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Secondary metabolites of the fungus *Monascus*: a review

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This review deals with polyketides produced by the filamentous fungus *Monascus* which include: 1) a group of yellow, orange and red pigments, 2) a group of antihypercholesterolemic agents including mevinolin and related compounds and 3) the newly discovered metabolite ankalactone. Biosynthesis, methods of production, isolation and biological activities of these secondary metabolites are discussed.

Keywords: *Monascus*; pigments; rubropunctatin; monascorubrin; rubropunctamine; monascorubramine; ankaflavin; monascin; mevinolin; monacolin; lovastatin; ankalactone

Introduction

The genus *Monascus*, which includes four species: *M. pilosus*, *M. purpureus*, *M. ruber* [39] and *M. floridanus* [7], belongs to the class *Ascomycetes* and the family *Monascaceae*. This fungus is a source of various secondary metabolites of polyketide structure. The aim of the present paper is to summarize the present state of knowledge of this diverse group of compounds some of which are applicable as food additives (the red pigments) or pharmaceuticals (mevinolin).

Pigments

Structure and biosynthesis

Organisms in the genus *Monascus* produce a mixture of six major pigments of polyketide origin [17,31,40,44] (Figure 1). In recent years, two novel yellow pigments have been discovered [86,100] (Figure 2).

The orange pigments, monascorubrin and rubropunctatin, are synthesized in the cytosol from acetyl coenzyme A (Figure 3) by the multienzyme complex of polyketide synthase I [43,85]. These compounds possess a unique structure responsible for their high affinity to compounds with primary amino groups (so called aminophiles). Reactions with amino acids (Figure 4) yield the water-soluble red pigments, monascorubramine and rubropunctamine [11,34,64].

The mechanism of formation of the yellow pigments, ankaflavin and monascin, has not yet been elucidated. Carels and Shepherd [14] supposed that these compounds originated from chemical oxidation of monascorubrin and rubropunctatin. However, their structures (Figure 1) strongly suggest that the yellow pigments are reduced derivatives of the orange ones. Thus, the suggestion of Yongsmith *et al* [101] that ankaflavin and monascin have their own biosynthetic pathway seems to be more probable.

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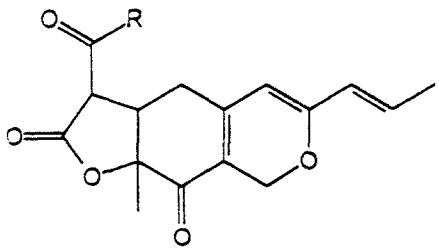
Utilization and biological activity

Red rice (ang-kak) obtained by cultivation of *Monascus* species on rice grains has been well-known as a food dye in Japan and China for centuries. Recent efforts to replace synthetic food dyes by natural colorants, *Monascus* pigments, has attracted worldwide attention.

Extracts from red rice have been suggested as a substitute for the nitrate/nitrite salts in meat products [30,33]. It has been demonstrated that extracts exert no acute toxic effects on mice [32]. Furthermore, treated animals exhibited favorable changes of lipid blood levels. However, this effect might be ascribed to the presence of other metabolites, eg mevinolin, in the crude extracts. Leistner *et al* [60] concluded that the genotoxic potential of extracts from *Monascus* species was much lower than that of nitrosamines which possibly occur in cured meats.

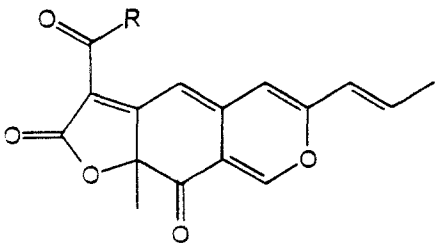
Monascus species, first mentioned in a monograph of Chinese medicine in 1590, were also used for the treatment of, eg indigestion, muscle bruises, dysentery and anthrax [99]. However, the first scientific report on the antibiotic activity of this fungus appeared in 1977 when Wong and Bau [97] found antibacterial effects of *M. purpureus* on *Bacillus*, *Streptococcus* and *Pseudomonas*. Two major active yellow-colored compounds, a yellow pigment of an unknown structure named monascidin A and a fluorescent yellow pigment, were isolated from a crude pigment extract by chromatography on silica gel columns and thin layers [99]. Leistner and Dresel [60] reported on the bacteriostatic action of the *Monascus* extract against *Staphylococcus aureus*. The active fractions were colorless and sterilizable.

