Polyketides

DOI: 10.1002/anie.201006165

Divergolides A–D from a Mangrove Endophyte Reveal an Unparalleled Plasticity in ansa-Macrolide Biosynthesis**

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Ansa macrolides (or ansamycins) comprise a diverse group of complex, often remarkably bioactive natural products that have been isolated predominantly from actinomycetes.[1] A hallmark of these compounds is a medium-sized to large macrolide or macrolactam "handle" fused to a mono- or bicyclic aromatic core. Among the most prominent representatives are the HSP90 inhibitor geldanamycin,[2] the antimycobacterial antibiotic rifamycin,[3] and the maytansinoid antitumor agents.[4] These valuable bacterial metabolites share a common biosynthesis involving a modular type I polyketide synthase (PKS).[5–8] In general, an aromatic starter unit—typically 3-amino-5-hydroxybenzoic acid (AHBA)—enters the thiotemplate assembly line.[9] Stepwise elongation and macrocylization give rise to a defined ansamycin skeleton, and further structural diversity is usually governed by enzymatic post-PKS modification steps, such as oxygenation, alkylation, amination, and halogenation.[10,11] Recent remarkable studies have been devoted to tailor ansamycin scaffolds through (semi)synthesis,[12,13] mutasynthesis,[14,15] and combinatorial biosynthesis.[16–19]

Herein we report the isolation, structure elucidation, and biological activities of four novel ansa macrolides, which point to a highly divergent biosynthetic pathway in an endophyte of the mangrove tree *Bruguiera gymnorrhiza*. *B. gymnorrhiza* is one of the dominant mangrove species along the Chinese coast, and in Chinese traditional medicine the bark and the root of the tree is used to treat diarrhea, throat inflammation, and hemostasia.[20] While several chemical constituents of the plant itself have been investigated, the biosynthetic potential of its endophytes has remained underexplored.[21] To address this gap of knowledge we investigated various *Streptomyces* spp. isolated from the stem of the mangrove tree. Metabolic profiling of a cultured endophyte strain (*Streptomyces* sp. HKI0576) by HPLC-MS revealed a complex metabolome. Since various structurally intriguing metabolites were only formed in trace amounts, the fermentation had to be scaled up to 200 L to allow for a full structure elucidation of these compounds. Through column chromatography on silica, size-exclusion chromatography, and preparative HPLC we succeeded in the purification of several components: **1** (32 mg), **2** (1 mg), **3** (5 mg), and **4** (15 mg). As their structures imply a highly divergent biogenesis, the new compounds were named divergolides (Scheme 1).

For compound **1**, high-resolution ESIMS (*m*/*z* 576.2549, $[M+Na]^+$) and ^{13}C NMR data established a molecular formula of $C_{31}H_{39}NO_8$. The ^1H NMR spectrum exhibited complicated signal patterns originating from various spin systems. Four substructures were deduced from the H,H COSY data,

