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# Engineered Biosynthesis of Geldanamycin Analogs for Hsp90 Inhibition

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## Summary

**Geldanamycin, a polyketide natural product, is of significant interest for development of new anticancer drugs that target the protein chaperone Hsp90. While the chemically reactive groups of geldanamycin have been exploited to make a number of synthetic analogs, including 17-allylamino-17-demethoxy geldanamycin (17-AAG), currently in clinical evaluation, the "inert" groups of the molecule remain unexplored for structure-activity relationships. We have used genetic engineering of the geldanamycin polyketide synthase (GdmPKS) gene cluster in *Streptomyces hygroscopicus* to modify geldanamycin at such positions. Substitutions of acyltransferase domains were made in six of the seven GdmPKS modules. Four of these led to production of 2-desmethyl, 6-desmethoxy, 8-desmethyl, and 14-desmethyl derivatives, including one analog with a four-fold enhanced affinity for Hsp90. The genetic tools developed for geldanamycin gene manipulation will be useful for engineering additional analogs that aid the development of this chemotherapeutic agent.**

## Introduction

Geldanamycin [1–4] (Figure 1) and several other benzoquinone microbial products classified as ansamycin antibiotics [5], herbimycin A [6, 7], macbecins [8], ansatrienins [9], and reblastatin [10, 11] were discovered between 1970 and 2000 in screens for antibacterial, antifungal, and antiviral compounds. Interest in geldanamycin and herbimycin A, however, increased greatly upon the discovery of their antitumor properties [12, 13].

Geldanamycin was believed initially to interfere with signal transduction pathways in tumor cells by direct inhibition of oncogenic protein tyrosine kinases. However, Neckers and coworkers [14] showed in 1994 that the principal cellular target is not a tyrosine kinase but Hsp90, a ubiquitous and abundant protein chaperone in mammalian cells [15]. Geldanamycin competes with ATP for the ATP binding site of Hsp90, inhibiting its ATP-dependent functions. One such function of Hsp90 is to chaperone nascent protein kinases that are critical components of signal transduction pathways, many of which are used by specific cancer cells [15]. In the presence of geldanamycin, the immature kinases undergo rapid degradation as a consequence of ubiquitination and subsequent catabolism by the proteosome. The depletion of mature kinases results in a cytostatic effect or, in some cases, apoptosis and cell death. Thus, as a potential new target for cancer therapy, the discovery that Hsp90 and one or more of its protein kinase cohorts are overproduced in several types of human cancers has led to further interest in geldanamycin and its analogs [16, 17].

Many geldanamycin analogs have been produced by replacement of the C-17 *O*-methoxy group with substituted amines [18, 19]. One such drug, 17-allylamino-17-demethoxygeldanamycin (17-AAG), is currently undergoing phase I and II clinical trials [20]. Experience with the behavior of geldanamycin and 17-AAG in animals and humans has pointed to the need for more water soluble and less hepatotoxic forms of this drug [21, 22]. Hence, we decided to explore ways to use the geldanamycin biosynthesis genes [23] for production of new geldanamycin analogs that could serve as the starting point for the chemical synthesis of improved anticancer drugs.

Geldanamycin is made in *Streptomyces hygroscopicus* by genes encoding a modular polyketide synthase (PKS) and several tailoring enzymes. The pathway begins with the formation of 3-amino-5-hydroxybenzoic acid (AHBA) from glucose via kanosamine (cf. [24]). This aromatic amino acid serves as the starter unit for polyketide biosynthesis by the geldanamycin polyketide synthase (GdmPKS), encoded by the *gdmA1-A3* PKS genes (Figure 2). Following elongation with the acyl-Coenzyme A substrates malonyl-CoA, methylmalonyl-CoA, and 2-methoxymalonyl-ACP [25–27], the presumed polyketide intermediate undergoes intramolecular lactamization by GdmF to form progeldanamycin [23]. This is then converted to geldanamycin by the action of at least five tailoring enzymes (Figure 2) whose gene-enzyme relationships have not been fully elucidated. GdmN encodes the C-7 carbamoyltransferase [23] and GdmM is likely to be involved in C-21 oxidation (unpublished observation), but the remaining oxidation and *O*-methylation steps have not been correlated with specific genes in the cluster.

Here, we report the production of several geldanamycin analogs by engineering of the GdmPKS. These compounds were made by substituting the substrate extender unit-encoding acyltransferase domains for those with different specificities. This approach enables alteration of carbon substituents around the macrolactam, which could not be changed easily by chemical modification. In the course of this work, an analog with increased binding affinity for Hsp90 was identified.

## Results

### Design of AT Substitutions

The GdmPKS consists of seven polyketide chain extension modules preceded by an AHBA loading domain (Figure 2). Each module contains an AT domain that loads a malonyl (module 6), methylmalonyl (modules 1,

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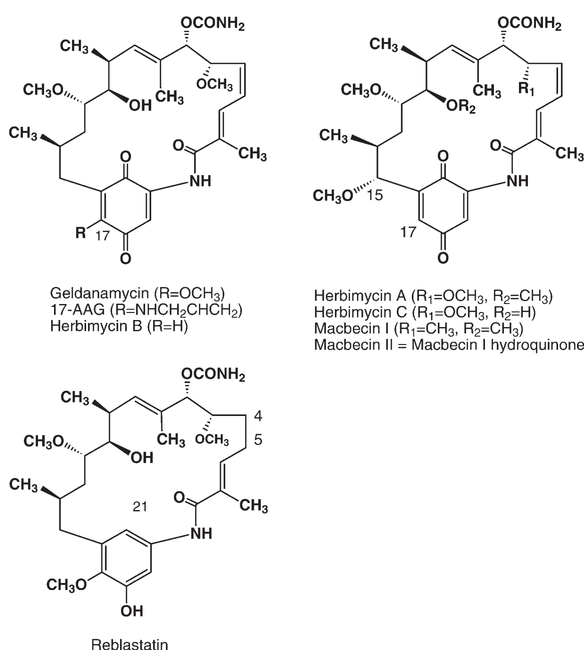


Figure 1. Geldanamycin, 17-AAG, and Related Ansamycin Polyketides

3, 4, and 7), or methoxymalonyl (modules 2 and 5) extender unit. Based on the precedent for substituting methylmalonate and/or methoxylmalonate with malonate extender units in the 6-deoxyerythronolide B synthase (DEBS) [28–32] and the FK520 PKS [33, 34], it was reasoned that substitutions in which either methylmalonyl or methoxylmalonyl ATs (i.e., modules 1–5 and 7) were replaced with a malonyl AT would have a high probability of generating productive PKS mutants. The rapAT2 domain, used extensively in AT domain swap experiments, and/or the rapAT14 domain, both from the rapamycin PKS [35], were therefore used to replace the AT domains in each of those modules. The boundaries between the AT and surrounding domains (Figure 3 and Table 1) used to create the hybrid modules were based on either those used in DEBS experiments [31] (modules 1, 4, 5, and 7), FK520 PKS experiments [34] (modules 2 and 3), or a new set of junctions determined through optimization experiments (to be reported elsewhere) (module 4). These replacements were expected to produce 2-desmethyl, 6-desmethoxy, 8-desmethyl, 10-desmethyl, 12-desmethoxy, and 14-desmethyl geldanamycin derivatives.

