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Abyssomicins, Inhibitors of the *para*-Aminobenzoic Acid Pathway Produced by the Marine *Verrucosispora* Strain AB-18-032†

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A screening method was established to detect inhibitors of the biosynthetic pathways of aromatic amino acids and *para*-aminobenzoic acid, the precursor of folic acid, using an agar plate diffusion assay modified as an antagonism test. By this screening method, a family of three novel polycyclic polyketides named as abyssomicins was isolated from a marine strain of *Verrucosispora*. The main component abyssomicin C inhibits the pathway between chorismate and *para*-aminobenzoic acid and is strongly active against Gram-positive bacteria, including multi-resistant clinical isolates of *Staphylococcus aureus*.

A successful search for novel antibacterial metabolites has to meet three criteria, first, a specific target which is essential for the metabolism of a bacterium and not yet provided with an known inhibitor. Second, a set of taxonomically characterized and dereplicated microorganisms as producers of secondary metabolites, and last but not least a lucky but experienced hand for strain isolation and cultivation. We have chosen the shikimate pathway as an essential target of bacterial metabolism, with special consideration of the biosynthesis of aromatic amino acids and *para*-aminobenzoic (*p*Aba) acid derived from the key-metabolite chorismate. Only a few antimetabolites are known as inhibitors of aromatic amino acids, such as L-2,5-dihydrophenylalanine[2], an antagonist of phenlyalanine, and glyphosate that inhibits 3-enolpyruvylshikimate-3-phosphate synthase[3,4]. To our knowledge, no natural product inhibitor of *p*Aba biosynthesis has been described in the literature. This pathway, which is catalyzed by two enzymes, 4-amino-4-deoxychorismic acid (ADC) synthase and ADC lyase, seems to be of considerable interest for the development of novel antibiotics since it is directly linked to folic acid biosynthesis, which is established in plants, fungi, prokaryotes and parasites of the apicomplexa group (*Plasmodium*, *Toxoplasma*) but not in vertebrates.

As suitable producers of bioactive metabolites we screened within the order *Actinomycetales* terrestrial and marine members of the families *Streptomycetaceae* and *Micromonosporaceae* and rare actinomycete genera. A total of 930 extracts derived from 201 actinomycetes were subjected to the screening. Among them, only AB-18-032, an extract from a marine isolate from a sediment collected from the Sea of Japan, was found to exhibit activity against

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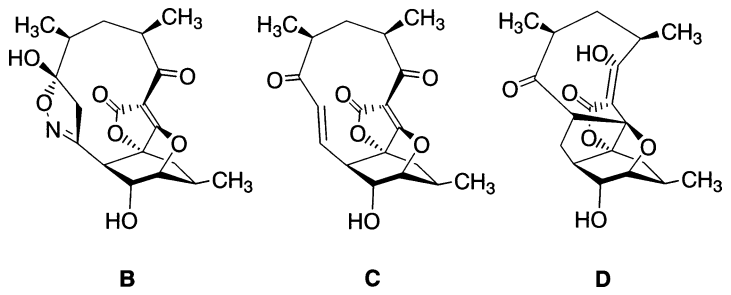
We dedicate this publication to Professor Dr. Axel Zeeck in honour of his 65th birthday.

Fig. 1. Structural formulae of abyssomicins.



*p*Aba biosynthesis. The secondary metabolite profile obtained by HPLC-diode array (HPLC-DAD) analysis could not be assigned to any known metabolites by means of our HPLC-UV-Vis Database that contains about 750 entries, most of them are antibiotics[5]. Therefore, strain AB-18-032 was further investigated for its active principle found in the extract. Three metabolites named as abyssomicins B, C and D were isolated and characterized by analytical methods as reported by BISTER *et al.*[6]. The chemical structures are shown in Fig. 1. Abyssomicin C, the main component of the abyssomicin complex, inhibits *p*Aba biosynthesis, whereas the other components showed no antibacterial activity[7].

