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Monaghan et al.

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[54] **HYPOCHOLESTEREMIC FERMENTATION PRODUCTS AND PROCESS OF PREPARATION**

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[51] **Int. Cl.**[3] **C07D 309/30**

[52] **U.S. Cl.** **260/343.5**; 560/256; 435/125

[58] **Field of Search** 260/343.5

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,983,140	9/1976	Endo et al.	260/343.5
4,049,495	9/1977	Endo et al.	195/36 R
4,137,322	1/1979	Endo et al.	424/273 R

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A. Endo et al., The Journal of Antibiotics, vol. XXIX, No. 12 (1976), pp. 1346–1348.

A. Endo et al., FEBS Letters, vol. 72, No. 2 (1976), pp. 323–326.

A. G. Brown et al., J.C.S. Perkin I (1976), pp. 1165–1169.

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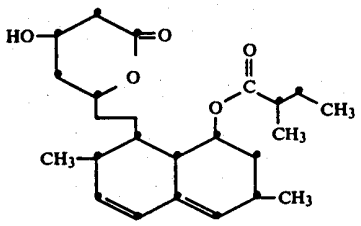
[57] **ABSTRACT**

Substances isolated after cultivation of a microorganism belonging to the genus Aspergillus in a culture medium comprise a compound designated MSD803 which has the lactone structure:



as well as its free hydroxy acid form. Together with salts and esters of the free acid form, these compounds form a class of highly active hypocholesteremic and hypolipemic medicaments.

