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Structure and Synthesis of the Methyl Ester of 3,4-Anhydroshikimic Acid, Isolated from a *Chalara* Sp.

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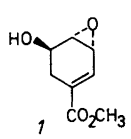
The methyl ester of (+)-3,4-anhydroshikimic acid has been isolated from the fungus *Chalara microspora* (Corda) Hughes. The structure was elucidated by spectroscopy, and was confirmed by synthesis of the title compound and its 5-epimer. Synthesis *via* a partially resolved intermediate allowed the absolute configuration to be assigned.

In a study of fungal metabolites antagonistic against *Fomes annosus* (Fr.) (root rot fungus),[1] a fungus identified as *Chalara microspora* (Corda) Hughes* has been found to produce two compounds (*1* and *2*) with an apparent antagonistic activity.** This paper deals with the structure and synthesis of *1*. The structure of *2* is described in the following paper.[2]



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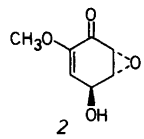

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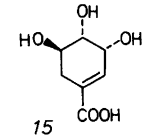


15

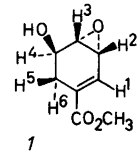
shikimic acid

Isolation and structure

The fungus was grown in a medium containing 2 % malt extract. Small amounts of two compounds, *1* and *2*, were isolated from the culture medium.

Compound *1* was amorphous. High resolution mass spectrometry did not give a molecular ion. The peak of highest molecular weight was 152.0485 ($C_8H_8O_3$). The appearance of the rest of the mass spectrum and the presence of 10 hydrogen atoms in the ^1H NMR spectrum suggested the molecular formula $C_8H_{10}O_4$. The molecule thus contained a total of four rings/double bonds.

The UV spectrum showed a maximum at 229 nm ($\varepsilon = 13000$), characteristic of an α,β-unsaturated carbonyl chromophore. The IR spectrum showed the presence of OH (3400 cm^{-1}), CO–O (1710 cm^{-1}, 1270 cm^{-1}) and C=C (3010 cm^{-1}, 1650 cm^{-1}). Furthermore, 100 MHz ^1H NMR established the chemical shifts and coupling constants for the various protons (Fig. 1). Taken together, the spectral data suggested the structure and relative configuration shown in Fig. 1. In deciding between this *trans* configuration and the corresponding *cis* isomer, it was noted that H^4 occurs at unusually low field for a cyclic CHOH group. This can best be explained if H^4 is equatorial and not axial.[3] It can be assumed that the most stable conformation of the compound has a



1

Fig. 1. ^1H NMR ($CDCl_3$): δ 7.25 (H^1), 3.50 (H^2), 3.55 (H^3), 4.65 (H^4), 2.9 (H^5), 2.4 (H^6), 3.85 (OCH_3); J (Hz): $H^1-H^2 = 4.1$, $H^1-H^6 = 3.0$, $H^2-H^3 = 4$, $H^3-H^4 = 2.5$, $H^3-H^5 = 1.8$, $H^4-H^5 = 1.8$, $H^4-H^6 = 5.0$, $H^5-H^6 = 17.0$.

*The identification was made at Centralbureau voor Schimmelkultures, Baarn, Netherlands, and the fungus is incorporated in their collection under that name.

**When prepared synthetically, these compounds were eventually found to be inactive, and we now know that minute amounts of truly active "impurities" were responsible for the observed activity. These "impurities" are being investigated.

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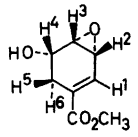


14

Fig. 2. ^{1}H NMR ($CDCl_3$): δ 7.05 (H^1), 3.55 (H^2), 3.70 (H^3), 4.20 (H^4), 2.95 (H^5), 2.15 (H^6), 3.90 (OCH_3); J (Hz): $H^1-H^2=3.8$, $H^1-H^6=3.2$, $H^2-H^3=4.4$, $H^3-H^4=6.4$, $H^3-H^5=1.6$, $H^4-H^5=6.4$, $H^4-H^6=10.0$, $H^5-H^6=16.4$.