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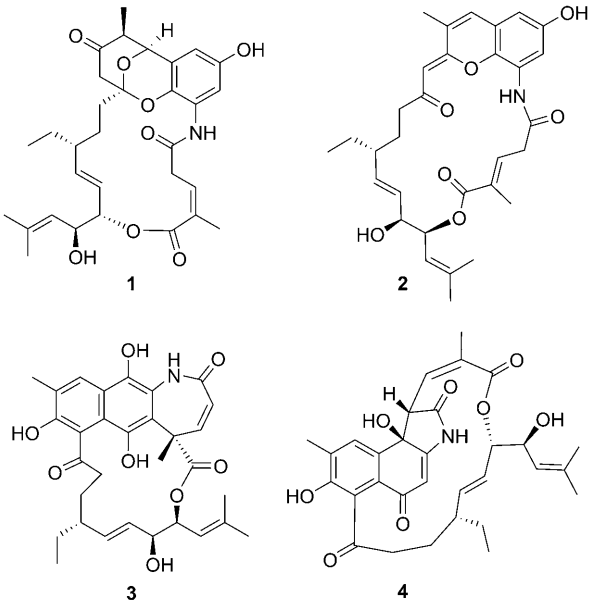
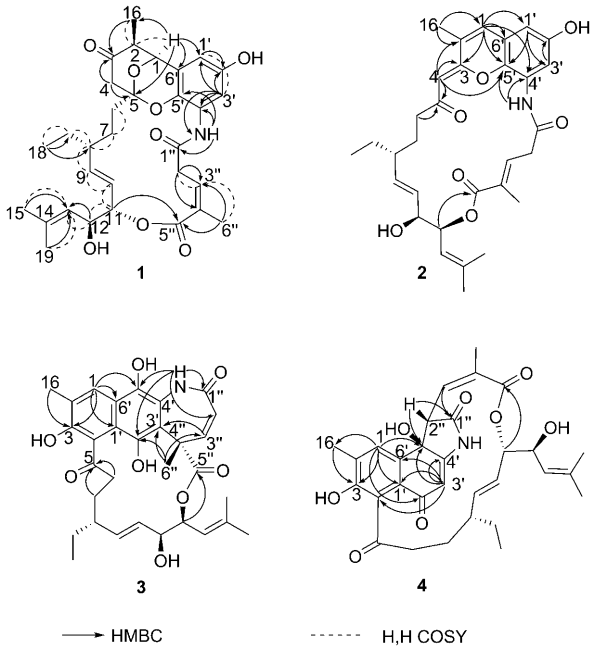
[**] We thank A. Perner, H. Heinecke, and F. Rhein for MS and NMR measurements, U. Valentin for SPE, C. Heiden and M. Steinacker for fermentation and downstream processing, and C. Weigel for performing biological assays. This research was financially supported by the Federal Ministry of Science and Technology (BMBF, Germany).

Supporting information for this article (including experimental details) is available on the WWW under http://dx.doi.org/10.1002/anie.201006165.



Scheme 1. Structures of divergolides A–D (**1**–**4**), novel ansa macrolides from an endophyte of the mangrove tree *Bruguiera gymnorrhiza*.



Scheme 2. Selected COSY and HMBC correlations of divergolides A–D (**1**–**4**).

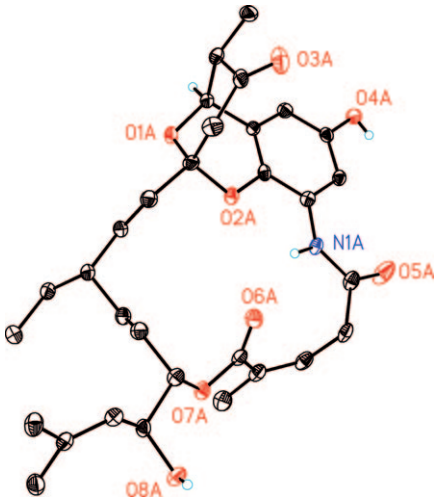


Figure 1. Molecular structure of **1**. The ellipsoids represent a probability of 40%, H atoms are drawn with arbitrary radii.

