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Venturia inaequalis-Inhibiting Diels−Alder Adducts from Morus Root Bark

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In organic apple orcharding there is a continuous need for natural fungicides effective against *Venturia inaequalis* (Cooke) Winter, the causal agent of apple scab. In this study an in vitro assay is presented for determining the germination inhibitory potential of extracts and pure compounds. From a screening of plant extracts, the methanol extract of Morus root bark revealed distinct *V. inaequalis* inhibiting qualities, which were subjected to a bioguided fractionation. Among the isolated metabolites [moracins M (**1**), O/P (**2**), kuwanon L (**3**), and sanggenons D (**4**), B (**5**), G (**6**), O (**7**), E (**8**), and C (**9**)] all the Diels−Alder adducts (**3**−**9**) showed an antifungal activity with IC_{50} values between 10 and 123 μM. The in vitro activity of the most active fraction (A5, IC_{50} 39.0 ± 4.2 μg/mL) was evaluated in vivo, confirming a distinct antifungal activity against *V. inaequalis* for the tested natural material.

KEYWORDS: Fungal pathogen; apple scab; in vitro and in vivo assay; Morus root bark; Sang-Bai-Pi; sanggenon; bioguided fractionation

INTRODUCTION

In the cultivation of apples, apple scab is the most serious disease because it causes yield decrease and loss of fruit quality. In conventional farming, the causative organism of apple scab, *Venturia inaequalis* (Cooke) Winter, is usually fought with synthetic fungicides. By contrast, in organically managed orchards only elementary and lime sulfur products as well as copper preparations may be employed (*1*). However, these "organic pesticides" are less effective than conventional pesticides and exhibit some problematic side effects such as a considerable phytotoxicity (*1*). More effective organic alternatives are therefore being called for, of which the use of plant extracts exhibiting antifungal activity seems most promising.

Low-molecular-weight secondary metabolites, which plants create in response to abiotic or biotic stress, are targeted against the stimulating pathogen (*2*). The antifungal capacity of the phytoalexin resveratrol and its glucoside piceid was successfully tested in vitro for the compounds' effectiveness to inhibit *V. inaequalis* (*3*). Moreover, a significant increase of the malusfuran content was observed in a scab-resistant apple cultivar after inoculation, an effect not found in a scab-susceptible apple cultivar (*4*). Another recent suggestion was using commercial grapefruit seed extracts (GSE) in organic scab control. A powerful antimicrobial activity was repeatedly reported (*5*−*7*). However, considerable amounts of preservatives were detected in all commercial GSE investigated so far (*8*−*11*).

In the present study, an in vitro assay was established to screen plant extracts for their inhibiting effect on the germination of *V. inaequalis* conidiospores. Among the extracts investigated, the methanol extract of the root bark of *Morus* sp. (Moraceae) emerged as the most promising one.

Morus root bark, which is listed in the Chinese Pharmacopoeia as Sang-Bai-Pi (*12*), has been used for centuries in folk medicine for the treatment of arthritis and rheumatism, as a diuretic, tonic, and sedative agent. A number of phytochemical and pharmacological investigations scrutinized the effects postulated by traditional medicine (e.g. *13*, *14*), and searched for the bioactive constituents (*15*−*18*). *Morus alba* L. (Moraceae), the main source of Sang-Bai-Pi, is characterized by a high content of prenylated flavanoids and prenylated benzofuran derivatives (*19*). The biosyntheses of some representatives of these chemical classes are stimulated by pathogen attack (*20*, *21*).

In this study, antifungal compounds were isolated from the methanol Morus root bark extract by bioguided fractionation. By this approach, the aim of this study was to identity the antifungal components within the active extract and to determine the germination inhibiting effect of the isolated single chemical entities. Finally, a further goal was to verify the in vitro activity

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by application and evaluation of the *V. inaequalis* inhibiting plant material in vivo on inoculated seedlings.

