TRS
TOXICOLOGY REGULATORY SERVICES

26 October 2017 via UPS

Paulette M. Gaynor, Ph.D.
Senior Regulatory Health Project Manager
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

Re: GRAS Notification for Long-Chain Glycolipids from *Dacryopinax spathularia*

Dear Dr. Gaynor,

In accordance with 21 CFR §170, Subpart E - Generally Recognized as Safe (GRAS) Notice, I am submitting, as the agent of the Notifier, IMD Natural Solutions GmbH, Otto-Hahn-Str. 15, 44227 Dortmund, Germany, a Notification regarding the conclusion of GRAS status for the use of long-chain glycolipids from *Dacryopinax spathularia* as an antimicrobial preservative in select non-alcoholic beverages.

Enclosed is one printed copy of the Notification as well as an electronic copy of the Notification on CD.

Please contact me with any questions.

Best regards,

(b) (6)

Andrey I. Nikiforov, Ph.D.
President

AIN/mor

Enclosures

154 Hansen Road, Suite 201, Charlottesville, VA 22911, USA
Telephone: +1 434-977-5957 ■ Facsimile: +1 434-977-1856 ■ trs@toxregserv.com ■ www.toxregserv.com

#740

SAFETY EVALUATION DOSSIER SUPPORTING A GENERALLY RECOGNIZED AS SAFE (GRAS) CONCLUSION FOR THE USE OF LONG-CHAIN GLYCOLIPIDS FROM *DACRYOPINAX SPATHULARIA* IN NON-ALCOHOLIC BEVERAGES

SUBMITTED BY:

IMD Natural Solutions GmbH
Otto-Hahn-Str. 15
44227 Dortmund, Germany

SUBMITTED TO:

U.S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety (HFS-200)
5001 Campus Drive
College Park, MD 20740

CONTACT FOR TECHNICAL OR OTHER INFORMATION:

Andrey I. Nikiforov, Ph.D.
Toxicology Regulatory Services (TRS), Inc.
154 Hansen Road, Suite 201
Charlottesville, VA 22911, U.S.A.

October 26, 2017

Table of Contents

Listing of Figures

Listing of Tables

Listing of Appendices

1. SIGNED STATEMENTS AND CERTIFICATION

The current GRAS Notice is hereby submitted in accordance with Title 21 of the U.S. Code of Federal Regulations (CFR), Chapter I, Subchapter B, Part 170, Subpart E to inform the Agency that the proposed uses of AM-1 described herein are considered to be generally recognized as safe (GRAS).

IMD Natural Solutions GmbH (INS) has determined that the ingredient, long-chain glycolipids from *Dacryopinax spathularia*, herein also referred to as "jelly mushroom glycolipids" or "AM-1", is GRAS under the conditions of its intended use in food as described below and therefore exempt from the pre-market approval requirements of the Federal Food, Drug, and Cosmetic Act. This determination is based on scientific procedures as described in the following sections, with consensus among experts qualified by scientific training and expertise.

(b) (6)

26 October 2017

Andrey I. Nikiforov, Ph.D.
President, Principal
Toxicology Regulatory Services, Inc.
Agent for IMD Natural Solutions GmbH

Date

A. Name and Address of Notifier:

Company:	IMD Natural Solutions GmbH
Name, Position:	Dr. Thomas Henkel, Managing Director
Address:	Otto-Hahn-Str. 15, 44227 Dortmund, Germany
Phone:	+49 231 9742 7400
Fax:	+49 231 9742 7401
Email:	info@imd-natural-solutions.com

B. Name of GRAS Substance

The subject of this GRAS Notice is a material identified as long-chain glycolipids from *Dacryopinax spathularia*, which is herein also referred to as "jelly mushroom glycolipids" or "AM-1".

The GRAS substance is obtained as purified mixture from the fermentation broth of the edible jelly mushroom *Dacryopinax spathularia* using glucose as feedstock. The production organism in also commonly known as sweet osmanthus ear mushroom in China or *Cantharellus spathularius.* The GRAS substance is recovered from the ferment by a food grade and solvent-free process using filtration and subsequent precipitation and washing steps.

