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Screening for metabolites of acarviostatin family aminooligosaccharides in rats using ultraperformance liquid chromatography coupled with electrospray ionization tandem mass spectrometry

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ABSTRACT

An ultraperformance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (UPLC/ESI–MS/MS) procedure was used to identify trace levels of metabolites after the administration of acarviostatin II03 or III03 to rats. Biosamples of the feces and urine of the treated rats as well as the intestinal sacs incubated with the drugs *in vitro* were pre-treated by cation-exchange extraction, and then applied to a reversed-phase C_{18} UPLC column with acetonitrile/1.5 mM aqueous ammonia as the mobile solvent. The parent drug and the potential metabolites were identified by two independent qualitative parameters, retention time (t_R) and MS/MS spectrum. Seven metabolites were successfully characterized from the intestinal sacs infused with acarviostatin II03 or III03. The metabolic pathways within the intestine were identified including glucose hydrolysis at the reducing terminus, and cyclohexitol hydrolysis at the non-reducing terminus of the parent acarviostatins. Subsequently, we determined that lower amounts of cyclohexitol-lost metabolites compared with the cyclohexitol-containing metabolites, as well as lower amounts of the acarviostatin III03 metabolites compared with the acarviostatin II03 metabolites, could be transferred by the intestinal walls. In the rat feces samples, although the parent compounds could not be found, acarviostatin II03 and III03 both yielded one-glucose-lost and four-glucose-lost types of metabolites. In the rat urine samples, no acarviostatin metabolites could be detected.

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1. Introduction

α-Amylase and α-glucodase inhibitors are well-known treatments and prophylactics for diabetes, obesity, or other secondary symptoms caused by these diseases [1]. Previously, in the course of screening for novel α-amylase inhibitors, we discovered a series of compounds secreted by *Streptomyces coelicoflavus* ZG0656, termed acarviostatins [2–4]. Among them, acarviostatins II03 and III03 are the dominant compounds acting as mixed non-competitive inhibitors of α-amylase. They showed 160 (II03) and 260 (III03) times, respectively, more potent inhibitory activities against porcine pancreatic α-amylase (PPA) than acarbose, and acarviostatin III03 is the most effective α-amylase inhibitor known to date [2,3].

The acarviostatin molecules are composed of 1–5 units of acarvisione and several units of glucopyranose. They have a repeating pseudotrisaccharide core formed by an acarviosine unit and a D-glucopyranose group through an α-(1 → 4) quinovosidic bond. Acarviosine is composed of a cyclohexitol unit (hydroxymethylconduritol residue) and a 4-amino-4,6-dideoxy-D-glucopyranose unit (4-amino-4-deoxy-D-quinovopyranose residue). The designation of "II03" indicates that the molecule is composed of two pseudotrisaccharide cores and three glucose units on the reducing end, with no glucopyranose units on the non-reducing end. The only difference between acarviostatin II03 and acarviostatin III03 is that the latter contains three pseudotrisaccharide cores (Fig. 1).

Because acarviostatins II03 and III03 are both effective and potentially leading drugs for diabetes and obesity, it is important to investigate their pharmacokinetic properties in mammalian systems. Metabolite screening requires detection techniques with

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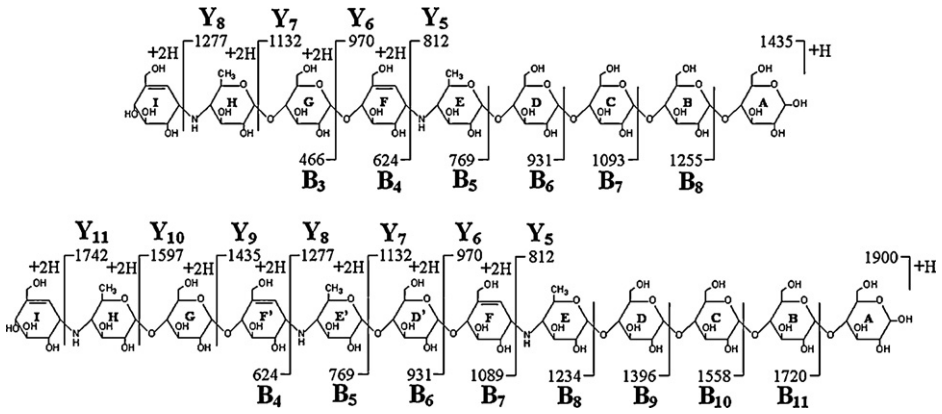


