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1,4-Dihydroxy-2-naphthoic Acid Sulfate: A Promising Constipation Preventive Agent

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Abstract 1,4-Dihydroxy-2-naphthoic acid (DHNA) is a metabolic by-product of fermentation by *Propionibacterium* and is thought to have a beneficial effect as a prebiotic. As it is unstable in air, we have developed a new stable DHNA derivative, DHNA-4-sulfate (DHNAS), which should be converted to DHNA *in vivo*. After DHNAS was administered to mice, fecal size enlargement and softening were observed. As a metabolite, DHNA-4-β-glucuronide (DHNAG) was detected in the urine, which suggested the sequential conversion, DHNAS→DHNA→DHNAG, *in vivo*. These 4-OH-blocked DHNA derivatives, DHNAS and DHNAG, were stable at pH 3 and pH 7.4, and in water, while DHNA decomposed rapidly within 20 hrs. DHNAS may be a useful food additive to improve human intestinal health, and a promising constipation preventive agent.

Keywords: *1,4-dihydroxy-2-naphthoic acid sulfate, DHNA, prebiotic, anti-constipation, laxative, vitamin K, food additive*

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1. Introduction

1,4-Dihydroxy-2-naphthoic acid (DHNA) has been detected as a main component of the bifidogenic growth stimulator in the culture broth of *Propionibacterium freudenreichii* ET-3, which grows in Swiss cheese [1]. Various prebiotics confer many beneficial effects on human health, such as the improvement of intestinal bacterial flora, constipation relief, osteoporosis prevention, immune system activation, and cancer prevention [2,3]. In some anaerobic bacteria, DHNA is a precursor of vitamin K [4,5]. In fact, DHNA-containing dairy products are sold commercially in Japan. However, DHNA easily decomposes in air due to its hydroquinone structure, and thus before the addition of a DHNA-containing extract, the oxygen must be removed from food products by introducing nitrogen gas or adding antioxidants, such as ascorbic acid, to prevent decomposition [6]. These processes hinder the efficiency of food production and can change taste and flavor. To address these problems, we developed a new stable DHNA derivative that should be converted to the unmodified DHNA in the intestine, by the action of a bacterial arylsulfatase.

2. Materials and Methods

2.1. Spectra Measurements

The mass spectra (FAB) were measured with a JEOL JMS-DX303 spectrometer. High-resolution mass spectra were measured with a Q Exactive LC-MS-MS Orbitrap (Thermo Fisher Scientific) apparatus. ^{1}H and ^{13}C NMR spectra were measured with a JEOL JNM-ECX400 spectrometer, using DMSO-d_6 as the solvent.

2.2. Synthesis of DHNA-4-sulfate (Na)

To a solution of DHNA (Tokyo Kasei Kogyo Co., Ltd.) (10.35 g, 50.69 mmol) in DMF (50 ml), a pyridine sulfur trioxide complex (Wako Pure Chemical Industries, Ltd.) (10.72 g, 67.35 mmol) was added and mixed for 4 hours at 25-28°C under argon gas in a four-necked flask. The reaction solution was concentrated under reduced pressure at 45°C, and the residue was dissolved in AcOEt/MeOH (5:1, v/v) (100 ml). The solution was cooled on ice, and the precipitated solid was collected, washed twice with cold AcOEt/MeOH (20 ml), and dried under reduced pressure to afford a crude DHNAS product. The crude DHNAS was suspended in water (50 ml), and a saturated sodium bicarbonate solution was added with stirring until the solution became clear. Activated charcoal (2 g) was added to the solution, which was then stirred for 30 min and filtered. The filtrate was passed through a Dowex 50W x8 (Na) (500 mL) ion-exchange column and washed with water. The DHNAS(Na)-containing fraction was collected and concentrated under reduced pressure at 45°C. An EtOH-toluene mixture was added to the residue and evaporated to dryness. This procedure was repeated to completely remove the water. Acetone (50 mL) was added to the residue, which was warmed at 40°C for 30 min, and

then cooled on ice. The precipitated solid was collected by filtration, washed twice with acetone (10 ml), and dried at 50°C under reduced pressure to afford a white solid of the DHNAS(Na):acetone (1:1)-complex (13.54 g, yield 73%). The product was dissolved in water (300 ml), concentrated under reduced pressure, and finally freeze-dried to afford the pure DHNAS(Na) (11.61 g, yield 73%, purity > 99.9%). The mass spectrum analyses yielded the following values (FAB, JEOL JMS-DX303, negative mode), M+Na-H=305, M-H=283.