MATERIALS AND METHODS

Plant Material. The dried root bark of *Morus* sp. L., Moraceae, was purchased from Plantasia (Import und Vertrieb asiatischer Heilkräuter, Oberndorf, Austria). Its quality was macro- and microscopically checked according to the monograph Sang-Bai-Pi of the Chinese Pharmacopoeia. Voucher specimens (JR-20020704A1) are deposited in the Herbarium of the Department of Pharmacognosy, Institute of Pharmacy, University of Innsbruck, Austria.

General. Optical rotation was measured on a Perkin-Elmer 341 polarimeter. Melting points were determined on a Kofler hot-stage microscope and are uncorrected. NMR spectra were recorded on a Bruker AM-300 spectrometer (Karlsruhe, Germany) at 300 and 75 MHz, respectively. Spectra were recorded in acetone-d_6 and calibrated to the residual solvent signal ($\delta_H = 2.05$ ppm and $\delta_C = 29.5$ ppm). Upon request, NMR spectra can be obtained from the corresponding author. Thin-layer chromatography (TLC) fractions from chromatographic separations were combined by similarity based on TLC investigations with Merck 40−63 μm silica gel 60 F_{254} TLC plates (Darmstadt, Germany), with a mixture of CH_2Cl_2 to CH_3OH (7/1 v/v) as the mobile phase. Moracins were detected as blue spots and sanggenons as red spots after spraying with vanillin (1% in MeOH) and sulfuric acid (5% in MeOH) and subsequent heating.

Liquid chromatography−mass spectrometry (LC-MS) HPLC parameter analyses were performed on an Agilent 1100 system, equipped with a photodiode array detector (DAD) and auto sampler. The LC was fitted with a Zorbax SB C-18 column, 150 × 4.6 mm i.d., 3.5 μm particle size (Agilent Technologies), guard column (Merck, Germany) RP-18; detection wavelength, 205 nm at a column temperature of 25 °C and a flow rate of 1.0 mL/min, injection volume 10 μL. The mobile phases consisted of A, water, and B, CH_3CN; linear gradient, 0 min 30% B, 5 min 45% B, 20 min 55% B, 22 min 98% B, stop time 30 min. MS parameters: the mass spectrometer, Finnigan MAT SSQ 7000 (Finnigan MAT, San Jose, CA), was equipped with a Digital DEC 3000 data station (Digital Equipment Corp., Maynard, MA), For electrospray ionization (ESI) (in negative or positive mode): LC flow split 1:5; capillary temperature 200 °C; for negative mode, spray voltage −4.5 kV, and for positive mode, 4.5 kV; for CID (collision-induced dissociation) 0 V in both cases; nebulizer 40 psi. For semipreparative HPLC, a Dionex system with a P580 pump, ASI-100 autosampler, UVD 170U detector (detection at 205 nm), and a Gilson 206 fraction collector was used. The system was fitted with an XTerra MS C_{18} column (5 μm; 7.8 × 100 mm) at a column temperature of 25 °C and a flow rate of 3.0 mL/min. All chemicals were analytical grade. Solvents were either analytical grade or puriss. grade and were distilled before use.

Extraction and Isolation. The dried root bark was crushed to coarse powder and premacerated with dichloromethane [3.5 L of CH_2Cl_2, at room temperature (RT), twice for 5 days]. The remaining plant material was macerated exhaustively with CH_3OH (3.0 L, at RT, twice for 5 days) to yield 46.0 g. This crude extract was separated by silica gel column chromatography (CC) as reported previously (*16*). From fraction A5, pure compounds **1**, **2**, **4**, **5**, **7**, **8**, and **9** have been isolated by different chromatographic methods and characterized as described previously (*16*). To isolate compounds **3** and **6**, newly prepared fraction A5 (3.1 g) was fractionated by silica gel flash CC employing a gradient of CH_2Cl_2 to CH_3OH, resulting in 12 fractions (B1−12). Fractions B7 and B8, enriched with **3** and **6**, were combined (270 mg) and further purified by Sephadex LH-20 (Pharmacia Biotech, Sweden) CC with CH_3OH as eluant to yield 12 fractions (C1−12). C6 (49.8 mg) was subjected to semipreparative HPLC with a mobile phase of water/CH_3CN (0 min, 49:51; 21 min, 40:60; 22 min, 10:90) to yield 12.3 mg of compound **6**. C7 (29.2 mg) was as well purified by semipreparative HPLC (water/acetonitrile; 0 min, 58:42, 15 min, 52:48, 20 min, 10:90) to yield 9.0 mg of compound **3**. Physical and spectroscopic data are consistent with data in literature (*22*, *23*).

