ORGANIC LETTERS

2013
Vol. 15, No. 17
4442–4445

# Bioreduction of Aryl Azides during Mutasynthesis of New Ansamitocins

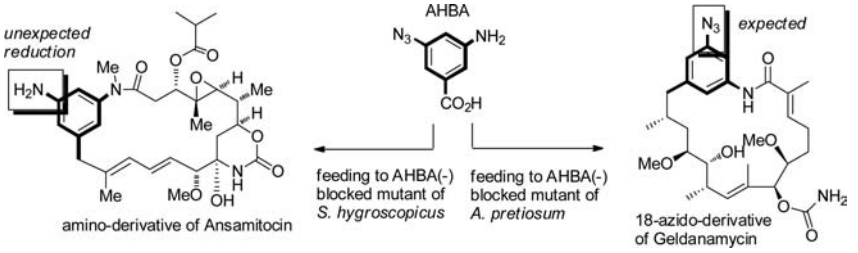
**Lena Mancuso,[†] Gerrit Jürjens,[†] Jekaterina Hermane,[†] Kirsten Harmrolfs,[†] Simone Eichner,[†] Jörg Fohrer,[†] Wera Collisi,[‡] Florenz Sasse,[‡] and Andreas Kirschning*[,†]**

*Institut für Organische Chemie und Biomolekulares Wikstoffzentrum (BMWZ) der Leibniz Universität Hannover, Schneiderberg 1B, 30167 Hannover, Germany, and Department of Chemical Biology, Helmholtz Center for Infectious Research (HZI), Inhoffenstrasse 7, D-38124 Braunschweig, Germany*

*andreas.kirschning@oci.uni-hannover.de*

**Received July 16, 2013**

## ABSTRACT




**Supplementing a culture of a mutant strain of *Actinosynnema pretiosum* that is unable to biosynthesize aminohydroxy benzoic acid (AHBA), with 3-azido-5-hydroxy-benzoic acid and 3-azido-5-amino-benzoic acid, unexpectedly yielded anilino ansamitocins instead of the expected azido derivatives. This is the first example of the bioreduction of organic azides. The unique nature of these results was demonstrated when 3-azido-5-amino-benzoic acid was fed to the corresponding AHBA blocked mutant of *Streptomyces hygroscopicus*, the geldanamycin producer. This mutasynthetic experiment yielded the fully processed azido derivative of geldanamycin.**

Mutasynthesis and related concepts based on interference of the biosynthesis of natural products have emerged as versatile strategies for the generation of novel natural products and can complement semi- and total syntheses in the generation of small libraries of structurally complex natural products (Scheme 1).[1]

Mutasynthesis[2] requires the generation of mutants of a producer organism that have had their biosynthetic pathway disrupted. Administration of mutasynthons, synthetic substrates resembling biosynthetic intermediates, to the blocked mutant then results in new metabolites which are formed in a predictable manner.

We demonstrated that mutasynthesis is a viable concept to create variants of ansamycin antibiotics by utilizing the mutant strains *Actinosynnema pretiosum*[3] HGF073 (the ansamitocin producer) and *Streptomyces hygroscopicus* K390-61-1 (the geldanamycin producer), both unable to produce 3-amino-5-hydroxybenzoic acid (AHBA, **1**), the starter unit for biosynthesis.[4,5] The ansamitocins P2−P4 (Scheme 1, **3a**−**3c**)[6] and geldanamycin (Scheme 1, **5**)[7] are highly toxic secondary metabolites that are of clinical interest as anticancer agents.[8,9]

These ansamycin antibiotics are polyketides that are assembled by a polyketide synthase (PKS). The PKS starter modules for both ansamycin antibiotics accept AHBA **1**.[3] After being extended by seven consecutive PKS modules, the polyketide chain is cyclized and released

[†] Leibniz Universität Hannover.
[‡] Helmholtz Center for Infectious Research (HZI).

