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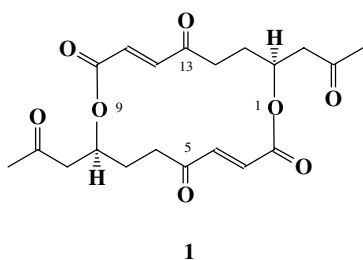
## Vermiculine: a diolide with immunoregulatory activity

B. PROKSA and J. FUSKA

Vermiculine (**1**), is a macrodiolide which displays a selective effect on certain reactions of cellular immunity. This paper summarizes data on its biological activity, isolation, microbial production, chemical properties and methods for its synthesis.

### 1. Natural sources and biosynthesis

The macrodiolide vermiculine, (8*S*,16*S*)-8,16-di(2-oxopropyl)-1,9-dioxo-3,11-cyclohexadecadiene-2,5,10,13-tetrone (**1**), is a microbial metabolite which has been isolated for the first time from a cultivation broth of a mould designated as 51-C<sub>1</sub>. This mould was collected in the old disused uranium mines in Jáchymov (Czech Republic) where it covered timber supports of adits [1]. The strain isolated belongs to the group of extremophile microorganisms as the growth conditions where it was abundant were quite harsh: even temperature at 5–12 °C, humidity around 80%, low background radiation and concentration of uranium salts in water up to 0.1 mg/l [2]. The strain 51-C<sub>1</sub> was deposited in the Czechoslovak Collection of Microorganisms as *Penicillium vermiculatum* Dangeard CCM F 276, but later it was renamed and the designation *Talaromyces flavus* (Kloecker) Stolk et Samson was given. The current taxonomic classification of this strain is as follows: Fungi – Ascomycota – Euscomycetes – Plectomycetes – Eurotiales – Trichocomaceae – *Talaromyces*. Later vermiculine (**1**) was identified in other species of *Talaromyces* e.g. *T. wortmannii* [3] or *T. ohiensis* [4].

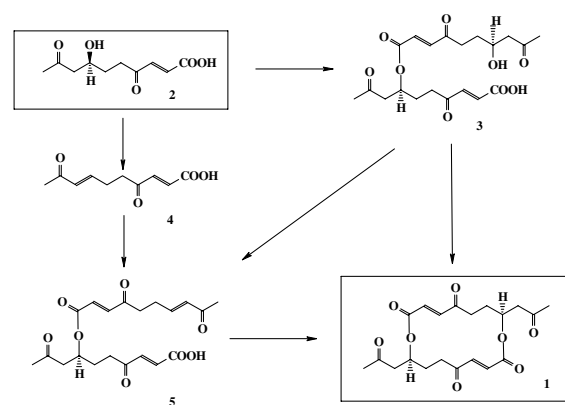


*T. flavus* is a recognized source of a range of secondary metabolites, but the strain 51-C<sub>1</sub> biosynthesized only two major metabolites depending on the carbon source in the cultivation medium – vermiculine (**1**) in the presence of sucrose and vermistatin in a medium with glucose [5, 6]. A number of minor metabolites were identified beside the two major ones, e.g. azaphilones [4, 7], vermixocins [8], vermilutine [9], dehydroaltenusin [10] etc. Determination of all these metabolites in cultivation media was by HPLC on a reversed-phase column [11, 12].

Production of the diolide **1** by *T. flavus* is controlled in addition to the concentration of sucrose by the quality of the nitrogen source (corn steep liquor), the concentration of trace elements (Fe<sup>3+</sup>, Cu<sup>2+</sup>) and organic acids, especially those involved in electron transport. This findings indicate a link between the biosynthesis of vermiculine (**1**) and oxidation processes in the stain producing it [13].

Vermiculine (**1**) is composed of two molecules of pentaketide acid **2**, biosynthesized from acetate, or malonate units. Theoretically two molecules of acid **2** are esterified head-to-tail and the secovermiculine **3** formed is then lactonized to diolide **1** (Scheme 1).