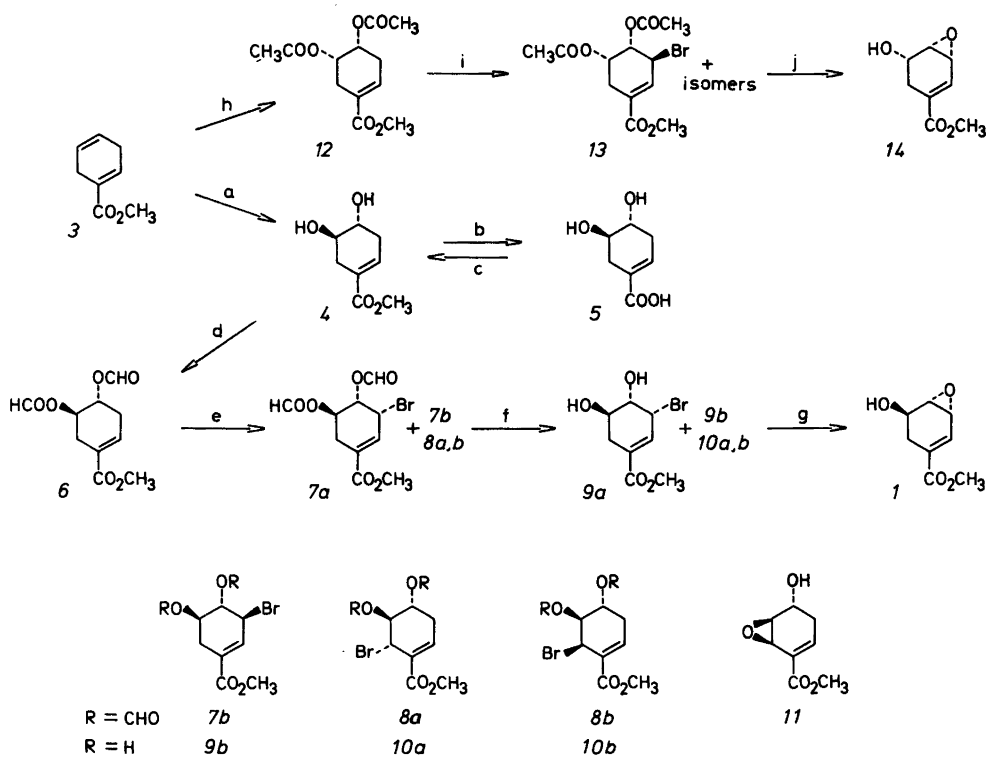
minimum of eclipsed groups, with the epoxide parallel to the double bond to give maximum overlap.[4] Dreiding models for the most probable conformations then show that when OH is *trans* to the epoxide, H^4 should be equatorial, while in the *cis* compound H^4 should be axial. The *trans* structure is thus the most likely. This was confirmed through a synthesis of both isomers. ^{1}H NMR assignments for the *cis* isomer *14* are given in Fig. 2. In addition, synthesis *via* a partially resolved intermediate established the absolute configuration to be that drawn in *1*. The close relation to shikimic acid *15* is obvious, and the compound is in fact the methyl ester of 3,4-anhydroshikimic acid.

Synthesis

Both the *trans* and *cis* isomers were synthesized (Scheme 1), largely following a route used by Grewe *et al.*[5,6] for the synthesis of shikimic acid.

Treatment of methyl 1,4-cyclohexadiene-1-carboxylate *3* with H_2O_2 in formic acid, followed by hydrolytic work up, yielded the *trans* dihydroxy compound *4*.[5] The hydroxyl groups were protected as easily cleaved formate esters. The formylation was performed in formic acid using polyphosphoric acid as a catalyst.[7]

The introduction of bromine in the 3-position was then desired. Grewe *et al.*,[5] working with the



Scheme 1. a: (1) H_2O_2, HCOOH; (2) H_2O. b: NaOH. c: CH_3OH, $BF_3 \times Et_2O$. d: HCOOH, $P_2O_5 \times nH_2O$. e: NBS, hν, CCl_4/CS_2 (1:1). f: CH_3OH + 5 % phosphate buffer (pH = 7.5). g: Na_2CO_3, NaBr, acetone, reflux. h: (1) I_2, AgOAc, HOAc; (2) H_2O; (3) Ac_2O, $HClO_4$. i: NBS, hν, CCl_4. j: $NaOCH_3$, CH_3OH.