(1) Reviews: (a) Kirschning, A.; Hahn, F. *Angew. Chem.* **2012**, *124*, 4086–4096. *Angew. Chem. Int. Ed* **2012**, *51*, 4012–4022. (b) Taft, F.; Eichner, S.; Knobloch.; Harmrolfs, K.; Hermane, J.; Kirschning, A. *Synlett* **2012**, 1416–1426. (c) Kennedy, J. *Nat. Prod. Rep.* **2008**, *25*, 25–34. (d) Kirschning, A.; Taft, F.; Knobloch, T. *Org. Biomol. Chem.* **2007**, 3245–3295.

(2) The term mutabiosynthesis or mutasynthesis was first suggested in: Rinehart, K. L. *Pure Appl. Chem.* **1977**, *49*, 1361–1384.

(3) Walsh, C. T.; Haynes, S. W.; Ames, B. D. *Nat. Prod. Rep.* **2012**, *29*, 37–59.

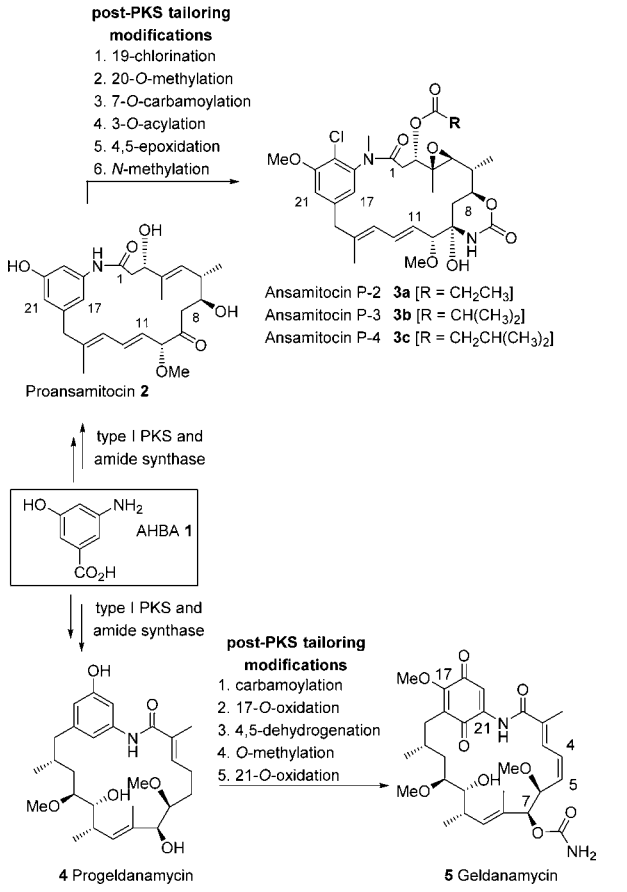
(4) HGF073 is a copy of HGF056: Yu, T. W.; Bai, L.; Clade, D.; Hoffmann, D.; Toelzer, S.; Trinh, K. Q.; Xu, J.; Moss, S. J.; Leistner, E.; Floss, H. G. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 7968–7973.

(5) For K390-61-1, see: Rascher, A.; Hu, Z.; Buchanan, G. O.; Reid, R.; Hutchinson, C. R. *Appl. Environ. Microbiol.* **2005**, *71*, 4862–4871.

(6) Reviews: (a) Cassady, J. M.; Chan, K. K.; Floss, H. G.; Leistner, E. *Chem. Pharm. Bull.* **2004**, *52*, 1–26. (b) Kirschning, A.; Harmrolfs, K.; Knobloch, T. *C. R. Chimie* **2008**, 1523–1543. (c) Floss, H. G.; Yu, T.-W.; Arakawa, K. *J. Antibiot.* **2011**, *64*, 35–44.

10.1021/ol401989e 
**Published on Web 08/27/2013**

**Scheme 1.** Biosyntheses of Ansamycin Antibiotics **3a**–**3c** and **5**




from the PKS by an amide synthase as proansamitocin (**2**). In a similar fashion, the PKS in *S. hygroscopicus* processes **1** to progeldanamycin **4**.[10] The biosyntheses are terminated by a set of post-PKS tailoring enzymes to furnish the ansamitocins **3a**–**3c**[11] and geldanamycin **5**[12,13] (Scheme 1).

