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New geldanamycin derivatives with anti Hsp properties by mutasynthesis†

Jekaterina Hermene,^{‡a} Simone Eichner,^{‡a} Lena Mancuso,^a Benjamin Schröder,^{‡a} Florenz Sasse,^{ib} Carsten Zeilinger^c and Andreas Kirschning^{ib}*^a

Mutasynthetic supplementation of the AHBA blocked mutant strain of *S. hygroscopicus*, the geldanamycin producer, with 21 aromatic and heteroaromatic amino acids provided new nonquinoid geldanamycin derivatives. Large scale (5 L) fermentation provided four new derivatives in sufficient quantity for full structural characterisation. Among these, the first thiophene derivative of rebastatin showed strong anti-proliferative activity towards several human cancer cell lines. Additionally, inhibitory effects on human heat shock protein Hsp90 α and bacterial heat shock protein from *H. pylori* HpHtpG were observed, revealing strong displacement properties for labelled ATP and demonstrating that the ATP-binding site of Hsps is the target site for the new geldanamycin derivatives.

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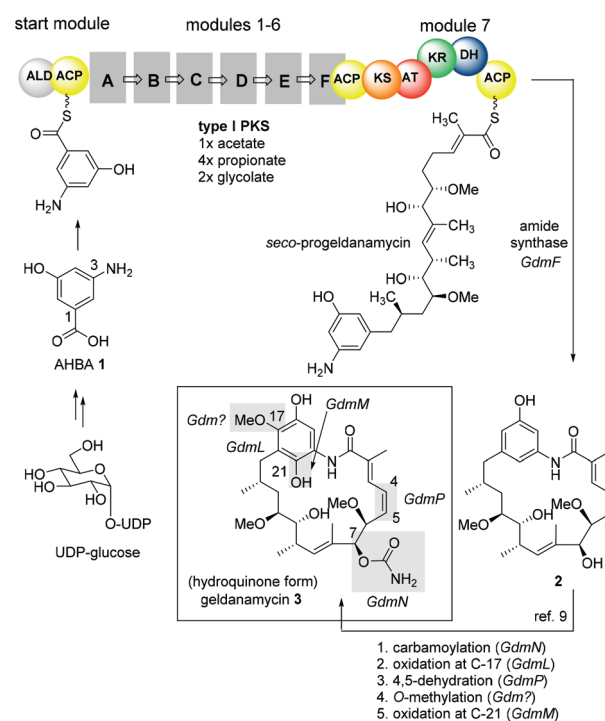
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Introduction

Besides classical concepts of natural product synthesis, namely total- and semisynthesis, strategies that rely on the manipulation of microbial secondary metabolites not after, but in the course of their biosynthesis, have broadened the synthetic portfolio of chemists and biotechnologists. One strategy employs mutant organisms blocked in the biosynthesis of endogenous key precursors on a genetic level. According to Rinehart,¹ mutational biosynthesis or mutasynthesis² aims to produce genetic mutants of an organism in which the formation of key biosynthetic building blocks required for the assembly of natural metabolites are blocked.³ In turn, administration of structurally modified building blocks, so-called mutasynthons, to the blocked mutant may result in their incorporation and lead to the synthesis of novel metabolites, which can be isolated and evaluated for their therapeutic potential.⁴ In principle, mutasynthesis allows access to compound libraries of pharmacologically relevant and complex novel products. While it is simple to produce gene knock-outs that are useful in the creation of blocked deletion mutants, it is critical that the modified organism performs enzymatic cata-

lysis with high levels of efficiency, selectivity and a broad substrate tolerance when presented with non-natural precursors.

Ansamycin antibiotics, specifically the ansamitocins, rifamycin and geldanamycin, have been a prime target to create natural product libraries through mutasynthesis.⁵ Geldanamycin (3; hydroquinone form in Scheme 1), produced by *Streptomyces*



Scheme 1 Key features of geldanamycin biosynthesis.^{8,9}

^aInstitute of Organic Chemistry, Leibniz Universität Hannover, Schneiderberg 1B, 30167 Hannover, Germany. E-mail: andreas.kirschning@oci.uni-hannover.de

^bHelmholtz-Center for Infection Research, Department of Microbial Communication, Inhoffenstr. 7, 38124 Braunschweig, Germany

^cLeibniz Universität Hannover, Institute of Biophysics and Center of Biomolecular Drug Research (BMWZ), Schneiderberg 38, D-30167 Hannover, Germany

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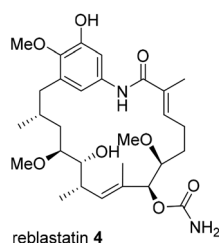
‡These authors contributed equally to the work.



hygroscopicus, is a potential antitumor drug⁶ that binds to the N-terminal ATP-binding domain of heat shock protein 90 (Hsp90) and inhibits its ATP-dependent chaperone activity.⁷

Heat shock proteins are essential for survival because they regulate a diverse set of cellular processes and consequently, geldanamycin has been a lead structure for at least two clinical candidates against cancer.

However, activity of the benzoquinone form of geldanamycin first depends on reductive activation to hydroquinone 3 by the enzyme NAD(P)H/quinone oxidoreductase 1 (NQO1).^{7,10–12} Thus, it is the hydroquinone itself that is regarded to be the motif, that binds to the ATP binding domain. The benzoquinone-based geldanamycins are far from being ideal antitumor drugs, because they show hepatotoxic side effects, in part, because of their Michael-acceptor properties toward natural thiols such as glutathione.^{12,13}



Reblastatin 4, a phenolic analogue of geldanamycin, shows similar Hsp90 inhibitory effects and antiproliferative activity. Beneficially, it lacks the redox-sensitive quinone or hydroquinone moiety¹⁴ and such macrolactam derivatives have emerged as promising alternatives in the development of cancer chemotherapies.¹⁵ The biosynthesis of geldanamycin (3)⁸ begins with 3-amino-5-hydroxybenzoic acid (AHBA 1), which is derived from D-glucose through a modified shikimate pathway. Loading onto the starter module and processing through seven PKS modules yields *seco*-progeldanamycin, which is subsequently cyclised and released from the PKS by an amide synthase. The resulting progeldanamycin (2) is then transformed to geldanamycin by a set of tailoring enzymes that induce amidination, several oxidation reactions of the aromatic moiety, O-methylation and desaturation at C4,C5.^{8,9}

As mentioned previously, mutasynthesis has been successfully employed to access new geldanamycin analogues, particularly by the groups of Lee, Hong,^{16,17} by Menzella¹³ and through our own efforts.^{9,18} All published mutasyntheses of new phenolic derivatives of geldanamycin, which we call rebblastatin derivatives in this work as they commonly lack the double bond at C4–C5 or a quinone/hydroquinone moiety, rely on the disruption of genes coding for AHBA formation and feeding of unnatural 3-aminobenzoic acid derivatives. In our preliminary studies, we demonstrated that aromatic mutasynthons 5–9 are successfully processed to new rebblastatin derivatives (Fig. 1). These results showed that modifications at positions 4–6 (AHBA numbering),¹⁹ as well as heteroaromatic mutasynthons, are in principle tolerated by the biosynthetic assembly line, although the scope is still unknown.