This report deals with the screening concept, the taxonomy of the producing strain, its fermentation, and the isolation and biological properties of abyssomicins.

Material and Methods

Screening for Inhibition of the Aromatic Amino Acid and *p*Aba Pathways

The screening was carried out in two stages: In the first stage, a growth inhibition reversal test was done. *Bacillus subtilis* DSM 10 was grown in an agar plate using a minimal medium consisted of glucose 0.5%, tri-sodium citrate$\times 2H_2O$ 0.05%, KH_2PO_4 0.3%, K_2HPO_4 0.7%, $MgSO_4\times 7H_2O$ 0.01% and $(NH_4)_2SO_4$ 0.1% in deionized water. In parallel, the *B. subtilis* strain was also grown in the same agar medium but supplemented with: L-tryptophan (Trp), L-phenylalanine (Phe), L-tyrosine (Tyr) and *p*Aba, each at a concentration of 5 mM. 10 μl of extract applied on a filter disk were tested on both agar plates. The extracts caused an inhibition zone exclusively on the agar plate without the addition of aromatic amino acids and *p*Aba were selected for further evaluation in the second stage of the screening.

In the second stage, an antagonism test was used. Two filter strips were placed on an agar plate across each other. The miminal medium was seeded with *B. subtilis* in the same manner as in the agar plate diffusion assay. One strip was soaked in the extract solution, the other in a solution of the suspected antagonist. The following antagonist combinations were used (50 μl in a concentration of 5 mg/ml of each antagonist), (a) Tyr+Trp+Phe+*p*Aba (control), (b) Tyr+Phe, (c) Trp, (d) *p*Aba. In the case of an antagonism, a competitive reversal of the growth inhibition will be observed.

Microorganisms

The producing strain AB-18-032 was isolated from a sediment sample collected from the Sea of Japan at a depth of 289 meters in August 1991. The strain is deposited as DSM 15899 in the culture collection of DSMZ, Braunschweig, Germany.

Standard strains for testing the biological activity spectrum were obtained from DSMZ and the stock collection of our laboratory at the University of Tübingen. Multi-resistant *Staphylococcus aureus* strains N315 and Mu50 were kindly provided by Professor HIRAMATSU, Department of Bacteriology, Juntendo University, Tokyo[8].

Taxonomy of the Producing Strain

Strain AB-18-032 was inoculated onto oatmeal agar[9] (ISP3), incubated at 27°C for 2 weeks, and examined visually for substrate mycelium pigmentation. Spore morphology and ornamentation were determined by scanning electron microscopy following the procedure described by O'DONNELL *et al.*[10]. The isomeric form of diaminopimelic acid (A_2pm) was determined by TLC

of a whole-organism hydrolysate following a standard procedure[11]. The composition of cell-wall sugars was analyzed by the method of CUMMINS & HARRIS[12], and menaquinones were determined by HPLC using the protocol described by COLLINS[13]. 16S rRNA gene amplification and sequencing were carried out after CHUN & GOODFELLOW[14] and KIM *et al.*[15].

Fermentation

Batch fermentations of strain AB-18-032 were carried out in 10-liter stirred tank fermenters (New Brunswick). The medium consisted of starch 1%, glucose 1%, glycerol 1%, corn steep powder (Marcor) 0.25%, peptone (Difco) 0.5%, yeast extract 0.2%, NaCl 0.1%, $CaCO_3$ 0.3% in tap water; pH 7.3 was adjusted prior to sterilization. The fermenter was inoculated with 5 vol.-% of shake cultures, grown in 500-ml Erlenmeyer flasks with one baffle for 48 hours on a rotary shaker at 120 rpm and 27°C using the same medium. The fermentations were carried out for 96 hours at 27°C with an aeration rate of 0.5 vvm and an agitation of 200 rpm.