A note describing substantial inhibitory effects of rubropunctatin and monascorubrin on *Bacillus subtilis* and *Escherichia coli* appeared in a paper by Nozaki *et al* [81]. Recently, we found [72] that the orange pigments were most probably responsible for not only antibacterial, but also antifungal, immunosuppressive, embryotoxic and teratogenic activities of extracts from submerged *Monascus* cultures. These samples impaired the concanavalin A-stimulated proliferation of mouse splenocytes and human peripheral blood cells and exhibited toxic and teratogenic effects on chicken embryos. On the contrary, the extracts from red rice were harmless to chicken embryos. The main



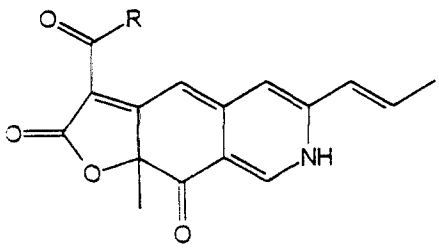
R = C_5H_{11} Monascin
R = C_7H_{15} Ankaflavin

Yellow pigments



R = C_5H_{11} Rubropunctatin
R = C_7H_{15} Monascorubrin

Orange pigments

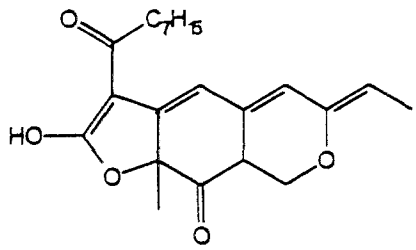


R = C_5H_{11} Rubropunctamine
R = C_7H_{15} Monascorubramine

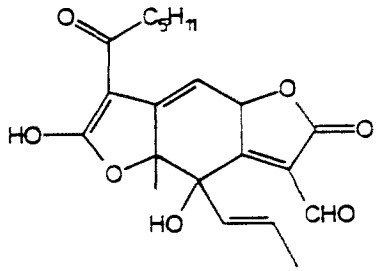
Red pigments

Figure 1 Major pigments produced by members of the genus *Monascus*.

colored components of red rice are probably pigment amino-acid complexes lacking toxic effects.



"Yellow II" [100]



Xanthomonascin A [86]

Figure 2 Novel yellow pigments produced by members of the genus *Monascus*.

Production

Solid state cultivation: The traditional manufacture of red rice includes the following steps: rice is washed, soaked in water for 24 h, drained, steamed, sterilized, fermented and dried [20].

One of the conditions of a successful cultivation is usually a low initial substrate humidity (25–30%, w/w) which prevents the risk of bacterial contamination, the sticking of rice grains together [41] and keeps a low glucoamylase activity of the fungus in favor of pigment production [68]. Nevertheless, some *Monascus* strains require a substantially higher water content in the substrate (approximately 50%, w/w) [46,49].

Another important factor is the oxygen supply. Han and Mudgett [38] recommended oxygen and carbon dioxide partial pressures of 0.5 and 0.02 atm, respectively. Pigment production was more sensitive than growth to oxygen and carbon dioxide concentrations in the atmosphere. In order to achieve a sufficient aeration of the mycelium it is also advisable to separate grains from agglomerates formed during sterilization or cultivation. This separation is quite easy when cultivation is carried out in plastic bags [68] or in a fermenter with a moving bed ('swing' fermenter) [57].