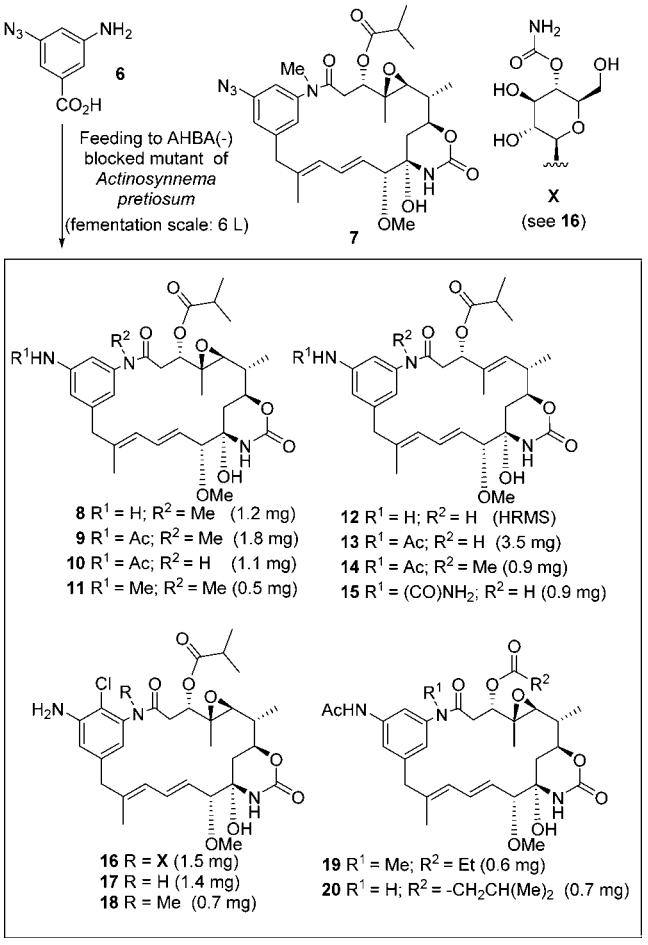



**Figure 1.** Feeding of azido benzoic acid **6** to the AHBA(–) blocked mutant of *A. pretiosum*.

Research in our group has been focused on the creation of ansamitocin conjugates for the selective treatment of tumors. Recently, we successfully prepared ansamitocins conjugated with folic acid for selectively targeting cancer[14] because tumor cells often overexpress folic acid receptors on their cell surface.

In order to expand this concept, we were interested in the mutasynthetic transformation[15] of aryl azides. "Click chemistry"[16] would allow the linkage of different biological probes such as fluorescent labels, monoclonal antibodies,[17] vitamins such as folic acid,[18] growth factors, and other groups that could specifically bind ansamitocins to tumor cells.[19]

In what follows we describe our unexpected findings on the unique azide reduction carried out by an AHBA(–)

(7) (a) P. Workman, P. *Curr. Cancer Drug Targets* **2003**, *3*, 297–300. (b) Neckers, L.; Neckers, K. *Expert Opin. Emerg. Drugs* **2005**, *10*, 137–149. (c) Whitesell, L.; Lindquist, S. L. *Nat. Rev. Cancer* **2005**, *5*, 761–772. (d) Prodromou, C.; Roe, S. M.; O'Brien, R.; Ladbury, J. E.; Piper, P. W.; Pearl, L. H. *Cell* **1997**, *90*, 65–75.

(8) (a) Thigpen, J. T.; C. E. Ehrlich, C. E.; Creasman, W. T.; Curry, S.; Blessing, J. A. *Am. J. Clin. Oncol. (CCT)* **1985**, *6*, 273–275. (b) Thigpen, J. T.; Ehrlich, C. E.; Conroy, J.; Blessing, J. A. *Am. J. Clin. Oncol. (CCT)* **1985**, *6*, 427–430. (c) Ravry, M. J.; Omura, G. A.; Birch, R. *Am J. Clin. Oncol. (CCT)* **1985**, *8*, 148–150. (d) Issell, B. F.; Crooke, S. T. *Cancer Treat. Rev.* **1978**, *5*, 199–207. (e) Kovtun, Y. V.; Audette, C. A.; Ye, Y.; Xie, H.; Ruberti, M. F.; Phinney, S. J.; Leece, B. A.; Chittenden, T.; Blättler, W. A.; Goldmacher, V. S. *Cancer Res.* **2006**, *66*, 3214–3221.