1 Claim, 2 Drawing Figures

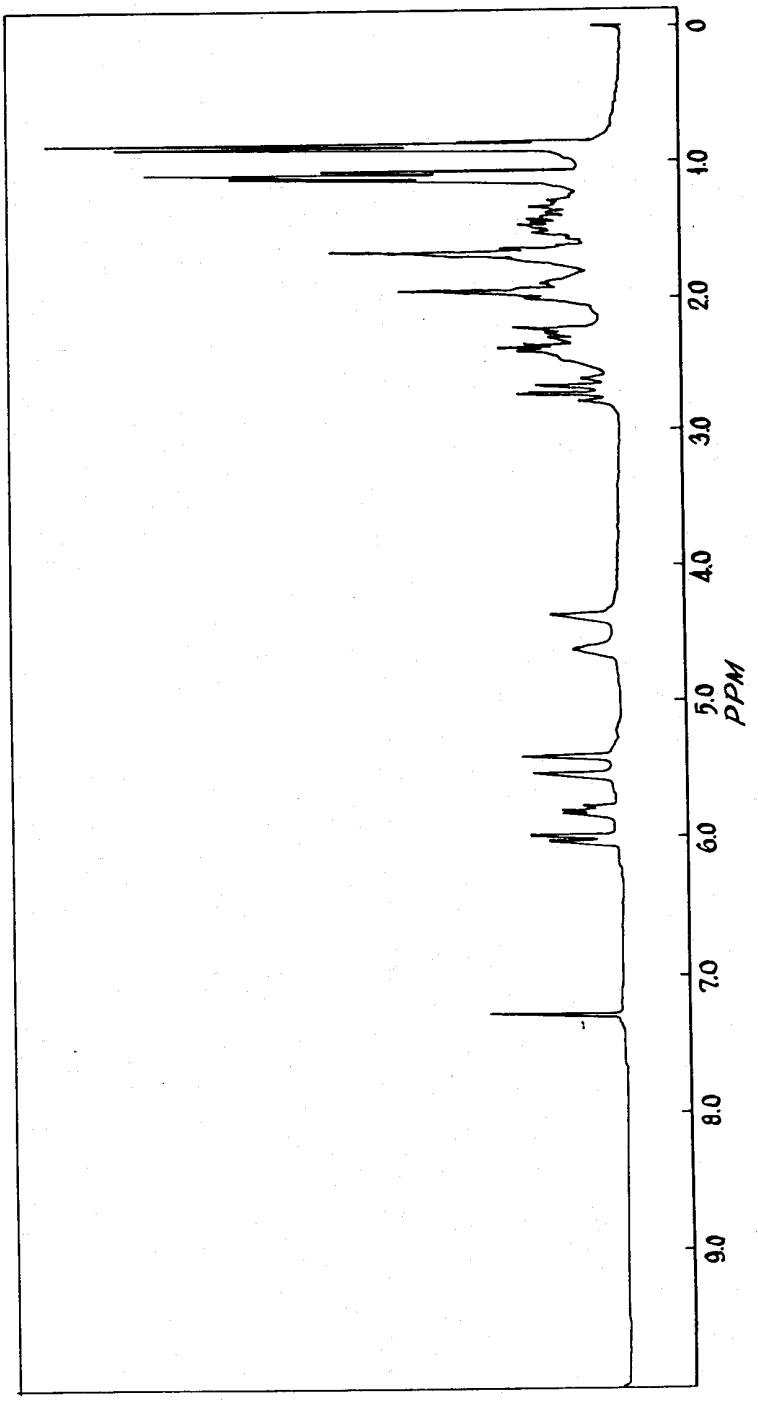
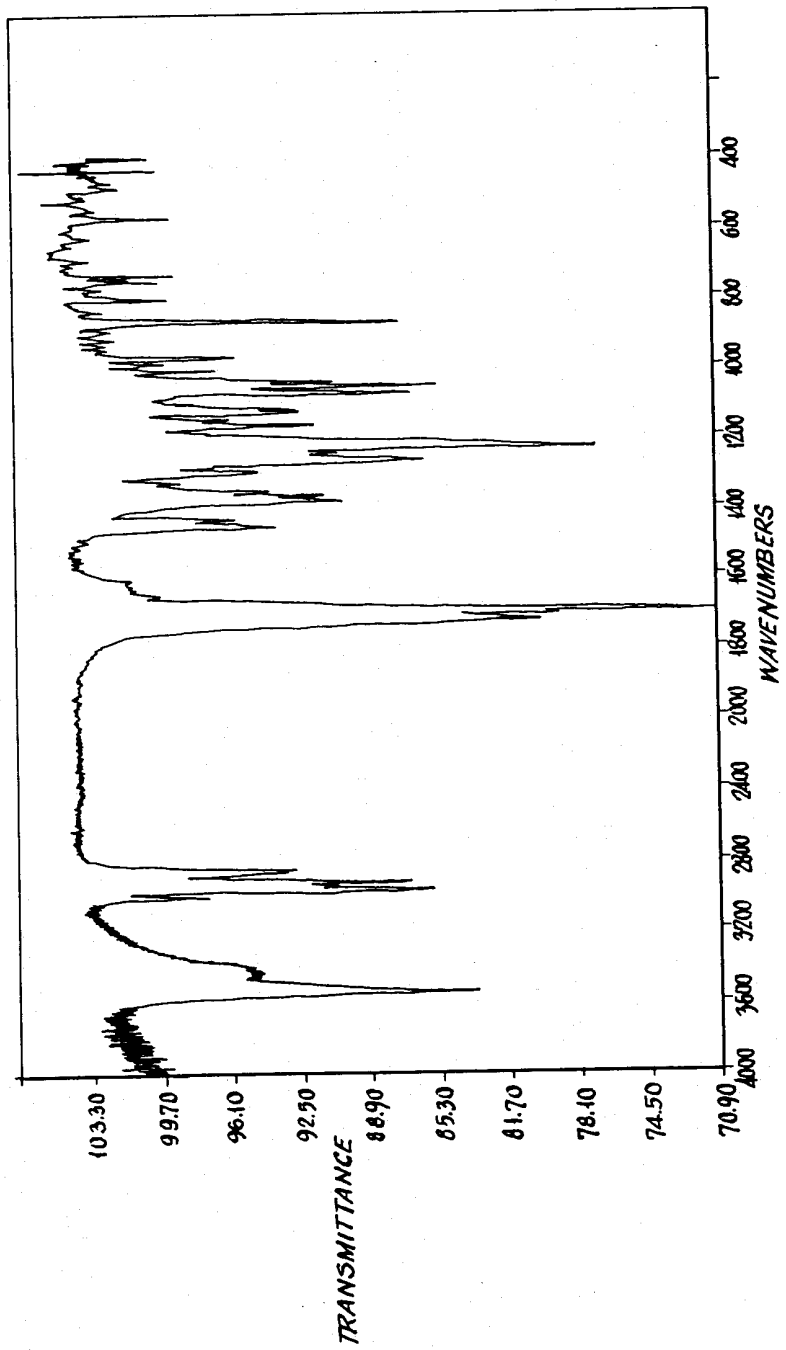


