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METABOLITES OF PYRENOMYCETES. XIV.[1] STRUCTURE AND PARTIAL STEREOCHEMISTRY OF THE ANTIBIOTIC MACROLIDES HYPOTHEMYCIN AND DIHYDROHYPOTHEMYCIN.

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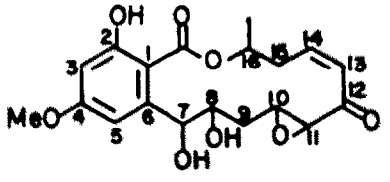
Abstract - Based on spectroscopic data and chemical degradation, hypothemycin and dihydrohypothemycin, two antibiotic metabolites of Hypomyces trichothecoides have been assigned the macrolide structures 1 and 2. Partial stereochemistry of these compounds was determined using Two-Dimensional J proton spectrum and extensive decoupling experiments.

In the course of our survey of pyrenomycetous fungi for biologically active metabolites, we studied the order Hypocreales extensively. Hypocrealean fungi are the source of a number of compounds with varied structures[4-10] which include acetogenins and terpenoids having antibiotic, antitumor, cardiotonic, anabolic and other interesting biological activities.

Hypomyces trichothecoides, one of the hypocrealean fungi selected for further study on the basis of its ability to produce antibiotics in our preliminary screening, when grown in liquid cultures, produced two new antibiotics (+) hypothemycin (1) and (+) dihydrohypothemycin (2). Recently in a short communication[9] we reported the isolation of hypothemycin. Here we present the structure and partial stereochemistry of these two compounds. Both hypothemycin and dihydrohypothemycin were active against the protozoan, Tetrahymena furgasoni and the plant pathogenic fungi Ustilago maydis and Botrytis allii.

Hypothemycin, $C_{19}H_{22}O_8$ and dihydrohypothemycin, $C_{19}H_{24}O_8$ showed maxima in the UV spectrum near 220, 265 and 305 nm typical of the 4-methoxy, resorcylic acid lactone macrolide chromophore present in radicicol derivatives.[11] IR spectrum also showed the highly chelated lactone carbonyl peak at 1620 cm^{-1}.



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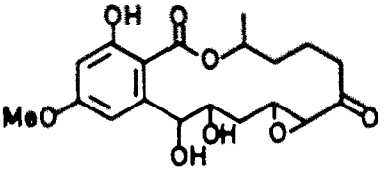

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The ^{13}C-NMR spectrum of hypothemycin showed signals for one methyl at δ 21.0, two methylenes at 34.6 and 36.9 and six sp^3 carbons carrying oxygen at 55.5 (OMe), 57.9 (C_{10}), 62.6* (C_8), 70.7* (C_{11}), 73.2 (C_7) and 81.2 (C_{16}) and sp^2 carbons at 101.3 (C_3), 103.6 (C_5), 104.0 (C_1), 126.4 (C_{13}), 142.3 (C_{14}), 145.3 (C_6), 165.2* (C_4), 166.2* (C_2), 171.2 (COO-lactone) and 199.9 (CO) ppm.

The ^1H-NMR spectrum (Table 1) showed among

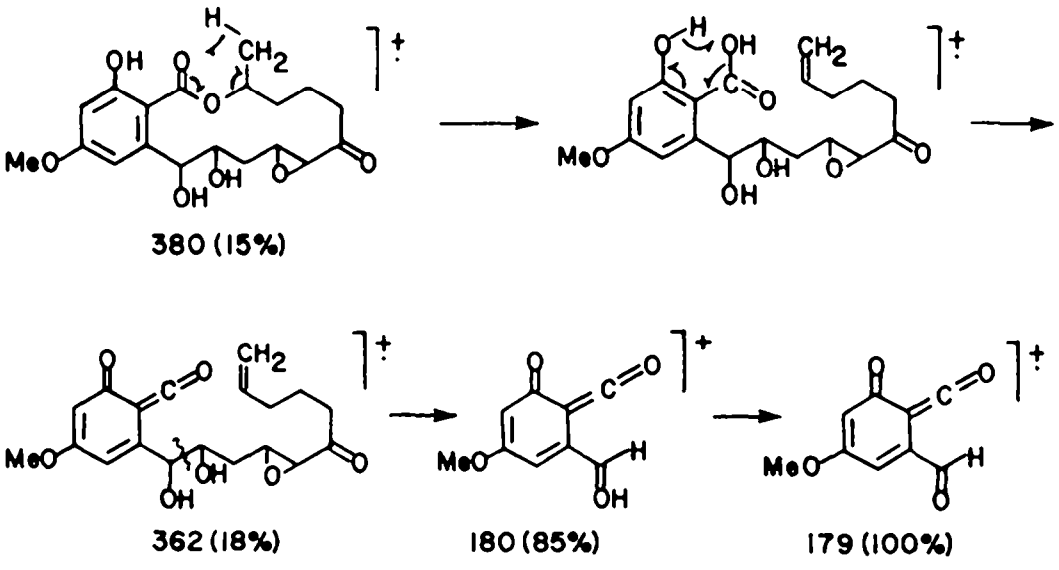
Table 1.