***Venturia inaequalis* Assays: (A) Origin of Conidiospores from *Venturia inaequalis* (Cooke) Winter.** The inoculum was collected in spring 2004 from an organic orchard of Golden Delicious cultivar owned by the Research Centre for Agriculture and Forestry Laimburg (Auer, Italy). Fifty scab lesions were cut out from 60−70 diseased leaves randomly collected from several trees. The lesions were placed into 60 mL of water and after vigorous shaking the conidia were filtered through a 30 μm mesh. The conidia suspension was adjusted to about 1×10^5 conidia/mL, and 28 seedlings were inoculated with the conidial suspension by use of a nebulizer. The inoculated plantlets were incubated in an experimental greenhouse containing eight independent glass chambers (3 × 4 × 3 m). Plantlets were kept for 24 h at 22 °C and 100% relative humidity (RH) (provided by a humidification nozzle, Jet D-ZW/3, Stielow, Beselich-Obertiefenbach, Germany). In the first experiment, plantlets were kept at an average of 80.9% RH (min 49.2%, max 90.2%) and 20.5 °C (min 16.5 °C, max 37.8 °C) until sporulation. In the second experiment, mean RH was 75.2% (min 36.2%, max 100%) and mean temperature 21.4 °C (min 16.0 °C, max 31.4 °C). Natural light was supplemented by a greenhouse lamp (HPS 400 W, Hortilux Schreder, Monster, The Netherlands) in an 8/16 h rhythm. Seedling leaves exhibiting sporulation lesions were stored at −30 °C. Spore suspensions for subsequent experiments were prepared from the stored inoculum.

(B) In Vitro Assay. The *V. inaequalis* bioassay employed in the current investigation represents a modification of the protocols of Olaya and Köller (*24*) and Gilliver (*25*). The advantages of the test model are cheapness, a minimum requirement for technical equipment, and suitability for minute amounts of test compounds. Furthermore, visual evaluation of the tests allows for quantitative and qualitative discrimination of the antimicrobial activity of the tested extracts and pure compounds.

Tests were performed on glass slides used for microscopy (Menzel-Gläser, Braunschweig, Germany, 76 × 26 mm). Extracts and pure compounds were dissolved in a suitable organic solvent (CH_2Cl_2 or CH_3OH). To obtain a test concentration of 5.00 mg/mL, aliquots of 30 μL of each test solution were placed on glass slides, three per slide. The positive control was made up of 30 μL of a 5.00% (m/v) solution of benzethonium chloride in either CH_2Cl_2 or CH_3OH, according to the solvent used for preparation of the test solutions. Each sample was tested in triplicate. Prior to incubation, all slides were kept for 4 h in a fume-hood to guarantee complete evaporation of the organic solvent. Spore solutions were obtained by swiveling 1 cm^2 of an infected apple leaf in distilled water for 10 min. The spore solution was filtered through a 30 μm net. Consecutively, 30 μL of spore solution were placed on each of the sample plaques. The control was made up of 30 μL of spore solution. Three glass slides at a time were placed in a polystyrene Petri dish (15 × 15 cm) along with four water-soaked sponge pieces (1 × 5 × 0.1 cm) and stored in an airtight plastic container in order to maintain the required high level of humidity. After 24 h of incubation at 22 °C (±1 °C), samples were evaluated by transmitted light microscopy (Reichert-Jung, Polyvar, Wetzlar, Germany). The samples were covered with a glass slip for microscopy and analyzed at 400-fold magnification.

From each object, 200 conidia in randomly chosen visual fields were counted and classified as either "fully germinated" (class 1, germination tube longer than conidiospore; **Figure 1a**), "not germinated at all" (class 2; **Figure 1b**), or "hardly germinated" (class 3, length of germination tube shorter than or equal to conidiospore; **Figure 1c**). Because none of the conidiaspores of the negative control showed class 3 status, classes 2 and 3 counted for inhibited germination. Antifungal activity of extracts and pure compounds was calculated by referring to the germination rate of the control sample employing Abbott's formula.