Isolation

Hypflo Super-cel (2%) was added to the fermentation broth (30 liters), which was separated by multiple sheet filtration into culture filtrate and mycelium. The culture filtrate (23 liters) was adjusted to pH 7 (5 N HCl) and extracted twice with 5 liters EtOAc. The organic extract was concentrated *in vacuo* to an oily residue (6.05 g), which was dissolved in 200 ml MeOH, split into four portions and concentrated to dryness. Each aliquot was dissolved in 5 ml MeOH and separated on a Sephadex LH-20 column (90×5 cm) using MeOH as eluent. Abyssomicin containing fractions were concentrated to dryness *in vacuo* and subjected to a LiChroprep Diol column (42×2.6 cm; Merck). The separation was accomplished by a linear gradient elution using CH_2Cl_2 - MeOH starting at CH_2Cl_2 to 10% MeOH within 4 hours at a flow rate of 5.6 ml/minute. Abyssomicin containing fractions were combined and concentrated *in vacuo* to dryness. Finally, pure abyssomicins were obtained by size-exclusion chromatography using Toyopearl HW-40F (90×2.5 cm; Tosoh Biosep) and MeOH as eluent. Abyssomicins were dissolved in small volumes of *tert*-BuOH and obtained as white powders after lyophilisation.

HPLC-DAD-Analysis

The chromatographic system consisted of an HP 1090M liquid chromatograph equipped with a diode-array detector and HP Kayak XM 600 ChemStation (HPLC-software revision A.08.03; Agilent Technologies). Multiple wavelength monitoring was performed at 210, 230, 260, 280, 310, 360, 435 and 500 nm. The spectrum measured was from 200 to 600 nm with a 2-nm step and a sampling rate of 640 mseconds.

A 5-ml sample of the culture filtrate was adjusted to pH 7 and extracted with the same volume of EtOAc. After centrifugation, the organic layer was concentrated to dryness *in vacuo* and the residue was resuspended in 0.5 ml MeOH. 10 μl of the samples were injected onto an HPLC column (125×4.6 mm), fitted with a guard-column (20×4.6 mm) which was packed with 5-μm Nucleosil-100 C-18 (Maisch). The samples were analyzed by linear gradient elution using 0.1% *ortho*-phosphoric acid as solvent A and acetonitrile as solvent B at a flow rate of 2 ml/minute. The gradient was from 0% to 100% solvent B and elution was made in 15 minutes with a 1-minute hold at 100% solvent B, followed by a 5-minute post-time at initial conditions.

Biological Assays

The antimicrobial spectrum of abyssomicins was determined by an agar plate diffusion assay. 10 μl of the samples were added on filter discs (6 mm diameter). The test plates were incubated for 24 hours at the temperature that permitted optimal growth of the test organisms.

For determining the minimal inhibition concentration of abyssomicin C a microtiter plate assay was used. The test strains *Staphylococcus aureus* N315 (MRSA strain) and *Staphylococcus aureus* Mu50 (MRSA including vancomycin resistance) were grown in 96-well microtiter plates in a medium consisting of glucose 0.1%, yeast extract 0.5%, soy peptone 1%, K_2HPO_4 0.1% and NaCl 0.5% in deionized water (pH adjusted to 7.2). The antibiotic was dissolved in DMSO; the final DMSO concentrations in the cultures did not exceed 5%. Growth inhibition was evaluated after incubation for 24 and 48 hours at 27°C on a rotary shaker by determining the optical density at 578 nm.

Results

The Screening Concept

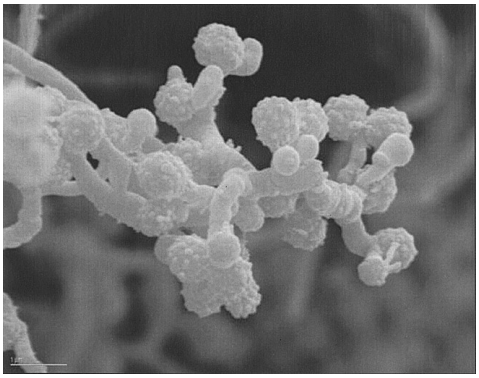
A simple biological assay was developed for the screening of antibacterial activities against a target that has not been previously utilized, which is the biosynthetic pathway from chorismate to *p*Aba. *p*Aba is a direct precursor of folic acid, the latter being an essential vitamin for bacteria and some protozoic parasites, *e.g. Plasmodium*

species. In a pre-screen using an agar plate diffusion assay with *Bacillus subtilis* as test organism, only extracts from microorganisms were of interest which showed a selective antibacterial activity against the test organism grown on a minimal medium, and no activity was observed when grown on a complex medium.