^{1}H NMR Spectra of Hypothemycin and Dihydrohypothemycin.[a]

	Hypothemycin		Dihydrohypothemycin	
Carbon #	δ ^{1}H ppm	J (Hz)[b]	δ ^{1}H ppm	J (Hz)
3	6.13 (s)		6.40 (d)	< 2
5	6.13 (s)		6.50 (d)	< 2
7	4.58 (d)	< 2	4.53 (d)	< 2
8	3.93 (m)	< 2, 8.5, < 2	4.22 (m)	
9	2.05 (m)	4, 8.5, 15	2.15 (m)	4, 8.5, 15
9'	1.12 (m)	< 2, 8.5, 15	1.12 (dd)	8.5, 15
10	2.89 (m)	< 2, < 2, 4	2.98 (m)	< 2, <2, 4
11	4.41 (d)	< 2	4.45 (d)	< 2
13	6.13 (dd)	11, < 2	2.80 (m), 2.62 (m)	
14	6.30 (m)	11, < 2, 11	1.65 (m), 1.73 (m)	
15	3.13 (ddd)	11, 6.5, 15	1.75 (m)	
15'	2.83 (m)	< 2, 4, 15	1.80 (m)	
16	5.48 (m)		5.28 (m)	
16-Me	1.40 (d)	6.5	1.41 (d)	6.5
OMe	3.83 (s)		3.82 (s)	

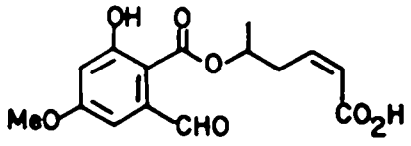
[a] D_2O exchanged spectrum, [b] Values obtained from a combination of 2D J spectrum and decoupling studies.

Scheme 1. Mass spectral fragmentation of dihydrohypothemycin.



other peaks, three protons exchangeable with D_2O and a methoxyl signal at δ 3.83 ppm. Thus seven of the eight oxygens were accounted for by three hydroxyls, one methoxyl, one carbonyl and a lactone moiety. The eighth oxygen has to be in an oxirane ring to accomodate the two remaining sp^3 carbons carrying oxygen in the ^{13}C-NMR spectrum as well as the molecular formula which needs nine unsaturations or rings.

Hypothemycin on oxidation with sodium metaperiodate gave a degradation product which was assigned the structure 3 on the basis of its ^1H-NMR spectrum and decoupling studies. Formation of this aldehyde acid defined the substitution pattern of the macrolide ring of hypothemycin, except for the four carbons lost during oxidation.



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By employing extensive decoupling experiments on a conventional 220 MHz spectrometer employing a Computer Averaging Transients and using relatively new Two Dimensional J spectroscopy on a 250 MHz PRFT-NMR spectrometer all the signals of both the compounds could be assigned and coupling constants could be determined (Table 1). In the former method, coupling constants could be arrived at accurately only when they were large and/or when the resolution was very good. In some cases sharpening of the peaks on decoupling and the resulting reduction of the width at half height was used to determine the coupling constants and at best, these values are only approximate. The 250 MHz 2 Dimensional J spectra of hypothemycin (a partial spectrum, the higher field, is shown in Fig. 1) and dihydrohypothemycin enabled accurate assignment of all the chemical shifts and determination of all but very low vicinal and long-range coupling constants. Theoretical aspects of this NMR technique have been extensively discussed in several recent papers.[12] The practical result is a 2D spectrum in which chemical shift information is contained along one frequency axis (f_2) and homonuclear coupling constant information in the other (f_1) axis. The J resolved spectrum (Fig. 1D) enables accurate measurements of coupling constants through greatly enhanced resolution. However, in this technique most of the long-range coupling and very low vicinal coupling are often not detected due to the limitations imposed by line broadening of slowly relaxing protons. In Fig. 1D, 9H' and