In Vivo Assay. The antifungal activity of A5 against the causal agent of apple scab was tested on leaves of Golden Delicious seedlings grown in a greenhouse, and 5−8-week-old plantlets were used for the experiments.

Jung leaves were sprayed with a solution of 10.0 and 1.0 mg of A5/mL of ethanol/water (1:1) of plant extract to runoff. For the negative control, leaves were sprayed with ethanol/water (1:1) only. The positive control was prepared with the commercial kresoxim methyl formulation Stroby WG (BASF AG, Ludwigshafen, Germany) at a final concentration of 2.0 μg kresoxim methyl/mL. Two hours after extract application (dried application film), the leaves were inoculated uniformly with a conidial suspension of 4×10^5 conidia/mL.

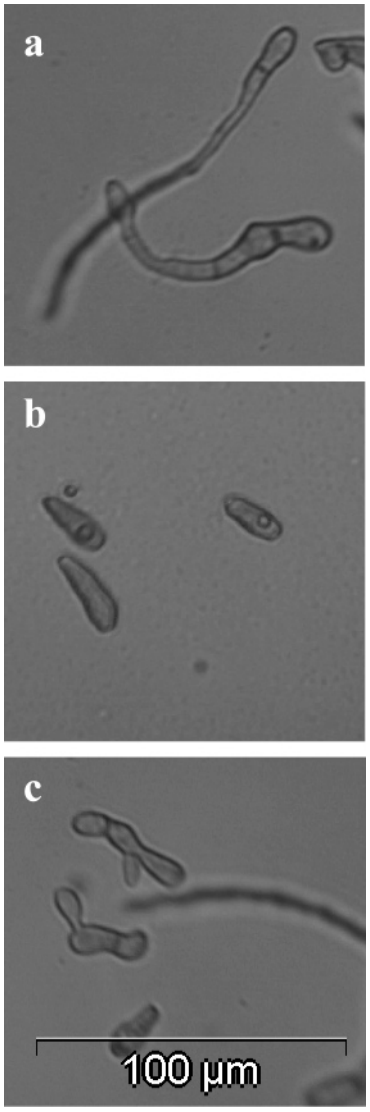

Figure 1. Different germination rates of *V. inaequalis* conidia: (**a**) class 1, germination tube longer than conidiospore; (**b**) class 2, not germinated at all; (**c**) class 3, length of germination tube shorter than or equal to conidiospore.

All inoculated seedlings were incubated in a greenhouse as described above. After 11 days of incubation, the assessment key developed by Croxall et al. (*26*) was used to assess the percentage of infected leaf area. The efficacy was calculated as follows: efficacy = [(infected leaf area of untreated plants − infected leaf area of treated plants)/ infected leaf area in untreated plants] × 100. The experiment was repeated twice.

RESULTS AND DISCUSSION

A bioassay used to explore the antifungal activity of plant crude extracts and pure compounds against the apple scab pathogen, *Venturia inaequalis*, was established. Compared to the methods applied by Olaya and Köller (*24*) and Gilliver (*25*), the test system applied in the investigation outlined in the present study at hand offers several advantages, since (i) very small amounts of test extracts/compounds (<0.5 mg/assay) are required, (ii) solubility of the test extracts/compounds is taken into account by using either CH_3OH or CH_2Cl_2 as a solvent, (iii) there are no interactions between the surface of the glass slides and the test solutions, and (iv) handling of the samples during microscopy is much easier.