In the second stage of testing these previously active extracts were submitted to a more detailed antagonism assay that allowed us to distinguish between the inhibition of the discrete biosynthetic pathways of Trp, Tyr/Phe and to *p*Aba branching off from chorismate. Similarly, the inhibition of the biosynthetic pathway prior to chorismate was tested. As a consequence, four agar plates in which *B. subtilis* was seeded in a minimal medium were necessary in the antagonism test for the evaluation of each extract. The filter paper strip which was placed on the agar plate across the antibiotic containing strip was soaked with solutions of (a) Tyr+Trp+Phe+*p*Aba, (b) Tyr+Phe, (c) Trp, and (d) *p*Aba, respectively. When the growth inhibition was competitivly reversed exclusively by (a), an inhibitor of an enzyme before chorismate can be assumed. An inhibitor of the biosynthetic pathway from chorismate to *p*Aba can be expected when the growth inhibition caused by the sample was competitivly reversed by (d). The observance of inhibition zones for (b) and (c) indicates the detection of inhibitors of the aromatic amino acid pathways, Tyr/Phe and Trp, respectively.

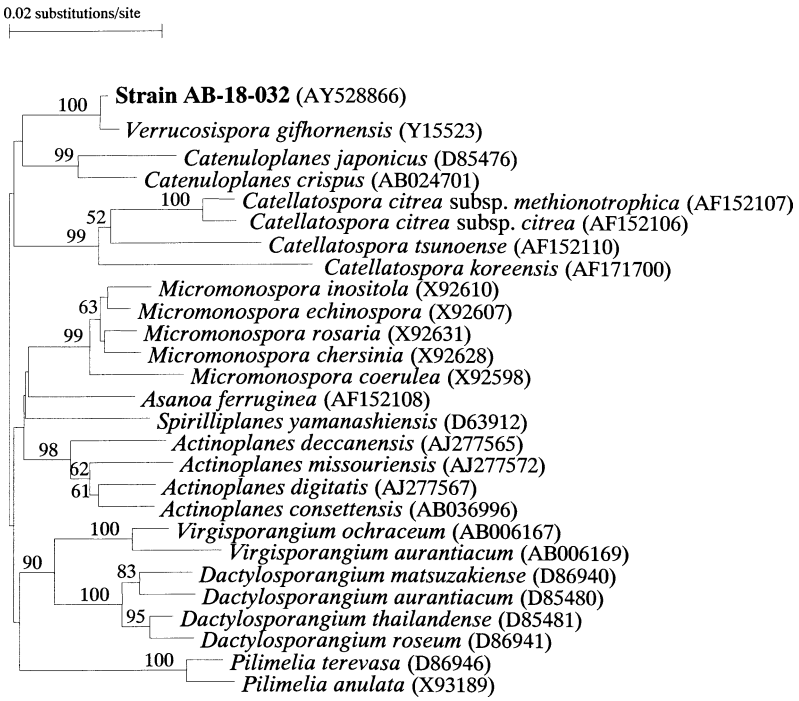
The screening concept was evaluated with extracts from the extract collection of our laboratory at the University of

Fig. 2. Scanning electron micrograph of strain AB-18-032.



Bar, 1 μm.

Fig. 3. Neighbour-joining phylogenetic tree based on nearly complete 16S rDNA sequences of members of the suborder *Micromonosporineae*.