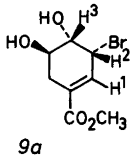
corresponding diacetate, used NBS in boiling CCl_4 to obtain a product containing about 20% of the desired epimers *7a, b*. We found that the combined yield of *7a* and *b* could be raised to approximately 40% by performing the reaction in a mixture of CCl_4 and CS_2 (1:1). The increased selectivity of the reaction may be attributed to the ability of CS_2 to stabilize radicals.[8] The bromination also gave some product *8a, b* with bromine in the 6-position.[5]

The mixture obtained after bromination was hydrolyzed under mild conditions, using methanol +5% phosphate buffer (pH=7.5). In early experiments the crude mixture after hydrolysis was treated with Na_2CO_3 in refluxing acetone. Under these conditions, both isomers *9a* and *b* gave the desired epoxide *1*, *9b* presumably *via* initial epimerization to *9a* by bromide ions in the solution. The resulting mixture of products was chromatographed on cellulose/H_2O using benzene as eluent. This gave the desired product *1*, but contaminated with 10–20% of *11* (as indicated by ^1H NMR), formed from *10a, b*. We were not able to separate the isomers by conventional chromatography. At this stage it became apparent that the epoxides were unstable to chromatography both on silica gel and alumina.

A separation of the crude mixture of bromohydroxy esters obtained after hydrolysis was therefore attempted. The mixture was subjected to a rapid and incomplete separation on cellulose/H_2O using ether/pentane (4:1) as eluent. This gave a fraction containing *9a*. From this fraction it was possible to crystallize pure *9a* in 16% yield starting from *6*. Evidence that the isolated isomer was *9a* and not *9b*, were the coupling constants (Fig. 3), which according to the Karplus curve [3] are consistent only with the two possible conformers of *9a*.

Pure *9a* afforded *1* on treatment with Na_2CO_3 in refluxing acetone. Some NaBr was added to increase the rate of initial epimerization. Spectral data of the synthetic product were identical with those of *1* obtained from the fungus.

In order to prepare the *cis* ester *14*, methyl 1,4-cyclohexadiene-1-carboxylate *3* was treated with iodine and silver acetate in wet acetic acid. The resulting product was acetylated to give *12*. Bromination with NBS in CCl_4 gave a mixture of isomers containing *13*. Hydrolysis of the mixture with NaOMe/MeOH and subsequent chromatography on alumina with ether gave a low yield of the *cis* compound *14*.



9a

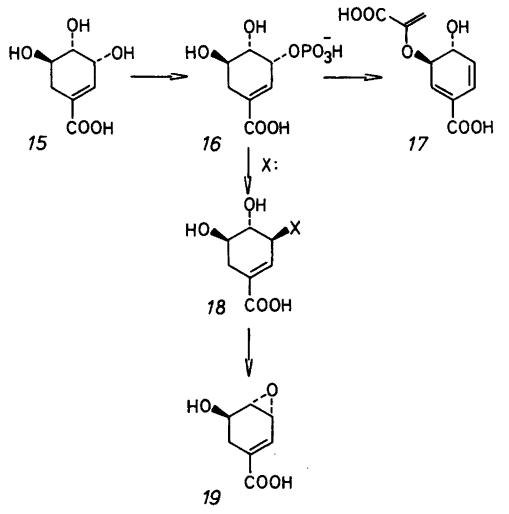
Fig. 3. J (Hz): $H^1-H^2=4.8$, $H^2-H^3=4$.

Absolute configuration

In order to establish the absolute configuration of natural compound *1*, we synthesized optically active *1*. The ester *4* was hydrolyzed with NaOH. The acid *5* was then partially resolved by fractional crystallization of its brucine salt in ethanol. The (−)-form was enriched, and this is known to have the absolute configuration shown in *5*.[9] Esterification [10] afforded optically active *4*, which was then carried through the synthesis indicated in Scheme 1 to yield optically active *1*, which showed a positive rotation. Natural compound *1* also exhibited a positive rotation, thus establishing its absolute configuration to be as depicted in *1*, and thus identical with that of shikimic acid *15*.

Biosynthesis

The structural and stereochemical similarities between shikimic acid and the metabolite *1* isolated from the fungus invites biosynthetic speculations (Scheme 2). In its well-established metabolism leading to a host of aromatic products, shikimic acid



Scheme 2.

is first converted to its 3-phosphate *16* which reacts with phosphoenolpyruvate and eliminates phosphate to form chorismic acid *17*. If instead the phosphate *16* were to undergo nucleophilic substitution with a nucleophile X, which were at the same time a reasonable leaving group, the *trans* intermediate *18* might expel HX to afford 3,4-anhydroshikimic acid *19*. It is implied that the methyl ester function might be introduced at any stage of this sequence.