Scheme 1

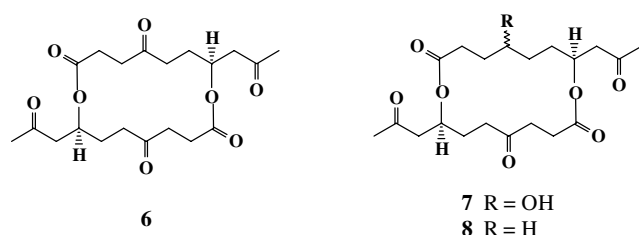


Biosynthesis of vermiculine by *Talaromyces flavus*

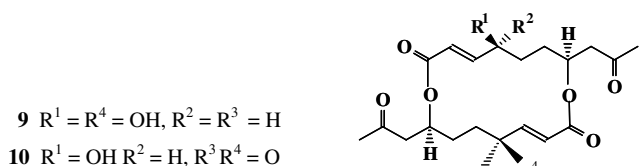
However, neither **2** nor **3** were identified in the cultivation medium but their dehydration products **4** and **5**, respectively, were [14]. Their concentration varied inversely with the concentration of vermiculine (**1**) and in the last stages of cultivation the acid **4** disappeared totally. Resting cells of *T. flavus* continued to biosynthesise vermiculine (**1**), but the resulting concentration of diolide **1** was very low. On the other hand addition of the acid **5** to a suspension of these cells resulted in a substantial increase in the concentration of vermiculine [15] indicating that *T. flavus* contains an enzyme catalyzing the cyclization. Probably this process is not a simple addition of carboxyl group on the double bond of the  $\alpha,\beta$ -unsaturated ketone moiety, but cyclization may proceed via an intermediate **3**, and this hydroxy acid is then lactonized. Similar stereoselective esterification coupled with lactonization was observed in the action of lipases on (*R,S*)-7-hydroxyoctanoic acids [16]. During static cultivation of *T. wortmannii* vermiculine (**1**) accumulated in the form of clusters of needle-like mostly rectangular flat crystals which were studied by electron scanning microscopy [17]. The diolide **1** is sparingly soluble in common solvents except chlorinated ones. It crystallizes from chloroform in the form of clear, large cubes belonging to the orthorhombic crystal class with space group P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>. According to the results of x-ray diffrac-

tion analysis the 16-membered macrocyclic ring has an approximate twofold axis [18]. Vermiculine (**1**) is stable in dilute neutral or acidic solution, but the diolide ring is decomposed in alkaline ones straight to a C<sub>10</sub> diketoacid **4** [18], which easily undergoes an aldol polycondensation. Vermiculine (**1**) heated in acetic acid is gradually decomposed to acid **4** and the intermediate **5** can be isolated from the reaction mixture [19].

The unsaturated bonds conjugated with carbonyl groups in vermiculine (**1**) are susceptible to hydrogenation; this reaction performed in acetic acid on Adams catalysts gave tetrahydrovermiculine (**6**) together with minor by-products **7**, **8** in which the carbonyl group was also attacked [20].



Oxidation reactions prevailing in the first stages of cultivation of *T. flavus* are replaced by reduction ones in the last period; in addition to vermiculine (**1**), metabolites **9** and **10**, products of stereoselective hydrogenation of the C-5 and C-13 carbon atoms were isolated after 30 days cultivation [21].



## 2. Synthesis

The first total synthesis of vermiculine (**1**) was worked out by Corey et al. [22] only one year after the structure of the novel diolide was published. In their approach (Scheme 2) dimethyl 2,2-dimethoxy-glutarate was transformed to aldehyde **11** and its condensation with dimethylallyl cadmium yielded the carbinol **12**. The hydroxyl group of **12** was protected with tribenzylsilylchloride and

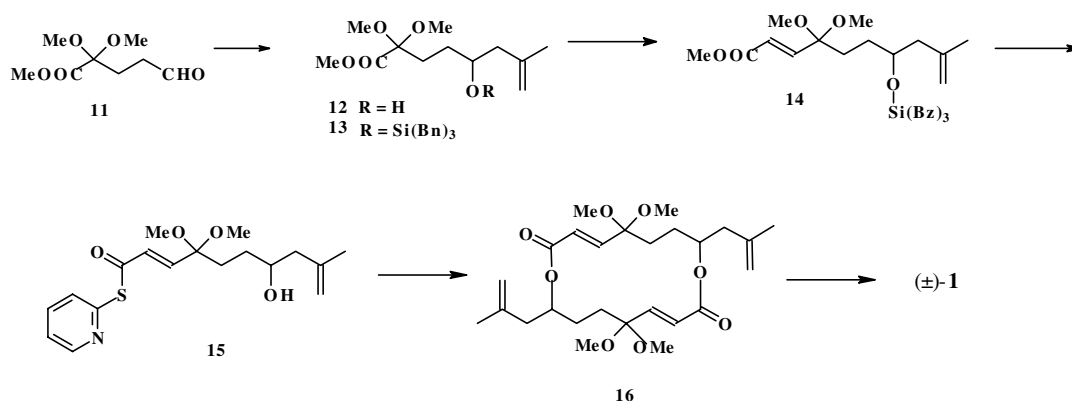
reduction of the methoxycarbonyl moiety of silyl ether **13** with diisobutylaluminum hydride gave an aldehyde. This compound gave the unsaturated ester **14** with diethyl methoxycarbonylmethane phosphonate (Wittig–Horner reaction). The ester methoxyl of **14** was replaced by a 2-pyridylsulfide moiety and the thioester **15** when refluxed in xylene/2,6-lutidine lactonized to diolide **16**; (±)-vermiculine was obtained after the oxidation of exomethylene groups with OsO<sub>4</sub>/NaIO<sub>4</sub> followed by deprotecting the C-5, C-13 carbonyls [22].