Lin [61] reported that solid state cultivation resulted in a higher pigment yield than cultivation in shaken flasks and concluded that this phenomenon could be due to a minor inhibition by the product. In solid state culture, pigments were released into grains while during submerged culti-

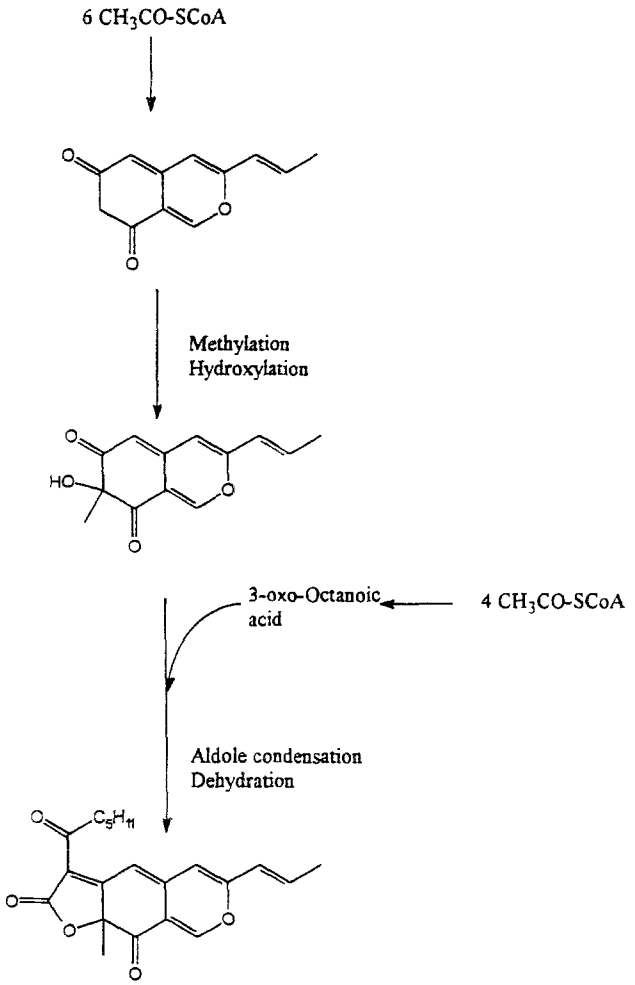


Figure 3 Probable mechanisms of the biosynthesis of rubropunctatin [39].

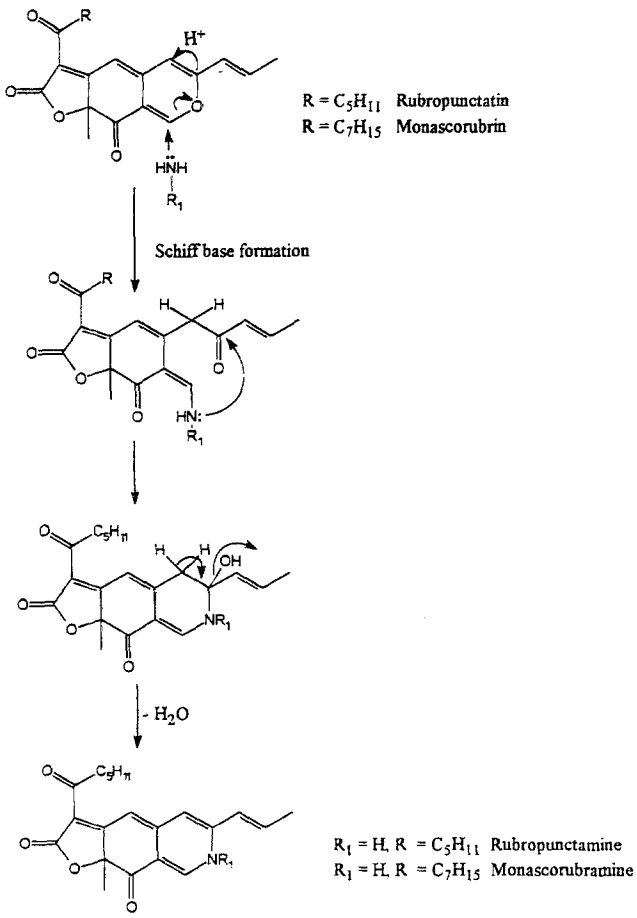


Figure 4 Formation of red pigments [64].

vation they accumulated in the mycelium. However, Evans and Wang [29] showed that better pigment yields by solid state cultures were probably not caused by the extractive effect of rice grains because addition of sorbent particles into submerged cultures did not result in an increase of pigment production. These authors suggested that the reason might be rather the attachment of the mycelium to the grains. Johns and Stuart [46] supposed that the microscopic porous structure of rice influenced the cultivation favorably because the substitution of this substrate for carrageenan particles containing all nutrients was not successful.