Thus, considering its chemical identity and biological source and manufacturing process, the GRAS substance may also be described by the following common names:

Jelly mushroom glycolipids,

Sweet osmanthus ear glycolipids,

Cantharellus spathularius ferment extract,

Mushroom ferment extract (*Dacryopinax spathularia*).

For labeling of foods, according to 21 CFR 101.22, the common name shall be followed by a description of its function, e.g. "Jelly mushroom glycolipids (to retard spoilage)" or "Sweet osmanthus ear glycolipids (to preserve freshness)".

C. Intended Use and Consumer Exposure

Jelly mushroom glycolipids (AM-1) is proposed for use as an antimicrobial preservative in select non-alcoholic beverages at use levels ranging from 2 to 100 ppm. Based on these use levels and daily estimates derived from the NHANES 2009-2010 and 2011-2012 database (NCHS, 2012, 2014), the conservative estimated daily intake (EDI) of AM-1 from all proposed uses (and assuming the maximum proposed use level for each food category) is less than or equal 0.51 mg/kg bw/day at the mean and 1.09 mg/kg bw/day at the 90th percentile of intake among users in the total U.S. population (equivalent to 28.99 mg/day and 58.56 mg/day, respectively).

D. Basis for GRAS Conclusion

Regulatory Framework

The regulatory framework for determining whether a substance can be considered generally recognized as safe (GRAS) in accordance with section 201(s) (21 U.S.C. § 321(s)) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. § 301 et. Seq.) (the "Act"), is set forth at 21 CFR 170.30, which states:

> General recognition of safety may be based only on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. The basis of such views may be either (1) scientific procedures or (2) in the case of a substance used in food prior to January 1, 1958, through experience based on common use in food. General recognition of safety requires common knowledge about the substance throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food.
>
> General recognition of safety based upon scientific procedures shall require the same quantity and quality of scientific evidence as is required to obtain approval of a food additive regulation for the ingredient. General recognition of safety through scientific procedures shall ordinarily be based upon published studies which may be corroborated by unpublished studies and other data and information.

GRAS Conclusion

The basis for the GRAS conclusion for long-chain glycolipids from *Dacryopinax spathularia*, which is herein also referred to as "jelly mushroom glycolipids" or "AM-1" is scientific procedures in accordance with 21 CFR §170.30(a) and (b). The criteria stated above are applied herein in an analysis of whether the use of jelly mushroom glycolipids is GRAS for the intended conditions of use, i.e. as an antimicrobial agent (21 CFR §170.3(o)(2)) in non-alcoholic beverages.

The safety and GRAS status of jelly mushroom glycolipids (AM-1) is based on the totality-of-the-evidence of published data, information, and methods regarding the AM-1 chemical structural characteristics, ADME profile, subchronic toxicity profile in multiple species, and low potential for carcinogenicity, mutagenicity, or developmental and reproductive toxicity. Unpublished data and information is also available as supplemental evidence regarding the safety of AM-1, which corroborate the conclusions that can be made based on generally available and accepted scientific data, information, or methods. The entire body of available information relevant to the safety of jelly mushroom glycolipids, including identity, specifications, manufacturing process, analytical characterization, stability, safety of production organism, probable consumer exposure, metabolic fate, and toxicology profile, provides a basis upon which to conclude that there is a reasonable certainty that AM-1 is not harmful under its intended conditions of use.

The members of an Expert Panel (the "Panel") were convened to evaluate the safety of AM-1 and the Panel's Opinion is provided as Exhibit I of this Notice. The Panel critically evaluated the information summarized herein and other information they deemed appropriate and relevant. The Panel unanimously concluded that the totality-of-the-evidence satisfies the safety standard of reasonable certainty of no harm for the intended conditions of uses of AM-1. In addition, because the information supporting safety is widely known and accepted by qualified experts, the Panel concluded that AM-1 is not only safe, but generally recognized as safe (GRAS) for the intended condition of use described herein.

