 and 28-32, **5** demonstrated a highly significant ($p<0.001$) antitumor activity against MAXF 401. Therapy clearly reduced tumor growth rates resulting in a minimum *T/C* value of 45.3% recorded on Day 35. In comparison to the vehicle treated control tumors there were remarkable growth delays of 6.4 and 15.2 days for the tumor doubling and the tumor quadrupling times. The antitumor effect of **5** i.p. therapy at the lower dose level of 42.5 mg/kg/d administered on the same days was only slightly less prominent than the antitumor activity of the higher dose level, resulting in a highly ($p<0.001$) significant minimal *T/C* value of 53.9% recorded on day 21. In this therapy group the growth delays for the tumor doubling and quadrupling times were 4.7 and 9.8 days. Therapy with 17-AAG at 50 mg/kg/d administered i.p. on the same days resulted in a slightly better minimal

T/C value of 35.6% on day 28. The difference of the antitumor activities of the two therapies tested to be statistically not significant. This data is shown below in Figure S5.

All animals treated with **5** at the dose level of 85 mg/kg/d i.p. survived therapy. There was only minimal and transient median body weight losses of 3.2% (85 mg/kg/d). 17-AAG therapy induced a single mortality on Day 19. There was a maximum median body weight loss of 4.4% recorded on Day 21. In conclusion it can be stated that **5** was administered well below the MTTD level in the antitumor efficacy experiment while 17-AAG therapy at 50 mg/kg/d i.p. represents the maximum tolerable dose level.

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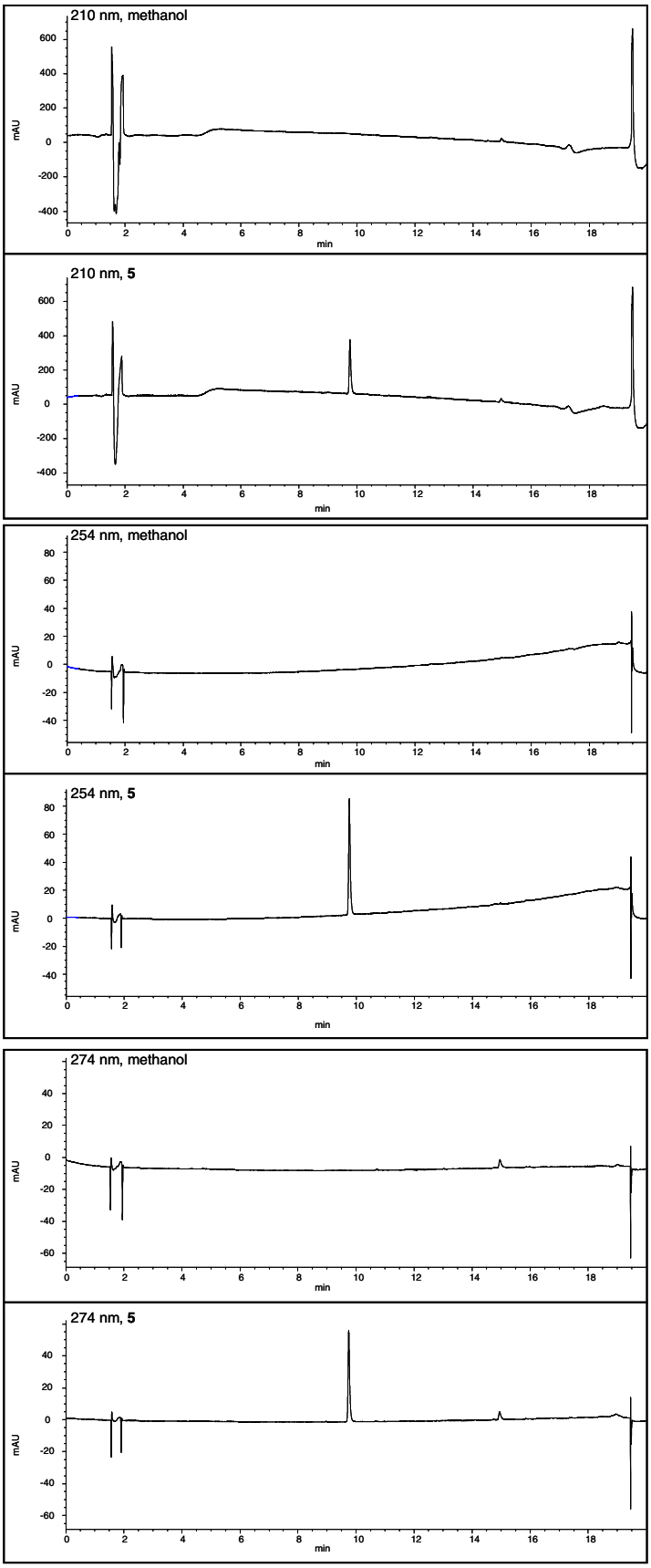

Figure S1. Purity of **5** determined at 210, 254 and 274 nm, 5μL of a 0.1 mg/mL was injected

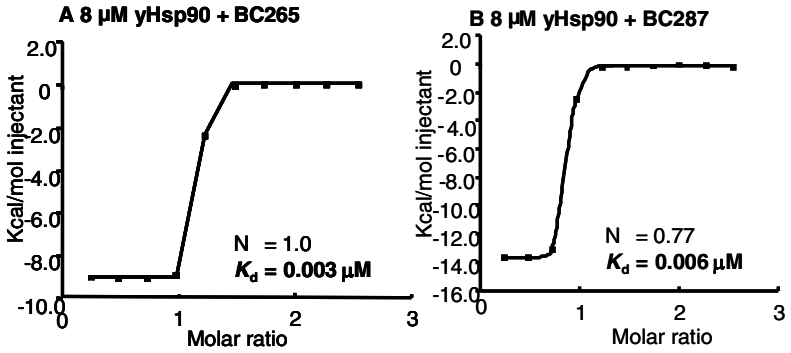

Figure S2. ITC determination of binding to Hsp90 of **5** (**A**) and **6** (**B**)