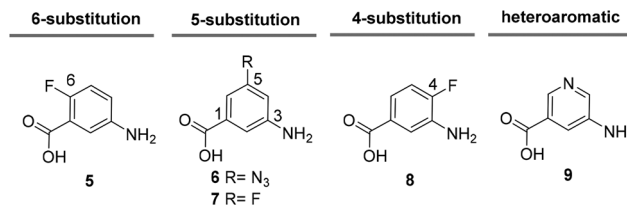


Fig. 1 Aromatic mutasynthons 5–9 successfully transformed into new rebblastatin derivatives in preparative amounts.

Herein, we provide a full evaluation of substrate tolerance for an AHBA blocked mutant strain of *S. hygroscopicus* using a wide variety of aromatic and heteroaromatic amino acids as mutasynthons, and subsequently report the preparation of new rebblastatin derivatives by mutasynthesis. Antiproliferative properties of the new metabolites, which were available in sufficient amounts, are compared with their inhibitory effects on the ATP binding domain in human and bacterial heat shock proteins using a microarray competition assay.²⁰

Results and discussion

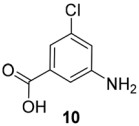
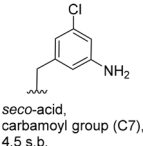
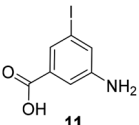
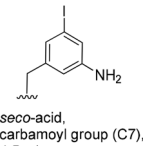
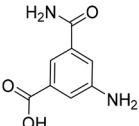
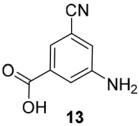
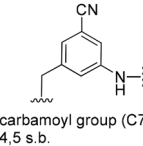
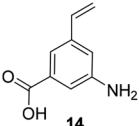
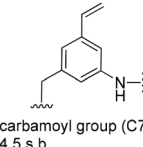
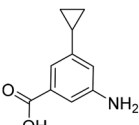
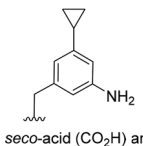
Mutasynthetic experiments (preliminary screening)

5-Substituted 3-aminobenzoic acids 6 and 7 were found to be the most promising candidates for accessing new rebblastatin derivatives using mutasynthesis.^{18a} We extended the scope of substrates to 5-substituted-3-aminobenzoic acids 10–15 (Table 1)²¹ that included halo-substitution (10 and 11) as well as C-substitution (12–15). Typically, the free acids can be administered as they are activated to the corresponding adenylates in *Streptomyces hygroscopicus*. Next, these are attacked by phosphopantetheine-thiol to form the PKS-bound thioesters. Preliminary experiments were performed on a small scale (0.038 mmol mutasynthon, 30 mL fermentation broth, 7 d). This set of mutasynthetic experiments revealed that carbamoylation always takes place, and in selected cases hydroxylation at C17 as tailoring modifications can occur. In our hands, desaturation at C4,C5 is a rarely observed transformation when feeding unnatural aminobenzoic acids and for the new examples reported here, it was not observed at all. Amide 12 was not processed as judged by UPLC-HRMS analysis but the functionally related nitrile 13 provided one macrolactam metabolite after feeding to the AHBA(–) mutant strain of *S. hygroscopicus*. Other C-substituents such as the vinyl group present in benzoic acid 14 or the cyclopropyl group as in 15 were also converted into the amidinated macrolactams. In several cases, we detected the presence of *seco* acids (for mutasynthons 10, 11 and 15) and in one case also the “*seco* amide” (for mutasynthon 15). The “*seco* amide” is the amide derivative of the corresponding *seco* acid, which cannot serve as substrate for macrolactamisation anymore.

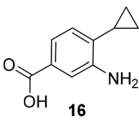
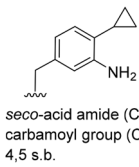
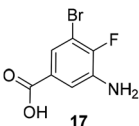
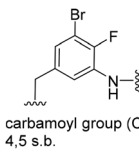
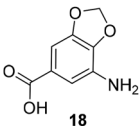
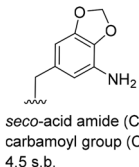
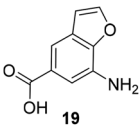
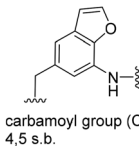
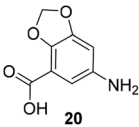
The second series of experiments utilised diversely substituted aminobenzoic acids 16–20 in which the third and in some cases a fourth substituent are located at positions 4 and



Table 1 Test mutasyntheses with (–)-AHBA blocked mutant of *S. hygroscopicus* using 5-substituted-3-aminobenzoic acids 10–15

Mutasynth ²¹	Formula	Proposed structure ^{a,b}
	C ₂₉ H ₄₄ N ₂ O ₇ Cl[M + H] ⁺ : calc.: 555.2837, found: 555.2834 C ₂₈ H ₄₂ N ₂ O ₇ Cl[M + H] ⁺ : calc.: 537.2731, found: 537.2748 C ₂₈ H ₄₁ N ₂ O ₆ ClNa[M + Na] ⁺ : calc.: 575.2500, found: 575.2505	 seco-acid, carbamoyl group (C7), 4,5 s.b.
	C ₂₈ H ₄₄ N ₂ O ₇ I[M + H] ⁺ : calc.: 647.2193, found: 647.2184 C ₂₈ H ₄₂ N ₂ O ₇ I[M + H] ⁺ : calc.: 629.2088, found: 629.2072	 seco-acid, carbamoyl group (C7), 4,5 s.b.
	—	Not processed
	C ₂₉ H ₄₂ N ₃ O ₆ [M + H] ⁺ : calc.: 528.3074, found: 528.3070	 carbamoyl group (C7), 4,5 s.b.
	C ₃₀ H ₄₄ N ₂ O ₆ Na[M + Na] ⁺ : calc.: 551.3097, found: 551.3105	 carbamoyl group (C7), 4,5 s.b.
	C ₃₁ H ₄₉ N ₂ O ₇ [M + H] ⁺ : calc.: 561.3540, found: 561.3527 C ₃₁ H ₅₀ N ₃ O ₆ [M + H] ⁺ : calc.: 560.3700, found: 560.3710 C ₃₁ H ₄₆ N ₂ O ₆ Na[M + Na] ⁺ : calc.: 565.3254, found: 565.3254 C ₃₁ H ₄₆ N ₂ O ₇ Na[M + Na] ⁺ : calc.: 581.3203, found: 581.3208	 seco-acid (CO ₂ H) and seco-amide (CONH ₂); carbamoyl group (C7), 4,5 s.b.