Seebach et al. [23] confirmed the absolute configuration of vermiculine by an enantioselective synthesis (Scheme 3) starting from (*S*)-bromobutyloxirane (**17**). Reaction of the bromine and oxirane part of **17** with 2-Li-1,3-dithiane and 2-Li-2-methyl-1,3-dithiane at −75 and −30 °C, respectively, gave the carbinol **18** which was formylated with DMF to the aldehyde **19**. This compound was converted to the methylester **20** with methyloxycarbonylmethylene phosphorane. The acid **21** prepared from the ester **20** was dimerized to diolide **22** according to Mitsunobu. This reaction proceeded with inversion at the chiral carbons, and therefore the vermiculine obtained possessed an unnatural *R,R*-configuration [23].

An analogue of the ester **20** was also synthesized from ethyl 2,4-dioxo-7-octenoate (**23**) (Scheme 4). An oxime prepared from **23** and hydroxylamine was cyclized to isoxazole in the presence of sulfuric acid; in the next step acetonitrile oxide added to the terminal double bond gave dihydroisoxazole **24**. While the former heterocycle was opened by hydrogenolysis on Pd/C giving an unsaturated amino ketone, the latter was transformed to β-hydroxyketone **25** by hydrogenation over Raney Ni. Subsequent acylation of **25** yielded the α-ketoester **26**, and its α-carbonyl regioselectively reduced with a complex hydride yielded the α-hydroxy diketoester **27**. After dehydration, and protection of carbonyls in the dithiane form compound **28** was suitable for dimerization to (±)-vermiculine [24].

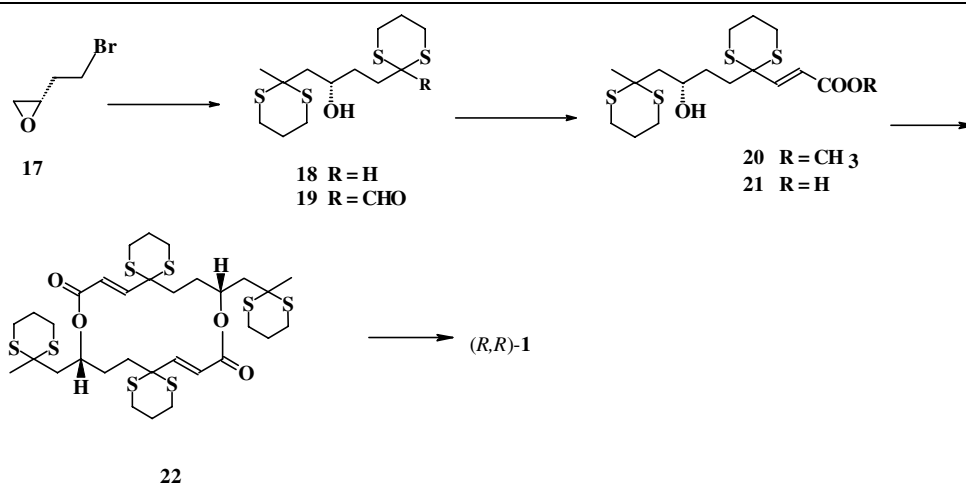
A C<sub>10</sub> synthon intended for preparation of (±)-vermiculine was synthesized from a vinyl ketoester **29** previously obtained from a substituted 2-siloxycyclo-propanecarboxylate [25] and nitrobutylacetal **30** by Reissig et al. [26]. The nitro group of the synthesized methyl 9,9-(ethylenedioxy)-7-nitro-4-oxodecanoate (**32**) was exchanged for a keto one by a modified Neff reaction with hydrogenium peroxide in presence of potassium carbonate (Scheme 5). The C<sub>10</sub> ketoester **33** is a suitable precursor for vermiculine after dehydrogenation and reduction of the C-7 carbonyl.

Scheme 2

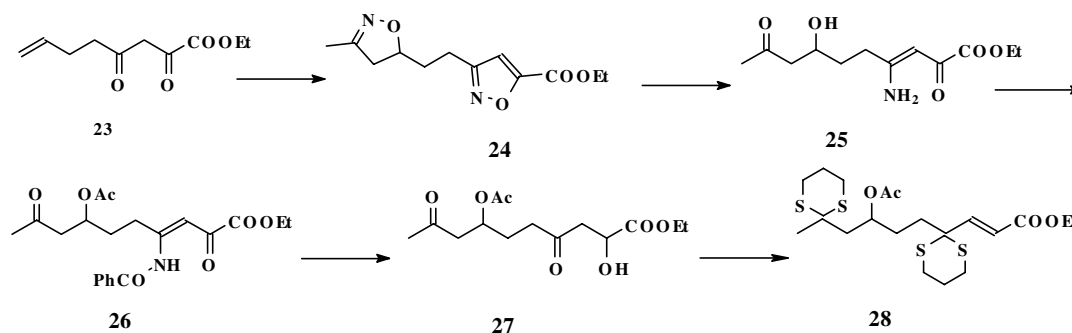


Synthesis of (±)-vermiculine from methyl 4-formyl-2-dimethoxybutanoate [22]

Scheme 3

Synthesis of (*R,R*)-vermiculine [23]