Based on the findings of the Expert Panel and our own knowledge of the information compiled in this Notice, we conclude that AM-1 is GRAS for the intended conditions of use described herein. To the best of our knowledge, the current GRAS Notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of AM-1.

E. Availability of Information

The data and information that serve as the basis for the GRAS conclusion are appended to this Notification. Questions or requests for additional information may be directed to: Toxicology Regulatory Services Inc., 154 Hansen Road, Suite 201, Charlottesville, VA 22911, USA (contact: Andrey Nikiforov (Agent for IMD Natural Solutions GmbH), telephone (434) 977-5957; email: anikiforov@toxregserv.com).

None of the data and information in Parts 2 through 7 of the current GRAS Notice are considered to be exempt from disclosure under FOIA.

2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT

This section of the GRAS notice fulfills the requirements of 21 CFR 170.230 by providing information in regard to the GRAS material identity, method of manufacture, specifications, and physical or technical effect including product characteristics and analytical data.

A. Identity

The subject of this GRAS Notice is the material identified as long-chain glycolipids from *Dacryopinax spathularia*, which is herein also referred to as "jelly mushroom glycolipids" or "AM-1".

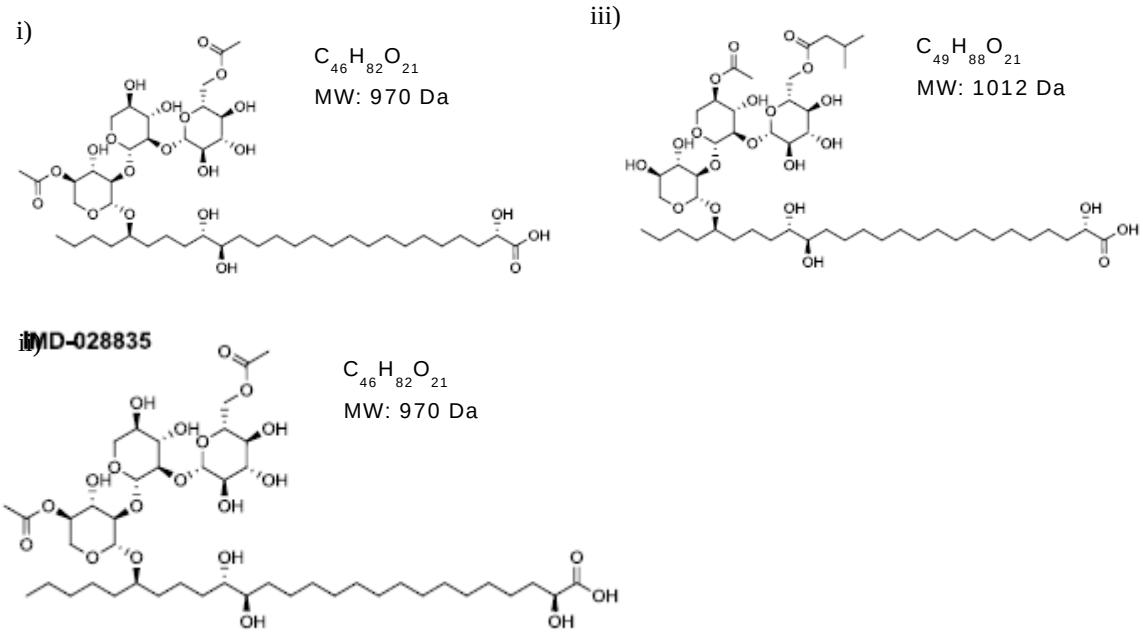
CAS Registry Number

To be determined.

Molecular and Structural Formula

The major components of jelly mushroom glycolipids are three structurally-related glycolipid congeners. Representative structure diagrams for these main components (i, ii, iii) are provided below in Figure 1. The remaining components are congeners of the major components, sharing the saturated C_{26} fatty acid and the glucopyranosyl-(1→2)-xylopyranosyl-(1→2)-xylopyranosyl trisaccharide moiety but differing in the acylation pattern, i.e. the number and position of acyl groups attached to the sugar units.