Fig. 1. Chemical structures and positive ESI–MS/MS fragmentation pathways of $[M+H]^+$ ions of acarviostatin II03 (top) and acarviostatin III03 (bottom).

high sensitivity and selectivity such as mass spectrometry (MS), because of the trace levels of the parent drugs and their metabolites [5,6]. The combination of electrospray ionization (ESI) MS with liquid chromatography (LC) provides not only the qualitative data of retention times (t_R), but also well-purified components from which good-quality MS/MS spectra can be acquired [7–10]. Ultra-performance liquid chromatography (UPLC) [11,12] is a promising separation technique with more advantages over regular high performance liquid chromatography (HPLC). When analyzing complex mixtures with LC/MS, UPLC can be used to detect more components than with HPLC [13].

We previously reported a simple UPLC/ESI–MS/MS procedure for the profiling of acarviostatin family secondary metabolites secreted by *S. coelicoflavus* ZG0656 with a limit of detection less than 1 pmol, which successfully resulted in the identification of 80 acarviostatins, including 65 novel components [14]. The acarviostatins showed good chromatographic separation and sufficient sensitivity under the given UPLC/ESI–MS/MS conditions. Here, we explored the use of UPLC/ESI–MS/MS for analyzing aminooligosaccharides in the presence of various biomatrices, and applied it to screen for metabolites of acarviostatins II03 and III03 in a mammalian system.

2. Experimental

2.1. Materials and chemicals

Acarviostatins II03 and III03 were isolated and purified from the culture filtrate of *S. coelicoflavus* ZG0656 as described previously [2,3]. Acetonitrile was chromatographic grade. Concentrated ammonia (25% in water) and diethyl ether were of analytical grade. Double distilled water was used throughout the study.

2.2. Animals

Male Wister rats (4–5 weeks of age; 120–140 g body weight) were provided by the Experimental Animal Center of Academy of Military Medical Sciences (Beijing, China).

2.3. Extraction, preparation and incubation of rat intestinal sacs

After an overnight fast, male Wister rats were anaesthetized with diethyl ether, and their abdominal cavities were dissected longitudinally. A length (approximately 10 cm) of intestine was excised, and the gut cavity was rinsed twice with ice-cold Krebs–Henseleit solution. The intestinal segment was tied off with cotton thread at each end to form a sac which was subsequently filled with 0.2 mL of 0.1% acarviostatin II03 or III03 in Krebs–Henseleit solution. Each sac preparation was suspended in a tube with 4 mL of Krebs–Henseleit solution and immersed in a water bath to incubate at 37 °C. After 1, 2, or 4 h from the infusion of test drugs, the reaction was terminated by adding 10 μL 1 mol L^{-1} HCl, and the solutions from the mucosal side (equivalent to reactions within the intestine) and the serosal side (equivalent to drug absorption and transference to blood) were both collected to be stored at −20 °C until analysis [15].

For analysis of metabolites, the samples from the treated intestinal sac for various times were transferred into 7 mL polypropylene snapcap tubes, which were then centrifuged for 20 min at 18,000 × *g*. The supernatants were transferred into 0.5 mL 001 × 7 cation-exchange resins (Chemical Plant of Nankai University, Tianjin, China). The resins were washed with 3 × 2 mL of water, and eluted with 2 mL of 1 M aqueous NH_3. For UPLC/MS analysis, 10 μL aliquots of the eluates were injected into the chromatographic system.

2.4. Preparation of rat urine and feces and extraction procedures

Male Wister rats were administered a single 2 mg kg^{-1} of body mass dose of acarviostatin II03 or III03 in 0.1% aqueous solution. Urine and feces were collected separately every 6 h after administration for 24 h. All samples were stored at −20 °C until analysis. Control rat urine and feces samples were collected before drug administration to determine whether they contain components that would interfere with identification of the acarviostatins or their metabolites.

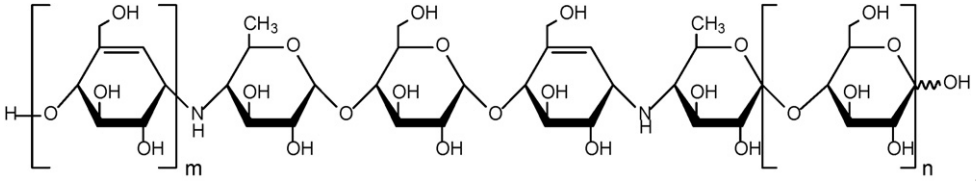
Urine samples (5 mL) were transferred into 7 mL polypropylene snapcap tubes, which were then centrifuged for 20 min at 18,000 × *g*. Feces samples (∼0.5 g) were suspended in 5 mL of water in 7 mL polypropylene snapcap tubes, vortexed for 1 min, ultrasonically extracted for 5 min, and centrifuged for 20 min at 18,000 × *g*. The supernatants were transferred into 0.5 mL 001 × 7 cation-exchange resins, and were extracted as described above for the intestinal sac samples. For UPLC/MS analysis, 10 μL aliquots of the eluates were injected into the chromatographic system.