Scheme 4

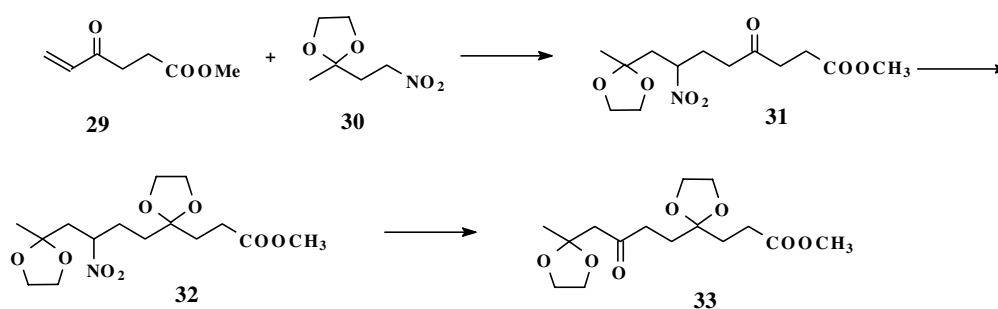
Synthesis of C<sub>10</sub> intermediate of (±)-vermiculine [24]

A simple procedure for the synthesis of a C<sub>10</sub> hydroxyacid leading to vermiculine was developed by Hase et al. [27]. Methyl 4-oxo-2-pentenoate (**34**) treated with allyl alcohol in presence of TsOH yielded diallyl acetal, which immediately rearranged to methyl 4-oxo-2,7-octadecadienoate (**35**). The terminal vinyl group of **35** was ozonized after masking the oxogroup to give methyl 6-formylhexenoate **36** which with methylpropenyl cadmium yielded the decenoate **37** (Scheme 6), a derivative of intermediate **14** (Scheme 2) used in the Corey synthesis of vermiculine. The terminal double bond of the C<sub>8</sub> oxoester **35** (Scheme 6) with its keto group protected in the form of dioxolane

was regioselectively oxidized, and the generated epoxide **38** hydrolyzed with LiOH to an unsaturated epoxy acid with 2-Li-2-Me-1,3-dithiane gave C<sub>10</sub> acid **39**. Its dimerization to masked vermiculine **40** was carried out in the presence of dimethyltin oxide in refluxing mesitylene (Scheme 7) [28].

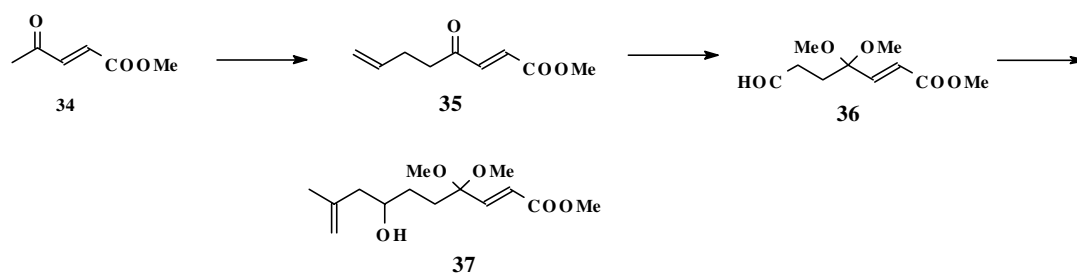
Fukuyama et al. [29] completed the synthesis of (±)-vermiculine from ethyl 2-methyl-4-oxocyclohexene-1-carboxylate (**41**) which was transformed in a series of reactions via 4-hydroxy-2-methyl-1-cyclohexene-1-carbaldehyde (**42**) to cyclohexenyl-2-propenoate **43**. This compound was esterified with bromoacetic bromide and the bromoester thus pre-

Scheme 5



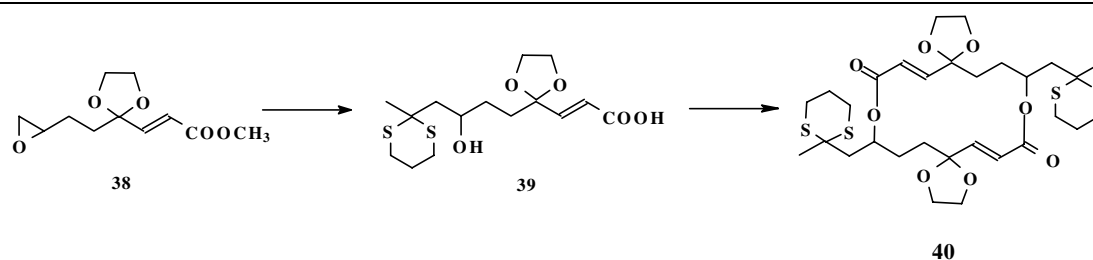
Preparation of protected methyl 7-oxo-decanoate [26]

## Scheme 6



Synthesis of C<sub>10</sub> intermediate of synthesis of vermiculine [27]