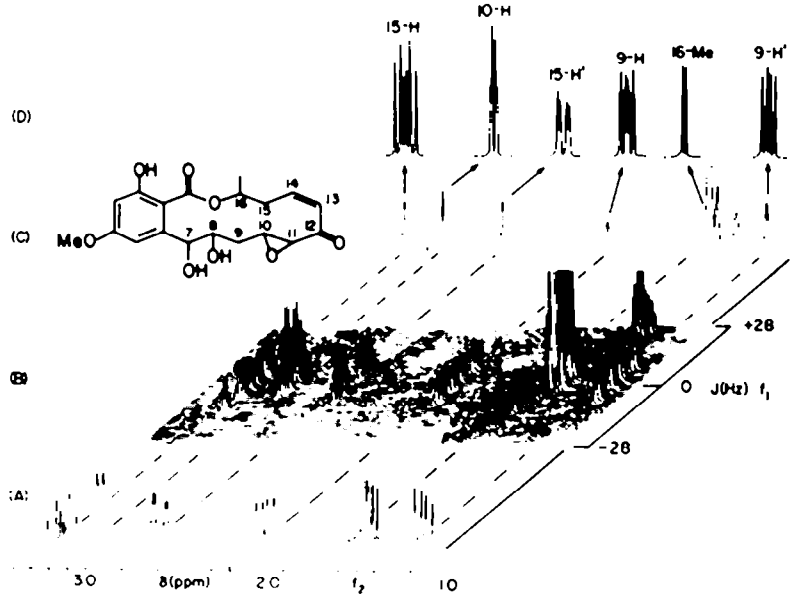
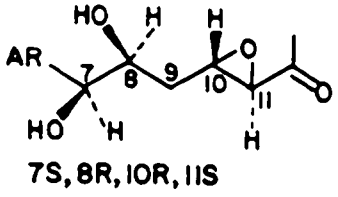


Fig. 1. Partial (high field) PMR spectrum of hypothemycin. A) Normal 250 MHz PMR spectrum. B) 2-D J spectrum. C) Proton-decoupled proton spectrum. D) Sections showing J resolved spectrum of high field protons.

15H' which are coupled to 10H and 16H with very low coupling constants, appear as a doublet of a doublet rather than as a doublet of a doublet of a doublet like their geminal counterparts.

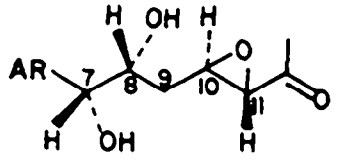
Both hypothemycin and dihydrohypothemycin gave the same main product on hydrogenation in presence of reduced platinum oxide catalyst indicating structure 2 for dihydrohypothemycin. The NMR data (Table 1) as well as the fragmentation pattern in the high resolution EI-mass spectrum (Scheme 1)confirmed this structure.

The complete analysis of the NMR spectrum also helped in assigning the relative stereo chemistry of carbons 7, 8, 10 and 11 (Fig. 2) of hypothemycin.



7S,8R,10R,11S

or



7R,8S,10S,11R

Fig. 2.

The protons of carbon 7 and 8 have a coupling constant of less than 2 Hz showing them to be threo and the low coupling constant, again less than 2 Hz between the oxirane ring protons show they are trans to each other. The high field 9H which is coupled to 8H (J=8.7Hz) is also coupled to 10H (2Hz). These coupling constants show that the glycol hydroxyls and the epoxide ring are on the opposite sides of the molecule, leading to the relative configuration as shown in Fig. 2.

The mycelial extract of this fungus was studied and gave mainly ergosterol, fatty material and the taxonomiclly important pigment skyrin.[13]

EXPERIMENTAL

Melting points were determined on a Koeffler hot stage and are uncorrected. IR spectra were determined in KBr pellets and UV spectra in 95% EtOH. 220 MHz NMR spectra were determined on a Varian NMR spectrometer equipped with CAT and 80 and 250 MHz and ^{13}C spectra were taken on Brucker WP-80 and WP-250 PRFT multinuclear spectrometers. All NMR spectra were taken in $CDCl_3$ with TMS as standard. High resolution MS was obtained from Cornell University Mass Spectrometry Facility using MS 902 and low resolution EI- and CI-MS on Finnigan 3300 mass spectrometers.

Specimens of the fungus Hypomyces trichothecoides are on deposit in the Herbarium of the New York Botanical Garden.

Culture: Two single-ascospore cultures of H. trichothecoides that had been isolated from an ascus were combined and grown on a modified dextrose-yeast medium in Fernbach flasks containing glass wool in still cultures in the dark at 25°C. It was harvested 22 days after innoculation.