and confirmed and connected by HMBC correlations (Scheme 2, and see also the Supporting Information). The HMBC correlations of NH ($\delta = 8.95$ ppm) to C4′ ($\delta =$ 126.9 ppm) and C5′(δ=132.0 ppm), H3′ ($\delta = 8.30$ ppm) to C1′ ($\delta = 107.6$ ppm), C2′ ($\delta = 149.9$ ppm), and C4′ ($\delta =$ 126.9 ppm) revealed that the nucleus is a 1-amino-3-hydroxybenzene derivative. The connections from the aliphatic bridge to the aromatic nucleus were established by HMBC correlations from H1 ($\delta = 4.99$ ppm) to C1′ ($\delta = 107.6$ ppm) and C6′ ($\delta = 118.3$ ppm), and NH to C1″ ($\delta = 169.8$ ppm). A large coupling constant ($J = 15.7$ Hz) between H9 and H10 led to the assignment of a 9-*E* configuration, while the downfield shift of C6″ ($\delta = 20.4$ ppm) indicated a 3″-*Z* configuration. The *anti* orientation of H11 and H12 was deduced from the relatively large coupling constant ($J = 7.4$ Hz), and a coupling constant of $J = 5.3$ Hz was indication of a *syn* orientation between H1 and H2. Eventually, we succeeded in crystallizing compound **1** from a mixture of CH_2Cl_2/CH_3OH. X-ray crystallography fully confirmed the proposed structure and the relative configuration (Figure 1). In sum, divergolide A represents a novel type of ansa macrolide with an unusual branched side chain and a disrupted polyketide backbone. Furthermore, the tricyclic chromophore is unprecedented for macrolides; related O-heterocylic substructures are only known from aromatic polyketides such as the nogalamycin aglycone[22] and chaetoxanthone.[23] The CD spectrum of the latter aided in establishing the absolute configuration of **1**.

For the first congener of **1**, divergolide B (**2**), high-resolution ESIMS and ^{13}C NMR spectroscopy established a molecular formula of $C_{31}H_{37}NO_7$. A large ansa bridge with a similar architecture as in **1** was deduced from the ^1H NMR and COSY data, which was further confirmed by HSQC and HMBC correlations. However, the chromophore differed greatly from the one found for **1**. Two signals characteristic for *meta*-positioned aromatic protons ($\delta = 8.39$ ppm, H3′; $\delta =$ 6.48 ppm, H1′) were detected in the ^1H NMR spectrum. The HMBC correlations revealed the same 1-amino-3-hydroxybenzene substructure as in divergolide A (Scheme 2). In the ^1H NMR spectrum, two additional aromatic signals ($\delta =$ 6.91 ppm, d, H1; $\delta = 5.57$ ppm, s, H4), and HMBC correlations from H1 to C1′ ($\delta = 106.1$ ppm), C4′ ($\delta = 126.1$ ppm), C5′ ($\delta = 134.2$ ppm), and C6′ ($\delta = 120.0$ ppm) elaborated the position of C1. Likewise, the position of Me16 ($\delta = 2.03$ ppm), which correlates with H1 in the COSY spectrum, was established. HMBC correlations from H4 to C2 ($\delta =$ 127.3 ppm), C3 ($\delta = 158.5$ ppm), C5 ($\delta = 197.4$ ppm), and C5′ resulted in the final proposal of a substituted benzopyran nucleus. The H-H coupling constants confirmed the 9-*E* conformation as well as the H11 and H12 *anti* orientation, as in **1**. Furthermore, the *E* configuration of the double bond at position C3″ was deduced from the relative upfield shift of the signal for the allylic methyl group C6″ ($\delta = 13.1$ ppm). Finally, based on the HMBC correlation between H12 ($\delta = 5.62$ ppm) and C5″ ($\delta = 166.3$ ppm), it was deduced that the aliphatic bridge was connected to the aromatic core. Divergolide B represents another novel type of ansa macrolide featuring an unprecedented benzopyran/chromene nucleus. Interestingly, related cryptic hydroquinone substructures can be found in various anti-inflammatory agents, for example, tocopherol and quercinol.[24]

Finally, we found that compounds **3** and **4** share substructures with **1** and **2**, but feature structurally intriguing tetracyclic scaffolds. Divergolide C (**3**) has a molecular formula of $C_{31}H_{33}NO_8$. The 1D and 2D NMR data revealed that this metabolite represents a homologue of hygrocin B, an unusual metabolite isolated from *Streptomyces hygroscopicus*.[25] Compound **3** differs from hygrocin B in the unprecedented isobutenyl side chain at C12, the oxidative state of the aminonaphthoquinone, and the site of ester linkage.