## Scheme 7



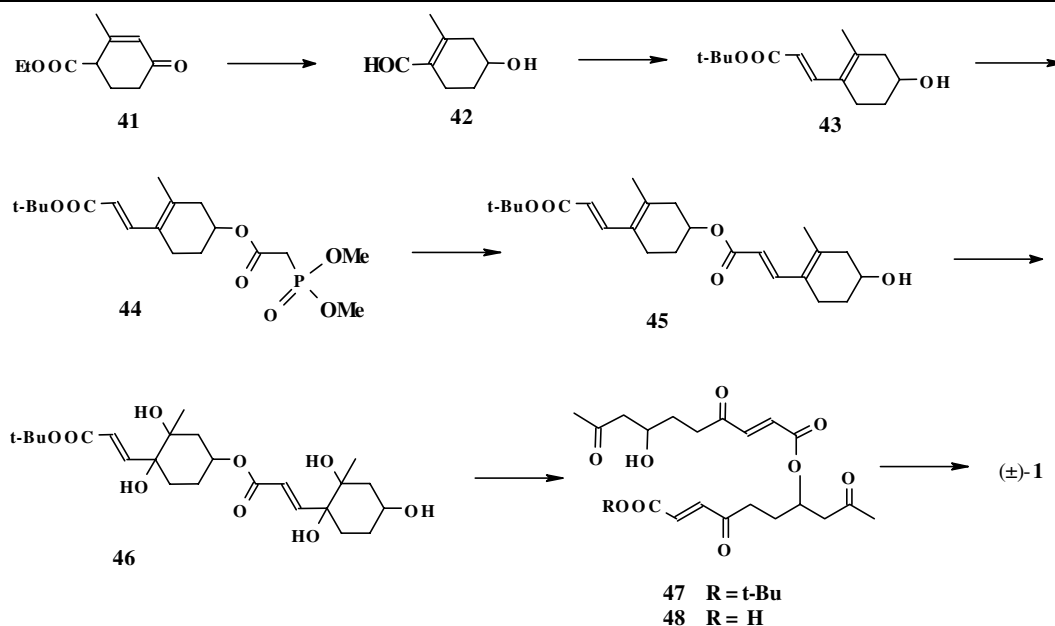
Synthesis of (±)-vermiculine derivative [28]

pared was coupled with trimethylphosphite to the phosphonate **44**. Wittig-Horner reaction of **44** with the aldehyde **42** yielded the diester **45**. Further procedure included selective epoxidation of the carbocyclic double bonds with *m*-chloroperbenzoic acid, opening the oxirane rings and oxidation of the formed diol **46** with Pb(OAc)<sub>4</sub>. This reaction gave the *t*-butyl ester of vermiculic acid (**47**). By selective hydrolysis with TFA prepared hydroxyacid **48** was lactonized to (±)-vermiculin in presence of triphenylphosphine and diethyl azodicarboxylate (Mitsunobu reaction) (Scheme 8).

The approach of Wakamatsu et al. [30] to the synthesis of (±)-vermiculine is noteworthy with an α,β-dehydrogena-

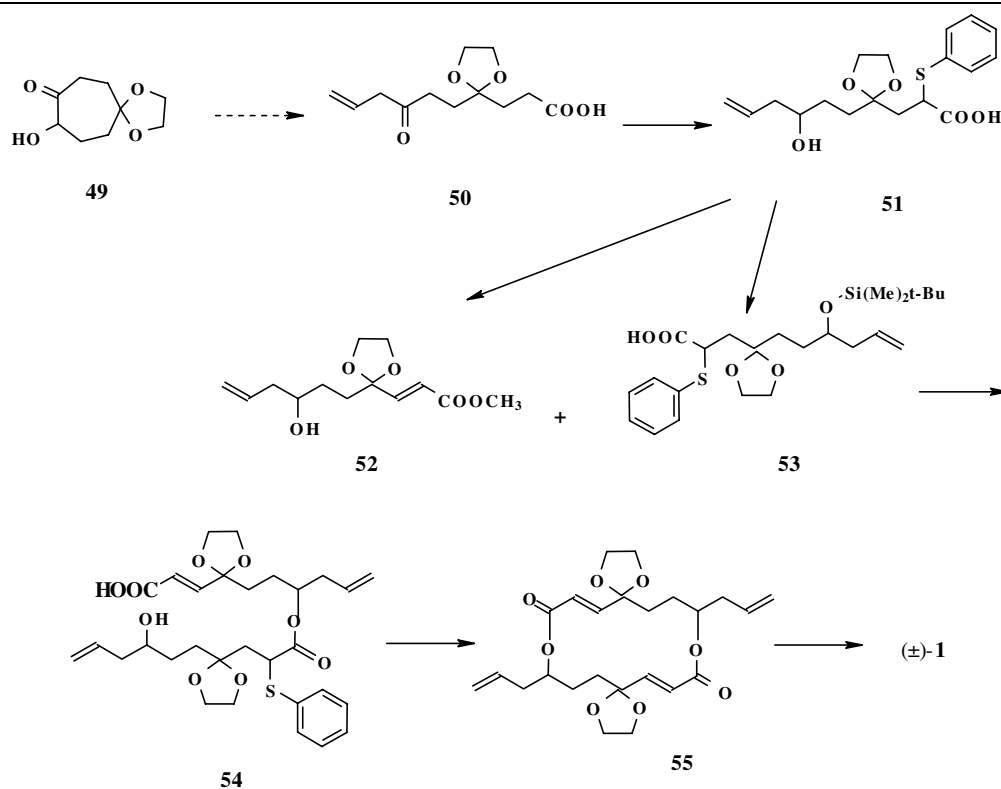
tion of a protected C<sub>10</sub> hydroxyacid using an arylsulfide. The substituted hydroxycycloheptanone **49** was transformed to the acid **50**; reduction of C-7 carbonyl with NaBH<sub>4</sub> followed by treatment of the 7-hydroxy-9-decanoic acid formed with PhSSPh yielded phenylthiocarboxylic acid **51**. This compound was the key intermediate of this synthesis because it was oxidatively desulfated to α,β-unsaturated C<sub>10</sub> hydroxy acid then methylated to ester **52** and silylated to phenylthioacid **53**. A mixture of **52** and **53** treated with diethyl phosphochloridate gave the secovermiculine derivative **54**. Its phenylthioether moiety was removed by oxidation with NaIO<sub>4</sub>, and the ring was