Figure 1. Representative structures for Jelly Mushroom Glycolipids (AM-1)



B. Production Process

Jelly mushroom glycolipids (AM-1) represents a natural glycolipid mixture obtained via fermentation of glucose by the edible jelly fungus *Dacryopinax spathularia* (Schwein.) (Martin, 1948). This mushroom species is also known as sweet osmanthus ear or under the scientific synonym *Cantharellus spathularius.* It belongs to the phylum Basidiomycota and builds edible, orange-colored, spatula-shaped fruiting bodies. A safety assessment of the AM-1 production organism is presented in Appendix 3 and summarized below in Section 6.

Fermentation of *Dacryopinax spathularia* strain MUCL 53181 is conducted using glucose as carbon source in aerobic submerged culture. Starting from a cryogenic Working Cell Bank (WCB), a series of seed cultures is conducted in shake flasks and then in bioreactors with increasing volumes in order to obtain sufficient living fungal cells to inoculate the main culture. The main culture is conducted in fed-batch mode, with start medium and feed medium both consisting of glucose and smaller amounts of yeast extract (commercial extract of autolysed Baker's yeast *Saccharomyces cerevisiae*). The culture is maintained at 30 °C for several days until maximum titer of glycolipids is achieved. The feed is stopped and the cells are further cultivated until no free glucose is found in the culture medium.

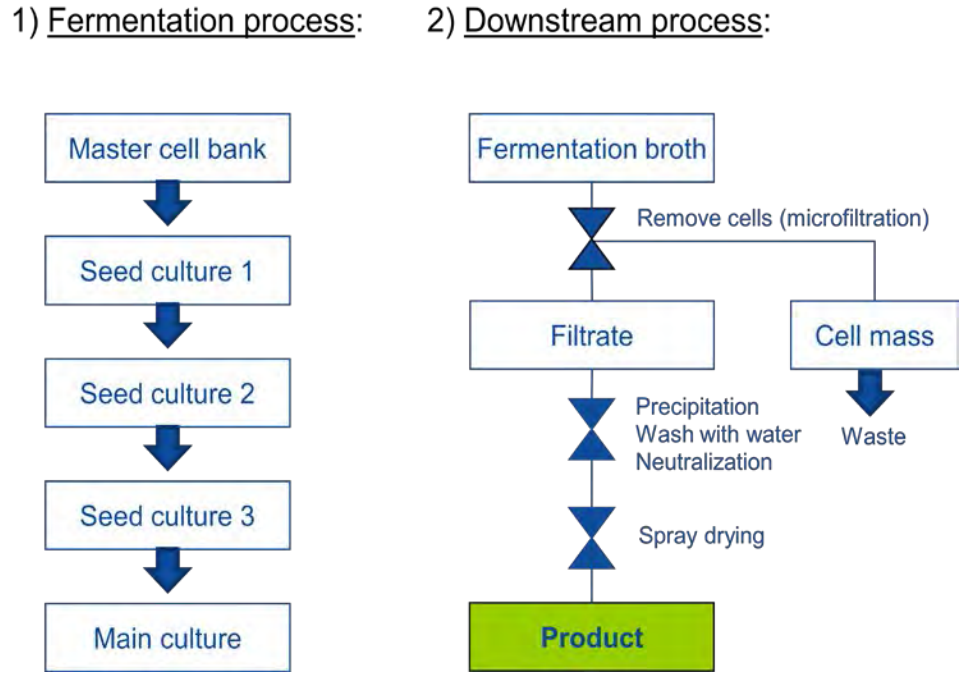
Then, the fungal cells are quantitatively removed by microfiltration, followed by acidic precipitation of the glycolipids. The precipitate is washed with water and neutralized using sodium hydroxide solution. Spray or freeze drying leads to the final product as an off-white, water soluble powder. The absence of any remaining intact fungal cells from the source organism *Dacryopinax spathularia* is technically excluded by the design of the microfiltration step and has been confirmed by viable fungal cell count (using preferred growth conditions for *Dacryopinax spathularia* strain MUCL 53181) as well as microscopic control of representative batches.

