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New, Highly Active Nonbenzoquinone Geldanamycin Derivatives by Using Mutasynthesis

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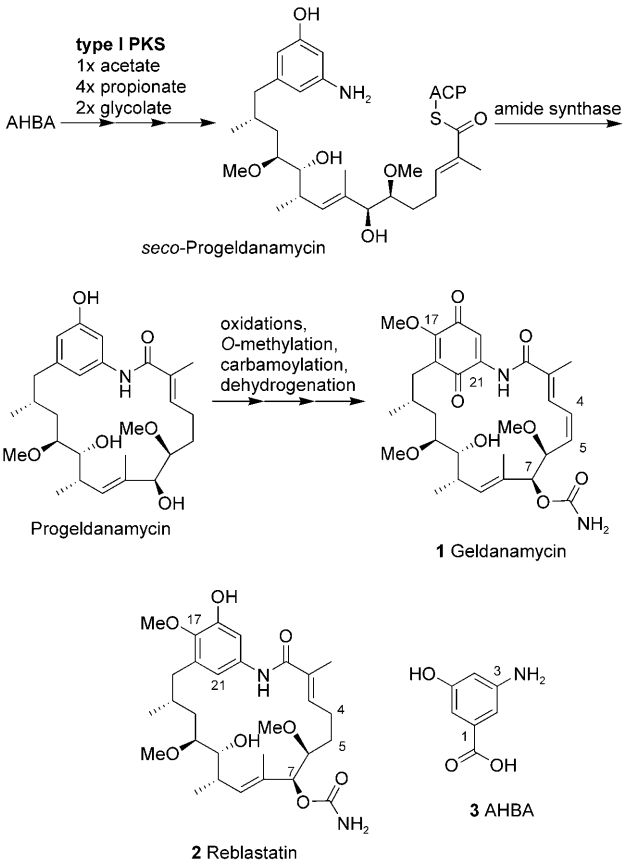
Since its invention by Rinehart and Gottlieb,[1] mutational biosynthesis ("mutasynthesis"[2]) has become a useful tool in the portfolio of the synthetic natural product chemist[3] for the preparation of complex natural product derivatives with pharmaceutical potential.[4] Mutasynthesis requires the generation of mutants of a producer organism that are blocked in the formation of a biosynthetic building block of the end-product. Administration of mutasynthons to the blocked mutant results in new metabolites.[5] A natural product suitable for mutasynthetic investigations is geldanamycin (**1**, Scheme 1), a potential antitumor drug[6] that binds to the N-terminal ATP-binding domain of heat shock protein 90 (Hsp90) and inhibits its ATP-dependent chaperone activities.[7] Most geldanamycin derivatives reported to date are 17-aminated compounds and were obtained by semisynthesis.[8] Recently, two groups have utilized blocked mutants of the microbial source of geldanamycin to prepare several new derivatives.[9–11]

Benzoquinone-containing Hsp90 inhibitors depend on reductive activation to the hydroquinone by the enzyme NAD(P)H/quinone oxidoreductase **1** (NQO1).[12–15] As the activity of this enzyme in different patients is variable, derivatives that show binding to the ATP binding pocket of Hsp90 without the need for activation by NQO1 are highly desirable. Additionally, the quinone moiety of geldanamycin is held responsible for undesired side effects (for example, hepatotoxicity). The Michael addition of the thiol moiety of glutathione to the quinone is regarded as one source of problems.[16] Related to geldanamycin **1** is reblastatin **2**, which is saturated across C4–C5 and has a benzene chromophore instead of a benzoquinone or a hydroquinone moiety.[17] Importantly, reblastatin shows lower cytotoxicity than geldanamycin but has a higher affinity for Hsp90.[17]