## Scheme 8



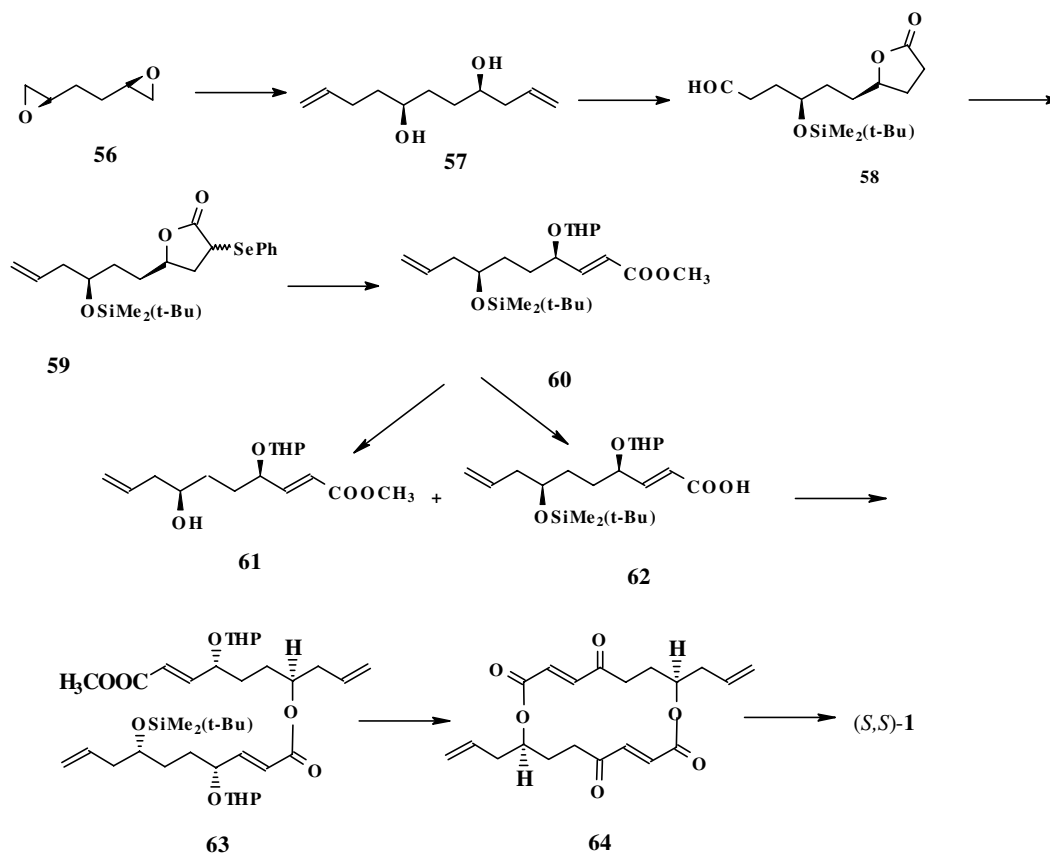
Synthesis of (±)-vermiculine from ethyl 2-methyl-4-oxocyclohexene-1-carboxylate [29]

Scheme 9



Transformation of hydroxycycloheptanone **49** to (±)-vermiculine [30]