Fig. 1.

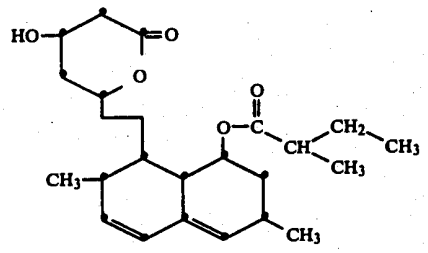
Fig.2.



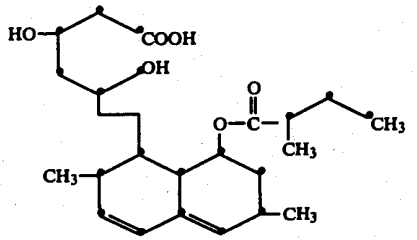
HYPOCHOLESTEREMIC FERMENTATION PRODUCTS AND PROCESS OF PREPARATION

SUMMARY OF THE INVENTION

This invention relates to hypocholesteremic products from the cultivation of a microfungus of the genus Aspergillus. More specifically, it relates to a compound of the formula:



and to the corresponding free hydroxyacid



as well as pharmaceutically acceptable salts of the latter and lower alkyl and substituted alkyl esters of the latter in which the possible substituent is phenyl, dimethylamino or acetylamino. The invention also relates to a process of cultivating the microfungus and isolating from the medium a hypocholesteremic compound of the above structures. These new compounds have excellent properties of inhibiting cholesterol biosynthesis and are useful against hypercholesteremia and hyperlipemia.

BACKGROUND OF THE INVENTION

Because of the possible connection between high blood cholesterol and atherosclerosis, many efforts have been made to find ways and substances which would reduce the cholesterol in the mammalian body. One of these ways is to inhibit in mammals the body's ability to synthesize cholesterol.

Recently, Endo et al., described (U.S. Pat. Nos. 4,049,495 and 3,983,140) a fermentation product obtained by cultivation of a microorganism of the genus Penicillium and isolation from the medium. They called it ML 236 B and determined its structure together with two related compounds 236 A and 236 C. Its structure, under the name compactin, was also determined by A. G. Brown, T. C. Smale, T. J. King, J. Chem. Soc. (Perkin I) 1165 (1975). This compound has been found to be a strong inhibitor in vivo of the biosynthesis of cholesterol.

DESCRIPTION OF THE INVENTION

We have found that unexpectedly, the cultivation of a very different microorganism, a microfungus of the genus Aspergillus produces a new substance that is also a very potent inhibitor of the biosynthesis of cholesterol in mammals. We have further found that this substance comprises principally the new compound, MSD803, of the above structure, accompanied by only traces of other compounds, none of which appears to be those isolated by Endo et al. This new compound of our invention does not appear to be formed in the fermentations described by Endo. The new compound, MSD803, is a much more potent inhibitor of cholesterol synthesis in vivo than is the compound, ML236B described by Endo.

The compounds of this invention are highly useful as antihypercholesteremic agents for the treatment of atherosclerosis, hyperlipemia and like diseases in humans. They may be administered orally or parentally in the form of a capsule, a tablet, an injectable preparation and the like. It is usually desirable to use the oral route. Doses may be varied, depending on the age, severity, body weight and other conditions of human patients but daily dosage for adults is within a range of from about 2 mg. to 2000 mg. (preferably 10 to 100 mg.) given in three or four divided doses. Higher doses may be favorably applied as required.