Lin and Iizuka [63] compared various kinds of substrates and found that the use of steamed bread (mantou) led to the best pigment yield. In addition to rice and bread, oat [84], corn or wheat grains [41,63] can serve as substrates for the solid state cultivation of *Monascus* species.

Submerged cultivation: In general, pigment production can be influenced by the medium composition, especially by the type of nitrogen source, whereas the suitability of the carbon source seems to be strain dependent, and dependent upon oxygen supply.

1) Effect of the carbon source. Glucose was held by most authors [13,65,82,102] to be a superior substrate for pigment production by *Monascus* species. However, others, [74] found glucose to be less suitable for this purpose. This may be caused by strain differences or by other differences in medium composition (glucose concentration, type of nitrogen source). A high glucose concentration (50 g L^{-1}) led to low growth rates, pigment synthesis and considerable ethanol production [19], perhaps due to induction of respirofermentative metabolism (Crabtree effect) in submerged aerobic cultures of *M. purpureus* by high glucose levels. It was recommended that glucose be maintained below 20 g L^{-1}.

Starch, maltose [56,61,65,74,102], sucrose and galactose [61,82,102] were suitable carbon sources for pigment production, whereas lactose, fructose and xylose were inferior substrates [61,65,82]. Nevertheless, we observed (unpublished results) that for a strain of *M. ruber* and a strain of *M. purpureus*, fructose gave pigment yields comparable to glucose. Some strains of *M. ruber* can be also grown on cellulose but pigment production is negligible. [12].

Stimulation of pigment production by ethanol in some *Monascus* strains [30,50,102] could originate from a higher cellular pool of acetyl CoA formed during cultivation on ethanol in comparison with that on sugars. Maltitol and glycerol were tested as substrates for pigment production; the former was a superior substrate [74] but the latter resulted in poor pigment production [61].

Fatty acids can be transformed by *Monascus* into methyl-

ketones [55,83] but cannot be utilized as sole carbon sources [47].

2) Effect of the nitrogen source. The effect of the nitrogen source on submerged cultures of glucose-utilizing *Monascus* species was described by Shepherd [89] and Carels and Shepherd [14]. Yeast extract-stimulated conidiation, repressed the sexual cycle and increased biomass production. Due to the formation of copious amounts of conidia, pigment production (calculated from spectrophotometric measurements) remained at a relatively low level. It was proposed that the orange pigments entered reactions with amino acids because the pH (above 5) in cultures assimilating organic nitrogen source was favorable for this interaction. Sodium nitrate supported sporulation, limited growth and gave intermediate pigment yields; the use of ammonium chloride resulted in a repression of conidiation and the sexual cycle and led to the best pigment yields. In this medium the dramatic pH decrease impaired the pigment-amine interactions giving origin to red pigments.

In addition to ammonium chloride, peptone also yielded superior growth and pigment amounts when compared with sodium nitrate [19]. Surprisingly, in this work monascorubramine was the major product in all media in spite of the low pH of some cultures.

For the formation of red pigments in a MOPS-buffered culture, monosodium glutamate was the most favorable nitrogen source [65]. In an unbuffered culture of another *Monascus* strain ammonium glutamate gave superior pigment yields [50].

3) Effect of other medium components. The only trace element which was reported to support growth and pigment production by *Monascus* species was zinc [8,47,73]. This effect could be due to the participation of zinc in the uptake and utilization of carbon sources.

Shepherd [89] found that addition of individual amino acids influenced neither growth nor pigment production. On the other hand McHan and Johnson [73] reported that almost all protein amino acids except lysine stimulated growth. Pigment production was also increased by the addition of nonprotein amino acids, especially methanproline and azetidinecarboxylic acid [58]. Leucine, valine, lysine and methionine had strong negative effects on the formation of hydrophilic red pigments, ie pigments containing an amino acid side-chain [66].

