Synthase Inhibitors

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Action of atrop-Abyssomicin C as an Inhibitor of 4-Amino-4-deoxychorismate Synthase PabB**

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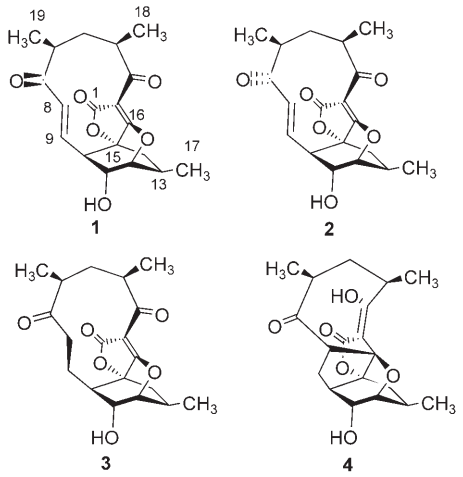
Dedicated to Professor Günther Jung on the occasion of his 70th birthday and to Professor Hans-Peter Fiedler on the occasion of his 60th birthday

Among biosynthetic pathways, chorismate is an important and central biosynthetic metabolite that branches off to the biosynthesis of aromatic amino acids Trp, Tyr, and Phe, but also to *p*-aminobenzoic acid (*p*ABA).[1] *p*-Aminobenzoic acid is a component of the biosynthesis of tetrahydrofolate. Corresponding biosynthesis enzymes of *p*ABA and tetrahydrofolate occur in many microorganisms and parasites, but not in humans, which makes *p*ABA biosynthesis an interesting target for anti-infective agents. Prominent synthetic inhibitors of the tetrahydrofolate pathway are sulfonamides and trimetoprim.[2]

Recent screening efforts in the *p*ABA biosynthesis pathway led to the isolation of abyssomicins B, C (**1**), and D (**4**)[3,4] from the gram-positive marine actinomycete *Verrucosispora* AB-18-032 (Scheme 1). Out of the three metabolites, abyssomicin C has been described as the only active component against gram-positive bacteria including pathogenic *Staphylococcus aureus* strains. Because of the attractive structure, several synthetic chemistry groups have directed their interests towards the total synthesis of abyssomicin C.[5–8] Two successful total syntheses have been published so far, the first by Sorensen and co-workers[5] and the second by Nicolaou and Harrison.[6,8]

Apart from abyssomicin C (**1**), Nicolaou and Harrisson described the synthesis of the isomer atrop-abyssomicin C (**2**),[6] which had been found to have an increased antibiotic activity. More recently, atrop-abyssomicin C was also detected in fermentations of *Verrucosispora* AB-18-032, and it was found that **2** is the main abyssomicin product synthesized by *Verrucosispora*, while abyssomicin C (**1**) is a minor by-product which is also formed from **2** under acidic conditions.[6,9] From the structures of abyssomicins and preliminary agar diffusion assays, the putative target and the mode of action were deduced.[3,4] To our knowledge, abyssomicin C (**1**) and atrop-abyssomicin C (**2**) are the first known natural inhibitors of *p*ABA biosynthesis.



Scheme 1. Structures of abyssomicin C (**1**), atrop-abyssomicin C (**2**), abyssomicin H (**3**), and abyssomicin D (**4**).

Herein we describe the inhibition and the molecular mode of action of atrop-abyssomicin C (**2**) as the key metabolite of *Verrucosispora* AB-18-032. Interestingly, the structures of **1** and **2**, more precisely the oxabicyclooctane ring system, show striking similarity to one solution conformation of chorismate,[10] which suggests that **1** and **2** are substrate mimetics. Furthermore, the structural resemblance of abyssomicins to chorismic acid transition-state analogues is evident.[11] Also, **1** and **2** feature an α,β-unsaturated ketone adjacent to the oxabicyclooctane system, which is absent in the inactive derivative abyssomicin H (**3**; Scheme 1),[9] suggesting that this Michael system plays a crucial role in antibiotic activity.

*p*ABA biosynthesis from chorismate requires two enzymes: 4-amino-4-deoxychorismate (ADC) synthase, which converts chorismate and glutamine into ADC and glutamate,[12] and ADC lyase, which catalyzes an elimination reaction of ADC to produce *p*ABA. ADC synthase is a heterodimer composed of two nonidentical subunits, PabA and PabB. PabA functions as a glutamine amidotransferase while PabB catalyzes the substitution of the chorismate 4-hydroxy group by an amino group while retaining the original configuration.