2.5. Instrumentation

2.5.1. UPLC

All separations were performed with a Waters Acquity UPLC system (Waters Co., USA), operated on a 50 mm × 2.1 mm (1.7 μm) Waters Beh C_{18} column (Waters Co., USA). The mobile phase was acetonitrile-1.5 mM aqueous ammonia (pH ∼ 9) with a flow rate of 0.2 mL/min. Using 3:97 (v/v), acarviostatin II series compounds were separated; and using 5:95 (v/v), acarviostatin III series compounds were separated [14].

Table 1
The detailed information of acarviostatin II03 metabolites and their relative abundances in rat systems.



II03 metabolites	*m*	*n*	Name	Format	MW	UPLC/ESI–MS/MS			*In situ* rat intestinal sac incubation						Feces
						t_R (min)	$[M+H]^+$ (*m*/*z*)	Fragment ions (*m*/*z*)	1 h		2 h		4 h		6–12 h
									Intestine	Blood	Intestine	Blood	Intestine	Blood	
M0	1	4	II03	$C_{56}H_{94}N_2O_{40}$	1434	9.66	1435	304(B_2), 769(B_5), 1132(Y_7)	++	+	++	++	++	++	–
M1	1	3	II02	$C_{50}H_{84}N_2O_{35}$	1272	8.87	1273	304(B_2), 769(B_5), 970(Y_6)	++	+	++	++	++	++	+
M2	1	2	II01	$C_{44}H_{74}N_2O_{30}$	1110	8.28	1111	304(B_2), 769(B_5), 808(Y_5)	++	+	++	++	++	++	–
M3	1	1	II00	$C_{38}H_{64}N_2O_{25}$	948	6.87	949	304(B_2), 769(B_5), 646(Y_4)	+	–	+	–	+	+	–
M4	1	0	II0(-1)	$C_{32}H_{54}N_2O_{20}$	786	3.75	769[a]	304(B_2), 466(Y_3)	++	++	++	++	++	++	+
M5	0	4	II(-1)3	$C_{49}H_{84}N_2O_{36}$	1276	5.13	1277	611(B_4), 1132(Y_7)	++	–	++	–	++	+	+
M6	0	3	II(-1)2	$C_{43}H_{74}N_2O_{31}$	1114	4.63	1115	611(B_4), 970(Y_6)	++	–	++	–	++	+	–
M7	0	2	II(-1)1	$C_{37}H_{64}N_2O_{26}$	952	4.01	953	611(B_4), 808(Y_5)	+	–	+	–	+	–	+

++: abundant; +: detected; –: undetected.
[a] $[M-OH]^+$.

2.5.2. Mass spectrometry

Mass spectrometric analysis was performed on a quadrupole-orthogonal time-of-flight (Q-TOF) premier mass spectrometer (Waters Co., USA) equipped with an ESI source and a mass range up to *m*/*z* 2000. The positive ion mode was employed, and the spray voltage was set at 4.5 kV. The capillary voltage was fixed at 5.0 kV, and the temperature was maintained at 220 °C. The solvent was nebulized using N_2 as both the sheath gas, at a flow rate of 0.80 L min^{-1}, and the auxiliary gas at a flow rate of 0.08 L min^{-1}. The collision energies in multistage MS experiments were set at 25–40 V [14].

3. Results and discussion

3.1. ESI–MS/MS fragmentation patterns of acarviostatins

Since the acarviostatins contain secondary amine residues with fairly strong basicity, they are highly sensitive to positive ion ESI, readily forming protonated molecules. In the given ESI–MS conditions, acarviostatin II03 generated a strong $[M+H]^+$ signal at *m*/*z* 1435. However, acarviostatins III03 showed a strong $[M+H]^+$ signal at *m*/*z* 1900 and another $[M+2H]^{2+}$ signal at *m*/*z* 951. The $[M+H]^+$ and $[M+2H]^{2+}$ ions of the acarviostatins both yielded MS/MS spectra that could be applied for structural determination purposes.