2.3. Administration of DHNAS to Mice for Analyses of Toxicity and Metabolites in the Urine

Six-week-old ICR female mice (n = 13) were purchased from Japan SLC, Inc. (Shizuoka, Japan). The mice were fed a commercial diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*. The mice were kept in a temperature-controlled room with a 12 h light/dark cycle. After one week for acclimatization, the mice were divided into two groups (7 mice for DHNAS administration and 6 mice for control). DHNAS (60 mg, dissolved in 200 µl of purified water) was orally administered to 7-week-old mice (2.14 g/kg body weight). The negative control group received purified water. After the DHNAS administration, two mice from the DHNAS group and one mouse from the control group were housed in metabolic cages for urine and feces collection for 3 days. The body weights were measured for 28 days, except for the mice in the metabolic cages. All procedures were performed according to the guidelines for the care and use of laboratory animals at the University of Occupational and Environmental Health, Japan.

2.4. Analysis of Metabolites from a DHNA-Treated Mouse

DHNA (60 mg dissolved in 200 µl of 1% Tween 80 solution) was orally administered to a 7-week-old ICR female mouse (2.34 g/kg body weight). After the DHNA administration, the mouse was housed in a metabolic cage for urine and feces collection for 3 days. A portion of the 24 hour urine sample from the DHNA administered mouse was analyzed by HPLC [column, GL Sciences, Inc., InertSustain C-18, 4.6 x 250 mm, 3 µm; elution, linear gradient (0-15 min), solution A (12.5 mM citric acid, 25 mM sodium acetate, 10 mM acetic acid, 30 mM sodium hydroxide) → solution A containing 20% acetonitrile; (15-35 min) solution A containing 20% acetonitrile; speed, 0.65 ml/min].

For the large-scale isolation of the metabolite (M1), the urine sample (0.7 ml) was fractionated by the following HPLC conditions (column, Shiseido Capcell Pak C-18, 10 x 250 mm, 5 µm; elution, 15% MeOH containing 10 mM ammonium formate, 3 ml/min). The peak corresponding to M1 (retention time, 12.4-14.3 min) was collected. This procedure was repeated 5 times. The pooled M1 fraction was purified by a second HPLC fractionation, to remove the ammonium formate [column, Shiseido Capcell Pak C18, 4.6 x 250 mm, 5 µm; elution, linear gradient (0-30 min), water → 10% acetonitrile; speed, 1 ml/min]. Evaporation of the M1 fraction (retention time, 5.5-7.5 min) gave a purified amorphous product (2.2 mg) (ca. 3% calculated from the administered DHNA).

2.5. Digestion of M1 (DHNAG) by β-glucuronidase.

The metabolite M1 (5 mg) was treated with 50 units of β-glucuronidase (Sigma, Type H-1) in 50 mM NaOAc buffer (pH 4.5) at 37°C for 30 min.

2.6. Administration of DHNAS to Examine Changes of Fecal Size and Weight

After one week for acclimatization, ICR female mice (7-week-old) were divided into three groups (n=5, each). DHNAS solutions, with concentrations of 6 mg/L and 300 mg/L, were administered to the mice *ad lib.* in their drinking water, for 4 and 3 weeks, respectively, while water was provided to the control group. After the administration periods, feces were collected from each mouse in a metabolic cage for a 2 h period (9:00-11:00 am), and were kept in a freezer until weight measurement. The weight of one fecal pellet was calculated by (weight of total feces / number of feces) for each mouse.

2.7. Stability Test

DHNA, DHNAS and DHNAG were dissolved in 0.1 M phosphate buffer (pH 7.4), 0.1 M NaH_2PO_4 solution (adjusted to pH 3.0) and water (final concentration, 25µg/ml). Each solution was kept at room temperature (23°C), and aliquots of the solutions were analyzed by HPLC at various time points [column, GL Sciences Inc., InertSustain C18 (4.6 x 250 mm, 3 µm); elution, 12% MeOH, 20% acetonitrile, 8 mM ammonium formate; speed, 0.65 ml/min; 40°C]. The solutions were also analyzed by TLC, under the following conditions: Merck TLC silica gel 60F254 (5x10 cm); solvent: 1-butanol: acetic acid:water (4:1:2); Rf values: DHNA (0.87), DHNAS (0.56), DHNAG (0.43). Compounds were detected as blue fluorescent spots by irradiation with 366 nm UV light.