^a Analysis by UPLC-HRMS. ^b s.b. = single bond.**Table 2** Test mutasyntheses with (–)-AHBA blocked mutant of *S. hygroscopicus* using diversely substituted 3-aminobenzoic acids 16–20

Mutasynth ²¹	Formula	Proposed structure ^{a,b}
	C ₃₁ H ₅₀ N ₃ O ₆ [M + H] ⁺ : calc.: 560.3700, found: 560.3701	 seco-acid amide (CONH ₂), carbamoyl group (C7), 4,5 s.b.
	C ₂₈ H ₄₀ N ₂ O ₆ FBrNa[M + Na] ⁺ : calc.: 621.1951, found: 621.2000	 carbamoyl group (C7), 4,5 s.b.
	C ₂₉ H ₄₆ N ₃ O ₈ [M + H] ⁺ : calc.: 564.3285, found: 564.3288	 seco-acid amide (CONH ₂), carbamoyl group (C7), 4,5 s.b.
	C ₃₀ H ₄₂ N ₂ O ₇ Na[M + Na] ⁺ : calc.: 565.2890, found: 565.2884	 carbamoyl group (C7), 4,5 s.b.
	—	Not processed

^a Analysis by UPLC-HRMS. ^b s.b. = single bond.

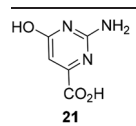
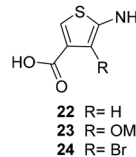
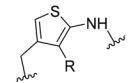
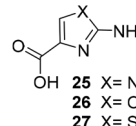
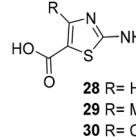
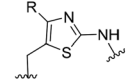
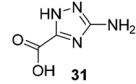
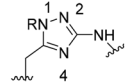
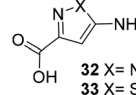
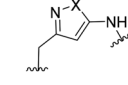
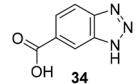
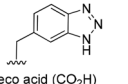
5 or 5 and 6, respectively (Table 2). With the successful bio-transformation of 4-fluoro-3-aminobenzoic acid **8**, precedence for the acceptance of 4-substituted mutasynthons was already at hand.^{18b} 3-Amino-4-cyclopropylbenzoic acid **16** was processed to the carbamoylated seco acid (amide form) so that macrolactamisation did not occur, which is likely due to steric hindrance in the *ortho*-position of the aniline group. 4,5-Disubstituted aminobenzoic acid **18** behaved in the same manner, whereas the 4-fluoro-5-bromo derivative **17** yielded the expected amidinated macrolactam. Obviously, a small fluorine substituent at the 4 position as is also the case for benzoic acid **8** does not hamper macrolactamisation by the amide synthase (*GdmF*; Scheme 1).

Subtleties concerning the type of substitution at C4 become evident when examining benzoic acid **19**, which also gave the corresponding macrolactam ring. Compared to the 2,2-dimethyl-1,3-dioxole ring in **18**, the furan moiety is planar and sterically less congested. Finally, substituents located at position 6 as in benzoic acid **20** suppress PKS processing probably due to inefficient loading onto the PKS starter module.



One important result of our previous studies was that, not only are 3-aminobenzoic acid derivatives processed by the PKS of *S. hygrosopicus*, 5-aminonicotinic acid **9** is also accepted as a PKS substrate. This unprecedented finding initiated a study in our laboratories on the scope and limitations of this new result using a series of six- and five-membered hetarenes **21–34** as mutasynthons (Table 3). The pyrimidine derivative **21**, bearing the same substitution pattern as AHBA, failed to serve as a mutasynthon. When exposing a series of five membered hetarenes to the typical

Table 3 Test mutasyntheses with (–)-AHBA blocked mutant of *S. hygrosopicus* heteroaromatic aminobenzoic acids **21–34**

Mutasynthon ²¹	Formula	Proposed structure ^{a,b}
 21	—	Not processed
 22 R = H 23 R = OMe 24 R = Br	For R = H C ₂₆ H ₄₀ N ₂ NaO ₆ S [M + Na] ⁺ : calc.: 551.2505, found: 551.2510	 R = H carbamoyl group (C7), 4,5 s.b. R = OMe not processed R = Br not processed
 25 X = NH 26 X = O 27 X = S	—	Not processed
 28 R = H 29 R = Me 30 R = CF ₃	For R = H C ₂₅ H ₃₇ N ₃ NaO ₆ S [M + Na] ⁺ : calc.: 530.2301, found: 530.2298	 R = H carbamoyl group (C7), 4,5 s.b. R = Me not processed R = CF ₃ not processed
 31	C ₂₆ H ₄₁ N ₅ NaO ₇ [M + Na] ⁺ : calc.: 558.2904, found: 558.2911	 R = Ac (or at position 2) carbamoyl group (C7), 4,5 s.b.
 32 X = N 33 X = S	For X = S C ₂₅ H ₃₉ N ₃ NaO ₆ S [M + H] ⁺ : calc.: 532.2457, found: 532.2451	 X = N not processed X = S carbamoyl group (C7), 4,5 s.b.
 34	C ₂₈ H ₄₃ N ₄ O ₇ [M + H] ⁺ : calc.: 547.6730, found: 547.6725	 seco acid (CO ₂ H) carbamoyl group (C7), 4,5 s.b.

^a Analysis by UPLC-HRMS. ^b s.b. = single bond.

fermentation conditions, we found the following general trends. One sulfur atom must be part of the five-membered ring in order to yield mutaproducts, either as macrolactam derivatives or as seco acids. Consequently, thiophene **22**, thiazole **28** and thiazine **33** were accepted and processed. However, in the case of the thiazole core the positions of the amino- and carboxyl substituents are crucial, because mutasynthon **27** did not provide new products as judged by HRMS. Therefore, also imidazole **25**, oxazole **26** and pyrazine **32** did not serve as mutasynthons. Additional substitution as present in thiophenes **23** and **24** as well as in thiazoles **29** and **30** is also not tolerated by *S. hygrosopicus*. In view of these findings triazole **31** represents an unusual case, as the sulfur atom is missing in the hetarene ring but UPLC-HRMS revealed formation of a macrocyclic mutaproduct. It has to be noted that the thiophene moiety is a bioisostere for the benzene ring²² and this is also reflected in the present work as 5-aminothiophene-3-carboxylic acid **22** was processed to the corresponding macrolactam by the polyketide synthase.