(9) Janin, Y. L. *J. Med. Chem.* **2005**, *48*, 7503–7512.

(10) (a) Rascher, A.; Hu, Z.; Viswanathan, N.; Schirmer, A.; Reid, R.; Nierman, W. C.; Lewis, M.; Hutchinson, C. R. *FEMS Microbiol. Lett.* **2003**, *218*, 223–230.

(11) Spiteller, P.; Bai, L.; Shang, G.; Carroll, B. J.; Yu, T.-W.; Floss, H. G. *J. Am. Chem. Soc.* **2003**, *125*, 14236–14237.

(12) (a) Hong, Y.-S.; Lee, D.; Kim, W.; Jeong, J. K.; Kim, C. G.; Sohng, J. K.; Lee, J. H.; Paik, S. G.; Lee, J. J. *J. Am. Chem. Soc.* **2004**, *126*, 11142–11143. (b) Lee, D.; Lee, K.; Cai, X. F.; Dat, N. T.; Boovanahalli, S. K.; Lee, M.; Shin, J. C.; Kim, W.; Jeong, J. K.; Lee, J. S.; Lee, C. H.; Lee, J. H.; Hong, Y.-S.; Lee, J. J. *ChemBioChem* **2006**, *7*, 246–248.

(13) Eichner, S.; Eichner, T.; Floss, H. G.; Fohrer, J.; Hofer, E.; Sasse, F.; Zeilinger, C.; Kirschning, A. *J. Am. Chem. Soc.* **2012**, *134*, 1673–1679.

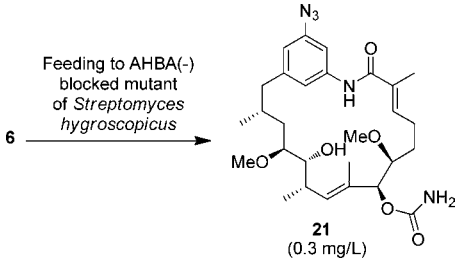
(14) Taft, F.; Harmrolfs, K.; Nickeleit, I.; Heutling, A.; Kiene, M.; Malek, N.; Sasse, F.; Kirschning, A. *Chem.—Eur. J.* **2012**, *18*, 880–886.

(15) (a) Taft, F.; Brünjes, M.; Floss, H. G.; Czempinski, N.; Grond, S.; Sasse, F.; Kirschning, A. *ChemBioChem* **2008**, *9*, 1057–1060. (b) Knobloch, T.; Floss, H. G.; Harmrolfs, K.; Sasse, F.; Taft, F.; Thomaszewski, B.; Kirschning, A. *ChemBioChem* **2011**, *12*, 540–547. (c) Eichner, S.; Knobloch, T.; Floss, H. G.; Fohrer, J.; Harmrolfs, K.; Hermane, J.; Schulz, A.; Sasse, F.; Spiteller, P.; Taft, F.; Kirschning, A. *Angew. Chem.* **2012**, *124*, 776–781. *Angew. Chem., Int. Ed.* **2012**, *51*, 752–757. (d) Eichner, S.; Floss, H. G.; Sasse, F.; Kirschning, A. *ChemBioChem* **2009**, *10*, 1801–1805.

blocked mutant of *A. pretiosum*. In fact, aminobenzoic acid **6**[20] was not loaded onto the PKS or processed by the polyketide synthase in *A. pretiosum* and thus formation of the desired ansamitocin derivative **7** was not encountered. Instead, when supplementing a culture of this AHBA blocked mutant with **6**, 13 new metabolites **8**–**20** were isolated as pure compounds after extensive preparative HPLC purification. Except for metabolite **12** for which only HRMS data were available all metabolites were characterized by NMR spectroscopy and HRMS. Remarkably, none of these metabolites contained an azido group. In all cases, the reduced anilines were formed and in several cases the amino group was further modified by acetylation (see **9**, **10**, **13**, **14**, **19**, and **20**), methylation (see **11**), or amidination to yield the corresponding urea derivative **15** (Figure 1).