3. Results and Discussion

3.1. Structural Confirmations of Synthetic DHNAS

The ^{13}C- and ^{1}H-NMR data of DHNAS are shown in Table 1. The signal assignments were accomplished by two-dimensional NMR techniques, including correlation spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC). The attachment site of the sulfate ester was proposed to be C-4-OH, for two reasons (Figure 1). First, the C-1-OH group may be inactivated for the sulfation reaction by the O-H---O=C hydrogen bonding between the 1-OH and the C-2-COOH in the ortho-position in the aromatic ring, as exemplified by the

published data [7]. Second, the computer simulated ^{13}C-chemical shift of the C-4-sulfate (136.5 ppm), rather than the C-1-sulfate (149.7 ppm), is closer to the observed chemical shift of the synthetic product (137.8 ppm) (ACD/C+H NMR Predictors 2012, v14.00, Advanced Chemistry Development Inc., Canada).

Table 1. NMR data of DHNAS

Position	δC(ppm)	δH(ppm) (J in Hz)
1	158.4	
2	111.2	
3	118.0	7.68 (s)
4	137.8	
5	123.0	8.00 (d, 7.8)
6	126.2	7.43 (ddd, 7.8, 6.9, 1.4)
7	123.7	7.35 (ddd, 8.3, 6.9, 1.4)
8	122.9	8.14 (d, 8.3)
9	125.9	
10	130.7	
11	172.4	
		*17.75

*Signal of COOH.

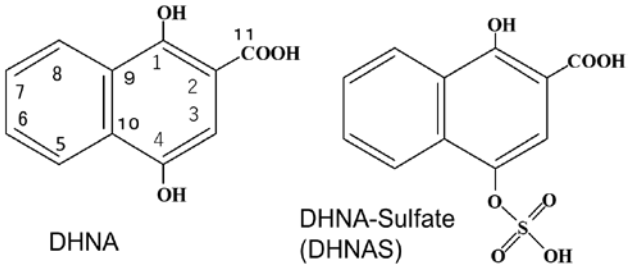

Figure 1. Structures of DHNA and DHNAS

3.2. Comparison of Urine Metabolites after DHNA and DHNAS Administration

3.2.1. Analysis of Urine Metabolites in the DHNA Administered Mouse

Two metabolites, M1 and M2, were detected in the urine of the DHNA administered mouse (24 h) (Figure 2).

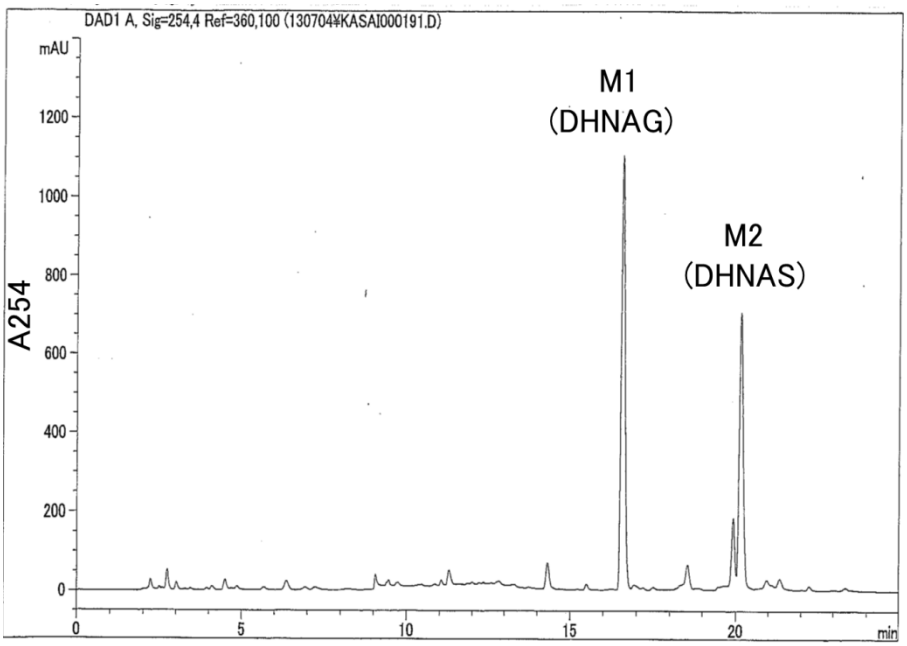

Figure 2. Analysis of DHNA metabolites in mouse urine. After DHNA administration to mice, a portion of the 24 h urine sample was analyzed by HPLC