To investigate the interaction of **2** with ADC synthase, we chose ADC synthase from abyssomicin-sensitive *Bacillus*

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subtilis and heterologously overexpressed and purified PabA (native) and PabB (N-terminal His_6-tag) from *E. coli.* An assay based on electrospray mass spectrometry was developed that monitors ADC formation directly by means of multiple-reaction-monitoring (MRM) experiments. The inhibition assay was performed at fixed concentrations of chorismate (5 mM) and ammonium sulfate (100 mM). Preincubation of ADC synthase, PabA, and PabB with **2** resulted in a time-dependent loss of activity (for inhibition data, see the Supporting Information). The addition of PabA was necessary owing to the instability of PabB if preincubated alone. Preincubation of PabA with **2** had no inhibiting effect on ADC formation. A series of experiments were carried out with varying inhibitor concentrations (50–500 μM) at a fixed enzyme concentration and various incubation times. The remaining activity was tested by monitoring ADC formation with MRM. When the observed rate constant for enzyme inactivation, k_{obs}, was plotted as a function of inhibitor concentration, a hyperbolic curve was obtained, which is characteristic of an irreversible inhibitor and indicative of covalent binding to the protein. An approximate K_I^{app} value of 390 μM and an approximate k_{inact} value of 0.8 min^{-1} were determined. Additional inhibition assays with abyssomicin C (**1**) show that **1** has a significantly lower potential to inactivate PabB than **2** (data in the Supporting Information).

To locate the protein-binding site of atrop-abyssomicin C on the corresponding amino acid side chain, PabB was incubated with **2**. Digestion of PabB with endoproteinase GluC yielded a doubly charged peptide at *m/z* 827.0 [*M* = 1651.7] that was not detected in the control digest without an inhibitor (Figure 1). By mass-spectrometric fragmentation (ESI-MS/MS) it was found that atrop-abyssomicin C had covalently bound to the thiol side chain of Cys263 of the peptide TPDFQIICGSPE (Figure 2).

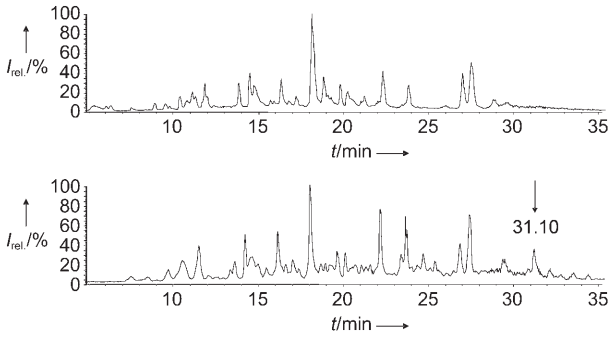


Figure 1. HPLC-ESI-MS chromatogram of peptides from a GluC digest of untreated PabB (top) and PabB (bottom) incubated with atrop-abyssomicin C, the latter showing an additional signal at R_t = 31.1 min.

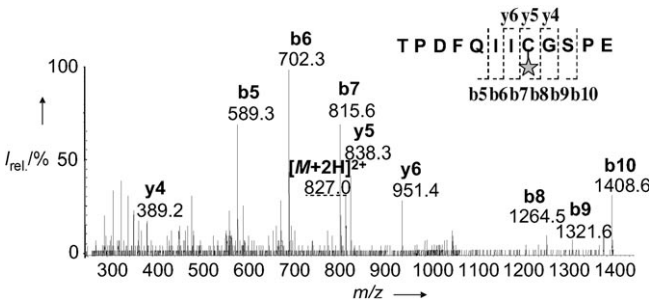
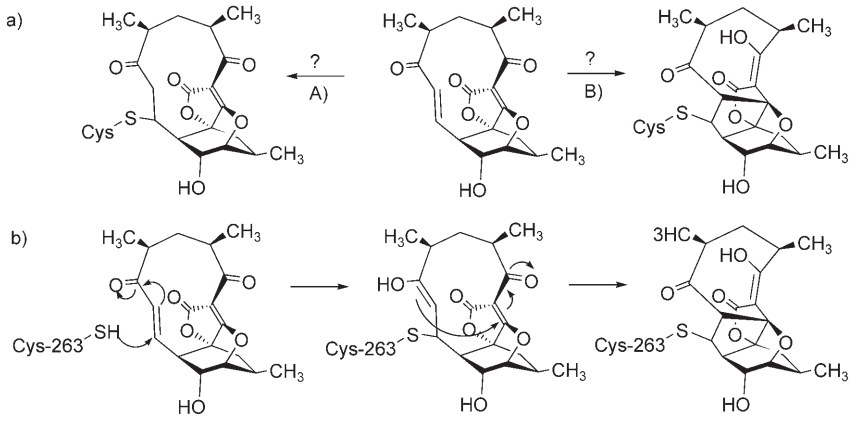