Three different methods of gene manipulation were developed to make the AT replacements in the geldanamycin-producing strain, *Streptomyces hygroscopicus* NRRL3602. For two methods, a set of donor cassettes was created containing the rapAT2 or rapAT14 cassette flanked on either side by ~1.0–1.3 kb of GdmPKS DNA. These donor cassettes were then moved into either the temperature-sensitive plasmid pKC1139 [36] or phage KOS305-114, an apramycin-resistant derivative of the phage KC515 [37] (described in Experimental Procedures). The donor vectors were introduced by conjugation from *E. coli* (pKC1139) or by transfection (KOS305-114), and replacements occurred by stepwise double crossing over as described in Experimental Procedures. It was found that the pKC1139 plasmid as well as the phage were highly unstable in this strain when integrated in the chromosome, and, once a bona fide single crossover was isolated, the second crossover occurred with high frequency (~50%) under nonselective conditions. AT swaps in modules 1, 2, 3, 5, and 7 were constructed in this manner (Table 2). The third approach employed a gene complementation host/vector system in which Red/ET *E. coli* recombination [38, 39] was used to construct the AT swaps in an expression plasmid (pSET152) containing the *gdmA2* and *gdmA3* genes and integrated into a Δ*gdmA2A3* deletion strain (see Experimental Procedures). AT replacements in modules 4 and 5 were constructed in this manner (Table 2).

**Production of Geldanamycin Analogs by AT Swaps**

Fermentation broth from each strain grown in production media was analyzed initially by LC-MS. Strains in which new compounds were detected were scaled up, and the abundant compound(s) were purified and characterized by MS and NMR spectroscopies. Functional AT substitutions were made successfully in four of the six modules: modules 1, 4, 5, and 7 (Table 2). The AT substitutions in modules 1, 4, and 5 with rapAT2 all led to the expected analogs, 14-desmethyl-geldanamycin (KOSN1877), 8-desmethyl-geldanamycin (KOSN1859), and 6-desmethoxy-geldanamycin (KOSN1631) (Figure 4). All three of these compounds were modified completely by the post-PKS tailoring reactions and were the major compounds present. A 4,5-dihydro derivative of 6-desmethoxy-geldanamycin (KOSN1630) was also isolated. Oxidation of the C4-C5 positions is believed to be the last step of the pathway (see Discussion). The yield of analog in each case was lower (>10-fold) than the amount of geldanamycin typically produced by the parent wild-type strain. Swaps using rapAT14 were also successful in modules 1 and 5 (Table 2) with no significant differences in production levels compared to the swaps using rapAT2.

The substitution in module 7 produced three 2-desmethyl compounds, KOSN1559, KOSN1558, and KOSN1619 (Table 2; Figure 4). The combined yield of all three compounds was substantially greater than the analogs described above and reached approximately half of the yield of geldanamycin normally observed. KOSN1559 and KOSN1558 were the two most abundant products, present in roughly equal amounts. Both lack the post-PKS oxidation reactions at C-17 and C-21 and the double bond at C4-C5. KOSN1558 appears to result from a polyketide pathway in which the dehydration of C2-C3, catalyzed by the dehydratase (DH) domain of module 7, does not occur. Finally, KOSN1619 is an analog of KOSN1558 that lacks a carbamoyl substituent. Reasons for the incomplete processing of these analogs are discussed below.

The AT substitutions that were attempted in modules 2 and 3 did not produce polyketide at detectable levels (Table 2).