The amounts of these metabolites were negligible in the urine samples from the second and third days, and in the feces. DHNA was not detected in the urine and feces (retention time of DHNA standard was 27 min), presumably due to decomposition during/after excretion. The mass spectrum of M1 (Q Exactive LC-MS-MS Orbitrap, negative mode) showed an M-H peak at m/z 379.0668 (379.0660 calcd. for $C_{17}H_{15}O_{10}$). The structure of M1 (MW=380) was proposed to be a glucuronide of DHNA (DHNAG) (Figure 3), and was confirmed by ^{1}H- and ^{13}C-NMR (Table 2). The attachment position of the glucuronyl group was determined to be C-4, due to the observed correlation between H-1' and C-4 in HMBC. The structure was also confirmed to be DHNA-β-glucuronide, as it was digested with β-glucuronidase to produce DHNA, based on its retention time in HPLC and UV spectrum (data not shown). The second metabolite, M2, was identified as DHNAS, because it showed an M-H peak at m/z 283 in the FAB Mass (negative mode), and its HPLC retention time and UV spectrum measured online were identical to those of the authentic DHNAS.

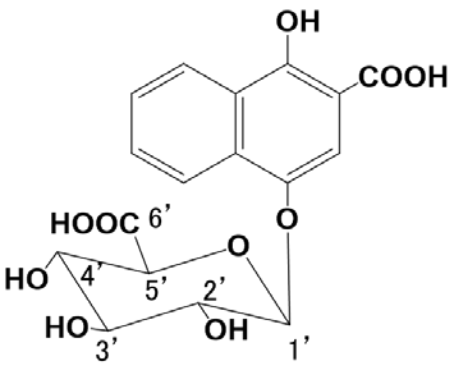

Figure 3. Structure of DHNA-4-β-glucuronide (DHNAG)

Table 2. NMR data of DHNAG

Position	δC(ppm)	δH(ppm) (J in Hz)
1	157.3	
2	111.2	
3	112.1	7.43 (s)
4	142.7	
5	122.2	8.23 (d, 8.2)
6	126.9	7.49 (t, 6.9)
7	124.7	7.43 (t, 6.9)
8	123.2	8.16 (d, 8.2)
9	126.0	
10	128.9	
11	172.6	
1’	103.1	4.77 (d, 7.3)
2’	73.6	3.37 (m)
3’	76.3	3.31 (m)
4’	72.0	3.32 (m)
5’	75.0	3.56 (d, 9.2)
6’	171.6	

3.2.2. Analysis of Urine Metabolites in the DHNAS Administered Mouse

In the urine from the DHNAS administered mouse (24 h), a DHNA-glucuronide-conjugate (DHNAG) peak was detected by HPLC, in addition to a large DHNAS peak, based on a comparison of the retention time and the UV spectrum with those of the authentic DHNAG sample (Figure 4). The amounts of DHNAG were negligible in the second- and third-day urine samples, and in the feces. DHNA was not detected in the urine and feces. This result suggests the sequential conversion, DHNAS → DHNA → DHNAG, *in vivo*.

3.3. Effects of DHNAS Administration to Mice

After a high dose (2.14 g/kg body weight) of DHNAS was administered to mice, no toxicity was detected in any of the mice for 28 days, while softening of the feces was observed for all of the treated mice. No differences in the body weight change profiles and general behavior were observed between the control and DHNAS administered groups (Figure 5). After DHNAS administration through the drinking water (6 mg/L or 300 mg/L), the size (Figure 6) and weight (Figure 7) of each fecal pellet were significantly increased as compared to the control.

