MICROBIAL PHOSPHORYLATION OF COMPACTIN (ML-236B) AND RELATED COMPOUNDS

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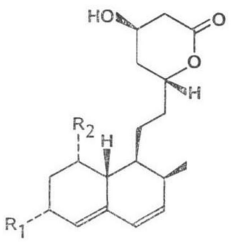
Several fungal strains were found to convert compactin (ML-236B) to 5′-phosphocompactic acid. The product was isolated by solvent extraction and column chromatography, and identified by IR, UV, ^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectroscopy. Related structures (monacolin K, L and X) were also transformed to their corresponding phosphorylated analogues. The products were re-converted back to respective parental compounds by treatment with alkaline phosphatase of calf intestine.

Compactin (ML-236B) and related compounds inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol synthetic pathway, thereby lowering plasma cholesterol levels in human as well as in animals[1,2]. Since these compounds are poorly absorbed in animals and human when administered orally, attempts have been made in the past to modify their structures and improve their bioavailability.

As seen in Fig. 1, the molecule of the compactin-related compounds contain a β-hydroxy-δ-lactone which can be readily opened by mild alkaline hydrolysis, leading to a dihydroxycarboxylic acid. The saponified forms (free acids) and their salts with alkali metals and esters with alkyl groups are, although superior to the corresponding lactone forms in both bioavailability and pharmacological activities, labile on storage at room temperature. The present communication deals with the microbial phosphorylation of the compactin related compounds at the C-5′ hydroxyl group (Fig. 2). The phosphorylated products were slightly less active *in vitro* but even more active *in vivo* (pharmacologically) than their respective parental compounds.

Fig. 1. Structures of the lactone forms of compactin (ML-236B) related compounds.



ML-236A R_1 = H, R_2 = OH

ML-236B (Compactin) R_1 = H, R_2 = O-(2-methylbutyryl)

ML-236C R_1 = H, R_2 = H

Monacolin J R_1 = CH_3, R_2 = OH

Monacolin K (Mevinolin) R_1 = CH_3, R_2 = O-(2-methylbutyryl)

Monacolin L R_1 = CH_3, R_2 = H

Materials and Methods

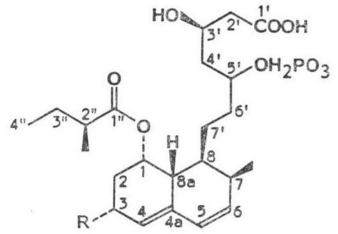
Microbial Strains

All microbial strains used in the present study were obtained from the Institute for Fermentation, Osaka (IFO).

Materials

ML-236A, ML-236B (compactin) and monacolins J, K and L were isolated as described previously[3~5]. Monacolin X was isolated from a mutant strain of *Monascus ruber* (NAKAMURA, KUNISHIMA, MASUDA and ENDO, in preparation). These compounds were converted to respective acid forms (sodium salts) by saponification prior to use.

Fig. 2. Structures of 5′-phosphocompactic acid and 5′-phosphomonacolic K acid.



5′-Phosphocompactic acid R = H

5′-Phosphomonacolic K acid R = CH_3

Growth and HPLC Analysis

Fungal strains were aerobically grown at 25°C in a medium consisting of glucose 1%, peptone (Daigo Eiyo) 0.2%, meat extract (Kyokuto Seiyaku) 0.1%, yeast extract (Difco) 0.1% and corn steep liquor 0.3%, after 3 days compactin (or related compounds) 0.05% was added to cultures. Growth was further continued. Aliquots were withdrawn at different time intervals and 2 ml portions of the culture filtrate were extracted twice at pH 3 with ethyl acetate (2 ml). The solvent layer was dried over Na_2SO_4 and evaporated to dryness. The resulting residue was dissolved in methanol (2 ml) and analyzed by HPLC (high performance liquid chromatography) on Silica ODS using 0.1% H_3PO_4 - acetonitrile (55: 45) as eluent. Under these conditions phosphorylated products derived from compactin and monacolin K appeared in elution volumes of 5.0 and 5.6 ml, respectively. On HPLC these products were assayed by measuring the absorbance at 237 nm.