Finally, the benzotriazole derivative **34**, which bears a meta orientation between the carboxylate and in this case a hetarene NH group, was also processed to the amidinated seco acid. We did not encounter macrolactamisation, likely due to the presence of a secondary sp²-hybridised amine instead of an aniline.

At this point, one can conclude that the PKS machinery involved in the biosynthesis of geldanamycin shows a rather broad substrate flexibility with respect to structural alterations in the aromatic starter group. Additional substituents at the 4, 5 and 6 positions as well as disubstitution of selected 3-aminobenzoic acids are tolerated as long as these substituents are not too large. In several cases, we encountered seco acid formation which included formation of carboxamides, a process that stops macrolactamisation. The open chain metabolites indicate that the amide synthase can act as a bottle neck for further processing, while substrate tolerance of the PKS up to that point must have been rather broad. Advanced intermediates, whether they are acyclic or cyclic, all serve as substrates for the tailoring amidination. Other post PKS transformations, such as oxidations of the aromatic core as well as 4,5-desaturation, are much more sensitive to structural variation in progeldanamycin precursors and in most cases, they do not occur.

Mutasynthetic experiments (preparative scale)

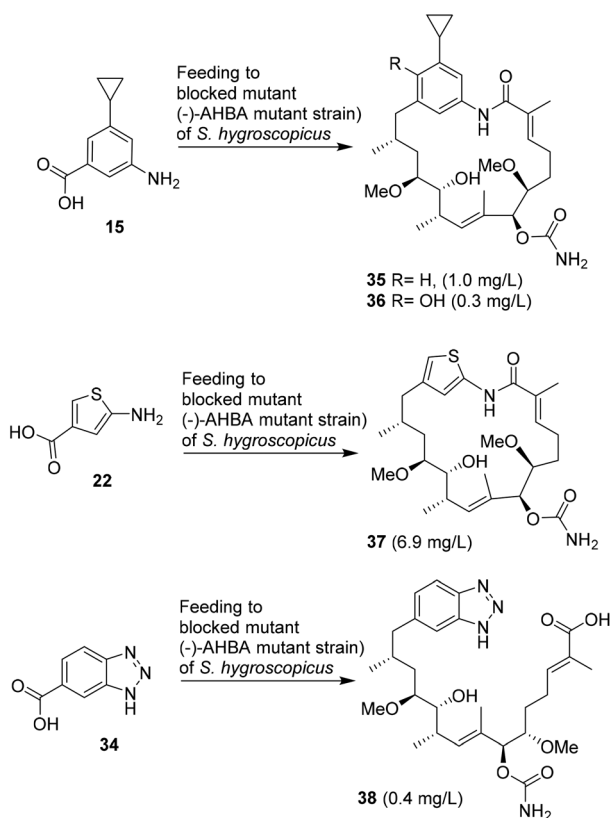
Principally, all new seco acids and ansa macrolactams that are listed in Tables 1–3 should be accessible as pure compounds, provided that the fermentation is appropriately upscaled. However, substantial HPLC purification is required to separate all metabolites and mg quantities are needed for full structural characterisation as well as biological evaluation. These requirements may therefore necessitate fermentation at a 10–50 L scale.

After close inspection of all chromatograms of the crude fermentation extracts, we decided that mutasynthons **15**, **22** and **34** provided sufficient amounts of new rebastatin deriva-



tives for complete structure elucidation and biological evaluation. For this purpose, the fermentations were repeated in up to 5 L scale.

After harvesting, the fermentation broths were extracted with ethyl acetate. Depending on the complexity of the metabolic spectrum, several purification steps using silica gel chromatography, Sephadex size exclusion chromatography and RP-HPLC were performed (see ESI†). In all cases, the fermentation yields of new mutaproducts were significantly lower than that obtained when the natural starter unit, AHBA **1**, was fed to yield geldanamycin **3**. Supplementation of cyclopropyl-substituted aminobenzoic acid **15** to a growing culture of the (–)AHBA mutant of *Streptomyces hygroscopicus* provided rebplastatin derivatives **35** and **36** in sufficient amounts for isolation and characterisation (Scheme 2). Besides amidination of the alcohol at position 7, oxidation at C17 also occurred as a tailoring modification. Likewise, thiophene **22** provided rebplastatin derivative **37** in excellent yield in comparison to those commonly observed for such mutasynthetic experiments.¹⁸ Finally, we carried out the supplementation of AHBA(–) *S. hygroscopicus* with benzotriazole **34** and were able to collect the chemically labile seco acid derivative **38**. This is the first example of a bicyclic mutasynthron undergoing successful loading onto the starter module and being fully processed by the PKS; although this proceeded without macrolactamisation by the amide synthase.



Scheme 2 Successful upscale mutasyntheses with (–) AHBA mutant strain of *S. hygroscopicus*.

Biological studies

Antiproliferative activities and Hsp binding affinities

For the evaluation of its antiproliferating potential the two new derivative **35** and **37** that were available in sufficient amounts in pure form were administered to cultured human tumour cell lines (Table 4). The data were compared to geldanamycin **3** and the 19-fluoro derivative **8** one of the most potent nonhydroquinone derivatives known so far. The branched cyclopropyl substituent at position 17 led to reduced activity. Remarkably, the thiophene analogue **37** of rebplastatin shows good antiproliferative activity against all human cancer cell lines tested. Still it is not as active as geldanamycin **3**. This can be ascribed to the fact that thiophene and benzene are bioisosteres and the sulfur atom is located at position 18 of geldanamycin **3** and rebplastatin **4**, respectively.

Recently, we reported a new displacement assay for the ATP domain of heat shock proteins such as for Hsp90α.²⁰ The inhibition is monitored using fluorescence-labelled Cy3- or Cy5-ATP in the presence of a potential inhibitor that binds to the ATP binding domain. This approach allows to distinguish between the bound and unbound inhibitor. The assay is performed by incubating different Hsp-loaded microarrays for 16 h at 4 °C with a solution containing a mixture consisting of 100 nM of the fluorescent-labelled ATP derivatives Cy3-ATP or Cy5-ATP, the binding buffer and the potential Hsp90 inhibitor (500 nM), including 17-aminoallyl geldanamycin (17-AAG) **39** (line 2), rebplastatin derivatives **40–43** (lines 3–6) and thiophene rebplastatin **37** (line 7). To compare different heat shock proteins from different sources, we chose human Hsp90α (Fig. 2a), Hsp90β (b), *Xanthomonas campestris* XcHtpG (c) and *Helicobacter pylori* HpHtpG (d). The results are presented as a heat map (Fig. 2). The simultaneous screening of different purified Hsps reveals that most of all synthesised rebplastatin derivatives, except for cyclopropyl derivative **35**, are capable of inhibiting human and HpHtpG heat shock proteins. Remarkably, the clinical candidate 17-AAG **39** only shows a minor activity for HpHtpG. The EC₅₀ of thiophene **37** against HpHtpG and HsHtpG were 59 nM and 128 nM, respectively. This comparison reveals that the thiophene group shows varying affinities to the ATP binding sites of different Hsps,

Table 4 IC₅₀ [nM] of the antiproliferative activity of **35**, **37**, **3** and **8** with human cancer cell lines (values are means of two determinations in parallel)^a

	IC ₅₀ [nM]			
	KB-3-1	PC-3	SK-OV-3	A-431
35	960	1500	1300	980
37	128	472	159	85
3	53	18	125	18
8	73	42	54	62

^a KB-3-1 = Cervix carcinoma; PC-3 = Prostate carcinoma; SK-OV-3 = Ovarian carcinoma; A-431 = Epidermoid carcinoma.