Fragmentation of the protonated molecules of acarviostatins II03 and III03 yielded two types of protonated product ions, B_i and Y_j, derived from a single glycosidic bond cleavage (Fig. 1). Careful inspection of the MS/MS spectra from $[M+H]^+$ of acarviostatin II03 at *m*/*z* 1435 and $[M+H]^+$ of acarviostatin III03 at *m*/*z* 1900 suggested that the cleavages of α-(1 → 4) quinovosidic bond between the quinovopyranose unit and glucose unit in the precursor acarviostatin molecules formed the most intense product ion signals. Therefore, these diagnostic signals could be applied for the interpretation of the linkage sequences and the differentiation of the possible positional isomers among acarviostatins and their metabolites [3,14].

3.2. UPLC/ESI–MS/MS analysis of acarviostatins and their metabolites

Sample pretreatment before LC/MS analysis is necessary to minimize interference and to improve the sensitivity and selectivity. At a low pH, the basic secondary amine residues in the acarviostatin molecules mediated their retention in the cation-exchange resin, and thus to their successful purification.

The rat urine and feces samples and the intestinal sacs were prepared as described in Section 2. Analysis with the present UPLC/ESI–MS/MS method provided two independent parameters, t_R and mass spectrometric information, for the identification of both parent molecules and possible *in vivo* metabolites in the biosamples.

3.3. Screening for in situ metabolites in rat intestinal sac incubated with acarviostatins

The acarviostatins are expected to be poorly absorbed following oral administration because of their relatively high molecular weight and high polarity [16]. Therefore, the metabolic fates of the acarviostatins in the alimentary gut were first studied using excised rat intestinal sacs. This approach involved the incubation of drugs within the rat intestinal sacs, and the detection of parent drugs and possible metabolites on both the mucosal/lumenal and serosal sides of the sacs by the extraction plus UPLC/ESI–MS/MS method as described in Section 2.

3.3.1. Metabolites of acarviostatin II03 in rat intestinal sac incubation

We identified seven metabolites besides the parent drug from incubation of 0.2 mg acarviostatin II03 within the rat intestinal sac (about 10 cm). Fig. 2 shows the multiple reaction monitoring (MRM) mode chromatograms and the MS/MS spectra of each acarviostatin II03 metabolite, and their detailed results are listed in Table 1 (left column).

From analysis of t_R and the characteristic fragment ions in the MS/MS spectra, we determined that M0 was the parent drug; M1, M2, M3 and M4 were derived from the loss of one, two, three and four glucose residues from the reducing end of the parent drug, respectively; M5 matched with the hydrolysis of the cyclohexitol unit in the non-reducing end of the parent drug; M6 and M7 resulted from the further loss of one and two glucose residues from the reducing end of M5, respectively. Among the metabolites, the M3 content was relatively low, which indicated that the fourth glucose unit from the reducing end is more easily hydrolyzed to form M4. That is, the intensity of the α-(1 → 4) quinovosidic