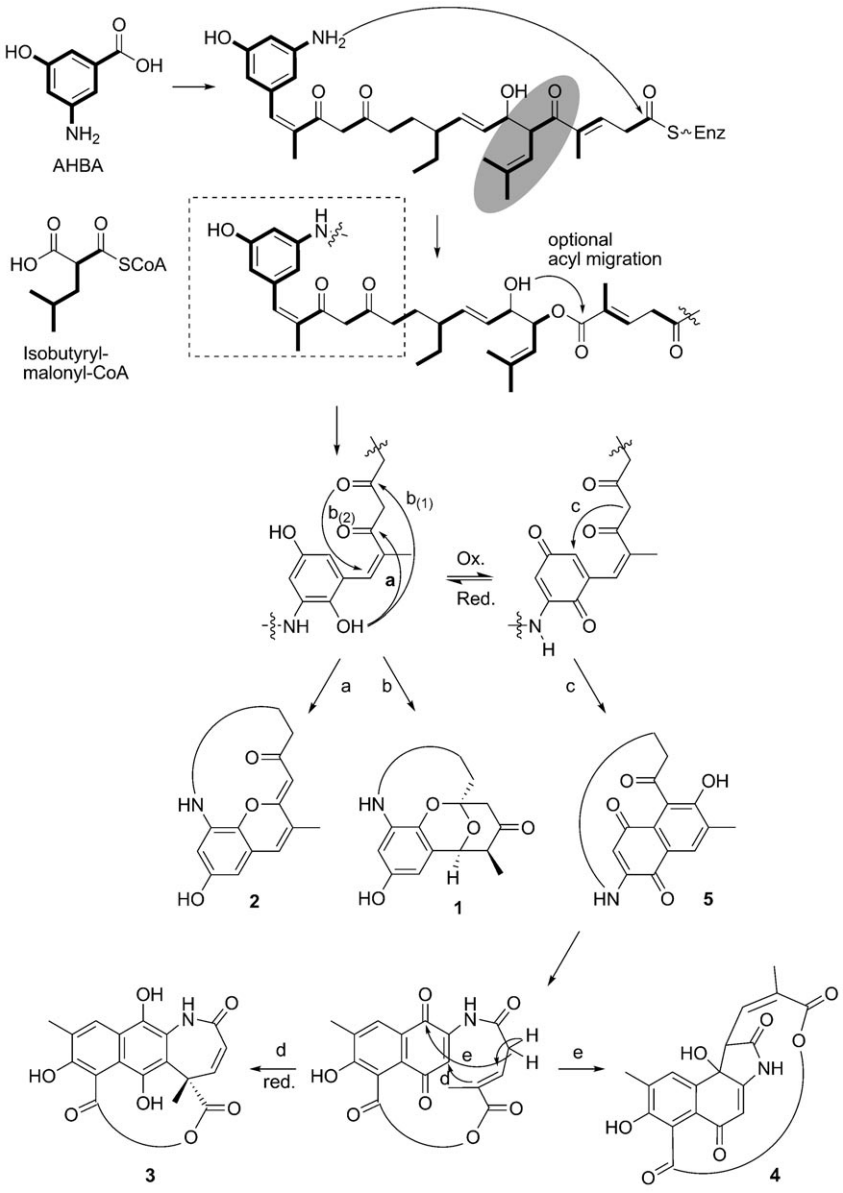
Another ring topology was found for divergolide D (**4**), which has a molecular formula of $C_{31}H_{35}NO_8$. According to NMR data, the ansa bridge of **3** remained largely conserved as