Nutrient media used in the fermentation process contain glucose and yeast extract. Hydrochloric acid and sodium hydroxide solution are used for pH adjustment during downstream processing. All nutrient components and solutions for pH adjustment are food-grade. The simple production process consists only of typical food-grade processing steps. No organic solvents are used. No chemical reaction or modification of the glycolipids is done.

The production process follows current Good Manufacturing Practices (cGMP) as defined in 21 CFR §110 and quality management compliant to FSSC22000, including a Hazard Analysis and Risk-based Preventive Controls (HARPC) plan as demanded by the FDA Food Safety Modernization Act (FSMA).

A Manufacturing Process Flow Diagram for jelly mushroom glycolipids (AM-1) is provided below in Figure 2.

Figure 2. Manufacturing Process Flow Diagram for Jelly Mushroom Glycolipids (AM-1)



C. Product Characteristics

Physical Description

Jelly mushroom glycolipids (AM-1) is a white to off-white / ivory solid with weak, characteristic odor.

Specifications and Composition

The specifications for jelly mushroom glycolipids (AM-1) are summarized below in Table 1, including a total glycolipids content of ≥ 93% (dry weight basis, calculated as sodium salt). The remaining ≤ 7% of dry weight is comprised of protein, fat, and sodium chloride.

The major components of jelly mushroom glycolipids are three structurally-related glycolipid congeners as depicted in Figure 1. The remaining glycolipid components are congeners of the major components, sharing the saturated C_{26} fatty acid and the glucopyranosyl-(1→2)-xylopyranosyl-(1→2)-xylopyranosyl trisaccharide moiety but differing in the acylation pattern, i.e. the number and position of acyl groups attached to the sugar units. The acyl groups are usually acetate and isovalerate. The presence of glycolipids containing the minor component 3-hydroxy-3-methylglutarate (HMG) as an acyl unit instead of acetate and isovalerate has also been noted in certain batches of AM-1 (average calculated concentration < 1.0%), as further described in Appendix 1-A and discussed within the AM-1 safety evaluation narrative below (Part 6).

Analytical Characterization

Analytical results for non-consecutive lots/batches of commercially representative AM-1 are provided in Appendix 1-B and demonstrate conformance to specifications and consistency of AM-1 manufacturing.

A robust, selective, accurate and precise HPLC-MS method has been developed and validated for qualitative and quantitative analysis of AM-1 in highly aqueous food matrices such as beverages.

Stability

When stored as a dry powder in a closed container at temperatures of 40°C and below, AM-1 was shown to be stable for at least three years without any detectable degradation or modification of its composition (see Appendix 1-C).

In aqueous solution, AM-1 is stable at room temperature for at least six months, with minor hydrolysis occurring for some of the glycolipid acyl moieties (<5%). Hydrolysis products are low concentrations of acetate and isovalerate. Antimicrobial efficacy is not influenced significantly. Aqueous solutions may be stored under refrigerated (4-8°C) conditions for up to 1 year.

AM-1 is stable in beverage applications of various pH under typical storage conditions, with hydrolysis of the ester moieties of the glycolipids dependent on the pH value and cloudiness of the individual beverage (see Appendix 1-D). At low pH, elevated temperature and extended

time, ester moieties of AM-1 can slowly hydrolyze resulting in a higher ratio of deacylated glycolipids compared to the original mixture. The preserving properties of this partially deacylated glycolipid mixture are slightly reduced but still acceptable. Degradation of the glycolipids to its principal components glucose, xylose and long-chain fatty acids (LCFA) did not occur in any beverage.