EXPERIMENTAL

Melting points were determined on a Kofler micro hot stage and are uncorrected. Specific rotations were measured with a Perkin Elmer 141 Polarimeter. UV spectra were recorded with a Bausch & Lamb Spectronic 505 instrument. IR spectra were recorded on a Perkin Elmer infrared spectrometer model 257. ^{1}H NMR spectra were recorded on a JEOL MH 100 and a JEOL PM 60 instrument. Merck Silica gel 60 F_{254} aluminum sheets were used for TLC. Alumina was Merck Neutral, activity III.

Fermentation. Isolation of 3,4-anhydroshikimic acid methyl ester (1). The fungus was grown as a shaking culture in a medium containing 2% malt extract (Difco) in deionized water. The temperature was kept below 20 °C, and the shaking rate controlled so as to make the fungus grow as small pellets. The activity of the brew was tested by mixing a filtered sample into agar plates (1.5% agar, 2.5% malt extract), and measuring the radial growth of *Fomes annosus* (Fr.) from an inoculated agar block. When the brew showed maximum activity (4 weeks), the mycelium was filtered off, and the filtrate was extracted with ethyl acetate. After drying and evaporation of the extract, the residue was chromatographed on a silica gel column using ether as eluent. The antifungal activity was found to reside in two chromatographically homogeneous fractions with $R_F = 0.42$ (containing *1*) and $R_F = 0.25$ (containing *2*) (TLC, silica gel/ether), which were obtained in a low yield (5 – 15 mg each from 3 l of culture medium). These fractions were subsequently shown to consist mainly (>95%, ^{1}H NMR) of the new compounds 3,4-anhydroshikimic acid methyl ester *1* * and compound *2*, respectively. The fraction containing *1* caused 50% inhibition of the growth of *Fomes annosus* (Fr.) at concentrations of about 1 μg/ml. Since the active principles constitute less than 5% of the fraction, they must be at least 20 times more active.

Physical data for 3,4-anhydroshikimic acid methyl ester 1. Oil, $[\alpha]_D^{20}$ +95° (*c* 0.5, abs. ethanol). High resolution MS [70 eV; *m/e* (rel. int.)]: 152.0485 (14, $C_8H_8O_3$), 139.0405 (36, $C_7H_7O_3$), 138.0312 (100, $C_7H_6O_3$), 127.0379 (21, $C_6H_7O_3$), 126.0323 (11, $C_6H_6O_3$), 124.0519 (11, $C_7H_8O_2$); IR (film): 3440, 3010, 2960, 2940, 1710, 1650, 1445, 1270 cm^{-1}; UV (abs. ethanol) 229 nm (ε = 13000). ^{1}H NMR is given in Fig. 1.

Methyl trans-*4,5-diformyloxy-cyklohexene-1-carboxylate* 6.[5] The ester *4* was dissolved in formic acid (98 – 100%) (25 ml). The catalyst was prepared through dropwise addition of water to dry P_2O_5, accompanied by shaking the mixture until pellets were formed. A few pellets were added to the reaction mixture, which was left at room temperature for 3 h. The mixture was poured into an ice-slurry and extracted with chloroform. After washing the extract with 1 M $NaHCO_3$ and drying, the solvent was removed to give crude formate *6* as an oil (2.20 g, 92%). ^{1}H NMR ($CDCl_3$): δ 8.1 (2H, s), 6.9 (1H, m), 5.3 (2H, m), 3.8 (3H, s), 3.2 – 2.2 (4H, m); IR (film) 2960, 1725 (broad), 1660, 1440, 1260, 1160 cm^{-1}.

*Methyl 3-bromo-*trans-*4,5-dihydroxy-cyclohexene-1-carboxylate* 9a. Formate ester *6* (1.82 g) was dissolved in CCl_4/CS_2 (1:1; 100 ml) and NBS (1.56 g, 1.1 ekv.) was added. The mixture was refluxed while being irradiated with a 500 W lamp for 30 min, cooled and filtered through a short column of alumina. The solvent was evaporated to give a crude mixture of bromides (2.44 g).