MeOH and CH_2Cl_2 extracts of four different organs of *Morus* sp. were prepared and tested in vitro for their potential to inhibit germination of the fungus *V. inaequalis*. Whereas both CH_2Cl_2 and MeOH extracts of the plant's leaves, twigs, fruits, and the CH_2Cl_2 extract of the root bark showed no significant effect at a test concentration of 5 mg/mL, the MeOH extract of the root bark showed a 100% germination inhibitory effect even at a concentration of 300 μg/mL. Thus, 46.0 g of this extract were subjected to a bioassay-guided fractionation. In this process active fractions are continuously further separated by chromatography and retested in an iterative way until the active ingredients are isolated. Twelve subfractions (A1−12) obtained by silica gel flash CC were tested at a concentration of 100 μg/mL revealing A5 and A6 as the most active fractions, with A5 showing 100% (SD ±0.0%) and A6 98.9% (SD ±1.7%) inhibition of the germination rate. HPLC/DAD analysis revealed a complex and similar composition of secondary metabolites in A5 (**Figure 2**) and A6.

In a previous study, a Morus root bark extract was phytochemically investigated because of its significant effect on cyclooxygenases 1 and 2 (*16*). Most of the metabolites of A5 and A6 detected by HPLC/DAD (**Figure 2**) may therefore be assigned to previously isolated and structurally elucidated compounds (**Figure 3**): moracin M (**1**), an inseparable mixture of isobars moracin O and P (**2**), and sanggenons D (**4**), B (**5**), O (**7**), E (**8**), and C (**9**).

In the newly prepared methanol extract of Morus root bark, two additional compounds (**3** and **6**) were detected by TLC and HPLC. By use of silica gel CC, Sephadex CC, and subsequent semipreparative HPLC, pure compounds **3** and **6** were obtained. By means of mass spectrometry and 1D and 2D NMR experiments, **3** and **6** were identified as Diels−Alder adducts of a chalcon and a flavanon moiety linked via a cyclohexene ring system. In contrast to **3**, NMR results of **6** showed a linkage of the two moieties via C_6 of the flavanon system, instead of C_3, as in the case of compound **3**, and an additional isoprenyl moiety at the methyl group of the cyclohexene ring (**Figure 3**). By deduction from physical and spectroscopic data and comparison with data in the literature, **3** could be assigned to kuwanon L (*22*) and **6** to sanggenon G (*23*).

The more polar constituents in A5 and A6 belong to the chemical class of benzofurans (**1** and **2**), called moracins, whereas the more apolar ingredients are Diels−Alder adducts of a chalcon and a dehydroprenyl-flavonoid moiety (**3**−**9**) forming a cyclohexene ring. Only compound **5** steps out of line, because of the missing benzoyl subunit of the chalcon (**Figure 3**).

All the isolates (**1**−**9**) and the most active subfraction A5 were tested in vitro at a concentration of 50 μg/mL. Their inhibitory effects on *V. inaequalis* are depicted in **Figure 4**. At the tested concentration, the moracins (**1** and **2**) showed no antifungal activity against *V. inaequalis*. Accordingly, they do not contribute to the pronounced inhibitory effect determined for fraction A5 (**Figure 4**). This may, however, be attributed to all the isolated Diels−Alder adducts (**3**−**9**). Their IC_{50} values are shown in **Figure 5**.

Among them, **3** clearly shows the weakest activity with an IC_{50} value of 122.9 ± 12.8 μM. Sanggenons **4**−**9** are in the range of 10−62 μM, revealing the most potent inhibition of germination for compound **6** (10.0 ± 0.6 μM) and **9** (17.7 ± 1.0 μM). On the basis of a structure−activity relationship among the tested Diels−Alder adducts, a distinctly better activity is evident when the two subunits are linked at position C_6 of the flavanoid moiety in contrast to a linkage in position C_3, (i.e., in