in the other divergolides, in particular **1**. However, the ^{13}C and ^{1}H NMR signals indicated that **4** features a different ansa nucleus. The main difference in the NMR spectra is one upfield-shifted aromatic signal ($\delta = 5.82$ ppm, s, H3′) and one signal for an oxygen-substituted quaternary carbon atom ($\delta =$ 74.9 ppm, C5′). The identity of the substituted phenol moiety was confirmed by HMBC correlations from H1 ($\delta =$ 7.36 ppm) and Me16 to an aromatic carbon atom at $\delta =$ 153.2 ppm (C3). The positions of C3′ and C5′ could be established by HMBC, where H1, H3′ ($\delta = 5.82$ ppm), and H3″ ($\delta = 6.52$ ppm) showed correlations to C5′, and H3′ showed correlations to C1′, C4′, and C6′. C5′ is connected to C2″, thus forming a tricyclic chromophore, which is fully supported by all the observed correlations. The HMBC correlation (Scheme 2) between H11 ($\delta = 5.19$ ppm) and C5″ ($\delta = 167.6$ ppm) led to the connection of the aliphatic bridge to the aromatic nucleus, similar to a degradation product of hygrocin A.[25] The double bond at C3″ was proposed as *Z* because of the relative downfield shift of the signal for the allylic methyl group at C6″ ($\delta = 21.6$ ppm).

Although the core structures of the novel ansa macrolides differ profoundly, a closer inspection clearly indicates that they originate from the same biosynthetic precursor. A retro-biosynthetic analysis (Scheme 3) strongly suggests that **1**–**4** derive from an AHBA-primed polyketide backbone that is disrupted by a Baeyer–Villigerase, as in mithramycin biosynthesis.[26] Apparently, the different size of the ansa bridge results from an optional acyl migration, and the terminal double bond may be shifted in analogy to what has been observed in ansamitocin,[27] bacillaene,[28] and rhizoxin biosynthesis.[29] The unusual branched side chain, which is uniformly found in all divergolides, implies that the requisite polyketide synthase utilizes a novel branched extender unit.[11,30] Unfortunately, all attempts to perform stable isotope labeling experiments were unsuccessful because of the low amounts of metabolites produced. However, the most plausible scenario would be that isobutyryl-CoA is elongated by a ketosynthase (KS III) to give the unsaturated homologue, which is then transformed to an isobutyrylmalonyl-CoA unit by a crotonyl reductase/carboxylase.[30] Future genetic and biochemical studies will test this hypothesis.

The most remarkable finding, however, is that all four divergolides fit into one biosynthetic scheme. Accordingly, the diversification of the ansa macrolides results from a degree of flexibility in the reactions of the polyketide chain. In the four divergolide structures, we observe three different types of cyclizations that lead to three alternative aromatic chromophores, and two options for the final regiodivergent heterocyclizations. Specifically, the exomethylene-2*H*-benzopyran O heterocycle as in **2** is produced when the phenolic hydroxy group attacks the carbonyl group adjacent to the double bond and subsequent elimination of water. In the second scenario, where the phenol attacks the more distant carbonyl group, an acetal is formed, and after addition of the hydroxy group to the side-chain double bond, the remarkable ring system of **1** is formed. Conversely, C–C bond formation of the reactive methylene to the aminoquinone results in the formation of a naphtho(hydro)quinone, the plausible precursor of **3** and **4** (Scheme 1, routes a–c).

The formation of the aminonaphthoquinone sets the stage for subsequent formation of N heterocycles. The carbonyl-



Scheme 3. Model for divergolide biosynthesis with tentative biosynthetic building blocks and proposed cyclization modes that lead to the diverse chromophores of **1**–**4**.

activated methylene may attack the quinone in a vinylogous fashion to yield a seven-membered lactam ring, as has been proposed for the biosynthesis of hygrocin B.[25] Alternatively, in an intramolecular aldol reaction, the γ-lactam is formed. Apparently, the size of the third ring is governed by the hard and soft reaction centers (Scheme 1, routes d and e, respectively). Notably, N heterocyclization is not observed in the compounds bearing the hydroquinone cores found in **1** and **2**.