Addition of a crystallization inducer, poly(oxyethylene)-sorbitane esters of palmitic acid (Tween), to the cultivation medium resulted in the production of extracellular microcrystalline pigments [93].

4) Effect of pH. pH change during cultivation depends on the nitrogen source [14,50] and, to a lesser extent, on the carbon source [49,50]. The optimal initial pH value must also be selected with respect to the carbon and nitrogen sources used [61].

Regardless of the initial pH, the final pH of the cultures utilizing the same carbon and nitrogen source was approximately the same [15,49,50]. Yoshimura *et al* [102] reported that maintenance of pH at a constant value during the entire cultivation was not profitable. On the contrary, Lin and Demain [65] carried out successful cultivations at a constant pH by using a MOPS buffer.

5) Effect of physical factors. The optimal cultivation temperature for individual *Monascus* strains varies from 25°C [89] to 37°C [65]. Nevertheless, the most frequently cited temperature is 30°C.

The fungus requires sufficient aeration and therefore submerged cultivation can proceed only in shaken, preferably baffled [49] flasks or in a well-stirred and aerated fermenter, eg a fermenter equipped with an airlift system [70]. Shear forces which may destroy the mycelium can be overcome by using roller bottles [69].

Pigment formation is independent of visible light. Broder and Koehler [13] recommended cultivation of *Monascus* species in total darkness. Irradiation of *Monascus* cultures by light of various wavelengths (blue, red, infrared light) did not affect pigment production [98].

Pigment determination and isolation: Owing to the complexity of the pigment mixture produced by *Monascus* species, in most of the previous studies pigment composition was determined spectrophotometrically, using the absorption maxima exhibited around 400 nm, 470 nm and 500 nm by the yellow, orange and red compounds, respectively.

Qualitative analysis of the pigments was carried out by TLC on silica gel plates (Merck) developed with a solvent system containing chloroform : methanol : acetic acid (285 : 21 : 9) [15].

Recently, HPLC was applied to pigment determination. The columns used were a Bondapak C_{18} or LichroCART 100 RP-18 and mobile phases were 60% acetonitrile–0.05% trifluoroacetic acid, 70% acetonitrile or a gradient from 15 to 80% acetonitrile–water [18,53,64]. It is notable that the results of HPLC pigment analysis differed from the absorbency measurement [18]. Whereas according to HPLC analyses monascorubramine concentration was much higher than the concentration of yellow pigments, the absorbency data indicated the opposite result. In addition, HPLC analysis showed maximum pigment concentrations at earlier stages of cultivation when compared with spectrophotometric measurements. The differences between spectrophotometric and HPLC analyses could be caused by formation of some unknown compound(s) that interfere(s) with absorption maxima of pigments from *Monascus* species.

Purification of the major *Monascus* pigments to homogeneity has been reported [11,37,44].

Strain improvement

Irradiation of wild *Monascus* strains by UV light, neutron- or X-rays, mutation using MNNG or combinations of these methods can result in mutants with advantageous properties (rapid growth, superior pigment production, elimination of ascospore formation) or albino mutants [42,62,98,99]. The latter strains can be reverted into pigment producers by further UV irradiation [98]. Lin and Iizuka [63] prepared a *Monascus* strain which produced mainly extracellular pigments by a series of mutations induced by chemical and physical mutagens. Yongsmith *et al* [100] obtained a mutant of a *Monascus* species which produced a high concentration of yellow pigments instead of the red pigments formed by its parent strains.

Mevinolin and related compounds

Structure and biosynthesis

Unlike pigments described above, the polyketide mevinolin (also referred to as Lovastatin, monacolin K, Mevacor, MB 530B, MK 803 or MSD 803) is produced not only by members of the genus *Monascus*, but also by a variety of other filamentous fungi including *Aspergillus terreus* and some species of *Penicillium* [2], *Hypomyces*, *Doratomyces*, *Phoma*, *Eupenicillium*, *Gymnoascus*, *Trichoderma* [25] and *Pleurotus ostreatus* [36].