The genes required for the biosynthesis of **1** have been cloned, sequenced, and independently analyzed in several streptomycetes.[19]The producing microorganism *Streptomyces hygroscopicus* var. *geldanus* NRRL 3602 creates geldanamycin through a biosynthetic machinery based on a polyketide synthase (PKS) and additional post-PKS enzymes. The biosynthesis of **1** is primed by the starter unit, 3-amino-5-hydroxybenzoic acid (AHBA, **3**), which originates from a shikimate-type biosynthetic pathway (Scheme 1).[20] The PKS generates *seco*-progeldanamycin which is cyclised and released from the PKS by an amide synthase. The resulting progeldanamycin is then further modified by a set of tailoring enzymes, starting with the oxidation of C21 and C17, followed by *O*-methylation at C17, introduction of the carbamoyl moiety and finalized by dehydrogenation across C4–C5.[19a,b] The oxidation of the hydroquinone moiety to the quinone only takes place after oxidation at C21.[19c] Disruption of genes coding for AHBA formation leads to blocked mutants without affecting the modules of the polyketide biosynthetic genes (PKS 1).[18]

After our successful application of the mutasynthesis methodology for the generation of ansamitocin P-3 derivatives[21]



Scheme 1. Principal biosynthetic pathway of geldanamycin (**1**) (ACP = acyl carrier protein of last PKS-module)[18] and structures of reblastatin (**2**) and 3-amino-5-hydroxybenzoic acid (AHBA, **3**).

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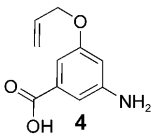
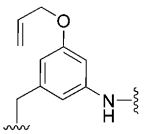
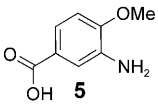
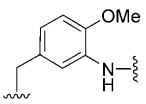
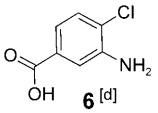
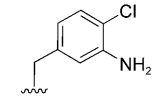
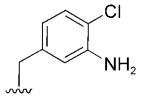
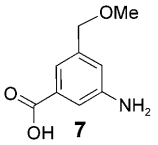
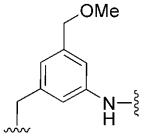
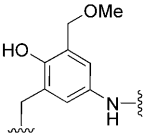
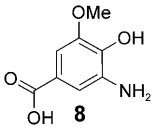
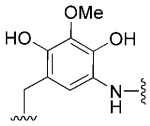
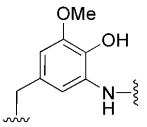
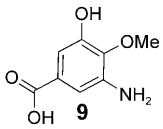
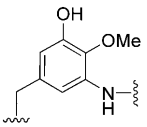
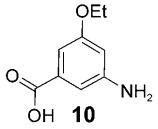
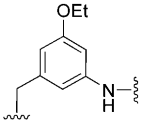
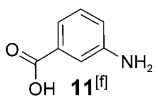
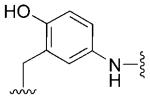
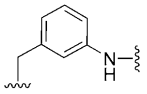
Table 1. Successful (not scaled-up) mutasyntheses with *S. hygroscopicus* K390-61-1 using 3-aminobenzoic acids **4**–**13**.

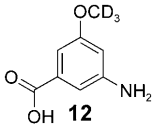
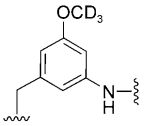
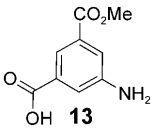
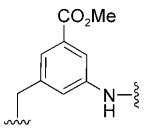
Mutasynthon[a]	t_R[b] [min]	Formula	Proposed structure[c]
 **4**	1.85	$C_{31}H_{47}N_2O_7$ $[M+H]^+$: calcd: 559.3383 found: 559.3397	
 **5**	1.66	$C_{29}H_{45}N_2O_7$ $[M+H]^+$: calcd: 533.3227 found: 533.3252	
 **6**[d]	n.d.[e]	$C_{27}H_{42}ClNNaO_6$ $[M+Na]^+$: calcd: 534.2598 found: 534.2579	 *seco*-acid and carbamoyl group at O-7
	n.d.	$C_{28}H_{43}ClN_2NaO_7$ $[M+Na]^+$: calcd: 577.2656 found: 577.2679	 *seco*-acid and carbamoyl groups at O-7 and O-11
 **7**	1.41	$C_{30}H_{47}N_2O_7$ $[M+H]^+$: calcd: 547.3383 found: 547.3364	
	1.68	$C_{30}H_{47}N_2O_8$ $[M+H]^+$: calcd: 563.3332 found: 563.3336	
 **8**	1.44	$C_{29}H_{44}N_2NaO_9$ $[M+Na]^+$: calcd: 587.2945 found: 587.2930	
	1.53	$C_{29}H_{45}N_2O_8$ $[M+H]^+$: calcd: 549.3176 found: 549.3193	
 **9**	1.36	$C_{29}H_{45}N_2O_8$ $[M+H]^+$: calcd: 549.3176 found: 549.3165	
 **10**	1.78	$C_{30}H_{46}N_2NaO_7$ $[M+Na]^+$: calcd: 569.3203 found: 569.3188	
 **11**[f]	1.38	$C_{28}H_{42}N_2NaO_7$ $[M+Na]^+$: calcd: 541.2890 found: 541.2894	
	1.69	$C_{28}H_{42}N_2NaO_6$ $[M+Na]^+$: calcd: 525.2941 found: 525.2932	