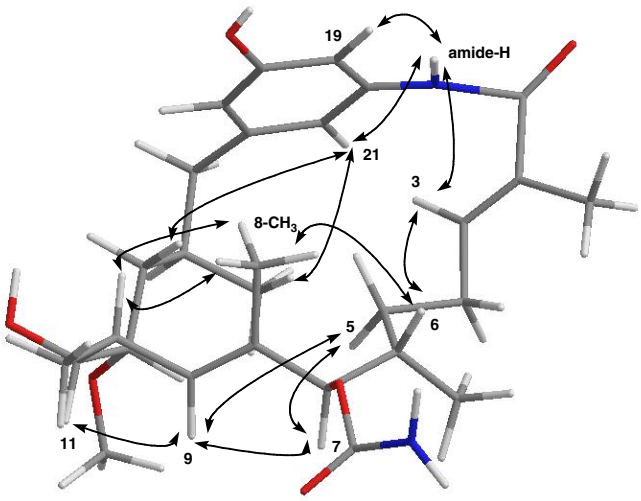

Figure S3. The key NOE correlations of **5** observed in NOESY spectrum

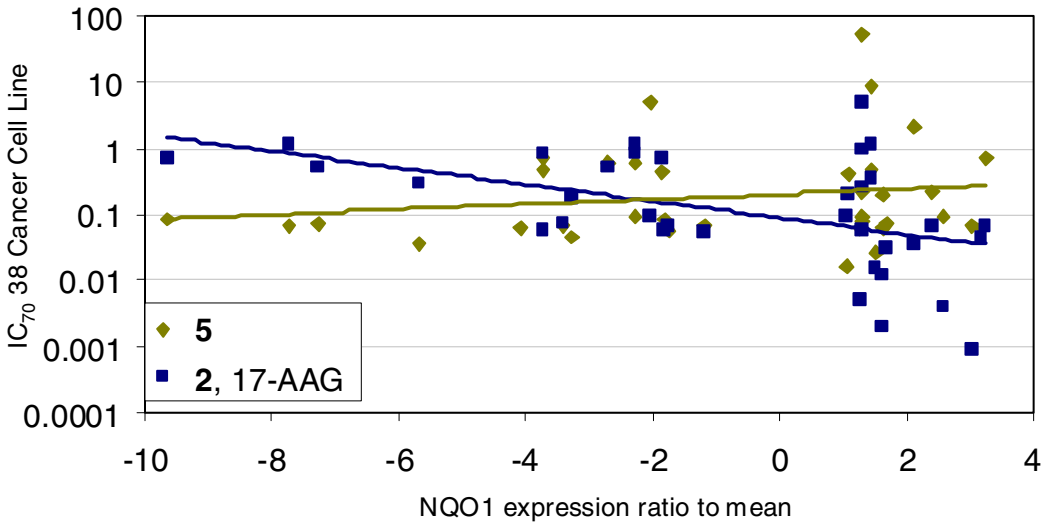

Figure S4. Removal of NQO1 dependency of activity: Comparison of IC_{70} values from a 38 cancer cell line panel inhibition experiment reveals that activity of **5** is not dependent on NQO1 expression, whereas the activity of **2**, 17-AAG is partially dependent on NQO1 expression.

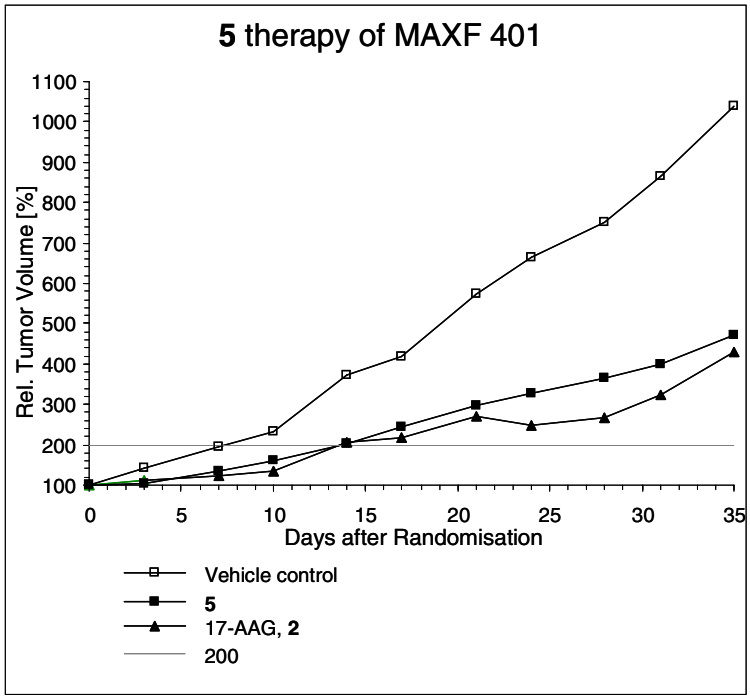

Figure S5. In vivo efficacy of **5** at 1/3rd MTTD and17-AAG at MTTD