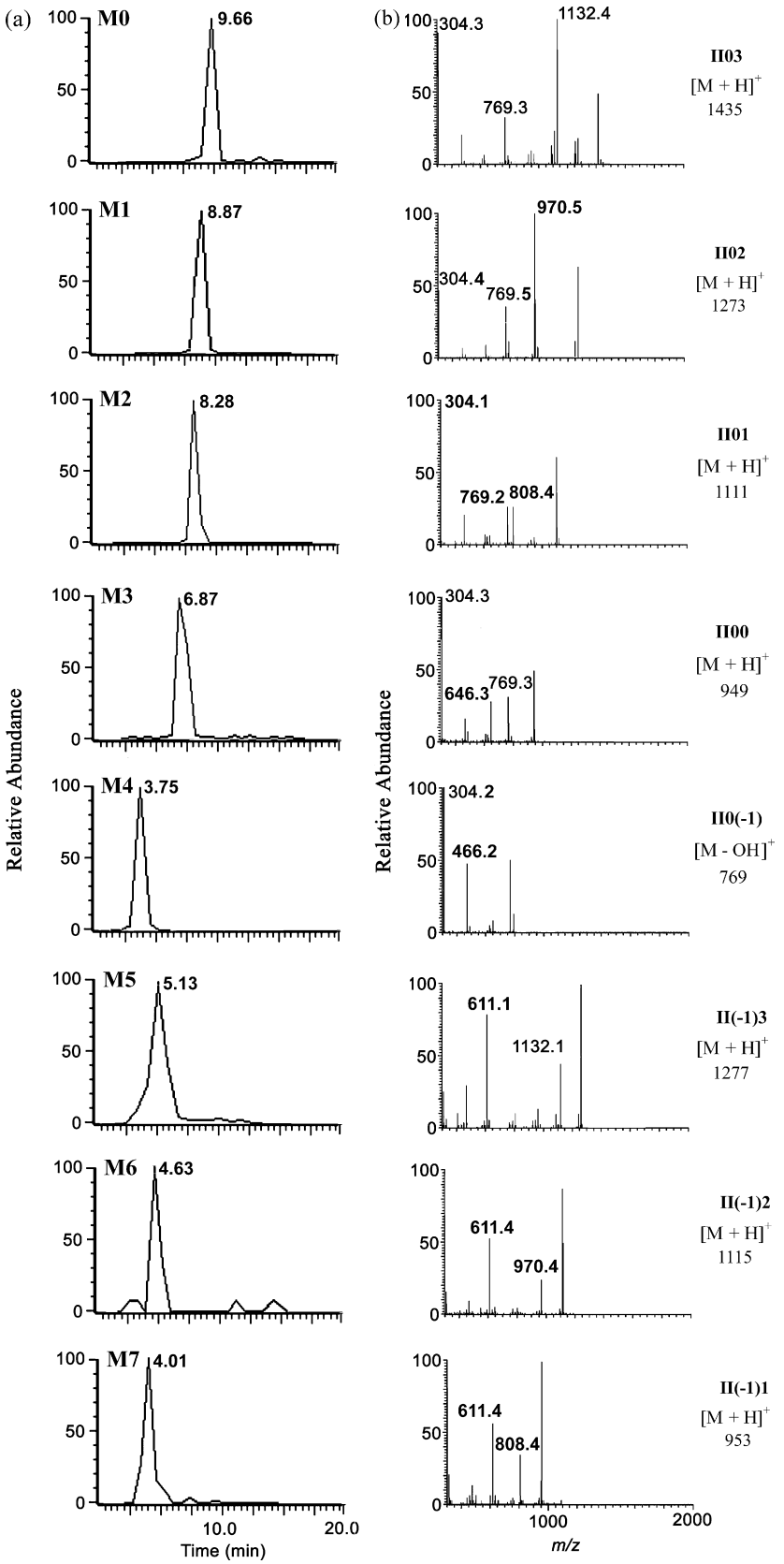


Fig. 2. UPLC/ESI–MS/MS traces for acarviostatin II03 metabolites, M0–M7, in rat intestinal sac incubation samples. (a) MRM chromatograms, separated with 3:97 (v/v) acetonitrile-aqueous ammonia and (b) ESI–MS/MS spectra of $[M+H]^+$ or $[M-OH]^+$. The standard name of every metabolite is labeled on the right.

bond between the quinovopyranose unit and glucose unit is relatively weak compared to the other ordinary glycosidic bonds in the molecule. This result is consistent with our previous results [3,4,14]. No other metabolites with lower molecular weights were detected, suggesting that the glycosidic bonds inside the acarviostatin molecules could not be enzymatically hydrolyzed.

3.3.2. Metabolites of acarviostatin III03 in rat intestinal sac incubation

Seven metabolites besides the parent drug were also identified after incubation of 0.2 mg acarviostatin III03 in the rat intestinal sac and showed a similar metabolic pathway to that of acarviostatin II03. Fig. 3 shows the MRM chromatograms and the MS/MS spectra of each acarviostatin III03 metabolite, and the corresponding detailed results are listed in Table 2 (left column).

Similar to the results obtained with acarviostatin II03, analysis of t_R and the diagnostic fragment ions in the MS/MS spectra demonstrated that M0 was the parent drug; M1, M2, M3 and M4 corresponded to the loss of one, two, three and four glucose residues, respectively, from the reducing end of the parent drug; M5 agreed with the hydrolysis of the cyclohexitol unit in the non-reducing end of the parent drug; M6 and M7 were produced by the further loss of one and two glucose residues, respectively, from the reducing end of M5. As with acarviostatin II03, the M3 content was relatively low in acarviostatin III03, and no other metabolites with lower molecular weights were detected.

3.4. Metabolic developments of acarviostatins vs. time incubated in the rat intestinal sacs

To determine the metabolic developments of acarviostatins vs. time in the incubated rat intestinal sacs, both the samples from the mucosal side (equivalent to reactions within the intestine) and the serosal side (equivalent to drug absorption and transference to blood) were collected at various periods and subsequently analyzed.