Scheme 10



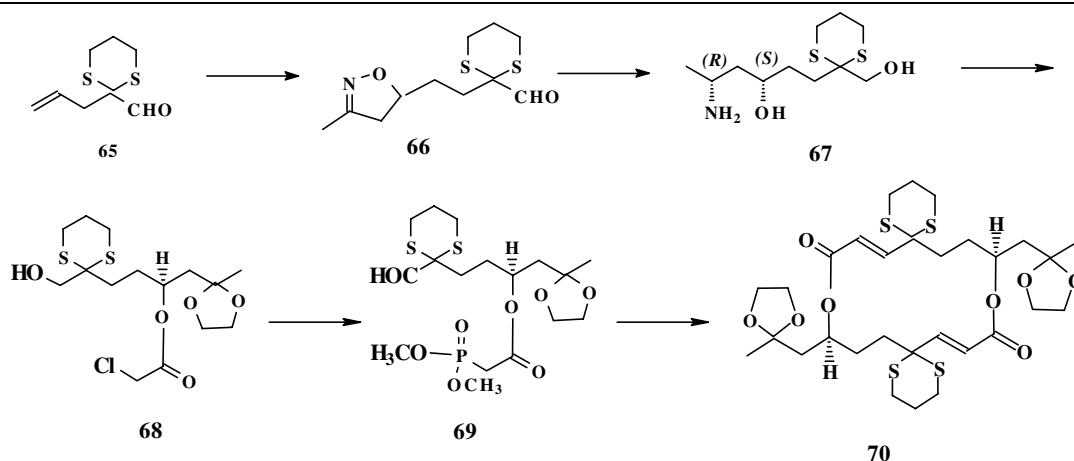
Synthesis of (S,S)-vermiculine from (R,R)-1,2:5,6-diepoxyhexane [31]

closed in the same way as the coupling of **52** and **53**. The diolide **55** obtained was converted to (±)-vermiculine after catalytic oxidation of terminal vinyl groups (PdCl<sub>2</sub>, CuCl, O<sub>2</sub>) and carbonyl deprotection with trifluoroacetic acid in wet dichloromethane (Scheme 9) [30].

(*R,R*)-1,2:5,6-diepoxyhexane (**56**) prepared from D-mannitol was a chiral synthon for an enantioselective preparation of vermiculine (**1**). The oxirane rings of **56** were opened with allylmagnesium chloride and the hydroxyl groups of diol **57** were selectively benzylated and silylated, then the double bonds were oxidized with OsO<sub>4</sub>/NaIO<sub>4</sub>, the benzyl group was selectively removed and synthesized γ-hydroxyaldehyde forming an acetal was oxidized to the lactone **58**. The remaining aldehyde group was converted to a double bond by a series of reactions and the butanolide part of the molecule was selenylated. Lactone **59** was opened and the carboxyl group generated was esterified with diazomethane. Elimination of the phenylselenene moiety by oxidation and protection of the hydroxyl group with tetrahydropyran resulted in formation of the unsaturated ester **60**; it was desilylated to **61** and hydrolysed to acid **62**. These two products were coupled (Mitsunobu reaction) to give ester **63**, which was desilylated and intramolecularly lactonized. Deprotection of secondary hydroxyls followed by their oxidation with pyridinium chlorochromate gave diolide **64** and catalytic (Wacker) oxidation of its terminal vinyl groups produced (*S,S*)-vermiculine (Scheme 10) [31].

Burri et al. [32] in contrast to previous developed a different strategy – they did not prepare an analogue of a C<sub>10</sub> hydroxyacid as a substrate for **1** lactonization, but they synthesized a chiral aldehyde, which was converted to (*S,S*)-vermiculin (Scheme 11). 1,3-Dithiane alkylated with acrylaldehyde followed by formylation yielded 2-allyl-2-formyl-1,3-dithiane (**65**). 1,3-Dipolar cycloaddition of acetonitrile oxide on the terminal double bond of **65** gave isoxazoline **66**, which was reduced to the racemate of an aminodiols and resolved with α-camporsulfonic acid to a compound **67** of the desired (3'*S*, 5'*R*)-configuration. The amino group of **67** was regioselectively oxidized with 3,5-di-*tert*-butyl-*o*-benzoquinone to a ketone which was masked in a dioxolane. The prepared diol was transformed to a chloroacetate **68**, the chlorine atom was replaced by a dimethylphosphonate group and oxidation of primary hydroxyl with DMSO yielded the aldehyde **69**. Subsequent dimerization in presence of NaH (Wittig-Horner reaction) gave diolide **70**, which was transformed to (–)-vermiculine [32].

Scheme 11



Synthesis of (–)-vermiculine according to Burri et al. [32]

### 3. Pharmacology

Vermiculine (**1**) suppressed growth of G+ bacteria in the concentration range of 10–30 µg/ml, and to a lesser extent was also active against yeasts, but it had a much more pronounced antiprotozoal effect on *Leishmania brasiliensis*, *Trypanosoma cruzi* or *Trichomonas foetus* [1]. The inhibition of growth of *T. foetus* was perceptible even at a concentration of 5 µg/ml; at higher concentrations diolide **1** completely blocked cell proliferation and caused lysis of protozoal cells. The minimum inhibitory concentration of this compound was comparable with that of therapeutically used agents e.g. amphotericin or metronidazole. The early onset of the inhibitory effect and its persistence after the removal of the agent indicated that vermiculine (**1**) penetrated into the host cells. According to the results of an experiment using labeled precursors, vermiculine interfered with the synthesis of nucleic acids, without affecting proteosynthesis [33].