It appears that the biosynthetic assembly line has left room for spontaneity in the diverging biosynthetic pathways through generating a reactive precursor. One may speculate that this "in-built diversification" does not lead to inactive shunt products, as in aromatic polyketide pathways,[11] but plays a functional role. To evaluate this, all new compounds were subjected to a panel of primary bioactivity screenings. We found that **1** exhibits strongest activity against *Mycobacterium vaccae*, whereas **4** is more active against *Bacillus subtilis* and *Staphylococcus aureus*. Of the four divergolides, **3** is the only compound with moderate activity against *Enterococcus faecalis* (Table 1). In a cytotoxicity screen against 40 tumor cell lines, only **4** displayed pronounced activity (Table 1). The most sensitive cell lines corresponded to lung cancer (LXFA 629L), pancreatic cancer (PANC-1), renal cancer (RXF 486L), and sarcoma (Saos-2), with IC_{50} values ranging from 1.0 to 2.0 μM. Taken together, the various ansa macrolides cover a range of antimicrobial and cytotoxic activities, which may contribute to regulating the endophytic microbiome and exclude pathogens from the mangrove tree. From a medicinal point of view, divergolides A and D, in particular, could be interesting candidates for further development as anti-infectives and antitumoral agents, respectively.

Table 1: Antibacterial and cytotoxic activities of divergolides A–D (**1**–**4**).

Cpd	Test strains[a] (mm inhibition zone)[b]				Cytotoxicity[c]
	Bs	*Mv*	MRSA	VRE	mean IC_{50} [μM]
1	11	19	11	0	>10
2	10	12	0	0	>10
3	13	11	13	14	>10
4	19	12	19	0	2.4

a] *Bs*: *Bacillus subtilis*, *Mv*: *Mycobacterium vaccae*, MRSA: methicillin-resistant *Staphylococcus aureus*, VRE: vancomycin-resistant *Enterococcus faecalis*. [b] Data in diameter; 50 μg per paper disk, $d = 7$ mm. [c] Test concentration in 10 half-log steps up to 10 μM.

Finally, it may be interesting to note that this is, to the best of our knowledge, the first report on the discovery of ansamycins from a tree endophyte. For a long time it has been hypothesized that endophytic actinomycetes may be involved in the biosynthesis of maytansin and maytansinoids isolated from trees, yet direct evidence is lacking.[4,31] This work may encourage further research in this direction.

In conclusion, we have isolated and fully characterized through extensive NMR spectroscopic and X-ray analysis four novel ansamycins from an endophyte of the mangrove tree *B. gymnorrhiza*. Despite the significant differences in the overall architectures of the divergolides, their substitution pattern point to a common biosynthetic precursor. The polyketide backbone likely derives from a novel branched polyketide synthase extender unit, which is disrupted through a Baeyer–Villiger oxidation. The inherent reactivity of the polyketide precursor allows various reaction channels to yield structurally intriguing ansa macrolides. The reactive amino-(hydro)quinone core sets the stage for three different core cyclizations and two final heterocyclizations, thus leading to macrolides with various ring sizes and overall topologies.[32] Not surprisingly, these diverging pathways result in metabolites that differ in their bioactivity profiles, covering antibacterial and cytotoxic properties. To the best of our knowledge, this degree of "in-built diversification" is unprecedented for complex polyketides and highlights the beauty of biosynthetic versatility in nature.

Received: October 1, 2010
Published online: January 11, 2011

Keywords: ansamycins · macrolides · natural products · polyketides · structure elucidation

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[32] Note added in proof: A related ansamycin with a spiro ring system and a similar branched side chain has been identified in a marine streptomycete: M. C. Wilson, S.-J. Nam, T. A. M. Gulder, C. A. Kauffman, P. R. Jensen, W. Fenical, B. S. Moore, *J. Am. Chem. Soc.* **2010** DOI: 10.1021/ja109226s.