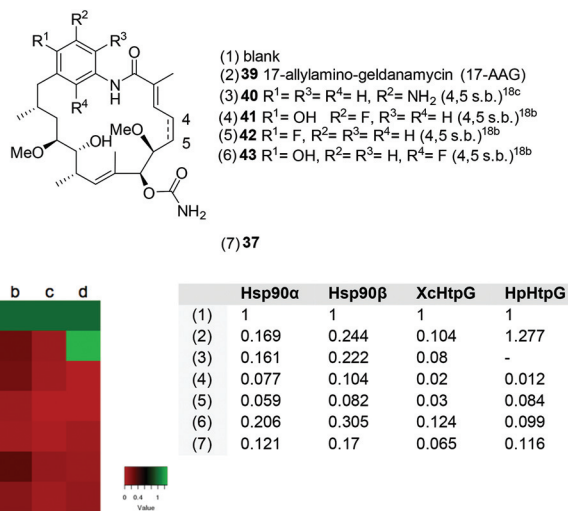


Fig. 2 Top: Structures of geldanamycin and rebblastatin derivatives screened. Bottom left: Heat map presentations of competitive Hsp activities tested for compounds **37** and **39–43** dedicated to lines 2–7 using a microarray-based assay with purified heat shock proteins in the presence of ATP. The normalised signals are ranked from 0 to 1 (line 1, blank) displaying different colours. Low signal values (red) indicate stronger competition against fluorescence dye labelled ATP and hence higher affinity for Hsps. No competition shown in position 1 provides a green colour. The concentrations of potential binders are 500 nM against (a) human Hsp90α, (b) Hsp90β, (c) *Xanthomonas campestris* XcHtpG and (d) *Helicobacter pylori* HpHtpG. These were spotted onto a microarray as described before.^{20a} Bottom left: Corresponding normalised values derived from the optical measurements.

which can serve as a starting point for developing Hsp specific inhibitors.

Additionally, the measurements were conducted at different concentrations (50 μM, 5 μM, 0.5 μM, 50 nM, 5 nM, 0.5 nM, 50 pM) to estimate the IC₅₀ value. Unbound fluorescently-labelled ATP was removed by several washing steps and then the amount of bound Cy3-labelled ATP was determined. The signal intensity was analysed and normalised for the unbound value and hence visualised. These studies^{18,20} reveal that the inhibitory properties found for new rebblastatin derivatives **35** and **37** match those of the antiproliferative activity (Tables 4 and 5).

Table 5 Summary of the calculated IC₅₀ values [nM] of Hsp90α from *H. sapiens* and of HpHtpG from *H. pylori* and the specific Z-factors²³ of each protein microarray (in brackets position on the microarray in Fig. 2)

	IC ₅₀ (nM)			
	Hsp90α	Z ²³	HpHtpG	Z ²³
35	n.b.	0.72	n.b.	0.53
39	30.03 ± 6.56	0.81	80.6 ± 7.34	0.53
40	59.41 ± 30.55	0.54	20.99 ± 5.91	0.79
41	57 ± 15.51	0.78	163.26 ± 31.77	0.69
42	18.22 ± 4.11	0.77	38.48 ± 7.48	0.55
43	10 ± 2.7	0.68	64 ± 2.34	0.78
37	127.5 ± 42.2	0.45	56.63 ± 25.0	0.77

Except for the cyclopropyl rebblastatin **35**, all derivatives show pronounced displacement properties for the heat shock proteins tested. Reblastatin derivative **39** bearing the simplest possible substitution pattern at the aromatic ring demonstrates that non-quinone and even non phenolic geldanamycin derivatives are very effective in replacing ATP in human Hsp90α (**39**, IC₅₀Hsp90α = 30 nM). However, in no case have we encountered a relevant selectivity difference between human Hsp90α and Hsp90β (Fig. 2). Interestingly, thiophene rebblastatin **37** exhibits a stronger activity for the bacterial Hsp homologue HpHtpG than for human Hsp90α. Therefore, it can serve as a starting point for developing antibiotics that target bacterial heat shock proteins, especially because it was recently shown that HtpG is required for adaptation through environmental stress.²⁴

Conclusions

In conclusion, we reported on the mutasynthetic generation and biological evaluation as heat shock protein inhibitors of several new rebblastatin derivatives. The cyclopropane derivatives **35** and **36** are remarkable in that this is the first example of introducing such structural elements into complex natural products by a mutasynthetic approach. More striking is the high yielding uploading and PKS-processing of 5-aminothiophene-3-carboxylic acid **22** to the corresponding thiophene rebblastatin derivative **37**, the first mutaproduct of this kind reported so far.²⁵

Importantly, this broad study also demonstrates the scope and limitations of mutasynthesis as a synthetic tool in natural product chemistry. The structural diversity of aromatic building blocks including hetarenes that are accepted by the starting module of Gdm-PKS and fully processed up to that stage of the macrolactam is astounding. A promising new candidate is the thiophene derivative of rebblastatin. It shows inhibitory activity against HtpG from *Helicobacter pylori* and this derivative clearly can serve as a starting point to develop new selective inhibitors against bacterial heat shock proteins. Finally, the limitation of producing sufficient quantities of new derivatives by mutasynthesis has to be kept in mind^{26–29} and is also in issue in the present study. This becomes particularly evident when calculating the isolated yields of new mutaproducts based on the amount of mutasynthons fed: **35** (0.15% from **15**), **36** (0.04% from **15**), **37** (1.1% from **22**) and **38** (0.09% from **34**).