HMG-CoA Reductase Assay

HMG-CoA reductase was isolated from rat liver and assayed as described previously[6].

Isolation of 5′-Phosphocompactic Acid

Circinella muscae IFO 4457 was grown at 25°C in Sakaguchi flasks containing 100 ml of the medium described above for 4 days at which time 0.05% compactin (Na salt) was added. After six additional days, incubation was terminated and the culture filtrate (2,250 ml) was adjusted to pH 3 with trifluoroacetic acid, followed by extraction thrice with one liter of ethyl acetate. The solvent layers were dried over Na_2SO_4 and evaporated to dryness. The resulting oily residue (1.95 g) was dissolved in 50 ml of ethyl acetate and extracted with an equal volume of 5% $NaHCO_3$. The aqueous layer was adjusted to pH 3 with 50% H_3PO_4 and extracted with 50 ml of ethyl acetate. The ethyl acetate layer was dried over Na_2SO_4 and evaporated to dryness, yielding 375 mg of oily residue. The dried residue was submitted to silica gel column chromatography (Wako gel C-200, 2×31 cm). The column was developed with a solvent system of *n*-hexane - acetone (9: 1, 200 ml; 8: 2, 1,000 ml; 7: 3, 400 ml; 6: 4, 700 ml; 5: 5, 200 ml; 4: 6, 200 ml) and fractions containing the transformation product were combined, evaporated; the combined fractions yielded 148 mg of solids. The solids were submitted to HPLC on a Silica ODS column (Nihon Bunko) by using a solvent mixture of 0.1% H_3PO_4 - acetonitrile (6: 4), yielding 62 mg of oil of 5′-phosphocompactic acid.

Isolation of 5′-Phosphomonacolin K Acid

C. muscae IFO 4457 was grown at 25°C in Erlenmeyer flasks (5 liters) containing 1,000 ml of the medium described above for 3 days and then incubated for an additional 6 days in the presence of 0.05% monacolin K (Na salt). The culture filtrate obtained from 2 flasks was processed as described above yielding 25 mg of oil of 5′-phosphomonacolic K acid.

Table 1. The ^{13}C NMR assignment for compactin and 5′-phosphocompactic acid (50 MHz, CD_3OD, TMS).

Carbon No.	Compactin (ppm)	5′-Phosphocompactin (ppm)	Carbon No.	Compactin (ppm)	5′-Phosphocompactin (ppm)
1	69.2 d	69.4 d	1′	173.4 s	175.3 s
2	27.2 t	27.1 t	2′	39.2 t	43.1 t
3	21.9 t	21.9 t	3′	63.3 d	66.6 d
4	124.4 d	124.2 d	4′	36.6 t	43.4 t $^3J_{cp}$=3 Hz
4a	135.2 s	135.2 s	5′	78.0 d	77.6 d $^2J_{cp}$=6 Hz
5	129.4 d	129.3 d	6′	34.1 t	33.8 t $^3J_{cp}$=5 Hz
6	133.6 d	133.8 d	7′	24.9 t	25.0 t
7	32.1 d	32.2 d	1″	178.2 s	178.4 s
7-CH_3	14.1 q	14.2 q	2″	43.1 d	43.1 d
8	38.2 d	38.5 d	2″-CH_3	17.4 q	17.3 q
8a	38.7 d	38.9 d	3″	27.9 t	28.0 t
			4″	12.2 q	12.2 q

Results and Discussion

Compactin Biotransformation

Approximately 2,000 strains of microorganisms were tested for their ability to modify compactin. A number of them were sensitive to and could not grow in the presence of 0.05% compactin, but some of them transformed it in each case to one bioactive product which was more polar than the substrate itself. *C. muscae* IFO 4457 and IFO 6410, *Absidia cylindrospora* IFO 4000 and *A. glauca* IFO 4003 carried out this conversion more rapidly and more efficiently than the others. Of the active strains *C. muscae* IFO 4457 was used for subsequent experiments. Under the conditions described in Materials and Methods, 20~30% of compactin added was converted to 5′-phosphocompactic acid after 4 to 7 days of incubation.