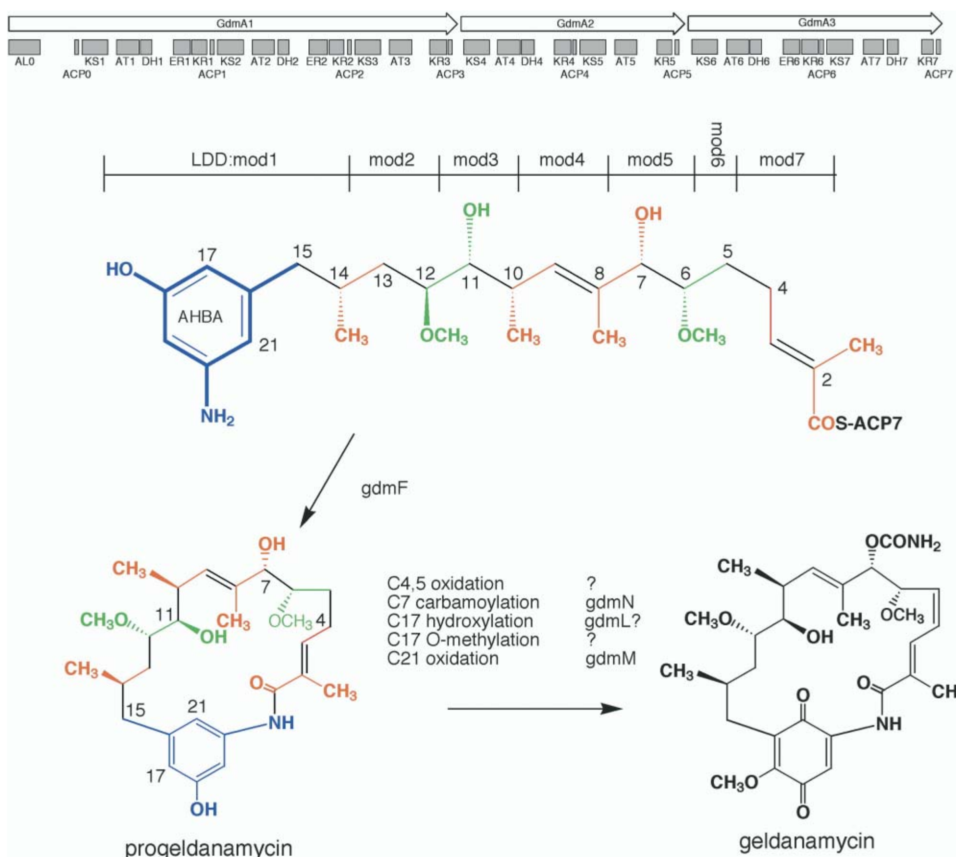


Figure 2. Genetics and Chemistry of Geldanamycin Biosynthesis

Progeldanamycin is the presumed polyketide product of the GdmPKS (encoded by *gdm*A1, A2, and A3) and GdmF (the lactam-forming amide synthase). The GdmPKS consists of an AHBA loading domain and seven extension modules. Each module catalyzes one round of chain elongation using malonyl-CoA (module 6), methylmalonyl-CoA (modules 1, 3, 5, and 7), or methoxymalonyl-ACP (modules 2 and 6). Each module also modifies the β-carbonyl from each elongation to a hydroxyl (modules 3 and 5), alkene (modules 4 and 7), or methylene (modules 1, 2, and 6). Progeldanamycin is converted to geldanamycin by five steps, the precise order of which has not been determined. (KS-ketosynthase, AT-acyltransferase, DH-dehydratase, ER-enolreductase, KR-ketoreductase, ACP-acyl carrier protein).

## Hsp90 Binding and Cytotoxic Activity of Geldanamycin Analogs

Cytotoxicity of the analogs was assessed in SKBr3 cell growth inhibition assays as previously described [19]. All of the compounds tested had substantially greater $IC_{50}$ values compared to geldanamycin and 17-AAG (Table 3). The compounds also had similar or reduced binding affinity for Hsp90 (Table 3). The exception was KOSN1559, which binds to Hsp90 with ~four-fold greater affinity than geldanamycin and ~eight-fold greater affinity than 17-AAG.

## Discussion

One of the major challenges faced with PKS engineering is the difficulty of performing genetic manipulations in the actinomycete or other host organism harboring the PKS gene cluster. This problem can sometimes be overcome by heterologous expression of the gene cluster in a more suitable organism, such as *Streptomyces coelicolor* [40, 41]. However, very large gene clusters or those for metabolites containing unusual precursors

| | | |
|---|---|---|
| gdmAT1→rapAT2 | TGQTGTDGGS/VFVFPGQ... | ...AFQHQRYW/LQRNTSAGD |
| gdmAT1→rapAT14 | TGQTGTDGGS/AFLFDGQ... | ...PFQHQSYW/LQRNTSAGD |
| gdmAT2→rapAT2 | RPWPETSHPR/RAGVSSF... | ...AFQHQRYW/LEATGHHGD |
| gdmAT2→rapAT14 | RPWPETSHPR/RAGVSSF... | ...PFQHQSYW/LEATGHHGD |
| gdmAT3→rapAT14 | RPWPETGHPR/RAGVSSF... | ...PFQHQSYW/LEPSEVPEA |
| gdmAT4→rapAT2 | TGQTGTDGGS/VFVFPGQ... | ...AFQHQRYW/LPRNTNAGD |
| gdmAT5→rapAT2 | TGETGSDGGS/VFVFPGQ... | ...AFQHQRYW/LQPARTPTR |
| gdmAT5→rapAT14 | TGETGSDGGS/AFLFDGQ... | ...PFQHQSYW/LQPARTPTR |
| gdmAT7→rapAT2 | TGHATTHGGS/VFVFPGQ... | ...AFQHQRYW/LQRGGPGDV |

Figure 3. Junctions Used for AT Domain Substitutions

Green sequences correspond to geldanamycin flanking regions, blue to rapamycin ATs, and red to amino acids introduced by restriction site engineering.

Table 1. Oligonucleotides Used in Plasmid Construction

| | |
|---|---|
| **AT1** | |
| Left flank | for 5′-TTGAATTCAGATCTGGTTCGCTGCACGGCACCGACGTC |
| | rev 5′-TTTCTAGAGGATCCGCCGTCTGTTCCGGTCTGTCCGGTG |
| Right flank | for 5′-TTTCTAGACTGCAGCGCAACACCAGCGCAGGCGATGTG |
| | rev 5′-TTAAGCTTATGCATCTCGGCGGTGTCGTGCACGGACAC |
| **AT2** | |
| Left flank | for 5′-TTGAATTCAGATCTGAGGACGCGGCCGGTTTCGACGCG |
| | rev 5′-TTTCTAGACCTAGGGTGGCTCGTCTCCGGCCAGGGCCG |
| Right flank | for 5′-TTTCTAGACTCGAGGCCACCGGCCACCACGGGGATGTC |
| | rev 5′-TTAAGCTTATGCATCGCGGCGAACGCCCGGGTGTCGGC |
| **AT3** | |
| Left flank | for 5′-TTGAATTCAGATCTGCCGGAGACACGGCCGCCCCTGCC |
| | rev 5′-TTTCTAGACCTAGGGTGTCCCGTCTCCGGCCAGGGCCG |
| Right flank | for 5′-TTTCTAGACTCGAGCCGTCCGAGGTACCGGAGGCGGTG |
| | rev 5′-TTAAGCTTATGCATCAGCCCGTCGAGGTAGGCGTTGCC |
| **AT5** | |
| Left flank | for 5′-TTGAATTCAGATCTGTGTTCGCCGGGGTCATCTACCAC |
| | rev 5′-TTTCTAGAGGATCCGCCGTCGCTGCCCGTCTCCCCGGTG |
| Right flank | for 5′-TTTCTAGACTGCAGCCCGCCAGGACACCGACGCGGGCC |
| | rev 5′-TTAAGCTTATGCATGGCGTTGCCCGCCGCGTACGGGGC |
| **AT7** | |
| Left flank | for 5′-TTGAATTCAGATCTACGTCACTGCGCGGACAGGAGGTC |
| | rev 5′-TTTCTAGAGGATCCGCCGTGGGTGGTGGCGTGGCCGGTG |
| | for 5′-TTTCTAGACTGCAGCGCGGCGGTCCGGGCGACGTCCGT |
| | rev 5′-TTAAGCTTATGCATCGGGTCGGTGACCTCGGCGGTGTC |

EcoRI, BglII, XbaI, BamHI, PstI, HindIII, and NsiI restriction sites are underlined

make this approach less practical in those cases. Here, three different methods of gene manipulation were developed for the geldanamycin-producing strain of *S. hygroscopicus*. The gene complementation method requires the greatest amount of development effort to construct vectors and host strains. However, it also provides the most flexibility and speed once established. Therefore, for constructing a large number of PKS gene manipulation experiments, this is the preferred method. In addition to the compounds reported here, the Red/ET cloning method has been used to produce geldanamycin analogs at the C6 ketone [42].