Further tailoring steps (epoxidation, chlorination, and N-methylation) did occur in all cases. Ansamitocin derivatives **16**–**18** bearing a chlorine substituent at C19 are rare examples of incorporation of chlorine into an unchlorinated substrate.

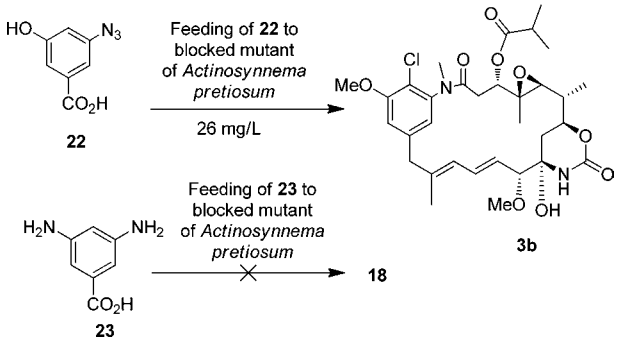
**Scheme 2.** Feeding of Azido Benzoic Acid **6** to the AHBA(−) Blocked Mutant of *S. hygroscopicus*




Commonly, the halogenase requires the phenolic group at C20 and even when the phenol at C20 is *O*-methylated chlorination is suppressed. Obviously, also a free amino group at C20 can act as a directing group, probably through H-bonding in the active site of the halogenase. It is noteworthy that feeding of 3,5-diaminobenzoic acid **23** to the mutant strain of *A. pretiosum* did not provide any of these anilino ansamitocins or other advanced metabolites.

The ability of *A. pretiosum* to reduce aryl azide **6** is unusual as was demonstrated by two additional experiments. First, feeding of aryl azide **6** to the AHBA(−) blocked mutant of the geldanamycin producer *S. hygroscopicus* yielded azido-geldanamcin derivative **21** (Scheme 2).

**Scheme 3.** Feeding of 3-Azido-5-hydroxybenzoic Acid **22** and 3,5-Diaminobenzoic acid **23** to the AHBA(−) Blocked Mutant of *A. pretiosum*




Second, we fed 2-azido-3-hydroxybenzoic acid **22** to *A. pretiosum* and found that AP3 (**3b**) was formed in good yield (Scheme 3). Again, the azido group must have been reduced during bioprocessing. However, when this experiment was repeated with the AHBA blocked mutant of *S. hygroscopicus*, geldanamycin was not formed. This further confirms the differences between *A. pretiosum* and *S. hygroscopicus*.

At this point, it remains unclear at which stage of the biosynthesis the reduction in *A. pretiosum* takes place. Either aryl azides **6** or **22** are loaded onto the PKS which means that, somewhere en route to the final PKS module, reduction of the azido moiety occurs so that the amide synthase can perform macrolactamization of the *seco*-acid.

Alternatively, and more likely, reductions of **6** and **22** occur prior to loading onto the PKS starter module, so that the whole process would proceed in the "natural" manner.

The fact that mutasynthon **23** (Scheme 3) was not further processed, though it is the reduction product of azide **6**, may be ascribed to its high polarity that could reduce its ability to penetrate the bacterial membrane. Alternatively, in vivo activation to the CoA ester may be hampered compared to **6**.

In order to rule out chemical reduction of the azide group by natural thiols we treated azide **22** with ethanethiol, thiophenol, and glutathione in degassed pH 7 buffer for 3 days. MS-analysis revealed no conversion of **22** by thiophenol, and only traces (less than 0.5%) of the reduction product was found in the presence of glutathione and ethanethiol.