There are no degradation products of safety concern associated with AM-1, as a bulk material ingredient or when formulated in low-pH beverage applications, under typical storage and use conditions. Consistent with the hydrolysis pathway of AM-1 in the gastrointestinal tract determined in experimental studies (Bitzer et al., 2017a), initial hydrolysis products are acetate and isovalerate which are normal constituents of the human diet and, if absorbed, are rapidly eliminated and may be ultimately metabolized to CO_2.

In the event that the 3-hydroxy-3-methylglutarate (HMG) acyl unit occurring in certain batches of AM-1 were to hydrolyze at a similar rate observed in the AM-1 beverage stability studies, a safety assessment of the potential consumer exposure to this minor constituent is presented below in Part 6.

Table 1. Specifications for Jelly Mushroom Glycolipids (AM-1)

Analytical Parameter	Acceptable Target/Range	Methods of Analysis
Appearance	Off-white to ivory powder	In-house method, based on RAL classic color scheme
Odor	Weak, characteristic	Olfactory assessment
Aqueous solubility	> 20 g/l	In-house method, shake-flask
Turbidity (0.1% in water)	< 8 NTU	Turbidity meter
pH value (1% in water)	5.0 – 7.0	pH meter
Water content	< 5.0%	Karl Fischer (USP 921)
Total protein	< 3.0%	Kjeldahl (USP 461, N × 6.25)
Total fat	< 2.0%	Gravimetric (AOAC, 2000)
Sodium	1.7 – 3.3%	AAS (USP 852)
Total glycolipids (dry weight basis, calcd. as sodium salt)	≥ 93.0%	HPLC-MS or GC, in-house method
Identity	Conform to standard chromatogram and mass spectra	HPLC-MS, in-house method
Heavy metals	< 1 ppm (As, Cd, Hg) < 2 ppm (Ni, Pb)	ICP-MS (USP 233)
TAMC (total aerial microbial count)	≤100 CFU/g	USP 61
TYMC (total yeast/mold count)	≤10 CFU/g	USP 61
Coliforms	≤ 3 MPN/g	AOAC 966.24
Escherichia coli	≤ 3 MPN/g	AOAC 966.24
Salmonella spec.	absent in 25 g	AOAC 967.26, AOAC 967.27

AAS = atomic absorption spectroscopy; AOAC = Association of Official Analytical Chemists; CFU = colony forming units; GC = gas chromatography; HPLC-MS = high-performance liquid chromatography-mass spectrometry; ICP-MS = inductively coupled plasma-mass spectrometry; MPN = Most Probable Number; NTU = Nephelometric Turbidity Unit; USP = United States Pharmacopeia

D. Physical or Technical Effect

Jelly mushroom glycolipids (AM-1) is intended for addition to beverages as an antimicrobial agent used to preserve food by preventing growth of microorganisms and subsequent spoilage (21 CFR §170.3(o)(2)).

Its prominent antifungal effect against common yeasts and molds makes jelly mushroom glycolipids particularly useful as a naturally derived preserving agent to prevent spoilage of beverages.

Thus, AM-1 can be used to prolong shelf life and guarantee the microbiological quality of beverages, making it a versatile alternative or complement to other techniques like heat treatment, which may be attributed with loss of nutritional value, vitamin content or taste. AM-1 may also substitute current chemical preservatives in beverages, such as sorbic acid, benzoic acid or sulfites at lower concentrations in use. For example, under identical test conditions (method DIN 58940-8), Minimum Inhibitory Concentrations (MICs) of 3.1 and 7.8 mg/L were determined for AM-1 against *Aspergillus niger* in SDB (Sabouraud Dextrose Broth) medium (pH 5.6) or clear apple juice medium (pH 3.3), respectively, compared to MICs of 250 mg/L (in SDB) or ≥1000 mg/L (in apple juice medium) for sorbic acid and benzoic acid, respectively (supporting details are provided Appendix 1-E). Since AM-1 is also active against yeast and mold strains which have adapted to current chemical preservatives, it represents an alternative or complement as to successfully assure shelf life of beverages.