Substitution of AT domains has to date provided the greatest number of polyketide analogs produced by modular PKSs. Modification of polyketide structures through AT engineering allows derivatives to be produced at positions that would otherwise be difficult or impossible to change by chemical modification. Here, we have altered a methyl or methoxyl group at four positions of geldanamycin previously unexplored for structure-activity relationship, further demonstrating the robustness of this technique for making unique polyketide analogs. Those AT swaps that failed to produce the desired analog likely resulted from a poor choice in AT boundaries used to create the hybrid. Recent experiments have indicated that the location of AT boundaries can make the difference between a functional and nonfunctional hybrid [32, 43]. In fact, the AT4 substitution reported here is a result of optimizing the location of the boundaries from an initially failed swap (details of this work will be reported elsewhere). Therefore, it is expected that the replacements in modules 2 and 3 could be made functional by repositioning the boundaries.

In many examples of PKS engineering, the changes made to the polyketide affect the activity or specificity of post-PKS enzymes [43–46]. Three of the four changes reported here were modified completely by the geldanamycin post-PKS enzymes. However, the substitution of methyl with hydrogen at C-2 of KOSN1559 has a dramatic impact on the oxidative modifications of the phenol. Disruption of *gdmM*, encoding a monooxygenase, produced the 17-demethoxy-21-desoxy derivative of geldanamycin, KOSN1806, which in turn affects formation of the C4-C5 double bond to produce the 4,5-dihydro form (unpublished results). No evidence of quinone-containing compounds was found in fermentation extracts of the AT7 substitution. Therefore, it appears that compounds lacking the C-2 methyl are very poor substrates for GdmM. The structure of KOSN1559 also supports the conclusion drawn from the *gdmM* disruption that the post-PKS steps occur in the order of carbamate attachment, oxidation of the phenol to the C-17 substituted quinone (the order of these two steps is still ambiguous), and formation of the C4-C5 alkene.

Both KOSN1558 and KOSN1619 appear to result from bypassing of the DH domain in module 7 and transfer of the β-hydroxy intermediate to the downstream enzyme, GdmF. Again, disruption of activity apparently results from the absence of a methyl substituent, in this case at the α-carbon of the ACP7-bound polyketide intermediate. Bypassing of β-keto processing activities has been observed previously in engineered PKSs [31]. However, this is believed to be an unprecedented example in which bypassing of an active DH domain has been characterized. Unused DH domains have been found often in sequenced PKSs;

Table 2. Engineered Strains Containing Substitution of AT Domains in the Gdm PKS and Polyketides Produced

| Strain | AT Substitution | Method of Construction[a] | Analogs Produced | Relative Yield[b] |
|---|---|---|---|---|
| K309-9 | AT1 → rapAT2 | 1 | KOSN1877 | <0.05 |
| K309-10 | AT1 → rapAT14 | 1 | KOSN1877 | <0.05 |
| K390-31A | AT2 → rapAT2 | 2 | — | |
| K390-31B | AT2 → rapAT14 | 2 | — | |
| K309-3 | AT3 → rapAT14 | 1 | — | |
| K279-248/pKOS367-79 | AT4 → rapAT2 | 3 | KOSN1859 | <0.05 |
| K309-2 | AT5 → rapAT2 | 1 | KOSN1630<br>KOSN1631 | 0.2 |
| K279-248/pKOS331-178 | AT5 → rapAT14 | 3 | KOSN1630<br>KOSN1631 | 0.2 |
| K309-1 | AT7 → rapAT2 | 1 | KOSN1559<br>KOSN1558<br>KOSN1619 | 0.5 |

[a] 1, KC1139 double crossover; 2, phage KOS305-114 double crossover; 3, gene complementation.
[b] Approximate combined yield relative to geldanamycin production under similar conditions.

however, it is not obvious in many of those cases whether the DH domain has been inactivated through mutation or is simply bypassed. Based on the above findings, it is possible that for many naturally occurring DH domains that are apparently not used, the domain remains active, but the rate of dehydration is slow relative to chain transfer to the next module. The relative stereochemistry of the C3 hydroxyl of KOSN1558 and KOSN1619 was not determined. However, based on signature sequences in the KR domain of module 7 that can correlated with product stereochemistry [47, 48], it is predicted that reduction of the β-keto intermediate would occur with D-configuration (B-type), leading to 3-*R* stereocenters for KOSN1558 and KOSN1619.

All of the analogs engineered in this study possess inferior cytotoxic activity toward SKBr3 cells compared to geldanamycin and 17-AAG. However, KOSN1559 binds to Hsp90 with at least four-fold greater affinity. It has become apparent in other studies [19], as well as with KOSN1559, that relative Hsp90 binding is not a predictive indicator of cytotoxicity. Preliminary experiments suggest that the discrepancy between the tight binding and low cytotoxicity of KOSN1559 may result from poor intracellular accumulation of KOSN1559 in SKBr3 cells (Z. Zhong, personal communication). KOSN1559 is an attractive analog because it lacks the quinone moiety believed to be a significant factor in hepatotoxicity encountered with geldanamycin [49].