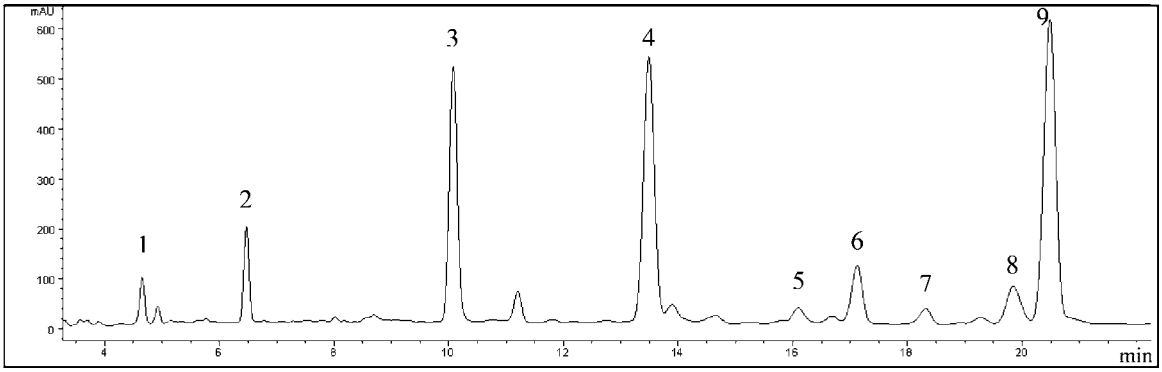

Figure 2. HPLC profile of fraction A5 of Morus root bark (*Morus* sp.). HPLC parameters: column, 150 × 4.6 mm i.d., particle size 3.5 μm, Zorbax SB C-18; guard column, Merck, RP-18; detection wavelength, 205 nm; injection volume, 10 μL; mobile phase A, water, and B, CH_3CN; flow rate, 1.00 mL/min; linear gradient, 0 min 30% B, 5 min 45% B, 20 min 55% B, 22 min 98% B, stop time 30 min; oven temperature, 25 °C. The peak assignment is according to the numbering in **Figure 3**.

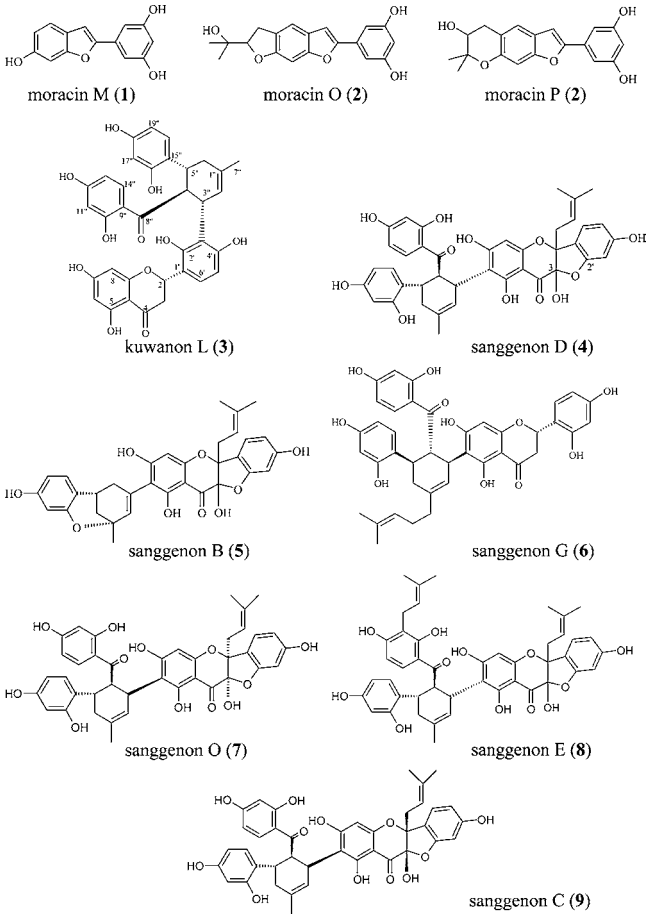

Figure 3. Chemical structures of isolates **1**–**9** from Morus root bark (*Morus* sp.)

3). The loss of rotational freedom between the flavanon's chromone and ring B by an additional ring cyclization (ether linkage) between C_3 and C_2, (i.e., in **4**, **5**, **7**, **8**, and **9** in contrast to **3** and **6**), however, seems not to influence the compounds' inhibitory potency.