AM-1 has good activity against Gram-positive bacteria (including spoilage organisms like *Bacillus cereus* or *Listeria* spp.) but weak or no activity against Gram-negative bacteria.

Additional data and figures illustrating the antimicrobial activity of jelly mushroom glycolipids and its application as an antimicrobial preservative in beverages are provided in Appendix 1-E.

3. DIETARY EXPOSURE

This section of the GRAS Notice fulfills requirements of 21 CFR 170.235 in regard to the dietary exposure of jelly mushroom glycolipids (AM-1) as a result of its intended uses and use levels in a variety of foods.

A. Intended Uses and Use Levels

Jelly mushroom glycolipids (AM-1) is proposed for use in a variety of beverage categories, including fruit drinks and juices where standards of identity do not preclude such use, at the levels shown in Table 2, below.

Table 2. Proposed Uses and Maximum Use Levels of Jelly Mushroom Glycolipids (AM-1)

Beverage Category	AM-1 (%)	AM-1 (ppm)
Carbonated soft drinks	0.0025	25
Fruit drinks	0.008	80
Sport drinks	0.005	50
Energy drinks	0.005	50
Enhanced waters	0.0025	25
Tea, RTD	0.005	50
Juice	0.01	100

RTD = ready-to-drink

B. Estimated Daily Intake (EDI) of Jelly Mushroom Glycolipids

Available Data and Methods

The estimated daily intake (EDI) of jelly mushroom glycolipids (AM-1) from the proposed uses was estimated based on the proposed use levels in Table 2, using Exponent Inc.'s Foods and Residue Evaluation Program (FARE™ version 11.14) software and data from the most recent National Health and Nutrition Examination Survey (NHANES 2009-2012). Individual food codes selected for inclusion in each proposed use category are provided in Appendix 2.

The NHANES 2009-2010 and 2011-2012 (NCHS, 2012, 2014) is a complex multistage probability sample designed to be representative of the civilian U.S. population. The survey collects two days of food intake data, in addition to nutrition, demographic, and health information. Exponent Inc. used the statistically weighted values from the survey in the analyses. The statistical weights compensate for variable probabilities of selection, adjust for

non-response, and provide intake estimates that are representative of the U.S. population and the selected age-gender subgroups.

Exponent Inc. estimated the daily intake on a per "user" basis. In this analysis, a "user" is anyone who reported consuming at least one category of beverage in which it is proposed to use AM-1 (AM-1 beverage category) on either of the survey days, i.e. United States Department of Agriculture's (USDA's) "user" definition. Each individual who reported consuming an AM-1 beverage on either of the survey days was identified, and that individual's responses for both survey days was used. Because AM-1 is likely to be consumed over a lifetime it is appropriate to average exposures over a longer period than one day. Therefore, Exponent Inc. used each respondent's food consumption averaged over the two days of the NHANES 2009-2010 and 2011-2012 surveys. A 2-day average typically overestimates lifetime average daily intake especially for foods/beverages consumed infrequently; however, only two nonconsecutive days' worth of food/beverage consumption data are available in the most recent NHANES 2009-2010 and 2011-2012 surveys database. It is well known that food/ beverage consumption data collected over longer periods of time, e.g., 14 days as in Market Research Corporation of America (MRCA) consumer surveys, yield estimates of daily intake that may be significantly lower than 2-day averages (Lambe et al., 2000). Therefore, actual consumer exposures to jelly mushroom glycolipids are anticipated to be lower than the estimates reported herein.

Estimated Daily Intake

The EDI of jelly mushroom glycolipids (AM-1) was calculated by multiplying each NHANES respondents' 2-day average beverage intake by the use levels described in Table 2, above. Each individual's intake of AM-1 was divided by his/her bodyweight to provide the per capita and per user intakes on a bodyweight basis. Mean and 90^{th} percentile daily intakes on a per user basis, as mg AM-1/kg bw/day, were estimated for the proposed uses of AM-1 for the U.S. population. Results are presented in Table 3, including per user mean and 90^{th} percentile intake of AM-1 for each proposed beverage category and total EDI over all beverage categories.