Furthermore, we fed benzyl azides **24**–**26** in order to probe the selectivity of *A. pretiosum*. None of these three arenes were processed by *A. pretiosum*. In contrast, benzyl azide **26** was transformed to *seco*-proreblastatinamide derivative **27** by the (−)AHBA mutant strain of *S. hygroscopicus* (Figure 2). In this case, almost complete processing took place; however, amide formation took place at

(16) For overviews on chemical ligation strategies, see: (a) Hackenberger, C. P. R.; Schwarzer, D. *Angew. Chem.* **2008**, *120*, 10182–10228. *Angew. Chem., Int. Ed.* **2008**, *47*, 10030–10074. (b) van Berkel, S. S.; van Eldijk, M. B.; van Hest, J. C. M. *Angew. Chem.* **2011**, *123*, 8968–8989. *Angew. Chem., Int. Ed.* **2011**, *50*, 8806–8827.

(17) (a) Wu, A. M.; Senter, P. D. *Nat. Biotechnol.* **2005**, *23*, 1137–1146. (b) Chari, R. V. *Acc. Chem. Res.* **2008**, *41*, 98–107. (c) S. C. Alley, S. C.; Okeley, N. M.; Senter, P. D. *Curr. Opin. Chem. Biol.* **2010**, *14*, 529–537.

(18) Low, P. S.; Henne, W. A.; Doorneweerd, D. D. *Acc. Chem. Res.* **2008**, *41*, 120–129.

(19) (a) Arap, W.; Pasqualini, R.; Ruoslahti, E. *Science* **1998**, *279*, 377–380. (b) Janssen, M. L.; Oyen, W. J.; Dijkgraaf, I.; Massuger, L. F.; Frielink, C.; Edwards, D. S.; Rajopadhye, M.; Boonstra, H.; Corstens, F. H.; Boerman, O. C. *Cancer Res.* **2002**, *62*, 6146–6151. (c) Haubner, R.; Wester, H. J.; Burkhart, F.; Senekowitsch-Schmidtke, R.; Weber, W.; Goodman, S. L.; Kessler, H.; Schwaiger, M. *Bioconjug. Chem.* **2001**, *12*, 84–91 and see also Supporting Information.

(20) Harmrolfs, K.; Brünjes, M.; Dräger, G.; Floss, H. G.; Sasse, F.; Taft, F.; Kirschning, A. *ChemBioChem* **2010**, *11*, 2517–2520.

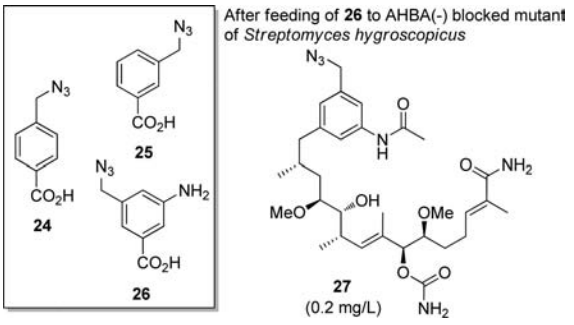



**Figure 2.** Feeding of aryl azides **24**–**26** and mutasynthetic transformation of benzyl azide **25** to seco acid **26** by *S. hygroscopicus* K390-61-1.

C-1 and *N*-acylation of the anilino group occurred instead. These are typical detoxification processes which made macrolactamization impossible.[15]

All new ansamycin metabolites (except for **12**) were subjected to *in vitro* biological testing with four different human cell lines obtained from cancer growths. The results from these tests are listed in Table 1 as the half-maximal inhibitory concentration of the respective ansamycin derivatives in comparison to the "gold standards" ansamitocin P-3 (**3b**) and geldanamycin (**5**). *N*-Glucosylation (in **16**) leads to complete loss of potency which has been noted before.[15b,21,22] In most cases exchange of the phenol to the anilino moiety only reduced the antiproliferative properties to a small extent (**8**–**11**), irrespective of whether the amino group was further methylated or acylated. The fully processed amino derivatives **17** and **18** show (compared ansamitocin P-3 (**3b**)) only slightly reduced antiproliferative effects on tumor cell lines. Metabolites that lack the epoxy group at C4–C5 (**13**–**15**) show no activity or only moderate activity. Finally, geldanamycin derivative **21** bearing an azido group at C18 was inactive.