we now wish to report similar results on geldanamycin; these new results complement and extend data recently published on this topic by Lee, Hong and coworkers[9] as well as Menzella et al.[10,11]

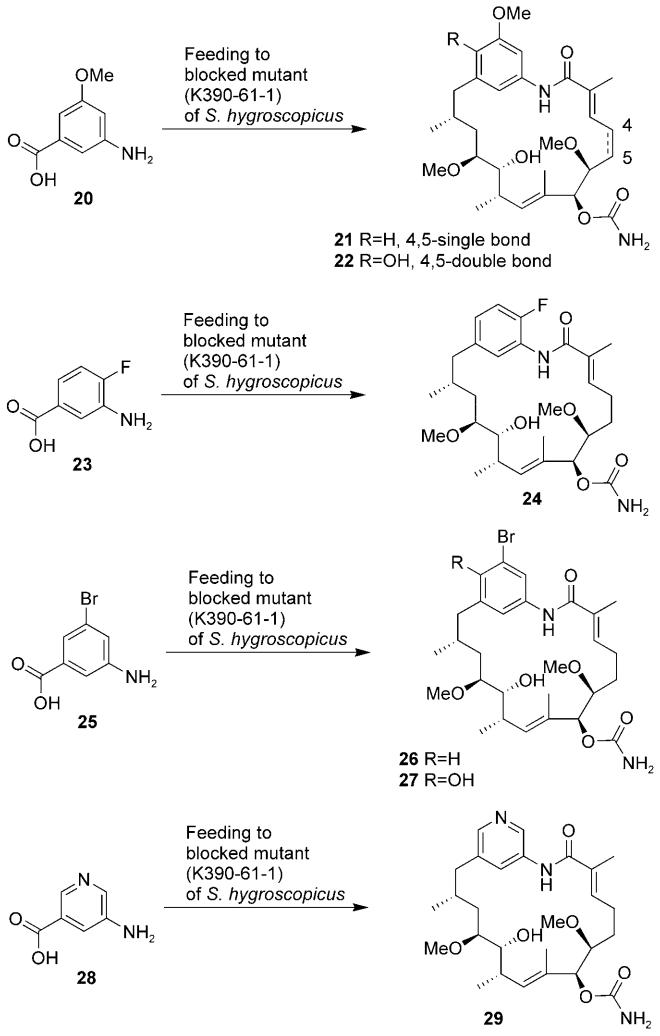
Here, we describe the generation and biological activity of new geldanamycin derivatives obtained by using mutational biosynthesis with an AHBA-blocked mutant of the geldanamycin producer, *Streptomyces hygroscopicus* K390-61-1.[18] Twenty different 3-aminobenzoic acids were chosen and individually added to cultures of strain K390-61-1. AHBA (**3**) and its derivatives are expected to be activated to the aryl adenylates, and then attacked by phosphopantetheine-thiol to form the PKS-bound thioesters.[18,21] Indeed, feeding the natural precursor **3** to cultures of strain K390-61-1 produced geldanamycin (**1**) in a yield of about 400 mg L^{-1}. Without supplementation with **3**, no geldanamycin was detected in the extracts.