To verify the significance of the in vitro results, the most active fraction of Morus root bark, A5, showing an IC_{50} of 39.0 ± 4.2 μg/mL, was tested in vivo. Therefore, leaves of 5−8-week-old Golden Delicious seedlings were sprayed with solutions of 10.0 and 1.0 mg of extract/mL. After inoculation with *V. inaequalis* conidia suspension, the antifungal activity of the A5-treated plantlets was compared with those of kresoxim methyl-treated and untreated plantlets by visual estimation. With

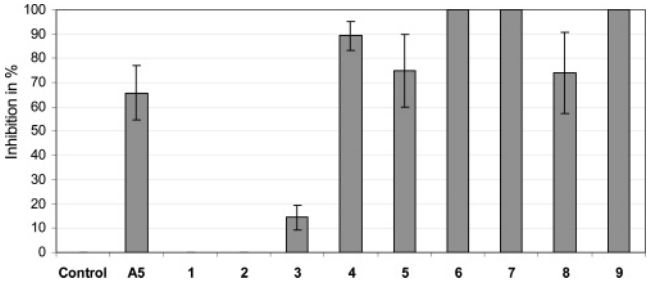

Figure 4. Inhibitory effect (±SD) of A5 and isolates **1**–**9** on the germination rate of *V. inaequalis* at a concentration of 50 μg/mL in comparison to the vehicle control.

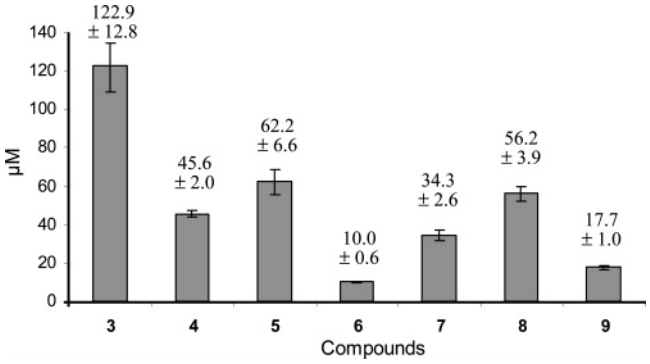

Figure 5. IC_{50} values (±SD) (micromolar) of Diels–Alder adducts **3**–**9** against *V. inaequalis*.

a time interval of 3 months, the experiment was repeated, showing almost identical results: After application of an extract concentration of 10.0 mg/mL an antifungal efficacy of 98% was obtained in both experiments. After treatment with 1.0 mg/mL, an efficacy of 73% and 72%, respectively, was achieved. The efficacy of kresoxim methyl at 2 μg/mL was 100% in both experiments.

Conclusion. On the basis of these preliminary in vivo results, evidence is given for (i) improved adaptation of the in vitro assay modified from previous protocols (*24*, *25*) and (ii) germination inhibitory potential of Morus root bark extract A5 against *V. inaequalis*. In this extract a number of prenylated flavanoids could be identified as the most active antifungal agents. Their common feature is a Diels−Alder adducted chalcon unit (**Figure 3**; sanggenons **4**−**9**). These unique natural products have been isolated from a limited number of plant species, all belonging to the Moraceae family, and are mainly accumulated in the genus Morus (*19* and literature cited therein). Although compounds **4**−**9** are preexisting secondary metabolites

and accordingly do not fit the definition of phytoalexins (*2*), it is most likely that their production is part of the plants' defense mechanism. Several earlier studies of the sanggenons described in the present paper were with good results scrutinized for a variety of pharmacological applications and benefits. They contributed to Sang-Bai-Pi being a valued source in traditional medicine: **9** and **6** could be shown to be protein tyrosine phosphatase 1B inhibitors (*15*), which therefore are effective against metabolic diseases. Anti-inflammatory activities have been demonstrated for **4**, **5**, **7**, **8**, and **9** by inhibition of cyclooxygenase and/or 5-lipoxygenase (*16*−*18*, *27*). Furthermore, compounds **4** and **5** were shown to exert a strong activity against gram-positive bacteria (*28*).

The findings of these investigations demonstrate a further interesting potential of Morus root bark extract and its constituents as a natural fungicide for organic pest control. The long beneficial application of Sang-Bai-Pi in folk medicine in combination with the pharmacological profiles previously described for Morus root bark extracts and constituents attest a safe profile for the investigated plant material. Further research is, however, called for in order to establish these compounds' underlying pharmacological mechanism of germination inhibition and for expanded in vivo experiments.

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