In conclusion, we report the unexpected bioreduction of aryl azides during mutasynthesis of new ansamitocin derivatives. This reduction is a rare phenomenon in nature; nitrogenases which are usually responsible for nitrogen fixation in selected classes of bacteria and archaea are able to reduce inorganic azide anions yielding $N_2$ and ammonia.[23] Furthermore, there is evidence that aryl azides are prone to metabolic degradation[24] and that Baker's yeast is able to reduce aryl azides.[25] Current research in our laboratories is focused on the search for this unknown biocatalyst that we expect to be present in *A. pretiosum*.

**Table 1.** Antiproliferative Activity against Different Cancer Cell Lines[a]

| | cell line | | | |
|---|---|---|---|---|
| | KB-3-1 | PC-3 | A-431 | SK-OV-3 |
| **3b** | **0.11** | **0.035** | **0.050** | **0.030** |
| **8** | 1.4 | 9.0 | 2.0 | 2.8 |
| **9** | 2.0 | 19 | 7.5 | 2.5 |
| **10** | 5.0 | 55 | 21 | 13 |
| **11** | 0.7 | 1.8 | 0.8 | 1.4 |
| **13** | 6000 | >10000 | >10000 | >10000 |
| **14** | 350 | n.d. | 350 | 650 |
| **15** | 10 | 70 | 26 | 20 |
| **16** | 2600 | 7500 | 7000 | 2800 |
| **17** | 0.28 | 1.5 | 0.28 | 0.45 |
| **18** | 0.35 | 1.4 | 0.4 | 0.35 |
| **19** | 20 | 220 | 120 | 54 |
| **20** | 58 | 320 | 260 | 210 |
| **5** | **2.7** | **21** | **2.0** | **4.1** |
| **21** | 280 | 990 | 550 | 600 |

[a] The values give the $IC_{50}$ [ng/mL] measured by an MTT assay. KB-3-1 = cervix carcinoma; PC-3 = prostate carcinoma; A-431 = epidermoid carcinoma; SK-OV-3 = ovarian carcinoma; entries labeled in bold are reference compounds ansamitocin P-3 and geldanamycin.

**Acknowledgment.** This work was supported by the Deutsche Forschungsgemeinschaft (Grant Ki 397/13-1) and by the Fonds der Chemischen Industrie.

**Supporting Information Available.** Experimental procedures for compounds; analytical characterization; details on the cell proliferation assays. This material is available free of charge via the Internet at http://pubs.acs.org.

(21) (a) Ma, J.; Zhao, P.-J.; Shen, Y.-M. *Arch. Pharm. Res.* **2007**, *30*, 670–673. (b) Lu, C.; Bai, L.; Shen, Y. *J. Antibiot.* **2004**, *57*, 348–350.

(22) Snipes, C. E.; Duebelbeis, D. O.; Olson, M.; Hahn, D. R.; Dent, W. H., III; Gilbert, J. R.; Werk, T. L.; Davis, G. E.; Lee-Lu, R.; Graupner, P. R. *J. Nat. Prod.* **2007**, *70*, 1578–1581.

(23) (a) Fisher, K.; Dilworth, M. J.; Newton, W. E. *Biochemistry* **2006**, *45*, 4190–4198. (b) Rivera-Ortiz, J. M.; Burris, R. H. *J. Bacteriol.* **1975**, *123*, 537–545.

(24) Nicholls, D.; Gescher, A.; Griffin, R. J. *Xenobiotica* **1991**, 935–943.

(25) (a) Kamal, A.; Damayanthi, Y.; Narayan Reddy, B. S.; Lakminarayana, B.; Praveen Reddy, B. S. *Chem. Commun.* **1997**, 1015–1016. (b) Kamal, A.; Laxminarayana, B.; Lakshmi Gayatri, N. *Tetrahedron Lett.* **1997**, *38*, 6871–6874.

The authors declare no competing financial interest.