The numbers at the nodes indicate the levels of bootstrap support based on a neighbour-joining analysis of 1,000 resampled datasets; only values above 50% are given. The scale bar indicates 0.02 substitutions per nucleotide position.

Tübingen which were exclusively distinguished by an antibacterial activity against test organisms grown on minimal medium. The resulting 930 extracts originated from 201 actinomycete strains, in detail 104 terrestrial members of the family *Streptomycetaceae*, 33 members of the family *Micromonosporaceae* (8 terrestrial, 25 marine), and 64 members of rare actinomycete taxa (55 terrestrial, 9 marine). Only the extracts from one actinomycete, the marine strain AB-18-032, showed an inihibitory activity which was competitivly reversed by (d) indicating an

Table 1. Phenotypic properties of strain AB-18-032 and *V. gifhornensis* DSM 44337.

	Strain AB-18-032	***V. gifhornensis* DSM 44337**
Carbon source utilization:		
Glycerol	+	–
L(+)-Arabinose	++	++
D(-)-Ribose	–	–
D(+)-Xylose	–	++
D(-)-Fructose	–	–
D(+)-Galactose	–	+
D-Glucose	+	++
D(+)-Mannose	++	±
L-Rhamnose	–	–
L-Sorbose	–	–
Dulcitol	+	–
meso-Inositol	+	–
D-Mannitol	+	–
D-Sorbitol	+	–
α-Lactose	+	–
D-Maltose	++	+
α-D-Melibiose	+	–
D-Sucrose	++	+
D(+)-Trehalose	++	+
D(+)-Melezitose	+	–
α-D-Raffinose	+	–
Salicin	+	+
Nitrogen source utilization:		
DL-Serine	++	+
L-Aspartic acid	+	+
L-Glutamic acid	++	+
L-Histidine	+	+
L-Arginine	++	+
L-Alanine	++	±
L-Valine	++	±
L-Methionine	++	–
L-Phenylalanine	++	+
L-Tryptophan	++	–

++, very good growth; +, good growth; ±, poor growth; –, no growth

inhibitor of the *p*Aba biosynthesis.

Taxonomy of the Producing Strain

Strain AB-18-032 was assigned to the family *Micromonosporaceae* on the basis of its morphological and chemotaxonomic properties. The organism forms a well developed, branched, red coloured substrate mycelium which carries single spores which have a warty ornamentation (Fig. 2). Aerial hyphae and sporangia were not found. The peptidoglycan is rich in *meso*-diaminopimelic acid and contains N-glycolated muramic acid. Mannose and xylose are the major sugars and hexahydrogenated menaquinones with nine isoprene units the predominant isoprenologue.

A nearly complete 16S rRNA gene sequence (1440 nucleotides) of strain AB-18-032 was compared with corresponding sequences of representatives of the suborder *Micromonosporineae* (Fig. 3). It is evident from the Figure that the tested strain is most closely related to the type strain of *Verrucosispora gifhornensis*[16], the sole representative of the genus *Verrucosispora*. It is evident from Table 1 that the two strains can be distinguished using a combination of phenotypic properties.

Fermentation and Isolation

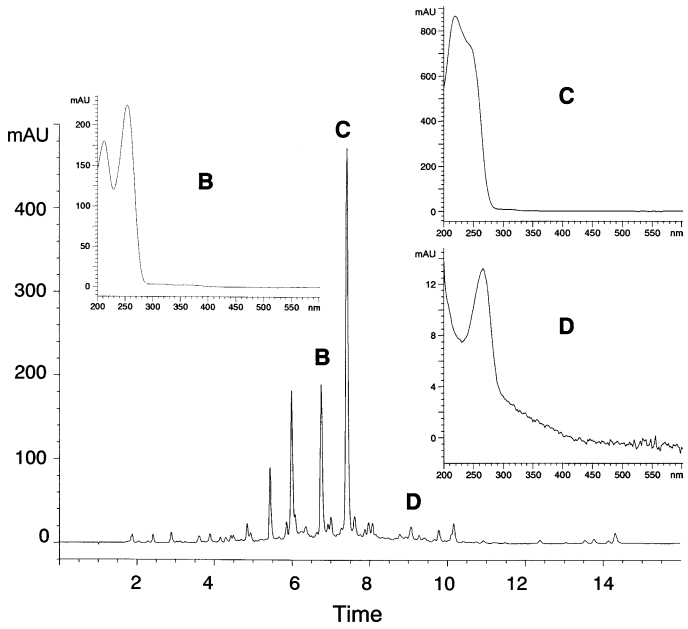
Batch fermentations were carried out in 10-liter stirred tank fermenters using a complex medium. The culture reached a maximal biomass of 10 vol-% after 48 hours of incubation, at which time abyssomicin production began, and maximal abyssomicin C yields reached 59 mg/liter after 96 hours. The HPLC analysis of the culture filtrate extract is shown in Fig. 4.