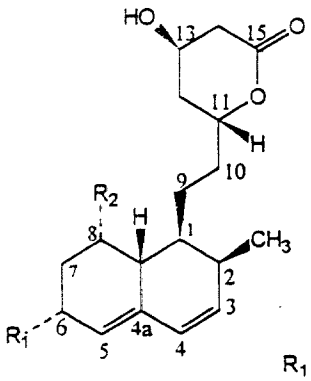
6-Demethylmevinolin (also referred to as compactin, Mevastatin, ML 236B, CS 500) was isolated in 1976 by Endo and colleagues and by researchers at Beecham Laboratories from *Penicillium citrinum* and *P. brevicompactum*, respectively [2]. Mevinolin was first reported from *M. ruber* by Endo [21] and, independently, by Alberts *et al* [3] from *Aspergillus terreus*.

Biosynthesis of mevinolin was detected in 17 of 124 *Monascus* strains tested [79]. The active strains belonged to *M. ruber*, *M. purpureus*, *M. pilosus*, *M. vitreus* and *M. pubigerus*. (According to the new taxonomy of this genus [39], *M. vitreus* and *M. pubigerus* belong to *M. ruber* and *M. pilosus*, respectively.) All mevinolin-producing strains were inferior in red pigment formation.

The biosynthesis of mevinolin was determined from studies using the fungus *Aspergillus terreus* [16,35,75,90]. Mevinolin (Figure 5) contains two polyketide chains, C_{18} and C_4 synthesized from acetate units coupled to each other in head-to-tail fashion. The C_{18}-chain is cyclized while bound to the polyketide synthase or immediately after dissociation from the enzyme, oxidized at the 8-carbon atom and esterified by the side chain. The 6α-methyl group and the methyl group on the side chain are derived from methionine. The methylations are sequential, the first one, on the 6 α-carbon atom, occurs before the closure of the rings. The methylation from L-methionine is typical of the fungal metabolism whereas propionate incorporation is generally used by actinomyces. Fatty acids with three and more carbon atoms are not incorporated into mevinolin. The oxygen atoms on the main chain are introduced successively on a deoxygenated precursor.

The biosynthesis of compactin and mevinolin by *Penicillium citrinum* and *Monascus ruber* proceeds in a similar way, ie the incorporation of acetate and methionine was observed, but not that of propionate. The enzymatic hydroxylation and subsequent esterification at the 8-carbon atom was also observed [22].

Mevinolin is produced as a mixture of a lactone and a free hydroxy acid [3]. Mevinolin-related compounds (Figure 5) vary in composition of the C_4 side chain (monacolins J [23], X [24] and M [26]) or lack this chain (monacolin L [23], dihydromonacolin L [23] and compactin derivative ML-236C [27]). Growth experiments with *M. ruber* using ^{14}C-labeled monacolin J or L suggested that both compounds are precursors of monacolin K [23]. The results of Komagata *et al* [54] indicated that monacolin L is the precursor of monacolin J, which, in turn, can be converted to monacolin K [52], and that a monooxygenase is involved in this reaction.



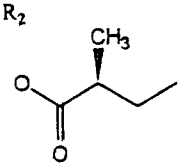
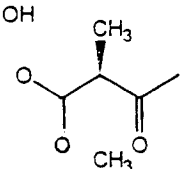
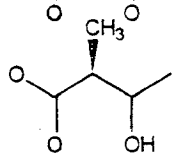
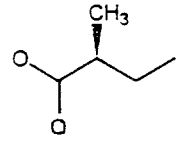
	R_1	R_2
mevinolin (monakolin K)	CH_3	
monakolin L	CH_3	H
monakolin J	CH_3	
monakolin X	CH_3	
monakolin M	CH_3	
compactin (ML-236B)	H	
ML-236A	H	OH
ML-236C	H	H

Figure 5 Mevinolin, compactin and related compounds.

Biological activity

Mevinolin, compactin and their derivatives obtained by chemical modifications (pravastatin, simvastatin) have provided a new mode of therapy for patients with hypercholesterolemia—a disease characterized by an elevated plasma concentration of the low density lipoprotein (LDL)/cholesterol complex.