Experimental procedures

Mutasynthetic experiments

General parameters. *Streptomyces hygroscopicus* mutant K390-61-1 was stored as stock cultures at –80 °C as a cryoculture in the refrigerator. 100 μL of the cryoculture of K390-61-1 mutant was used to inoculate the GYP precultures which were incubated at 28 °C for 2 days. Precultures were prepared in GYP-medium (40 mL per flask, 2.5 g L^{–1} yeast extract, 10 g L^{–1} peptone, 10 g L^{–1} glucose, 3 g L^{–1} xanthane gum, distilled



water). Main cultures were prepared in GP-medium (40 g L⁻¹ glucose, 2.5 g L⁻¹ peptone, 2.5 g L⁻¹ tryptone, 5 g L⁻¹ oatmeal, 2.5 g L⁻¹ yeast extract, 3 g L⁻¹ xanthane gum, distilled water). Liquid culture fermentations were incubated at 28 °C with vigorous shaking (200 rpm) in 500 mL Erlenmeyer flasks equipped with a baffle. Main cultures were inoculated with 1 mL preculture per 30 mL culture broth. The production cultures were harvested after 5 d of fermentation and extracted twice with EtOAc. The EtOAc extracts were concentrated, and the residue was dissolved in MeOH (1 mL) and used directly for ESI-MS analysis.

Feeding experiments for small scale transformations

Benzoic acid derivatives **10–20** and hetarenes **21–34** (37.5 µmol, in 2 mL sterile solution in DMSO/H₂O = 1 : 1) were added in three 670 µL portions at 48, 72 and 96 h after inoculation to 30 mL of the main culture of *S. hygroscopicus* mutant K390-61-1. After having fed precursors according to the fermentation conditions described above, UPLC-ESI-HRMS analysis was employed for detecting new products as [M + H]⁺ or [M + Na]⁺ peaks.

Feeding of benzoic acid 15 and formation of reblastatin derivatives 35 and 36. For scale-up fermentation 3-amino-5-cyclopropylbenzoic acid **15** (287.0 mg, 1.50 mmol, 20 mL sterile solution in DMSO/H₂O = 1 : 1) was fed in three equal portions at 48, 72 and 96 h after inoculation to sixteen 75 mL main cultures of *S. hygroscopicus* mutant K390-61-1. Extraction with EtOAc afforded 17-demethoxy-18-cyclopropyl-reblastatin **35** (1.3 mg, 2.3 µmol) and 17-demethyl-18-cyclopropyl-reblastatin **36** (0.3 mg, 0.5 µmol) after silica gel filtration and three HPLC purification steps.

HPLC conditions employed for the first purification step were (a) column: Reprosil-Pur 120 C18 AQ, 250 × 25 mm, 5 µm, endc.; (b) guard: Reprosil-Pur 120 C18 AQ, 30 × 20 mm, 10 µm, endc.; (c) solvents: methanol/H₂O and (d) program: H₂O : MeOH = 60 : 40 {5 min}, 60 : 40 → 50 : 50 {15 min}, H₂O : MeOH = 50 : 50 → 30 : 70 {65 min}, H₂O : MeOH = 30 : 70 → 0 : 100 {15 min}, H₂O : MeOH = 0 : 100 {10 min}, (15.0 mL min⁻¹) (t_R = 65.0 min). For the second purification step the conditions were (a) column: Reprosil-Pur 120 C18-ISIS AQ, 250 × 8.0 mm, 5 µm, endc.; (b) guard: Reprosil-Pur 120 C18-ISIS AQ, 40 × 8 mm, 5 µm, endc.; (c) solvents: MeOH/H₂O and (d) program: H₂O : MeOH = 80 : 20 → 65 : 35 {5 min}, H₂O : MeOH = 65 : 35 → 40 : 60 {65 min}, H₂O : MeOH = 40 : 60 → 0 : 100 {10 min}, H₂O : MeOH = 0 : 100 {10 min}, (2.5 mL min⁻¹) (t_R = 63.0 min). For the third purification step the conditions were (a) column: Reprosil-Pur 120 C18-ISIS AQ, 250 × 8.0 mm, 5 µm, endc.; (b) guard: Reprosil-Pur 120 C18-ISIS AQ, 40 × 8 mm, 5 µm, endc.; (c) solvents: methanol/H₂O and (d) program: H₂O : MeOH = 80 : 20 → 65 : 35 {5 min}, H₂O : MeOH = 65 : 35 → 40 : 60 {80 min}, H₂O : MeOH = 40 : 60 → 0 : 100 {5 min}, (3.0 mL min⁻¹) (t_R = 64.4 min).

17-Demethoxy-18-cyclopropyl-reblastatin 35

¹H NMR (500 MHz, T = 323 K, THF-d₈, THF-d₇ = 1.73 ppm): δ 8.43 (s, 1H, NH), 7.02 (s, 1H, Ar-H), 6.61 (s, 1H, Ar-H), 6.58

(s, 1H, Ar-H), 5.88–5.80 (m, 1H, 3-H), 5.69 (br. s, 2H, CONH₂), 5.30 (d, J = 9.9 Hz, 1H, 9-H), 5.06 (d, J = 5.8 Hz, 1H, 7-H), 4.54 (br. s., 1H, 11-OH), 3.54–3.48 (m, 1H, 11-H), 3.35 (s, 3H, 6-OMe), 3.31 (s, 3H, 12-OMe), 3.30–3.24 (m, 1H, 6-H), 3.15–3.08 (m, 1H, 12-H), 2.65 (dd, J = 13.2, 5.0 Hz, 1H, 15-Ha), 2.47–2.40 (m, 1H, 10-H), 2.37–2.25 (m, 1H, 15-Hb), 2.37–2.25 (m, 1H, 4-Ha), 2.17–2.05 (m, 1H, 4-Hb), 1.91–1.82 (m, 1H, 14-H), 1.91–1.82 (m, 1H, CH-cyclopropyl) 1.79 (s, 3H, 2-Me), 1.60–1.52 (m, 1H, 13-Ha), 1.48 (s, 3H, 8-Me), 1.40–1.32 (m, 2H, 5-H), 1.27–1.20 (m, 1H, 13-Hb), 1.00 (d, J = 6.6 Hz, 3H, 10-Me), 0.90–0.86 (m, 2H, CH₂-cyclopropyl), 0.82 (d, J = 6.8 Hz, 3H, 14-Me), 0.66–0.61 (m, 2H, CH₂-cyclopropyl) ppm; ¹³C NMR (125 MHz, T = 323 K, THF-d₈, THF-d₈ = 25.5 ppm): δ 171.2 (s, C-1), 157.3 (s, CONH₂), 145.4 (s, C-Ar), 141.8 (s, C-Ar), 141.3 (s, C-Ar), 134.7 (d, C-3), 133.0 (s, C-2), 133.0 (s, C-9), 131.7 (d, C-8), 124.3 (d, C-Ar), 123.5 (d, C-Ar), 116.3 (d, C-Ar), 82.7 (d, C-12), 81.6 (d, C-6), 81.3 (d, C-7), 75.1 (d, C-11), 58.9 (q, 6-OMe), 57.0 (q, 12-OMe), 43.9 (t, C-15), 35.5 (d, C-10), 34.6 (t, C-13), 32.8 (d, C-14), 31.0 (t, C-5), 25.5 (t, C-4), 19.8 (q, 14-Me), 17.3 (d, CH-cyclopropyl), 16.2 (q, 10-Me), 13.6 (q, 2-Me), 13.2 (q, 8-Me), 9.7 (t, CH₂-cyclopropyl), 9.6 (t, CH₂-cyclopropyl) ppm; HRMS (ESI) m/z for C₃₁H₄₆N₂O₆Na [M + Na]⁺: calculated: 565.3254 found: 565.3254.