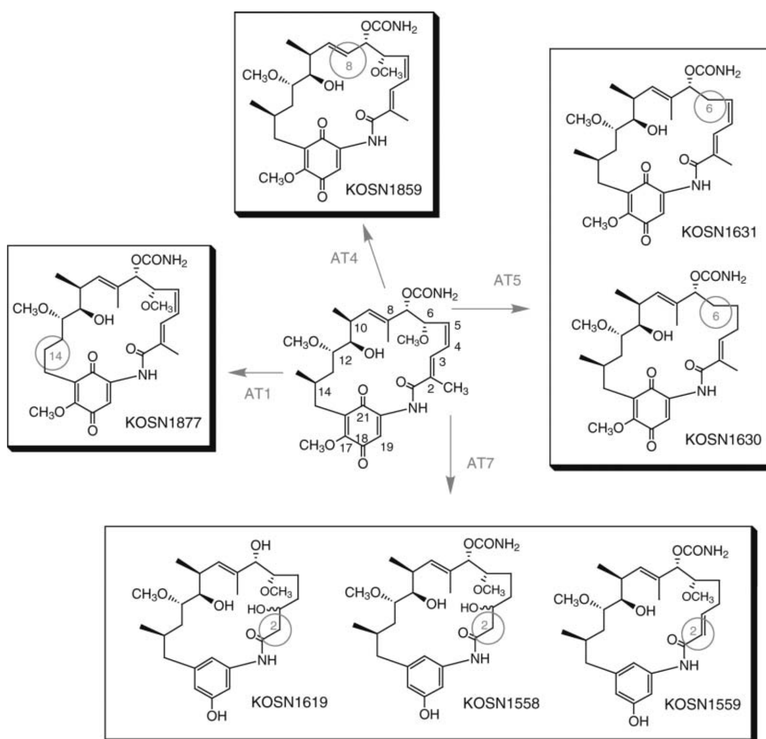


Figure 4. Geldanamycin Analogs Produced by AT Substitutions in the GdmPKS

Table 3. Cytotoxicity and Hsp90 Binding Affinity of Geldanamycin and Analogs

| Compound | SKBr3 $IC_{50}$ (nM) | Hsp90 $K_d$ (nM) |
|---|---|---|
| Geldanamycin | 41 | 670 |
| 17-AAG | 33 | 1300 |
| KOSN1631 (6-desmethoxy) | 3200 | 5000 |
| KOSN-1630 | 4900 | 3000 |
| KOSN1859 (8-desmethyl) | 480 | 5200 |
| KOSN1877 (14-desmethyl) | 470 | 660 |
| KOSN1558 | >5000 | 1000 |
| KOSN1559 | 860 | 16 |

Therefore, it may be possible to create more potent and safer analogs of geldanamycin through chemical modifications that improve cellular uptake while maintaining enhanced Hsp90 binding affinity of KOSN1559.

## Significance

**17-AAG, an analog of geldanamycin, is a promising anticancer agent that acts through a novel target, Hsp90. In order to access new structures of geldanamycin that might improve the activity or pharmacological profile of 17-AAG, three different methods to manipulate the polyketide biosynthetic synthase genes of geldanamycin were developed. These methods were used successfully to exchange acyltransferase domains of the geldanamycin PKS, leading to production of geldanamycin analogs at positions that would be very difficult to make by conventional chemical modification. One of these methods, which uses a modified cloning procedure based Red/ET recombination in *E. coli* to introduce genetic modifications, along with similar approaches developed recently by others [50, 51], offers a potentially more efficient way to engineer PKS gene clusters. Finally, a 2-demethyl-4,5-dihydro-17-demethoxy-21-desoxy derivative of geldanamycin was generated that binds to Hsp90 with eight-fold greater binding affinity than 17-AAG.**

## Experimental Procedures

### Bacterial Strains and Culture Conditions

*S. hygroscopicus* NRRL3602 was the strain used for chromosomal substitution of AT domains. Strain K279-248 (*gdmA2,gdmA3*::*neo*), used for gene complementation experiments, will be described elsewhere. Plasmids were introduced into *S. hygroscopicus* by conjugation using *E. coli* ET12657/pUZ8002 as donor on R5 agar plates containing 100 mg/l nalidixic acid. After incubating at 30°C for ~16 hr, exconjugants were selected with 3 ml soft nutrient agar containing 1.5 mg apramycin. Liquid cultures were grown in R5 media [37] for 24–48 hr. Cells were grown on tomato agar plates (per liter, 160 g tomato paste [Hunts], 80 g soy flour, 16 g calcium carbonate, 8 g peptone, 160 g agar; post-sterilization: 80 g glucose, 160 g sucrose, 8 g $KH_2PO_4$) for 7–10 days for sporulation. *S. hygroscopicus* cultures and plates were incubated at 30°C or 37°C to control the replication/propagation of temperature-sensitive pKC1139 plasmids. *S. hygroscopicus* cultures grew more slowly and eventually lysed after ~36 hr at 37°C. Production of geldanamycin and desired analogs was determined in geldanamycin-production medium (GPM) [2]. Seed cultures were grown in liquid R5 medium for 2 days at 30°C, and 2 ml was used to inoculate 40 ml of GPM in 250 ml shake flasks at 30°C for 4 days.

### Replacement of AT Domains in Modules 1, 2, 3, 5, and 7 of GdmPKS

DNA fragments, ~1.0–1.3 kb each, flanking the AT domains were PCR amplified from cosmid pKOS256-107-3 with the oligonucleotides in Table 1. The PCR fragments for each targeted AT were digested and cloned together using EcoRI/XbaI (left flank) and XbaI/HindIII (right flank) sites into pUC19. The rapAT2 or rapAT14 cassettes were then inserted between the two flanking sequences of each plasmid with BamHI and PstI restriction sites (AT1, AT5, and AT7) or AvrII and XhoI sites (AT2 and AT3). The ATs and flanking fragments were moved into the delivery vectors pKC1139 [36] with EcoRI and HindIII restriction sites or into phage KOS305-114 using BglII/NsiI. This phage was constructed by replacing the *tsr* cassette between the BamHI and BglII sites of KC515 [37] with the *aac(3)IV* apramycin-resistance gene excised from plasmid pHP45 [37] with BamHI. The corresponding AT swap cassettes were cloned into circularized and digested phage DNA, and phage propagation was performed as described by Kieser et al. [37]. The recombinant phage constructs were confirmed by restriction analysis and introduced into *S. hygroscopicus* NRRL3602 by transfection [37]. Lysogens were selected on solid R5 using 2 ml of 1 mg/ml apramycin overlay.

pKC1139 plasmids were introduced into *S. hygroscopicus* NRRL3602 by conjugation using *E. coli* ET12657/pUZ8002 as donor [37]. Primary exconjugants were first grown in 5 ml liquid R5 containing 100 mg/l apramycin at 30°C for 2 days. To generate the first crossover, 0.2 ml of these cells was used to inoculate 5 ml R5 with apramycin and grown at 37°C for 36 hr. This step was repeated once, and cells were plated on R5 agar with apramycin or tomato agar with apramycin at 37°C. Single colonies from these plates were grown, and their DNA was analyzed by Southern blot for integration of the delivery plasmid by homologous recombination. Confirmed single crossovers were propagated in R5 without antibiotic selection at 37°C for ~32 hr, plated on tomato agar plates at 30°C, and allowed to sporulate (~10–14 days). Spores were harvested, plated on R5, and single colonies were screened for sensitivity to apramycin. To identify the products of second crossovers, sensitive colonies were screened for production of new geldanamycin compounds. Those strains producing new metabolites were further analyzed by PCR and/or Southern blot to verify the expected replacement of the targeted AT domain in the *gdm* gene cluster.