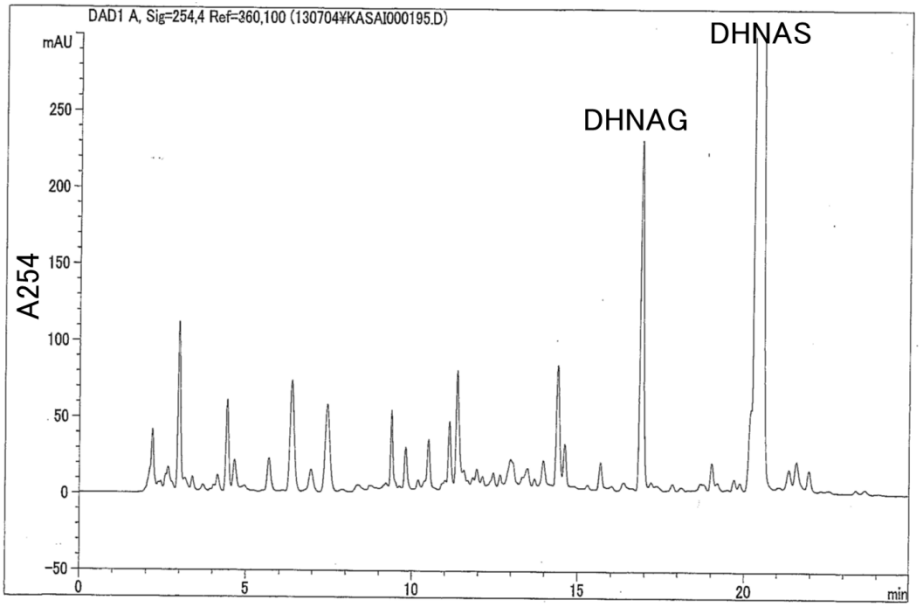

Figure 4. Analysis of metabolites in mouse urine after DHNAS administration. After DHNAS administration to mice, a portion of the 24 h urine sample was analyzed by HPLC

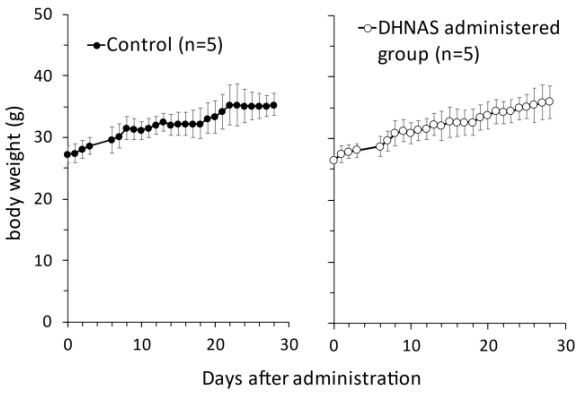

Figure 5. Body weight (mean ± SD) change after DHNAS administration to mice

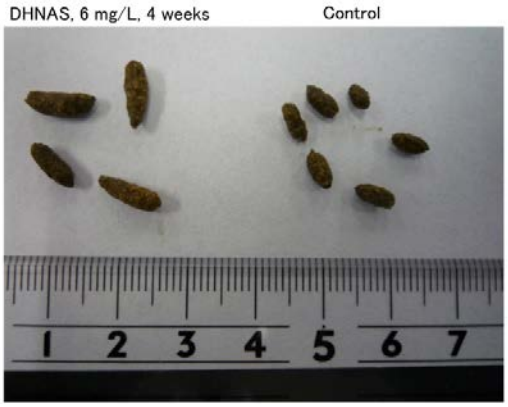

Figure 6. Examples of feces from control and DHNAS administered mice

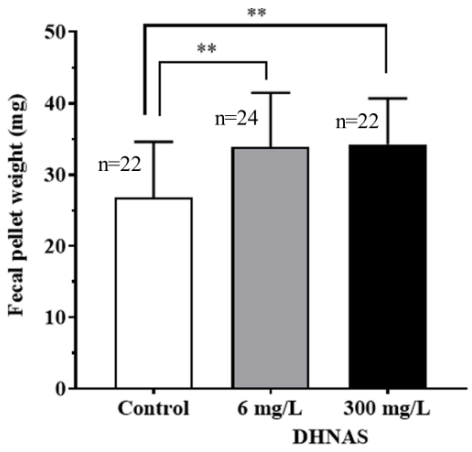

Figure 7. Weight of one fecal piece (mean ± SD) from control and DHNAS administered mice. ** P<0.01

3.4. Comparison of the Stabilities of DHNA and 4-substituted DHNA Derivatives

The stabilities of DHNA, DHNAS, and DHNAG were tested at pH 3.0, pH 7.4 and in water at room temperature (23°C) (Figure 8). The time courses of the decomposition were monitored by HPLC and TLC. DHNA decomposed rapidly within 24 h, especially at pH 7.4. In contrast, no decomposition of DHNAS and DHNAG was observed during 6 days, at any pH.