Extraction and isolation: The culture liquid (10 l) was separated from the mycelia and glass wool by straining through cheesecloth and was extracted three times with 5, 2.5 and 2.5 liters of EtOAc. The extracts were combined and taken down to dryness in vacuo and the residue (ca. 5 g) was partitioned in a 60 tube (150 ml) counter-current distribution unit using a four-solvent system obtained by equilibrating equal volumes of H_2O-MeOH (1:1) and ether-petroleum ether (2:1). The more polar fractions 6-22 were combined and most of the organic solvents were removed at 25° under vacuum when a crystalline material precipitated in the water. These crystals, mp 166-168°C, showed an unexpectedly high M+2 peak in MS suggesting an impurity of the dihydro compound. However, purification could not be achieved by open column chromatography, TLC or repeated crystallizations. Separation was finally effected by reverse phase liquid chromatography on a micro C_{18} column (Waters Associates) using 65% aqueous methanol. However, the poor solubility of the mixture in this solvent system , which was the only one to give a reasonable resolution of the mixture made the separation very tedious.

Hypothemycin, 1, mp 173-174°C, $C_{19}H_{22}O_8$ (elemental analysis) had MW 378 (MS) and $[\alpha]365 = +109°$ (0.136% MeOH). λ_{max} at 220 (34,000), 267 (14,000) and 307 nm (7,000); ν_{max} at 3350 (b), 1695, 1653, 1620, 1593 and 1250 cm^{-1} and MS peaks at 378, 360, 180 and 179 (base peak); CD maxima, [θ],(MeOH) at 335 (+ 3,351), 305 (- 8,987), 262 (- 40,000), 234 (+ 29,000) and 212 nm (+ 77,000).

Dihydrohypothemycin, 2, mp 178-179°C, $C_{19}H_{24}O_8$, MW 380.1501 (MS) had λ_{max} 305 (7,000), 265 (14,900) and 219 nm (31,000); ν_{max} 1703, 1650, 1620, 1595 cm^{-1}. ^1H-NMR is given in Table 1 and mass spectrum in Scheme 1. Crystals of both hypothemycin and the dihydro compound were unstable even at -20° in the dark under N_2 and showed several small peaks in the LC trace after a few days.

Hydrogenation of hypothemycin and dihydrohypothemycin: Twenty mg of both the compounds in 10 ml ethanol were hydrogenated in presence of reduced platinum oxide catalyst. The major component isolated by TLC in both the cases was the same, mp and mixed mp 112°C and mass spectra were the same, M^+ = 382. Hydrogenation opens the epoxide and probably the hydroxyl is on the carbon α to the ketone. This reaction was done when PRFT-NMR was not available to us.

Periodate degradation of hypothemycin: Hypothemycin (20 mg) was dissolved in 10 ml methanol, and sodium metaperiodate (100 mg) in 3 ml methanol was added at 50°C under N_2 and the mixture was stirred for 30 min. The solution was centrifuged and the clear liquid was

evaporated to remove the bulk of methanol. Dilution with 10 ml of water and extraction with ethylacetate and separation of the extract on TLC plate gave an aldehyde acid. The PRFT-NMR of the compound combined with decoupling experiments proved it to be 3. NMR peaks at δ 1.41 (3H, J=6.5) for C-Me, 3.1 (2H, dd, J=6.5, 8.0) for C_{15} protons, 3.83 (3H, s) for OMe, 5.45 (1H, qt, J=6.5, 6.5) for C_{16} proton, 5.95 (1H, d, J=11) for C_{13} proton, 6.40 (1H,dd, J=8, 11) for C_{14} proton and an AB quartet at 6.6 and 6.8 (J=2Hz) for the aromatic protons. The CI-MS showed the molecular weight to be 312, in agreement with this structure.

Antibiotic activity: In all cases binary serial dilution method of Kavanagh[14] was employed, and the compounds were dissolved in 20% ethanol.

1) Tetrahymena furgasoni, (ATCC 10542): the organism was grown in a protease peptone-yeast medium at 25°C and chloromycetin was used as positive control. Water was inactive and 20% ethanol inactive beyond the second tube. Hypothemycin and dihydrohypothemycin were active at 30 ppm (LD_{100}) and 1 ppm (LD_{50}). LD_{50} was determined by counting the motile cells.

2) Ustilago maydis, (ATCC 14826) and Botrytis allii (ATCC 9435): Spores of these fungi were suspended in the yeast medium consisting of dextrose, ammonium sulfate, potassium dihydrogen phosphate and magnesium sulfate in deionized water. Nystatin was used as positive control. The negative controls, water was inactive in the first tube and 20% ethanol beyond the first tube. Hypothemycin was active (LD_{50}) at 60 and 120 ppm against U. maydis and B. allii, respectively.

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