Figure 2. MS/MS sequencing of the abyssomicin-bound PabB target peptide (*m/z* 827.0) after GluC digest. Cys263 has been covalently modified by atrop-abyssomicin C.



Scheme 2. Function of atrop-abyssomicin C as a Michael acceptor. a) Michael addition (pathway A) and dual Michael addition with subsequent rearrangement to an abyssomicin D derivative (pathway B). b) Proposed reaction with Cys263 based on conversion with S nucleophiles 2-sulfanylethanol and *N*-acetylcysteine, respectively.

Since atrop-abyssomicin (**2**) binds to the side chain of Cys263, the question was raised whether a simple Michael addition occurs, or if a rearrangement to an abyssomicin-D-like structure takes place, similar to that of addition of a hydride equivalent to **2** (Scheme 2).[8,9] To test this hypothesis, we investigated the performance of **2** with S nucleophiles. A sample of **2** in tetrahydrofuran was incubated with 2-sulfanylethanol or *N*-acetylcysteine. The products were purified by preparative HPLC and analyzed by 2D NMR. Strikingly, the nucleophilic attack of 2-sulfanylethanol as well as that of *N*-acetylcysteine lead to the conversion of **2** into a derivative of abyssomicin D (data in the Supporting Information).

In conclusion, we have shown that the PabB of ADC synthase from *B. subtilis* is a molecular target of atrop-abyssomicin C (**2**). It acts as a covalent binder to the side chain of Cys263, located in the proximity of the active site of PabB.[13] Covalent binding to other potentially nucleophilic side chains of Ser, Thr, and Lys has not been observed under the reaction conditions described. The micromolar range of the inhibition constant K_I^{app} (390 μM) determined for **2** is not uncommon for irreversible inhibitors. Aspirin, which is without doubt a potent drug, has a K_I^{app} value of 14 mM.[14] The most potent inhibitor known for *E. coli* ADC synthase is 2-fluorochorismate, which is a specific synthetic inhibitor of

PabB and was described by Abell and co-workers.[15] 2-Fluorochorismate shows a k_i value of 1.0 min^{-1} and a K_I value of 130 μM. As compared to atrop-abyssomicin C, abyssomicin C has a significantly lower potential to inhibit ADC biosynthesis in vitro. This finding is consistent with the stronger inhibitory effect found in minimum inhibitory concentration (MIC) tests of **2** as compared to **1**.[6,9] This result is presumably due to **2** being a more powerful Michael acceptor than **1**, as discussed by Harrison and Nicolaou.[6] Conformational differences of **1** and **2** that alter their affinity towards the active site of the enzyme might play a role as well. The in vitro reaction of **2** with S nucleophiles under rearrangement towards a structure of the abyssomicin D type suggests that this observation is also valid for the reaction with PabB (Scheme 2). It may be speculated that the distortion of the abyssomicin structure induces a conformational change of the target protein. Current experiments by our group are directed towards investigation of other ADC synthases as well as towards determining the crystal structure of ADC synthase bound to atrop-abyssomicin C.

Experimental Section

NMR experiments were measured on a DRX500 NMR spectrometer (Bruker) equipped with a 5-mm-diameter broad-band inverse probe head with *z* gradients. Spectra were recorded in and referenced to [D_4]methanol ($\delta = 3.30$ ppm; $\delta = 49.0$ ppm). 2D COSY, NOESY, HMQC, and HMBC experiments were measured with standard Bruker parameters (XWinNMR 3.2). LC-MS experiments were performed on a 2000 Q Trap mass spectrometer (Applied Biosystems/MDS Sciex) coupled to an Agilent 1100 HPLC system (Agilent).

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