The compounds of this invention also have useful antifungal activities. For example, they may be used to control strains of *Penicillium sp., Aspergillus niger, Cladosporium sp., Cochliobolus miyabeorus* and *Hilminthosporium cynodnotis.* For those utilities they are admixed with suitable formulating agents, powders, emulsifying agents or solvents such as aqueous ethanol and sprayed or dusted on the plants to be protected.

In another aspect of this invention, it relates to a process for producing the compounds of this invention which comprises cultivating a microorganism belonging to the genus Aspergillus and then recovering said compounds of this invention from the cultured broth. Based upon taxonomic studies, this Aspergillus, isolated and identified as a hitherto undescribed microorganism, has been designated MF-4833 in the culture collection of Merck and Co., Inc., Rahway, N.J. and a culture thereof has been placed on permanent deposit with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, and has been assigned accession No. ATCC No. 20541. Another sample, of a similar organism, designated MF-4845 in the Merck culture collection, has likewise been placed on deposit and has been given the accession number ATCC 20542. The latter organism is the one giving the better yield. Although the use of these is described in connection with the process of this invention, other organisms of the genus Aspergillus including mutants of the above ones are also capable of producing MSD803 and their use is contemplated in carrying out the process of this invention.

The morphological characteristics of the microorganisms MF-4833 and MF-4845 have been found to be those of the genus Aspergillus. Using the criteria specified in the standard authority "Manual of the Aspergilli", Charles Thom and Kenneth B. Rasper, published by the Williams and Wilkins Company, Baltimore, Md., 1945, and by comparison with know species, it has been determined that both strains are *Aspergillus terreus.*

The culture of these organisms to produce MSD803 is carried out in aqueous media such as those employed for the production of other fermentation products. Such media contain sources of carbon, nitrogen and inorganic salts assimilable by the microorganism.

In general, carbohydrates such as sugars, for example, glucose, fructose, maltose, sucrose, xylose, mannitol and the like and starches such as grains, for example, oats, ryes, cornstarch, corn meal and the like can be used either alone or in combination as sources of assimilable carbon in the nutrient medium. The exact quantity of the carbohydrate source of sources utilized in the medium depend in part upon the other ingredients of the medium but, in general, the amount of carbohydrate usually varies between about 1% and 6% by weight of the medium. These carbon sources can be used individually, or several such carbon sources may be combined in the medium. In general, many proteinaceous materials may be used as nitrogen sources in the fermentation process. Suitable nitrogen sources include for example, yeast hydrolysates, primary yeast, soybean meal, cottonseed flour, hydrolysates of casein, corn steep liquor, distiller's solubles or tomato paste and the like. The sources of nitrogen either alone or in combination, are used in amounts ranging from about 0.2% to 6% by weight of the aqueous medium.

Among the nutrient inorganic salts which can be incorporated in the culture media are the customary salts capable of yielding sodium, potassium, ammonium, calcium, phosphate, sulfate, chloride, carbonate, and like ions. Also included are trace metals such as cobalt, manganese, iron and magnesium.

It should be noted that the media described in the Examples are merely illustrative of the wide variety of media which may be employed, and yet are not intended to be limitative. Specifically, the carbon sources used in the culture media to produce MSD803 included dextrose, dextrin, oat flour, oatmeal, molasses, citrate, soybean oil, glycerol, malt extract, cod liver oil, starch, ethanol, figs, sodium ascorbate and lard oil. Included as nitrogen sources were peptonized milk, autolyzed yeast, yeast RNA, tomato paste, casein, primary yeast, peanut meal, distillers solubles, corn steep liquor, soybean mean, corn meal, NZ amine, beef extract, aspargine, cottonseed meal and ammonium sulfate. The major ionic components were $CaCO_3$, KH_2PO_4, $MgSO_4.7H_2O$ and NaCl and small amounts of $CoCl_2.6H_2O$ and traces of Fe, Mn, Mo, B and Cu were also present.