### Production of Geldanamycin and Analogs by Gene Complementation in *S. hygroscopicus* NRRL3602

Plasmid pKOS279-69 (to be described elsewhere) contains the *gdmA2* and *gdmA3* genes under control of the ermE* promoter in pSET152 [36]. Plasmids pKOS331-178 and pKOS367-79 are derivatives of pKOS279-69 containing the AT5→rapAT14 and AT4→rapAT2 substitutions, respectively. Both plasmids were constructed using a modified two-step Red/ET cloning procedure [38, 39]. The first step introduces a selectable marker with unique restriction sites in pKOS279-69 that are used subsequently to linearize the plasmid for the second recombination step. For the second step, the linearized recipient plasmid and a linear donor AT cassette are used for transformation, thereby selecting for circularized plasmids formed by recombination between the two linear fragments.

Plasmid pKOS331-178 was constructed as follows. Plasmid pKOS309-6a contains the left and right 1.3 kb fragments flanking the AT5 domain, which are described in the preceding section, joined together at their XbaI sites and inserted between the HindIII and EcoRI sites of pUC19. A 1.5 kb SpeI/XbaI fragment containing the neomycin (*neo*) resistance marker of Tn5 was inserted into the XbaI site of pKOS309-6a between the AT5 flanking fragments to make pKOS331-74A. A 3.1 kb linear DNA fragment was excised from pKOS331-74a with SacI and HincII. This fragment contains 0.5 kb of the AT5 left flank, the 1.5 kb *neo* cassette, and 1.1 kb of the AT5 right flank. Approximately 0.1 μg of the purified linear DNA fragment was cointroduced with pKOS279-69 by electroporation into *E. coli* HS996/pSC101/BAD/γβαA (Gene Bridges), and colonies were selected with apramycin and neomycin. Colonies were checked by restriction enzyme analysis; approximately 60% were found to contain the *neo* marker recombined at the appropriate

location of pKOS279-69. One clone was designated pKOS331-124 and contains the *gdmA2* and *gdmA3* (AT5::*neo*) genes in pSET152. The unique NsiI and AvrII restriction sites in the *neo* cassette were used to linearize pKOS331-124 plasmid for the second cotransformation/recombination step. The delivery vector for this step, pKOS305-124A, was constructed by inserting the rapAT14 cassette into the BamHI and PstI restriction sites of pKOS309-6a. A 3.6 kb linear DNA fragment containing the 1.3 kb AT5 left flank, the rapAT14 cassette, and the 1.3 kb AT5 right flank was excised from pKOS305-124A with HindIII and EcoRI. Approximately 0.5 μg of the purified linear DNA fragment was cointroduced with ∼0.3 μg of linearized pKOS305-124A by electroporation into *E. coli* HS996/pSC101/BAD/γβαA, and colonies were selected with apramycin. One of eight colonies screened by restriction analysis was found to contain the correct AT5→rapAT14 substitution and was designated pKOS331-178. Plasmid pKOS367-79 containing the AT5→rapAT2 substitution was constructed by an analogous procedure, and details will be reported elsewhere.

### Purification and Characterization of KOSN1559, KOSN1558, KOSN1619, KOSN1630, and KOSN1631

Strains K309-1 and K309-2 were grown in flasks containing 38 ml each GPM with 1.6 g XAD-16 (Rohm-Haas) beads inoculated with 2 ml seed culture each and grown at 30°C for 4 days. Contents of flasks were pooled, centrifuged, and the supernatant was decanted without losing any XAD. To separate cells from XAD beads, the mixture was resuspended in water and the XAD beads were allowed to settle. The supernatant with suspended cells was decanted. This procedure was repeated two to three times until most of the mycelium was gone. The XAD was then filtered using a separation funnel with Whatman filter paper and washed briefly with water. XAD was collected and extracted in 100% methanol (∼1/10 of total fermentation volume) with stirring for ∼30 min. The methanol was decanted, and the extraction was repeated twice. The combined aqueous methanolic extracts were concentrated on a rotary evaporator and freeze dried. The resultant brown residue was resuspended in 120 ml of methanol and filtered. The filtrate was evaporated to give a brown solid, which was redissolved in dichloromethane/methanol. Silica gel was added to the solution, and the mixture was evaporated to give a free-flowing powder. This powder was loaded on a silica gel column and eluted with 0%–20% methanol in dichloromethane. Major fractions were collected and further purified by HPLC using a C-18 column (MetaChem Polaris 5μ C18-A 150 × 21.2 mm ID) and eluted using a gradient of acetonitrile in water. Pooling of the column fractions was guided by LC/MS. Three new compounds were obtained as white solids after lyophilization of pooled fractions. Yields of purified compounds were 35 mg/l KOSN1559, 15 mg/l KOSN1558, 10 mg/l KOSN1619, 12 mg/l KOSN1630, and 10 mg/l KOSN1631.

***2-Desmethylprogeldanamycin-7-Carbamate (KOSN-1559)***

$^1$H NMR (DMSO-$d_6$, 400 MHz) δ (relative to DMSO-$d_5$ at 2.49 ppm) 0.76 (d, 3, *J* = 6.4 Hz), 0.89 (d, 3, *J* = 6.4 Hz), 0.99 (m, 1), 1.29 (s, 3), 1.24–1.36 (m, 2), 1.49 (m, 1), 1.77 (m, 1), 2.14–2.24 (m, 4), 2.75 (br d, 1, *J* = 10.0 Hz), 2.95 (br d, 1, *J* = 9.6 Hz), 3.19 (s, 3), 3.21 (m, 1), 3.27 (s, 3), 3.42 (m, 1), 4.47 (br s, 1), 4.82 (d, 1, *J* = 8.8 Hz), 5.19 (d, 1, *J* = 10.4 Hz), 5.90 (d, 1, *J* = 15.6 Hz), 6.13 (s, 1), 6.26 (s, 1), 6.37 (s, 1), 6.46 (br s, 2), 6.76 (dt, 1, *J* = 5.6, 15.6 Hz), 9.46 (s, 1), 9.48 (s, 1). $^{13}$C NMR (DMSO-$d_6$, 100 MHz) δ (relative to DMSO-$d_6$ at 39.5 ppm) 11.2, 17.7, 19.2, 26.1, 27.9, 31.6, 32.9, 34.7, 42.9, 56, 58.4, 72.4, 78.5, 81.2, 81.8, 108.3, 114.3, 118, 122.2, 129.4, 134.2, 138.6, 142.2, 143.7, 156, 157.7, 165.9. ESI TOF MS *m/z* 527.2712, calculated for $C_{27}H_{40}N_2O_7Na$ ([M + Na]$^+$), 527.2728.