The microsome enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (EC 1.1.1.34) catalyzes an early step in cholesterogenesis, ie the reduction of HMG-CoA to mevalonic acid. The specific inhibitory effect of mevinolin on this enzyme is caused by a structural relation between the 5-carbon hydroxy acid fragment of mevinolin and HMG-CoA [78].

Mevinolin inhibits sterol synthesis not only in hepatocytes and other types of mammalian cells [76,92,96] but also in fungi [6,9,28,51,59,67,94] and in plants [4–6,91].

Maltese *et al* [71] reported that mevinolin can suppress tumor growth *in vivo* owing to its capability to inhibit the

synthesis of nonsterol isoprenoid compounds such as dolichol, ubiquinone and isopentenyl-tRNA. Recently, attention has been paid to mevinolin and related compounds as potential therapeutic agents for the treatment of various types of tumors [48,77,80,95].

Production and isolation

Cultivation of *M. ruber* for the production of monacolins proceeds for 10–11 days at 25°C in complex media consisting, eg of either glucose, peptone, corn steep and ammonium chloride [23] or glycerol, glucose, soy bean powder, peptone, sodium nitrate, zinc nitrate and olive oil [24]. An elevated temperature (35°C) inhibits mevinolin production [79].

Isolation of mevinolin is usually carried out by extraction of the culture broth with ethyl acetate [3] at a pH of 4–4.2. For easier purification, the hydroxy acid is transformed into lactone [1].

Separation can also be started by sorption of the desired compounds on the polymeric resin XAD-2 at a pH of 6–8 and their elution by a mixture of isopropanol : ethyl acetate : dichlormethane (25 : 45 : 30) [88].

Alternatively, preparative HPLC can be applied to purification of mevinolin, using, eg Nucleosil$_5$C$_{18}$ or Lobar (Si60, Merck, Darmstadt, Germany) and mobile phases consisting of 0.1% H_3PO_4 : acetonitrile (1 : 1) or benzene : acetone (7 : 3), respectively [23,24,87].

Ankalactone

Nozaki *et al* [81] isolated a novel α,β-unsaturated γ-lactone derivative (Figure 6), named ankalactone, from a culture filtrate of *M. anka* (ie *M. purpureus* [39]) grown in a glucose-peptone medium for 7 days. Using extraction with ethanol and repeated chromatography on silica gel with chloroform : ethyl acetate (4 : 1), the product was isolated as colorless crystals.

Ankalactone showed a gross inhibitory effect against *Escherichia coli* and *Bacillus subtilis*, although its action was weaker than that of monascorubrin or rubropunctatin.

Conclusions

Some biological effects of the secondary metabolites of *Monascus* species remain to be investigated. In this respect, additional studies on the antimicrobial and immunosuppressive effects of the pigments merits further research.

Because *Monascus* species are used in the production of food additives, it is necessary to take into account the toxicity of some of their metabolites. Based on their reactivity with amines, the orange pigments undergo detoxification during cultivation on solid substrates. Nevertheless, formation of the orange compounds is possible in submerged cultures utilizing inorganic nitrogen sources. Dyes prepared from *Monascus* species by this method must therefore be subjected to reaction with amino acids, aminopolysaccharides or amino alcohols [14] prior to use. According to Blanc *et al* [10] there is some risk of contamination of the colorants with citrinin which could be avoided, however, either by detoxification of the pigments, use of citrinin-non-producing species or by submerged fermentative conditions of citrinin non-production.

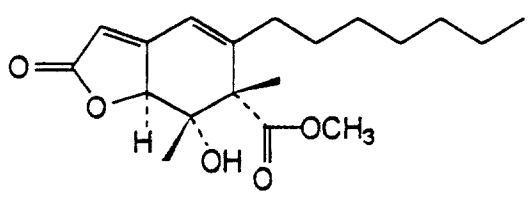


Figure 6 Ankalactone [81].

Ishiwata *et al* [45] simulated behavior of colorants from *Monascus* species during digestion and concluded that the main product formed from a pigment–protein complex was probably monascorublysine. However, this is, to the best of our knowledge, the only work concerning this topic. Thus, this problem may also provide an opportunity for further studies.

Acknowledgements

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