3.4.1. Metabolic developments of acarviostatin II03 incubated in the rat intestinal sacs

The relative abundances of each acarviostatin II03 metabolite after different incubation periods and on both sides (luminal or serosal) of the intestinal sacs are listed in Table 1 (middle column). In the samples taken from the intestinal lumen, all metabolites including M0–M7 were found after 1 h of incubation, and showed no decrease after 4 h. Among them, M3 and M7 were relatively low in abundance in all cases. In the samples taken from the serosal side of the intestine representing the blood, however, the situation was more complex. M4 could be detected in all periods, while M0, M1 and M2 were more abundant after the 2 and 4 h incubation periods compared to the 1 h treatment. M3, M5 and M6 appeared only after 4 h. No M7 was found in all cases. In general, both the types and the amounts of acarviostatin II03 metabolites transferred across the intestinal wall increased with time. Additionally, M5–M7, the cyclohexitol-lost metabolites, were not as easily transferred by the intestinal walls as the cyclohexitol-containing metabolites, such as M0–M4. The poor absorption could be due to the higher polarities of the cyclohexitol-lost metabolites which retained a primary amine residue after hydrolysis.

3.4.2. Metabolic developments of acarviostatin III03 incubated in the intestinal sacs

Table 2(middle column) shows the relative abundances of each acarviostatin III03 metabolite after different incubation periods and on both sides (luminal or serosal) of the intestinal sacs. The metabolic developments of acarviostatin III03 was quite similar to

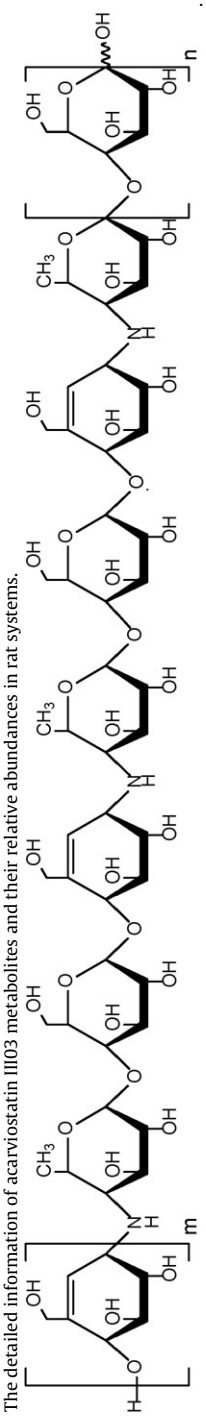


Table 2
The detailed information of acarviostatin III03 metabolites and their relative abundances in rat systems.

III03 metabolites	*m*	*n*	Name	Format	MW	UPLC/ESI–MS/MS			*In situ* rat intestinal sac incubation						Feces
									1 h		2 h		4 h		6–12 h
						t_R (min)	$[M+H]^+$ (*m/z*)	Fragment ions (*m/z*)	Intestine	Blood	Intestine	Blood	Intestine	Blood	
M0	1	4	III03	$C_{75}H_{125}N_3O_{52}$	1899	6.20	1900	304(B_2), 769(B_5), 1234(B_8), 1132(Y_7), 1597(Y_{10})	++	+	++	++	++	++	–
M1	1	3	III02	$C_{69}H_{115}N_3O_{47}$	1737	5.87	1738	304(B_2), 769(B_5), 1234(B_8), 970(Y_6), 1435(Y_9)	++	–	++	++	++	++	+
M2	1	2	III01	$C_{63}H_{105}N_3O_{42}$	1575	5.47	1576	304(B_2), 769(B_5), 1234(B_8), 808(Y_5), 1273(Y_8)	++	–	++	++	++	++	++
M3	1	1	III00	$C_{57}H_{95}N_3O_{37}$	1413	5.30	1414	304(B_2), 769(B_5), 1234(B_8), 646(Y_4), 1111(Y_7)	–	–	+	–	+	–	–
M4	1	0	III0(-1)	$C_{51}H_{85}N_3O_{32}$	1251	4.00	1234[a]	304(B_2), 769(B_5), 466(Y_3), 931(Y_6)	++	–	++	++	++	++	++
M5	0	4	II(-1)3	$C_{68}H_{115}N_3O_{48}$	1741	5.24	1742	611(B_4), 1076(B_7), 1132(Y_7), 1597(Y_{10})	++	–	++	–	++	–	–
M6	0	3	II(-1)2	$C_{62}H_{105}N_3O_{43}$	1579	5.07	1580	611(B_4), 1076(B_7), 970(Y_6), 1435(Y_9)	++	–	++	–	++	–	–
M7	0	2	II(-1)1	$C_{56}H_{95}N_3O_{38}$	1417	4.96	1418	611(B_4), 1076(B_7), 808(Y_5), 1273(Y_8)	+	–	+	–	+	–	–

++: abundant; +: detected; –: undetected.
[a] $[M-OH]^+$.