Structure Determination

Mass spectral analysis (SIMS) of the compactin product showed ion peaks at *m/z* 489 $(M+H)^+$, 511 $(M+Na)^+$ and 527 $(M+K)^+$ which corresponded to elemental composition $C_{23}H_{37}O_6P$ (calcd 488.5137). UV spectra in methanol were identical to those of compactin. IR spectra (KBr) showed an absorption at 1010 cm^{-1} (C-O-P stetch). ^{31}P NMR spectra (in $CDCl_3$, trimethylphosphate as internal standard) showed one phosphate (C-O-P) at −5.43 ppm.

As in compactin, the ^{13}C NMR spectrum of the new compound had 23 lines, thus showing that the carbon skeleton of the new product remained intact. This conclusion was further supported by the data that the new compound was quantitatively converted to compactin by treatment with alkaline phosphatase of calf intestine (see below). Since the ^{13}C NMR spectrum of compactin has been completely assigned[7], a comparison of the two spectra (Table 1) allowed assignment of all carbons. These data indicated that one of the two hydroxyl groups at C3′ or C5′ in compactin was phosphorylated. Furthermore as shown in Table 1, ^{13}C-^{31}P coupling was seen with C4′, C5′ and C6′ in the new compound, but not with C2′ and C3′, indicating that it was the hydroxyl group at C5′ of compactin which was phosphorylated.

Monacolin K Biotransformation

The ^{31}P NMR spectrum of the monacolin K product showed a peak at −5.89 ppm. The ^{13}C NMR spectrum showed a ^{13}C-^{31}P coupling at C4′ ($^3J_{cp}$=3 Hz), C5′ ($^2J_{cp}$=5 Hz) and C6′ ($^3J_{cp}$=5 Hz). These

data indicated that by analogy to compactin the hydroxyl group at C5′ of the substrate was phosphorylated.

Table 2. Phosphorylation at C5′ of compactin-related compounds by *C. muscae* IFO 4457*.

Substrate	Phosphorylation (%)
Compactin (acid)	51.0
Compactin (lactone)	3.1
ML-236A (acid)	<1
Monacolin K (acid)	43.5
Monacolin L (acid)	7.1
Monacolin X (acid)	12.3

* Experimental conditions are described in the text.

Bioconversion of Other Related Compounds

C. muscae IFO 4457 was grown at 25°C in a medium consisting of glucose 1%, peptone 0.2%, meat extract 0.1%, yeast extract 0.1%, corn steep liquor 0.3% and NaH_2PO_4 0.1% and after 4 days 0.05% of a compactin related compound was added to the culture which was further incubated for an additional 4 days. An aliquot of culture filtrate was assayed for a phosphorylated product as described in Materials and Methods. Under these conditions, compactin and monacolin K in acid form were converted to their respective phosphorylated products by 51.0 and 43.5%, respectively, while compactin (lactone form) was transformed only slightly. Monacolin L (acid form) was converted to some extent, but ML-236A (acid form) was not detectably phosphorylated (Table 2).

Hydrolysis by Alkaline Phosphatase

When 0.3 mg of 5′-phosphocompactic acid was incubated at 37°C with 0.05 U alkaline phosphatase of calf intestine (Sigma) in one ml of 0.1 M glycine - NaOH buffer (pH 10.4) containing 1.0 mM $ZnCl_2$ and 1.0 mM $MgCl_2$, the substrate was converted to compactin by 45.9% after 10 minutes and by 75.9% after 30 minutes, respectively. Essentially the same result was obtained with 5′-phosphomonacolic K acid.

Biological Activity

The incorporation of [^{14}C]acetate into non-saponifiable lipids was determined as described previously[8]. Concentrations required for 50% inhibition of the reaction were 0.24 μM for both 5′-phosphocompactic acid and 5′-phosphomonacolic K acid, respectively, while these for compactin and monacolin K were 17.1 and 6.9 nM, respectively. The phosphorylated derivatives of compactin and monacolin K inhibited HMG-CoA reductase activity approximately 50% at 7.9 and 4.0 μM, respectively. Under the same conditions, compactin and monacolin K inhibited reductase 50% at 0.71 and 0.22 μM, respectively. However, hypocholesterolemic activity of the phosphorylated compounds in animals, which will be reported elsewhere, were comparable to or even higher than their respective parental compounds.

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