Abyssomicins were isolated from the culture filtrate by extraction with ethyl acetate and were purified by subsequent chromatography on Sephadex LH-20, diol-modified silica gel and Toyopearl HW-40F. Abyssomicins were obtained as white powders after lyophilization.

Biological Properties

The antimicrobial spectra of abyssomicins B, C and D were examined by an agar plate diffusion assay. Only abyssomicin C was antibiotically active and showed a strong inhibitory activity against Gram-positive bacteria (Table 2). Gram-negative bacteria and fungi were not sensitive to abyssomicin C. As could be expected, the inhibitory activity of abyssomicin C was significantly stronger when the test organisms were cultivated in minimal media, as shown for the representative test

Fig. 4. HPLC analysis of a culture filtrate extract from strain AB-18-032 at a fermentation time of 96 hours, monitored at 260 nm.



Inserts: UV-visible spectra of abyssomicins B, C and D.

Table 2. Antibacterial spectrum of abyssomycin C, determined by the agar plate diffusion assay (inhibition zones in mm).

Organisms	Abyssomicin C (mg/ml)		
	1	0.3	0.1
Arthrobacter aurescens DSM 20166 [a]	14	10	–
Brevibacillus brevis DSM 30 [a]	17	12	9
Brevibacillus brevis DSM 30 [b]	30	24	19
Bacillus subtilis DSM 10 [a]	16	12	10
Bacillus subtilis DSM 10 [c]	26	19	14
Micrococcus luteus ATCC 381 [a]	–	–	–
Mycobacterium phlei DSM 750 [a]	–	–	–
Staphylococcus aureus DSM 20231[a]	19	11	9
Rhodococcus erythropolis DSM 1069 [a]	27	18	15
Rhodococcus erythropolis DSM 1069 [c]	30	28	21
Streptomyces viridochromogenes Tü 57 [a]	11	9	–

[a] complex medium

[b] chemically defined medium (per liter): glucose 8 g, *di*-ammonium tartrate 4 g, NaCl 5 g, K_2HPO_4 2 g, $MgSO_4 \times 7H_2O$ 1 g, $CaCl_2$ 0.2 g, $MnSO_4 \times 1H_2O$ 0.01 g, ferrioxamine B 0.02 g, Bacto agar 15 g

[c] chemically defined medium (per liter): glucose 5 g, *tri*-sodium citrate$\times 2H_2O$ 0.5 g, KH_2PO_4 3 g, K_2HPO_4 7 g, $MgSO_4 \times 7H_2O$ 0.1 g, $(NH_4)_2SO_4$ 1 g, Bacto agar 15 g