***3-Hydroxy-2,3-Dihydro-2-Desmethylprogeldanamycin-7-Carbamate (KOSN-1558)***

$^1$H NMR (DMSO-$d_6$, 400 MHz) δ (relative to DMSO-$d_5$ at 2.49 ppm) 0.84 (br. m, 6), 1.19 (m, 1), 1.32 (m, 1), 1.38 (m, 1), 1.41 (s, 3), 1.49 (m, 2), 1.56 (m, 1), 1.65 (m, 1), 2.10 (m, 1), 2.28 (m, 1), 2.31 (m, 1), 2.43 (m, 1), 2.47 (m, 1), 3.07 (m, 1), 3.20 (br s, 6), 3.27 (m, 1), 3.38 (m, 1), 3.87 (m, 1), 3.98 (br s, 1), 4.81 (s, 1), 4.91 (s, 1), 5.22 (d, 1, *J* = 8.0 Hz), 6.23 (s, 1), 6.37 (br s, 2), 6.66 (s, 1), 6.87 (s, 1), 9.20 (s, 1), 9.68 (s, 1). $^{13}$C NMR (DMSO-$d_6$, 100 MHz) δ (relative to DMSO-$d_6$ at 39.5 ppm) 12.6, 16.3, 21, 25, 31.9, 32.7, 33.7, 34.4, 42.6, 45.5, 56.1, 57, 67.4, 73.3, 76.9, 79, 81.7, 104.8, 112, 112.7, 130.3, 130.5, 139.2, 142.87, 156.4, 157, 168.9. ESI TOF MS *m/z* 545.2831, calculated for $C_{27}H_{42}N_2O_8Na$ ([M + Na]$^+$) 545.2833.

***3-Hydroxy-2,3-Dihydro-2-Desmethylprogeldanamycin (KOSN-1619)***

$^1$H NMR (DMSO-$d_6$, 400 MHz) δ (relative to DMSO-$d_5$ at 2.49 ppm) 0.85 (d, 3, *J* = 6.0 Hz), 0.87 (d, 3, *J* = 6.0 Hz), 1.23 (m, 1), 1.27–1.35 (m, 2), 1.29 (s, 3), 1.39 (m, 1), 1.57–1.63 (m, 2), 1.68 (m, 1), 2.19 (m, 1), 2.28 (m, 1), 2.31 (m, 1), 2.41–2.49 (m, 2), 3.09 (m, 1), 3.17 (m, 1), 3.20 (s, 3), 3.22 (s, 3), 3.32 (m, 1), 3.69 (br s, 1), 3.84 (m, 1), 3.99 (br s, 1), 4.52 (d, 1, *J* = 6.0 Hz), 4.68 (br s, 1), 5.26 (d, 1, *J* = 8.8 Hz), 6.23 (s, 1), 6.71 (s, 1), 6.77 (s, 1), 9.25 (br s, 1), 9.61 (br s, 1). $^{13}$C NMR (DMSO-$d_6$, 100 MHz) δ (relative to DMSO-$d_6$ at 39.5 ppm) 12.3, 16.5, 20.7, 24.8, 31.6, 32.1, 33.7, 34.1, 42.8, 45.7, 56.2, 57.1, 67.7, 73.6, 75.3, 80.9, 81.4, 104.6, 111.6, 112.0, 128.8, 133.8, 139.4, 142.4, 157.1, 169.0. ESI TOF MS *m/z* 502.2783, calculated for $C_{26}H_{41}NO_7Na$ ([M + Na]$^+$) 502.2775.

***4,5-Dihydro-6-Desmethoxygeldanamycin (KOSN-1630)***

$^1$H NMR ($CDCl_3$, 400 MHz) δ (relative to $CHCl_3$ at 7.26 ppm) 0.90 (d, 3, *J* = 6.8 Hz), 0.95 (d, 3, *J* = 6.8 Hz), 1.63 (s, 3), 1.55–1.73 (m, 7), 1.85 (s, 3), 2.14 (m, 1), 2.37–2.43 (m, 2), 2.65–2.71 (m, 2), 3.32 (s, 3), 3.35 (m, 1), 3.54 (m, 1), 4.06 (s, 3), 4.92 (br s, 2), 5.12 (m, 1), 5.76 (d, 1, *J* = 5.6 Hz), 6.23 (t, 1, *J* = 6.4 Hz), 7.12 (s, 1), 8.66 (s, 1). $^{13}$C NMR ($CDCl_3$, 100 MHz) δ (relative to $CDCl_3$ at 77.0 ppm) 11.0, 12.3, 13.0, 22.3, 24.6, 26.5, 28.4, 32.3, 32.4, 33.2, 35.0, 56.7, 61.5, 73.3, 80.5, 81.2, 111.0, 127.4, 132.4, 132.5, 132.8, 135.6, 138.1, 156.7, 156.9, 168.3, 184.1, 184.6. ESI TOF MS *m/z* 550.3102, calculated for $C_{28}H_{44}N_3O_8$ ([M + $NH_4$]$^+$) 550.3123.

***6-Desmethoxygeldanamycin (KOSN-1631)***

$^1$H NMR ($CDCl_3$, 400 MHz) δ (relative to $CHCl_3$ at 7.26 ppm) 0.87 (d, 3, *J* = 6.8 Hz), 0.95 (d, 3, *J* = 6.8 Hz), 1.59 (s, 3), 1.58–1.68 (m, 3), 1.96 (s, 3), 2.34–2.49 (m, 2), 2.52–2.57 (m, 2), 2.67 (m, 1), 3.32 (s, 3), 3.34 (m, 1), 3.64 (m, 1), 4.08 (s, 3), 4.87 (br s, 2), 5.10 (m, 1), 6.65 (d, 1, *J* = 5.6 Hz), 5.99 (m, 1), 6.41 (t, 1, *J* = 11.2 Hz), 6.97 (d, 1, *J* = 11.2 Hz), 7.20 (s, 1), 8.73 (s, 1). $^{13}$C NMR ($CDCl_3$, 100 MHz) δ (relative to $CDCl_3$ at 77.0 ppm) 11.0, 12.3, 12.7, 22.5, 28.2, 31.9, 32.4, 33.4, 34.8, 56.6, 61.6, 73.0, 80.6, 81.2, 111.2, 124.9, 127.6, 128.7, 132.6, 133.1, 133.7, 136.9, 138.0, 156.5, 156.9, 168.5, 184.1, 184.8. ESI-TOF-MS *m/z* 548.2928, calculated for $C_{28}H_{42}N_3O_8$ ([M + $NH_4$]$^+$) 548.2966.