The fermentation is carried out at temperatures ranging from about 20° to 37° C.; however, for optimum results it is preferable to conduct the fermentation at temperatures of from about 22° to 30° C. The pH of the nutrient media suitable for growing the Aspergillus culture and producing MSD803 can vary from about 6.0 to 8.0.

Although the novel compound is produced by both surfce and submerged culture, it is preferred to carry out the fermentation in the submerged state. A small scale fermentation is conveniently carried out by inoculating a suitable nutrient medium with the Aspergillus culture and, after transfer to a production medium, permitting the fermentation to proceed at a constand temperature of about 28° C. on a shaker for several days.

The fermenation is initiated in a sterilized flask of medium via one or more stages of seed development. The nutrient medium for the seed stage may be any suitable combination of carbon and nitrogen sources. The seed flask is shaken in a constant temperature chamber at about 28° C. for 2 days, or until growth is satisfactory, and some of the resulting growth is used to inoculate either a second stage seed or the production medium. Intermediate stage seed flasks, when used, are developed in essentially the same manner, that is, part of the contents of the flask from the last seed stage are used to inoculate the production medium. The inoculated flasks are shaken at a constant temperature for several days, and at the end of the incubation period the contents of the flasks are centrifuged or filtered.

For large scale work, it is preferable to conduct the fermentation in suitable tanks provided with an agitator and a means of aerating the fermentation medium. According to this method, the nutrient medium is made up in the tank and sterilized by heating at temperatures of up to about 120° C. Upon cooling, the sterilized medium is inoculated with a previously grown seed of the producing culture, and the fermentation is permitted to proceed for a period of time as, for example, from 3 to 5 days while agitating and/or aerating the nutrient medium and maintaining the temperature at about 28° C. This method of producing MSD803 is particularly suited for the preparation of large quantities.

The compound is conveniently isolated from the fermentation broth as the lactone. However, MSD803 is present in the fermentation broth largely as the hydroxycarboxylate (open lactone) form. It is possible to isolate this form and its salts. Alternatively, the lactone form can be hydrolyzed with bases such as NaOH to yield the corresponding salts such as the sodium salts. The use of bases with the pharmaceutically acceptable cations affords salts of these cations. Careful acidification of the salts affords the hydroxy acid form. Conversely, the hydroxy acid can be converted to the lactone form at acidic pH. Opening the lactone, under catalysis, with methanol, ethanol, propanol, or butanol or with phenyl, dimethylamino, or acetylamino alkanols yields the corresponding esters of this invention.

The physico-chemical properties of MSD803 in its lactone form are summarized as follows:

1.	Melting point	170°–171°
2.	Molecular Weight (mass spectrum)	404
3.	Formula	$C_{24}H_{36}O_5$
	(found by mass spectrometry	404.2555
	calculated	404.2563
4.	UV Spectrum (in acetonitrile) :	Maxima
		230.5 nm with E% 505.7
		237.5 nm with E% 576.6
		246 nm with E% 395.2

5. ^{13}C NMR chemical shifts. The spectrum has been recorded in $CDCl_3$ solution (20.1 mg in 0.35 ml). Chemical shifts are given relative to internal tetramethylsilane at zero ppm; under the experimental conditions the solvent ($CDCl_3$) signal appears centered at 70.0 ppm. In agreement with mass spectral data 24 carbon atoms are observed; their chemical shifts are: 11.5, 13.6, 16.0, 22.6, 24.1, 26.6, 27.2, 30.5, 32.8, 35.9, 36.4, 37.1, 38.4, 41.3, 62.4, 67.8, 76.4, 128.4, 129.7, 131.7, 133.2, 170.8 and 177.2 ppm.
6. ^1H NMR Spectrum. The spectrum was recorded in $CDCl_3$ solution and chemical shifts are shown in FIG. 1 in ppm relative to internal tetramethylsilane at zero ppm.
7. IR Spectrum. The infra red spectrum was recorded in a KBr pallet preparation of a sample. It is shown in FIG. 2.

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