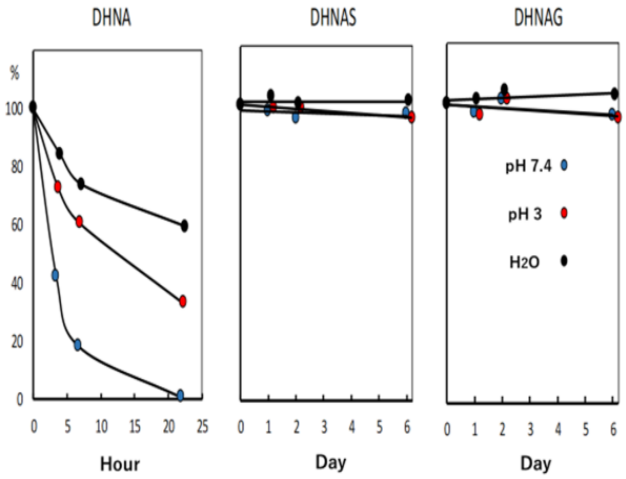

Figure 8. Comparison of the stabilities of DHNA, DHNAS and DHNAG

3.5. Discussion

The widely used laxative, sodium picosulfate, is a typical example of a phenolic compound stabilized by sulfation [8]. It is hydrolyzed to free 4,4'-dihydroxydiphenyl-(2-pyridyl)methane, with a laxative effect, by a sulfatase produced by the intestinal bacteria. Based on this information, we attempted to stabilize DHNA by sulfation.

DHNAS induced fecal softening after administration to mice, by both single oral administration and *ad lib*. drinking of the solutions. Increases in the size and weight of the feces were observed, as shown in Figure 6 and Figure 7, respectively. Liang et al. reported that the size and weight of mouse feces are good indicators to judge constipation [9]. Namely, during a treatment for constipation, the fecal size changed from small to large. Concomitantly, a long defection time changed to a shorter duration [9]. Therefore, our data suggest that DHNAS is a promising constipation preventive agent.

Based on the body weight change (Figure 5) and behavioral data, DHNAS does not seem to be toxic to mice. The toxicity of DHNA has been examined under various conditions in humans and rats. In a human study, no toxicity was detected with the administration of a DHNA-containing extract of a *Propionibacterium* culture to adults under two different conditions (22.5 μg/day of DHNA, 13 weeks, n=11; 283.5 μg/day, 2 weeks, n=11) [10]. In a rat experiment, the oral administration of 6 g *P. freudenreichii* ET-3 culture/kg (570 μg DHNA/kg) did not generate any adverse effects [11]. Furthermore, *in vitro* mutagenicity testing demonstrated that the *P. freudenreichii* ET-3 culture was non-mutagenic in the bacterial reverse mutation assay, using *Salmonella typhimurium* and *Escherichia coli* strains, and was non-clastogenic in Chinese hamster lung cells in a chromosome aberration test [11].

It is quite possible that the administration of DHNA to humans would result in the DHNA being primarily converted to DHNAS and DHNAG. As DHNA was non-toxic in the human studies, DHNAS and DHNAG, which were presumably formed as the major metabolites, may also be non-toxic in humans.

In addition to DHNAS, DHNAG, a metabolite of DHNA, was stable at pH 3.0, pH 7.4 and in water. Many phenolic compounds exist as stable glycosides in plants [12]. After food consumption, the polyphenol glycosides exert their biological activities as aglycones, after hydrolysis of the sugar moiety by glycosidases from the intestinal flora [13]. It is worthy to mention that the glucoside of DHNA has been identified in a Chinese herbal medicine [14]. Because this medicine has been used for a very long time, the DHNA glucoside may also be a safe and promising anti-constipation agent, in addition to DHNAS.

4. Conclusion

We have developed a new, stable DHNA derivative, DHNA-4-sulfate (DHNAS), which should be converted to DHNA *in vivo*. After DHNAS was administered to mice, the enlargement and softening of feces were observed. DHNAS may be a useful food additive to improve human intestinal health, and a promising constipation preventive agent.

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