### Purification and Characterization of KOSN1859

Strain K279-69/pKOS331-178 was grown in 50 ml of YPD medium (Sigma-Aldrich, St. Louis, MO) in a 250 ml flask for 1 day at 30°C, and 25 ml was transferred into 500 ml YPD medium in a 2.8 l Fernbach flask. The culture was incubated at 30°C for 1 day, then used to inoculate two 20 l bioreactors at 4% (v/v) each with 12 l of fermentation medium (per liter, 2.5 g peptone [Difco], 2.5 g tryptone [Difco], 2.5 g yeast extract [Difco], 5 g baby oatmeal [Gerber], and 10 g beet molasses [Minn-dak]). Glucose was added to a final concentration of 36.4 g/l with a sterile 650 g/l stock solution. Temperature was maintained at 30°C. The pH was maintained at 6.5 with 2.5 N sulfuric acid or 2.5 N sodium hydroxide for 2 days after inoculation and then at 6.0 thereafter. Dissolved oxygen was maintained above 30% with agitation (200–400 rpm). Overhead pressure was manually set at 6 PSI, and airflow was controlled at 1 vvm. Foam was controlled by automatic addition of 50% (v/v) Antifoam B (JT Baker).

Four days after inoculation, Celite (50 g/l) and Cycep 349 (2.5 ml/l) were added to the fermentation broth before filtering. The clarified solution (23 l) was loaded on to a preconditioned HP20 column (10 × 13 cm, 1 l). The column was eluted with 100% MeOH, and the resulting mixture (4 l) was concentrated in vacuo to 250 ml. The mixture was diluted to 25% MeOH and loaded on to a preconditioned C-18 column (5 × 20 cm, 400 ml). The column was washed with 50% MeOH (800 ml) and eluted with 60% MeOH. Fractions containing the compound were pooled and dried to yield a dark brown solid. The partially purified compound was further purified by preparative HPLC on a Polaris C-18 column using 60% MeOH as the mobile phase. KOSN1859 (470 mg) was isolated as a brown solid.

***8-Desmethylgeldanamycin (KOSN1859)***

$^1$H NMR ($CDCl_3$, 400 MHz) δ (relative to $CHCl_3$ at 7.26 ppm) 0.92 (d, 3, *J* = 6.0 Hz), 1.11 (s, 3), 1.53 (m, 1), 1.56 (m, 1), 1.83 (m, 1), 2.01

(s, 3), 2.40 (m, 1), 2.44 (m, 1), 2.52 (m, 1), 3.35 (s, 3), 3.36 (s, 3), 3.60 (m, 1), 3.70 (t, 1, $J$ = 6.0 Hz), 4.11 (s, 3), 4.26 (d, 1, $J$ = 8.0 Hz), 4.90 (bs, 1), 5.38 (d, 1, $J$ = 8.0 Hz), 5.84 (dd, 1, $J$ = 8.0, 15.5 Hz), 5.86 (dd, 1, $J$ = 8.0, 11.5 Hz), 6.22 (dd, 1, $J$ = 7.0, 15.2 Hz), 6.54 (dd, 1, $J$ = 11.0, 11.4 Hz), 6.94 (d, 1, $J$ = 11.5), 7.23 (s, 1), 8.8 (s, 1). $^{13}$C NMR ($CDCl_3$, 400 MHz) $\delta$ (relative to $CHCl_3$ at 77.0 ppm) 12.3, 13.5, 21.8, 30.1, 30.9, 35.8, 36.9, 56.4, 57.8, 61.6, 74.0, 77.9, 79.9, 80.6, 111.4, 125.9, 126.3, 127.3, 127.6, 134.6, 136.8, 137.8, 139.1, 156.0, 157.1, 168.2, 184.1, 184.4. ESI TOF MS *m/z* 569.2470, calculated for $C_{28}H_{38}N_2O_9Na$ ([M +Na]$^+$) 569.2466.

### Purification and Characterization of KOSN1877

One 20 liter bioreactor was used to produce KOSN1877. The fermentation of strain K309-9 was conducted as above for KOSN1859, except the pH was controlled at 6.0 throughout the process, and the airflow was set at 0.75 vvm. The filtered broth was loaded on to a preconditioned HP20 column, washed with 40% methanol (2 l), and eluted with 100% methanol (5 l). Fractions containing the compound were pooled and concentrated to 425 ml. The crude mixture was diluted with 850 ml water and loaded on to a C-18 column (3 × 15.5 cm, 110 ml). The column was washed with 40% MeOH (100 ml), eluted with 1 l 50% methanol, and rechromatographed over C-18 using the same conditions. Fractions containing compound were pooled and dried to a dark brown solid. The solid was dissolved in dichloromethane and extracted once with water. The organic extract was dried to a give a yellow solid, dissolved in dichloromethane:MeOH (2:1), and further purified by preparative HPLC using 55% MeOH:water as the mobile phase. KOSN1877 (164 mg) was isolated as a yellow-colored solid.

***14-Desmethylgeldanamycin (KOSN1877)***

$^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ (relative to $CHCl_3$ at 7.26 ppm) 0.88 (d, 3, $J$ = 7.0 Hz), 1.41 (m, 1), 1.52 (m, 1), 1.57 (m, 1), 1.79 (s, 3), 1.98 (m, 1), 2.0 (s, 3), 2.42 (m, 1), 2.64 (m, 1), 2.78 (m, 1), 3.29 (s, 3), 3.29 (m, 1), 3.31 (s, 3), 3.56 (d, 1, $J$ = 7.0 Hz), 4.10 (s, 3), 4.31 (d, 1, $J$ = 9.0 Hz), 4.95 (bs, 2), 5.16 (s, 1), 5.73 (d, 1, $J$ = 9.0 Hz), 5.86 (dd, 1, $J$ = 9.5, 10.0 Hz), 6.55 (dd, 1, $J$ = 11.0, 11.0 Hz), 6.96 (d, 1, $J$ = 9.5), 7.22 (s, 1), 8.73 (s, 1). $^{13}$C NMR ($CDCl_3$, 400 MHz) $\delta$ (relative to $CHCl_3$ at 77.0 ppm) 12.4, 12.6, 12.9, 20.4, 23.7, 25.9, 32.3, 56.6, 57.3, 61.7, 72.3, 80.4, 81.0, 81.3, 111.4, 126.3, 127.6, 128.2, 132.5, 133.5, 134.7, 136.3, 138.1, 156.1, 156.4, 168.2, 184.1, 184.5. ESI-TOF-MS *m/z* 569.2469, calculated for $C_{28}$ $H_{38}N_2O_9Na$ ([M+Na]$^+$) 569.2466.

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