17-Demethyl-18-cyclopropyl-reblastatin 36

HPLC conditions employed for the first purification step were (a) column: Reprosil-Pur 120 C18 AQ, 250 × 25 mm, 5 µm, endc.; (b) guard: Reprosil-Pur 120 C18 AQ, 30 × 20 mm, 10 µm, endc.; (c) solvents: methanol/H₂O and (d) program: H₂O : MeOH = 60 : 40 {5 min}, 60 : 40 → 50 : 50 {15 min}, H₂O : MeOH = 50 : 50 → 30 : 70 {65 min}, H₂O : MeOH = 30 : 70 → 0 : 100 {15 min}, H₂O : MeOH = 0 : 100 {10 min}, (15.0 mL min⁻¹) (t_R = 65.0 min). For the second purification step the conditions were (a) column: Reprosil-Pur 120 C18-ISIS AQ, 250 × 8.0 mm, 5 µm, endc.; (b) guard: Reprosil-Pur 120 C18-ISIS AQ, 40 × 8 mm, 5 µm, endc.; (c) solvents: MeOH/H₂O and (d) program: H₂O : MeOH = 80 : 20 → 65 : 35 {5 min}, H₂O : MeOH = 65 : 35 → 40 : 60 {65 min}, H₂O : MeOH = 40 : 60 → 0 : 100 {10 min}, H₂O : MeOH = 0 : 100 {10 min}, (2.5 mL min⁻¹) (t_R = 61.0 min). For the third purification step the conditions were (a) column: Reprosil-Pur 120 C18-ISIS AQ, 250 × 8.0 mm, 5 µm, endc.; (b) guard: Reprosil-Pur 120 C18-ISIS AQ, 40 × 8 mm, 5 µm, endc.; (c) solvents: MeOH/H₂O and (d) program: H₂O : MeOH = 80 : 20 → 70 : 30 {5 min}, H₂O : MeOH = 70 : 30 → 40 : 60 {80 min}, H₂O : MeOH = 40 : 60 → 0 : 100 {5 min}, (3.0 mL min⁻¹) (t_R = 60.2 min).

Feeding of benzoic acid 22 and formation of reblastatin derivatives 37. For scale-up fermentation 2-amino-4-carboxythiophene **22** (215 mg, 1.50 mmol, in 20 mL sterile solution in DMSO/H₂O = 1 : 1) was fed in three equal portions at 48, 72 and 96 h after inoculation to sixteen 75 mL main cultures of *S. hygroscopicus* mutant K390-61-1. Extraction with EtOAc afforded thiageldanamycin-derivative **37** (8.3 mg, 16.3 µmol) after silica gel filtration and three HPLC purification steps. HPLC conditions employed for the first purification step were (a) column: Reprosil-Pur 120 C18 AQ,



250 × 25 mm, 5 µm, endc.; (b) guard: Reprosil-Pur 120 C18 AQ, 30 × 20 mm, 10 µm, endc.; (c) solvents: methanol/H₂O and (d) program: H₂O:MeOH = 60:40 {5 min}, 60:40 → 50:50 {15 min}, H₂O:MeOH = 50:50 → 30:70 {65 min}, H₂O:MeOH = 30:70 → 0:100 {15 min}, H₂O:MeOH = 0:100 {10 min}, (15.0 ml min⁻¹) (*t_R* = 55.0 min). For the second purification step the conditions were (a) column: Reprosil-Pur 120 C18-ISIS AQ, 250 × 8.0 mm, 5 µm, endc.; (b) guard: Reprosil-Pur 120 C18-ISIS AQ, 40 × 8 mm, 5 µm, endc.; (c) solvents: MeOH/H₂O and (d) program: H₂O:MeOH = 80:20 → 65:35 {5 min}, H₂O:MeOH = 65:35 → 40:60 {65 min}, H₂O:MeOH = 40:60 → 0:100 {10 min}, H₂O:MeOH = 0:100 {10 min}, (2.5 mL min⁻¹) (*t_R* = 47.0 min). For the third purification step the conditions were (a) column: Reprosil-Pur 120 CN AQ, 250 × 8.0 mm, 5 µm, endc.; (b) guard: Reprosil-Pur 120 CN AQ, 40 × 8 mm, 5 µm, endc.; (c) solvents: MeCN/H₂O and (d) program: H₂O:MeCN = 90:10 → 85:15 {20 min}, H₂O:MeCN = 85:15 {40 min}, H₂O:MeCN = 85:15 → 75:25 {20 min}, H₂O:MeCN = 75:25 → 55:45 {20 min}, H₂O:MeCN = 55:45 → 0:100 {15 min}, (4.0 ml min⁻¹) (*t_R* = 40.0 min).