Crude bromination product (2.09 g) was hydrolyzed in methanol (20 ml) + phosphate buffer (pH = 7.5) (1 ml) for 20 min at room temperature. Ether (100 ml) and some dry $MgSO_4$ were added. The inorganic salts were filtered off, and the solvents removed at low temperature. The residue was chromatographed, with a high flow rate, on cellulose/H_2O, using ether/pentane (4:1) as eluent. The fractions containing the desired compound *9a* ($R_F = 0.37$, silica gel/ether) were collected, and from these *9a* (0.28 g) could be crystallized from chloroform/acetone. The overall yield of *9a* from *6* was 16%; m.p. 110 – 139 °C, dec.; MS [70 eV; *m/e* (% rel. int.)]: 252 (0.7, M), 250 (0.7, M); ^{1}H NMR ($CDCl_3$): δ 7.0 (1H, dd), 5.1 (1H, dd), 4.35 – 3.9 (2H, m), 3.8 (3H, s), 3.3 – 2.2 (2H, m); *J* (Hz): $H^1 - H^2 = 4.8$, $H^2 - H^3 = 4$ (see Fig. 3); IR (KBr): 3410, 3085, 3020, 3010, 2960, 2920, 2860, 1695, 1650, 1450, 1260 cm^{-1}; UV (abs. ethanol) 224 nm (ε = 22600).

Methyl trans-*3,4-epoxy-5-hydroxy-cyclohexene-1-carboxylate* 1. Bromoester *9a* (75 mg) was treated with excess Na_2CO_3 + NaBr in refluxing acetone (4 ml) for 5 h. After dilution with ether the mixture was filtered through a short column of alumina.

* Later experiments have shown that *1* is not quite stable on silica gel, and that the isolated yield may be improved 3 – 5 times by partition chromatography on cellulose/H_2O with ether pentane (4:1) as eluent.

Evaporation gave *1* (41 mg, 81 %) as an oil. Spectral data were identical with those of isolated natural *1*. Anal. $C_8H_{10}O_4$: C, H.

Methyl cis-*4,5-diacetoxy-cyclohexene-1-carboxylate* *12*, and the bromination of *12* with NBS in CCl_4 to afford bromoester *13* and isomers, have been described by Grewe *et al.*[6]

Methyl cis-*3,4-epoxy-5-hydroxy-cyclohexene-1-carboxylate* 14. The crude bromination mixture (2.0 g) above (*13* and isomers) was hydrolyzed in methanol with sodium methoxide until TLC showed no starting material. Ether was added and the mixture washed with water. The ether phase was dried and concentrated, and the residue was chromatographed on alumina (grade IV) using ether as eluent. This gave a low yield of *14* (0.15 g, 14 %) as an oil. (The product was unstable to alumina). MS [70 eV; *m/e* (rel. int.)]: 170 (2, M); IR (film): 3410, 3000, 2960, 2940, 1720, 1650, 1440, 1260 cm^{-1}; UV (abs. ethanol) 232 nm ($\varepsilon = 11400$). ^1H NMR is given in Fig. 2.

trans-*4,5-Dihydroxy-cyclohexene-1-carboxylic acid* 5. Ester *4* (5.5 g) was dissolved in 2 M NaOH (45 ml) and stirred at 60 °C for 2 h. The mixture was acidified with HCl, and filtered through carbon/celite (2:1) (40 g), which retained the acid *5*. After washing with water until neutrality, the product was eluted with acetone. Evaporation yielded *5* (4.2 g, 83 %), m.p. 185–187 °C (dioxane/toluene).

The acid was partially resolved as its brucine salt by fractional crystallization from ethanol (95 %). To recover the acid, the salt was dissolved in 1 M NaOH, and brucine removed by extraction with chloroform. The water phase was made acidic with HCl, and the acid was isolated as described above, m.p. 197–199 °C (dioxane/toluene); $[\alpha]_D^{20}$ −45° (*c* 0.6, abs. ethanol); Anal. $C_7H_{12}O_4$: C, H.

Methyl trans-*4,5-dihydroxy-cyclohexene-1-carboxylate* 4.[10] Acid *5* (0.40 g) was dissolved in methanol (15 ml), $BF_3 \times Et_2O$ (0.5 ml) was added and the mixture refluxed for 6 h. The methanol was evaporated and the residue dissolved in 1 M Na_2CO_3. Several extractions with ethyl acetate, drying and evaporation gave *4* (0.38 g, 87 %), m.p. 85–93 °C; $[\alpha]_D^{20}$ −41° (*c* 0.6, abs. ethanol).

Methyl trans-*3,4-epoxy-5-hydroxy-cyclohexene-1-carboxylate* 1. Optically active ester *4* was carried through the steps described earlier to give optically active *1*. $[\alpha]_D^{20}$ +18° (*c* 0.5, abs. ethanol).

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