Vermiculine (**1**) was originally isolated as a cytotoxic compound; an EAC assay was used to monitor the process of isolation. Further studies of the cytotoxicity of vermiculine were performed with EAC, L5178, P-388 cells (ID<sub>50</sub> 1.25, 0.5 and 9.3 µg/ml, respectively) [34, 35], HeLa and L5178Y cells. In these latter models diolide **1** reduced mitosis of cancer cells and interfered with the cellular content of nucleic acids and proteins. It induced an increase in cell size which was accompanied by accumulation of proteins with concurrent lowering of the concentration of nucleic acids, especially of cellular DNA. At the same time consumption of glucose by cancer cells was only slightly decreased, showing that the energy metabolism was not affected [36]. Interference with glycolysis or cell respiration was not observed [37]. Tests on a battery of carcinoma models were performed at the National Cancer Institute in Bethesda, USA, where activity on leukemia, colon, renal and breast carcinoma was confirmed. As well as vermiculine (**1**) its derivatives were also tested and it was concluded, that the prerequisite structural requirements for cytotoxic activity were the closed dilactone ring and the α,β-unsaturated ketone moiety conjugated with the lactone carbonyl group.

Many compounds interfering with the synthesis of nucleic acids in cancer cells have also shown this effect in lymphoid cells. The immunosuppressive properties of vermiculine (**1**) were examined in two systems aimed at rapid screening of current immunosuppressive agents (nucleolar test and the reactivity of mouse lymphocytes to T (phyto-

hemagglutinin, PHA) and B (lipopolysaccharide, LPS) mitogens). Vermiculine distinctly suppressed the increase in number of active lymphocytes with compact nucleoli in the popliteal lymph node activated by SRBC. Incorporation of uridine into PHA-stimulated T lymphocytes was suppressed, but incorporation into LPS-stimulated B lymphocytes was enhanced. Compound **1** was also tested in the Jerne test and graft-versus-host (GVH) reaction, representing humoral and cell mediated immune responses, respectively. Vermiculine in doses markedly inhibitory for GVH reaction did not suppress but significantly increased the number of hemolytic plaques in spleens of SRBC-immunized mice. Therefore, this compound showed a specific effect on individual types of immune response. Vermiculine probably did not stimulate the B lymphocytes directly, but behaved like an inhibitor of regulatory T suppressor cells [38].

Human peripheral neutrophils represent the basic component of defense of the immune system and are involved in the mechanism of natural immunity of organism. At a concentration of 100 pg per single neutrophil, vermiculine (**1**) significantly inhibited the metabolic activation (reduction of INT), and superoxide production of both unstimulated and stimulated neutrophils, when opsonized zymosan or phorbol derivative were used as the stimulating agents. At 100 µg/ml diolide **1** lowered the phagocytic and candidacidal activity of the neutrophilic granulocytes.

Lymphocytes could be activated in presence of monoclonal antibody anti-CD3, anti CD2/CD2R and polyclonal activators e.g. PHA, concanavalin A (ConA) and pokeweed mitogen (PWM). Vermiculine (**1**) did not affect the activity of untreated lymphocytes, but on the other hand it did reduce the stimulation of lymphocytes caused by ConA and PWA, while no effect was observed with lymphocytes stimulated by PHA. Stimulated lymphocyte subpopulations were analysed by flow cytometry, and in particular the expressivity of activation antigens CD69, CD25 and CD71 was studied. Vermiculine (**1**) diminished the activation of CD8<sup>+</sup> lymphocytes and the intensity of inhibition was more marked at a concentration of 0.01 µg/ml. These results indicate immunosuppressive properties of vermiculine [39].

Collagen-induced arthritis is a T cell-dependent animal model of rheumatoid arthritis useful to study of the etiology of inflammatory synovitis. Vermiculine (**1**) in this model positively affected body weight gain in arthritic rats and this value was closer to the value for the healthy control group of rats; diolide **1** significantly reduced hindpaw swelling, and prevented the decrease of serum albumine level observed in untreated arthritic rats. On the other hand, it had no effect on the concentration of hyaluronic acid in serum and no statistically significant difference of radiographic score between the arthritic and vermiculine treated groups of rats was observed [40].

Vermiculine (**1**) prevented the development of spontaneous autoimmune disease in NZB/W F mice. A dose of 25 mg/kg of **1** resulted in the improvement of clinical parameters (weight increase, significant inhibition of swelling and X-ray bone lesion and mild improvement of mobility of joints) in adjuvant arthritis. However, these effects were dose dependent: while the dose of 25 mg/kg was the most effective, a higher dose (100 mg/kg) had a detrimental effect on the course of the disease. Higher doses lowered the number of both T<sub>H</sub> and T<sub>S</sub> lymphocytes in the spleen, while a dose of 25 mg/kg increased the numbers of T<sub>S</sub> only. These latter lymphocytes are considered to be capable of inhibiting the autoimmune reaction [41].

Results of these experiments proved, that vermiculine (**1**) is a drug which has a selective effect on certain reactions of cellular immunity, and it may be an effective agent in conditions where suppression of cellular immunity is required.

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Received March 13, 2000  
Accepted April 22, 2000

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