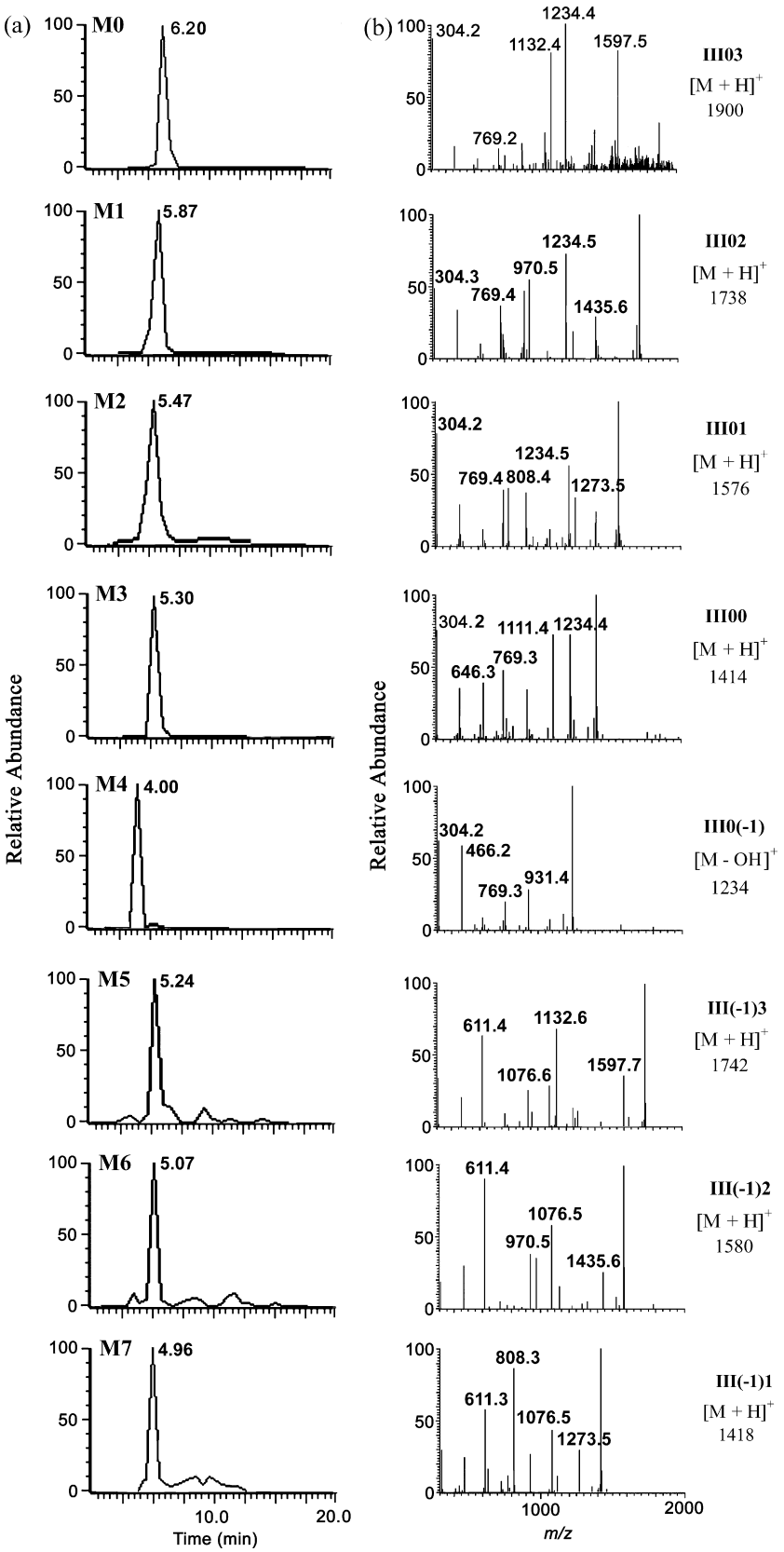


Fig. 3. UPLC/ESI–MS/MS traces for acarviostatin III03 metabolites, M0–M7, in rat intestinal sac incubation samples. (a) MRM chromatograms, separated with 5:95 (v/v) acetonitrile-aqueous ammonia and (b) ESI–MS/MS spectra of $[M+H]^+$ or $[M-OH]^+$. The standard name of every metabolite is labeled on the right.