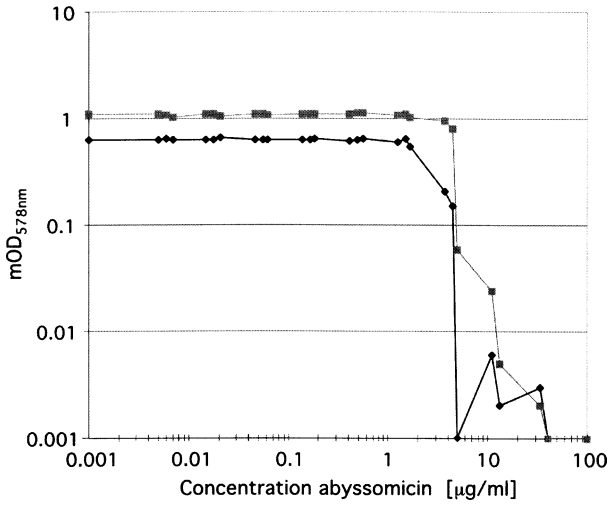
organisms *Brevibacillus brevis*, *Bacillus subtilis* and *Rhodococcus erythropolis*.

The minimal inhibitory concentration of abyssomicin C against two clinical isolates of *Staphylococcus aureus* are shown in Fig. 5. Strain *S. aureus* N315 represents a multi-resistant isolate (MRSA), and strain *S. aureus* Mu50 is provided in addition with an intermediate resistance against vancomycin. The MIC value of abyssomicin C against strains *S. aureus* N315 and *S. aureus* Mu50 are in the range of 4 μg/ml and 13 μg/ml, respectively.

Discussion

Our screening concept for the detection of novel antimicrobial metabolites was designed and intended for the screening of sample numbers that can be handled without the use of expensive robotic systems and, for the assays that do not require high-throughput operation systems. The shikimate pathway that leads to the biosynthesis of aromatic amino acids and *p*Aba was chosen as our target. These biosynthetic pathways are both absent in humans, therefore, inhibitors of enzymes involved in these pathways are expected to be excellent candidates as selective antibacterial, antifungal and antiprotozoic agents. A whole-cell system *versus* an enzyme assay was chosen to prevent the tremendous efforts needed for the isolation of the numerous enzymes involved in these biosynthetic pathways. The early pathway steps leading from shikimate to chorismate and that from chorismate to *p*Aba seemed to be especially promising targets. No inhibitor is known of the biosynthesis of *p*Aba, which is an essential pre-cursor in the biosynthesis of folic acid, whereas several synthetic and natural products are known as potent antifolate

Fig. 5. Minimal inhibition concentration (μg/ml) of abyssomicin C against multi-resistant *S. aureus* N315 (—◆—) and *S. aureus* Mu50 (—■—).



inihibitors. Sulfa drugs inhibit strongly dihydropteroate synthase[17] whereas trimethoprim[18], methotrexate[19] and pyrimethamine[20] inhibit dihydrofolate reductase and are used clinically as antibacterial, anticancer and antimalarial drugs, respectively. Diazaquinomycins[21], antibiotic AM-8402[22] and 7-hydro-8-methylpteroylgutamylglutamic acid[23] inhibit folate metabolism at the site of thymidylate synthase.

A second pre-condition in the strategy for developing a successful screening concept is based on the selection of producing organisms. Not only terrestrial streptomycete strains, but also dereplicated terrestrial and marine members of the family *Micromonosporaceae* and especially of rare actinomycete taxa were included in the screening program. It was an astonishing result that the only strain that produced an inhibitory activity of *p*Aba was a new and the second member of the genus *Verrucosispora*, isolated from a marine sediment. The strain produced a family of three novel polycyclic polyketides which were elucidated in structure and named as abyssomicins B, C and D[6]. X-Ray crystal data suggested that the antibiotically active member of the abyssomicin complex, abyssomycin C, represents a substrate mimic of chorismate. The comparison of all three abyssomicins suggests further a Michael-addition based enzyme-trapping mechanism, acting as antagonists of chorismate to the catalyzing enzymes ADC synthase and/or ADC lyase[6]. The target and the mode of action in the inhibition of the *p*Aba biosynthesis implies a potential application of abyssomicin C as an antimalarial drug and as a novel antiinfective drug against multi-resistant Gram-positive bacteria, especially staphylococci which show resistance against all clinically used antibiotics including vancomycin. These promising applications are currently under investigation.

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