Remarkably, the majority of the 3-aminobenzoic acids tested were converted into new geldanamycin derivatives. Table 1 and Scheme 2 show the successful complementation examples, while Figure 1 depicts compounds **14**–**19**, which were not transformed into new geldanamycin derivatives as judged by UPLC-MS (ultra performance LC coupled ESI-MS). After supplementation with aminobenzoic acids **6**–**13**, **20**, **23**, **25** and **28** and harvest after seven days, UPLC-MS analysis clearly revealed *m/z* peaks consistent with suggested geldanamycin analogues.[22] These were commonly not oxidized at

Table 1. (Continued)

Mutasynthon[a]	t_R[b] [min]	Formula	Proposed structure[c]
 **12**	1.69	$C_{29}H_{42}D_3N_2O_7$ $[M+H]^+$: calcd: 536.3412 found: 536.3417	
 **13**	1.70	$C_{30}H_{44}N_2NaO_8$ $[M+Na]^+$: calcd: 583.2995 found: 583.2999	

[a] The preparation of mutasynthons are described in the Supporting Information. [b] Analysis by UPLC-HRMS. [c] If not otherwise noted, the 4,5-hydro derivatives are proposed. [d] Mutaproducts obtained from **6** have been described in ref. [9]. [e] n.d. = not determined, mass determined by ESI-mass spectrometry without LC column. [f] Mutaproducts obtained from **11** have been described in ref. [11].



Scheme 2. Successful (scaled-up) mutasyntheses with *S. hygroscopicus* K390-61-1.

C4–C5, and thus lacked the additional olefinic double bond. Oxidation of the aromatic moiety was fully or partially suppressed. Only for mutasynthons **7**, **8**, and **11** (Table 1) as well as **20** and **25** (Scheme 2) were the 17-hydroxylated products detected. It was our goal to generate sufficient amounts of new geldanamycin analogues for isolation and biological evaluation in order to demonstrate the viability of mutational biosynthesis as a powerful synthetic tool for natural product chemists. Mutasynthons **20**, **23**, **25** and the aminonicotinic acid **28** (Scheme 2) turned out to be the most promising candidates with respect to yields. Fermentations were repeated with these aminobenzoic acid derivatives on a

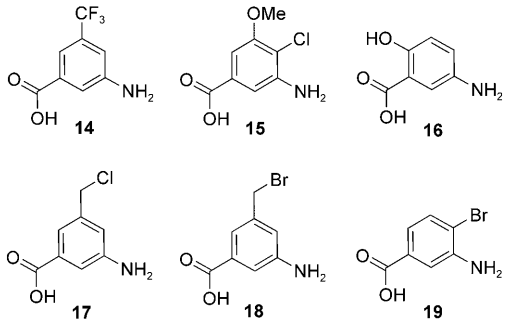


Figure 1. Aminobenzoic acids **14–19**, which were not accepted as mutasynthons.

larger scale to obtain sufficient amounts of the new geldanamycin derivatives for NMR-analysis and bioassay. After harvesting, the fermentation broths were extracted with ethyl acetate. The extracts were subjected to three chromatographic purification steps (silica gel chromatography, Sephadex size exclusion chromatography and RP-HPLC). Fermentation yields of the new geldanamycin derivatives were significantly lower than for the natural product **1**.[23] Methoxy-derivative **20** and 3-amino-4-fluorobenzoic acid **23** were processed in moderate yields (isolation of **21**, **22**: 7 mg $L^{-1}$and 2.4 mg L^{-1}, respectively; **24**: 8 mg L^{-1}). Lee, Hong and co-workers reported that their AHBA-blocked mutant *S. hygroscopicus* AC2 with unnatural starter units produces 4,5-dihydrogeldanamycin derivatives.[9] Commonly, we also encountered inhibition of the last dehydrogenation step (see Table 1). However, in the case of mutasynthons **20** and **21** (17-demethoxy-18-*O*-methylreblastatin) the 4,5-desaturated product (**22**) was also isolated.[24] 3-Amino-5-bromobenzoic acid (**25**) also yielded two major new fermentation products, namely 18-bromo-17-demethoxy reblastatin (**26**) and 18-bromo-17-demethyl reblastatin (**27**); however, isolated yields were rather low for both mutaproducts (0.6 mg L^{-1}). Remarkably, heteroarene **28** was also accepted by the mutant *S. hygroscopicus* K390-61-1 and yielded 18-aza-reblastatin (**29**, 1.4 mg L^{-1}), the first among all ansamycin antibiotics described in which the polyketide chain spans a heteroaromatic moiety.[25]