those of acarviostatin II03 due to their analogous chemical structures, although there were still a few differences. On one hand, in the intestinal lumen, all metabolites including M0–M7 were found after 1 h incubation except M3, and showed no decrease after 4 h. The relatively low abundance of M3 led to its detection only after accumulating for 2 h, and the M7 contents was poor in all cases. On the other hand, in the samples taken from the serosal side representing the blood, only the parent drug M0 appeared after 1 h of incubation. M1, M2 and M4 were confirmed on the serosal side only after 2 h, indicating that their transference by the intestinal walls required additional time. M3, M5, M6 and M7 were not detected at all in each condition. Similar to the results with acarviostatin II03, more types and amounts of acarviostatin III03 metabolites were transferred to blood over time. However, the cyclohexitol-lost acarviostatin III03 metabolites, M5–M7, could not be transferred at all by the intestinal walls. Overall, due to the relatively higher molecular weights, the acarviostatin III03 metabolites were not easily transferred by the intestinal walls compared to the acarviostatin II03 metabolites. Since acarviostatins exhibit their function mainly within the intestine, acarviostatin III03 with apparently poor absorption to the blood might be a better candidate as a prodrug than acarviostatin II03. Therefore, as the most effective α-amylase inhibitor known to date [2,3], acarviostatin III03 showed an obvious dominant advantage compared to its competitor.

3.5. Detection of acarviostatins metabolites in rat feces and urine

On the basis of the *in situ* rat intestinal sac incubation tests, several *in vivo* analyses were performed to screen for metabolites in rat feces and urine after oral administration of acarviostatins.

3.5.1. Metabolites of acarviostatins in rat feces

After oral administration of 2 mg kg^{-1} of the parent drugs, the metabolites were detected in the rat feces collected during the 6–12 h period following administration. Although acarviostatin II03 and III03 displayed similar metabolic pathways on the mucosal sides of the incubated rat intestinal sacs *in situ*, their final metabolites had quite different spectra in the *in vivo* rat feces tests. The acarviostatin II03 metabolites were M1, M4, M5 and M7 (Table 1, right column), including both glucose-lost and cyclohexitol-lost products However, the acarviostatin III03 metabolites consisted of glucose-lost derivatives only, such as M1, M2 and M4 (Table 2, right column). The common observations of metabolism of the two acarviostatins in rat feces are that no parent drugs are retained, and both M1 (one glucose unit lost) and M4 (four glucose units lost) type metabolites are found. It is unknown what may account for the differences.

3.5.2. Metabolites of acarviostatins in rat urine

After oral administration of 2 mg kg^{-1} acarviostatin II03 or III03, no metabolites could be detected in any of the urine samples within the 6–12 h time period. Given that acarviostatins metabolites could be transferred into the serosal side (blood) in the *in situ* rat intestinal sac incubation tests but not in the urine, we speculated that there might be other metabolic process on acarviostatin type compounds within the animal's hemal system. Further research on the metabolic pathway and mechanisms of acarviostatin elimination are now in progress in our group.

4. Conclusions

The acarviostatins as aminooligosaccharides can be well-separated and identified using a UPLC/ESI–MS/MS procedure. By adding a simple pre-treatment of the biosamples, this UPLC/ESI–MS/MS approach was used to explore metabolite profiling of acarviostatins in a mammalian (rat) system. Two independent qualitative parameters, retention time and MS/MS fragmentation spectra, were used to identify both parent compounds and the potential metabolites in the biosamples. Applying this method, seven metabolites of either acarviostatin II03 or III03 were successfully characterized from rat intestinal sacs incubated with these drugs. These biotransformation products resulted from glucose hydrolysis at the reducing terminus, and cyclohexitol hydrolysis at the non-reducing terminus of the parent acarviostatins. Furthermore, the cyclohexitol-lost metabolites were more difficult to transfer by the intestinal walls compared to the cyclohexitol-containing metabolites. The acarviostatin III03 metabolites were also more difficult to be transferred compared to the acarviostatin II03 metabolites. Finally, acarviostatins II03 and III03 both produced one-glucose-lost and four-glucose-lost types of metabolites in rat feces, while their parent compounds could not be detected.

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