Thiophene-reblastatin 37

¹H-NMR (500 MHz, *T* = 323 K, THF-d₈, THF-d₇ = 1.73 ppm): δ 8.61 (s, 1H, NH), 6.57 (s, 1H, Ar), 6.38 (s, 1H, Ar), 5.72 (br. s., 1H, 3-H), 5.57 (br. s, 2H, CONH₂), 5.22 (d, *J* = 10.0 Hz, 1H, 9-H), 4.99 (d, *J* = 7.0 Hz, 1H, 7-H), 3.56–3.51 (m, 1H, 11-H), 3.37 (s, 3H, 6-OMe), 3.31 (s, 3H, 12-OMe), 3.28–3.19 (m, 1H, 6-H), 3.11–3.05 (m, 1H, 12-H), 2.65 (dd, *J* = 13.6, 5.4 Hz, 1H, 15-Ha), 2.45–2.35 (m, 1H, 10-H), 2.45–2.35 (m, 1H, 15-Hb), 2.22–2.05 (m, 2H, 4-H), 2.05–1.91 (m, 1H, 14-H), 1.75 (s, 3H, 2-Me), 1.66–1.58 (m, 1H, 13-Ha), 1.55 (s, 3H, 8-Me), 1.24–1.14 (m, 2H, 5-H), 1.20–1.11 (m, 1H, 13-Hb), 1.00 (d, *J* = 6.6 Hz, 3H, 10-Me), 0.75 (d, *J* = 6.8 Hz, 3H, 14-Me) ppm; ¹³C-NMR (125 MHz, *T* = 323 K, THF-d₈, THF-d₈ = 25.5 ppm): δ 172.3 (s, C-1), 156.9 (s, CONH₂), 143.2 (s, C-Ar), 139.6 (s, C-Ar), 135.3 (d, C-3), 134.0 (d, C-9), 131.8 (s, C-2), 131.7 (s, C-8), 124.4 (d, C-Ar), 117.3 (d, C-Ar), 82.1 (d, C-12), 81.8 (d, C-7), 81.4 (d, C-6), 74.2 (d, C-11), 59.1 (q, 6-OMe), 57.0 (q, 12-OMe), 39.0 (t, C-15), 35.7 (d, C-10), 33.3 (t, C-13), 31.4 (t, C-5), 30.5 (d, C-14), 24.5 (t, C-4), 18.7 (q, 14-Me), 17.9 (q, 10-Me), 14.1 (q, 2-Me), 12.7 (q, 8-Me) ppm; HRMS (ESI) *m/z* für C₂₆H₄₁N₂O₆S [M + H]⁺: calculated: 509.2685 found: 509.2666.

Feeding of benzoic acid 34 and formation of seco acid 38.

For scale-up fermentation benzotriazole 34 (245 mg, 1.50 mmol), in 20 mL sterile solution in DMSO/H₂O = 1:1 was fed in three equal portions at 48, 72 and 96 h after inoculation to sixteen 75 mL main cultures of *S. hygroscopicus* mutant K390-61-1. Extraction with EtOAc afforded *seco*-progeldanamycin acid derivative 38 (0.5 mg, 0.9 µmol) after silica gel filtration and three HPLC purification steps. HPLC conditions employed for the first purification step were (a) column: Reprosil-Pur 120 C18-ISIS AQ, 250 × 25 mm, 5 µm, endc.; (b) guard: Reprosil-Pur 120 C18-ISIS AQ, 30 × 20 mm, 10 µm, endc.; (c) solvents: MeOH/H₂O and (d) program: H₂O:MeOH = 60:40 {5 min}, 60:40 → 50:50 {15 min}, H₂O:MeOH = 50:50 → 30:70 {65 min}, H₂O:MeOH = 30:70 → 0:100 {15 min},

H₂O:MeOH = 0:100 {10 min}, (15.0 ml min⁻¹) (*t_R* = 55.0 min). For the second purification step the conditions were (a) column: Reprosil-Pur 120 C18-ISIS AQ, 250 × 8.0 mm, 5 µm, endc.; (b) guard: Reprosil-Pur 120 C18-ISIS AQ, 40 × 8 mm, 5 µm, endc.; (c) solvents: methanol/H₂O and (d) program: H₂O:MeOH = 80:20 → 65:35 {5 min}, H₂O:MeOH = 65:35 → 40:60 {65 min}, H₂O:MeOH = 40:60 → 0:100 {10 min}, H₂O:MeOH = 0:100 {10 min}, (2.5 mL min⁻¹) (*t_R* = 52.0 min). For the third purification step the conditions were (a) column: Reprosil-Pur 120 CN AQ, 250 × 8.0 mm, 5 µm, endc.; (b) guard: Reprosil-Pur 120 CN AQ, 40 × 8 mm, 5 µm, endc.; (c) solvents: MeCN/H₂O and (d) program: H₂O:MeCN = 90:10 → 85:15 {20 min}, H₂O:MeCN = 85:15 {40 min}, H₂O:MeCN = 85:15 → 75:25 {20 min}, H₂O:MeCN = 75:25 → 55:45 {20 min}, H₂O:MeCN = 55:45 → 0:100 {15 min}, (4.0 ml min⁻¹) (*t_R* = 53.5 min).

¹H-NMR (500 MHz, *T* = 323 K, THF-d₈, THF-d₇ = 1.73 ppm): δ 7.72 (d, *J* = 7.0 Hz, 1H, Ph), 7.56 (s, 1H, Ph), 7.20 (d, *J* = 8.0 Hz, 1H, Ph), 6.77–6.69 (m, 1H, 3-H), 5.53 (br. s, 2H, CONH₂), 5.28 (d, *J* = 9.4 Hz, 1H, 9-H), 4.90 (dd, *J* = 8.6, 2.9 Hz, 1H, 7-H), 3.86 (d, *J* = 6.3 Hz, 1H, 11-H), 3.42 (s, 3H, 6-OMe), 3.30 (s, 3H, 12-OMe), 3.35–3.25 (m, 1H, 6-H), 3.25–3.12 (m, 1H, 12-H), 2.84 (dd, *J* = 13.5, 5.7 Hz, 1H, 15-Ha), 2.79–2.69 (m, 1H, 10-H), 2.61–2.50 (m, 1H, 15-Hb), 2.28–2.17 (m, 2H, 4-H), 2.14–1.98 (m, 1H, 14-H), 1.80 (s, 3H, 2-Me), 1.75–1.02 (m, 1H, 5-Ha), 1.70–1.62 (m, 1H, 13-Ha), 1.60 (s, 3H, 8-Me), 1.50–1.36 (m, 1H, 13-Hb), 1.17–1.07 (m, 1H, 5-Hb), 0.97 (d, *J* = 6.6 Hz, 3H, 10-Me), 0.83 (d, *J* = 6.6 Hz, 3H, 14-Me) ppm; HRMS (ESI) *m/z* for C₂₈H₄₃N₄O₇ [M + H]⁺: calculated: 547.3132 found: 547.3137.

Preparation of Hsps and competitive microarray assay

Recombinant Hsps were produced as previously reported.^{18,20} Hsp microarrays were produced and the binding with fluorescently labelled ATP and displacement with inhibitors was performed as described before.^{18,20}

Experimental details on assay (Fig. 2)

The assay was performed by incubating the Hsp-loaded microarrays for 16 h at 4 °C with a solution containing a mixture consisting of 100 nM Cy3-ATP or Cy5-ATP, the binding buffer and the potential Hsp90 inhibitor at 500 nM for comparative screen on different Hsps or at different concentrations (50 µM, 5 µM, 0.5 µM, 50 nM, 5 nM, 0.5 nM, 50 pM) for estimation of the IC₅₀ value. Unbound fluorescently-labelled ATP was removed by several washing steps and then the amount of bound labelled ATP was determined. The signal intensity was analysed and normalised for the unbound value.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

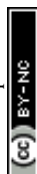
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