The position of the 17-hydroxyl group in reblastatin derivatives **22** and **27** was determined by heteronuclear multiple bond correlation (HMBC) cross peaks for NH/C19, C20, and C21. NMR analysis of the geldanamycin derivative **21** proved to be difficult due to substantial signal broadening. The main reason for this observation is a *cis*- and *trans*-amide isomerisation around the C1–N bond, which equilibrates slowly at room temperature relative to the NMR time-scale. After thorough solvent screening (CD_3OD, $CDCl_3$, CD_2Cl_2) and adjustment of the recording temperature (295, 300, 310, 320 K) we were able to collect sufficiently resolved spectra (see Supporting Information). For the evaluation of their biological profiles as anticancer agents, the new derivatives were administered to cultured human tumour cell lines; except for the pyridine derivative **29**, all showed strong antiproliferative activity, and most of them had IC_{50} values in the nM range (Table 2). The most active compounds, **24** and **27,** compare favourably with geldanamycin (**1**). The MCF-7 breast cancer cells were generally the most sensitive, but there were marked differences between the compounds tested in their activity profiles against different cell lines. This indicates that it is possible to generate derivatives with a certain degree of cell specificity.

Table 2. Antiproliferative activity IC_{50} [nM] of **21**, **22**, **24**, **26**, **27** and **29**. (Values shown are means of two determinations in parallel.)

Cell line Origin	**21**	**22**	**24**	**26**	**27**	**29**	**2**
KB-3-1 cervix carcinoma	109	470	73	123	34	>8000	53
U-937 lymphoma	188	93	62	142	21	2600	9
PC-3 prostate carcinoma	n.d.	300	42	118	36	2800	18
SK-OV-3 ovarian carcinoma	113	1060	54	264	46	4000	125
MCF-7 breast carcinoma	38	320	18	123	120	870	n.d.
A-431 epidermoid carcinoma	60	840	62	228	17	1900	18

n. d. = not determined.

In their detailed biological evaluation of nonbenzoquinone ansamycins, Menzella et al. showed that unlike quinone-based geldanamycin derivatives, they exert activity that is independent of reductive activation by NAD(P)H/quinone oxidoreductase 1 (NQO1).[11] Along this line, our nonbenzoquinone ansamycins maintained strong antiproliferative activity. In the present case, the new nonbenzoquinone geldanamycin derivatives **21**, **24** and **27** show in vitro potencies comparable to those of the lead Hsp90 inhibitors tanespimycin (IC_{50} SK-OV-3: 240 nM; MCF-7: 58 nM) and alvespimycin (IC_{50} SK-OV-3: 122 nM; MCF-7: 71 nM), which are under clinical evaluation.[11]

In conclusion, we have prepared new geldanamycin/reblastatin derivatives by exploiting the concept of mutational biosynthesis. Six new compounds were isolated in amounts sufficient for full structural characterization and for preliminary biological testing. Except for the first aza-analogue of geldanamycin (**29**) all new mutaproducts showed a pronounced to strong inhibitory effect on cell growth. Thus, this mutasynthetic strategy has great potential for accessing compound libraries of highly potent and complex natural products like geldanamycin. The bromo-derivatives **26** and **27** are ideal precursors for further synthetic Pd-catalyzed transformations; thus, these derivatives expand the opportunities to access new geldanamycin analogues. The combination of mutasynthesis with semisynthesis has already successfully been achieved for ansamitocin derivatives.[21a]

Acknowledgements

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Keywords: antitumor agents · geldanamycin · Hsp90 · mutasynthesis · polyketides · reblastatin

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[24] As most of the isolated derivatives rather resemble reblastatin and not geldanamycin (because of the absence of the C21 hydroxyl group and the quinone moiety) we choose to name these metabolites reblastatins.
[25] It is worth noting that reblastatin (**2**) does not contain the quinone moiety found in geldanamycin (**1**) and thus lacks the 4,5-unsaturation.
[26] Interestingly, Menzella et al. also fed amino acid **28** to the Δ-AHBA strain K554–161 but could not detect formation of **29** (see the Supporting Information in ref. [11]).

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