Table 3. Estimated Daily Intake of Jelly Mushroom Glycolipids (AM-1) from Proposed Uses at Maximum Use Levels (Total U.S. Population)

Beverage Category		***Per User* Intake of AM-1 (mg/kg bw/day)**	
	N (Unweighted)	**Mean**	**90th Percentile**
Carbonated soft drinks	8202	0.17	0.34
Energy drinks	267	0.17	0.33
Enhanced waters	235	0.13	0.24
Fruit drinks	4470	0.40	0.83
Sport drinks	1005	0.31	0.63
Tea, RTD	2258	0.25	0.50
Juice	6767	0.48	1.01
Total (all beverage categories)	**13,504**	**0.51**	**1.09**

Source: Appendix 2 (Exponent, 2016).

The total EDI for AM-1 from all proposed uses assuming the maximum proposed use level for each beverage category is not more than 0.51 mg/kg bw/day at the mean and 1.09 mg/kg bw/day at the 90th percentile of intake among users in the total U.S. population (equivalent to 28.99 mg/day and 58.56 mg/day, respectively).

A further breakout of estimated AM-1 intakes by age/sex subgroups is shown below in Table 4.

Table 4. Estimated Daily Intake of Jelly Mushroom Glycolipids (AM-1) from All Proposed Uses (Maximum Use Levels) by Population Subgroup (Infants, Children, Teenagers and Adults)

Population	**Unweighted Users**	***Per User* Intake of AM-1 (mg/kg bw/day)**	
		Mean	**90th Percentile**
Infants 0-11 months	132	1.02	2.26
Children 1-6 years	1995	1.52	3.29
Children 7-12 years	1783	0.64	1.27
Teenagers (13-19 years)	1713	0.50	1.03
Adults >20 years	7882	0.37	0.75

Source: Appendix 2 (Exponent, 2016).

On a bodyweight basis, the highest per user mean and 90th percentile intake estimates for AM-1 are among children 1-6 years at 1.52 mg/kg bw/day and 3.29 mg/kg bw/day, respectively. The second highest per user intake levels on a bodyweight basis are among infants 0-11 months with mean and 90th percentile estimates of 1.02 mg/kg bw/day and 2.26 mg/kg bw/day, respectively. Per user mean and 90th percentile intake estimates on a bodyweight basis among children 7-12 years are 0.64 mg/kg bw/day and 1.27 mg/kg bw/day, respectively. Although these 90th percentile estimates for children and infants are up to approximately 3-fold higher than the 90th percentile total population EDI (i.e. 3.29 versus 1.09 mg/kg bw/day), they are sufficiently below the acceptable daily intake determined for AM-1 (discussed below in Part 6). The range of exposures among remaining population subgroups is similar to the total population EDI, e.g., 0.37 to 0.50 mg/kg bw/day (mean) and 0.75 to 1.03 mg/kg bw/day (90th percentile).

As noted in the EDI report (Appendix 2), the above estimates based on 2-day average intakes do not necessarily represent long-term intakes, because (1) they may not capture infrequent consumers of foods proposed to contain AM-1, (2) assume that subjects who consumed AM-1-containing products on both survey days actually consume these AM-1 products every day of the year, and (3) do not adjust for potential day-to-day variation in AM-1 intake. A 2-day average typically overestimates long-term (chronic) daily intake and does not necessarily represent long-term intakes. Additionally, results of the *in vivo* absorption, distribution, metabolism and excretion (ADME) study with AM-1 (Bitzer et al., 2017a; discussed below in Part 6) support a conclusion of limited absorption of AM-1 or its major hydrolysis product, LCFA, further minimizing potential consumer exposure to AM-1.

4. SELF-LIMITING LEVELS OF USE

This section of the GRAS Notice fulfills requirements of 21 CFR 170.240 by providing information about any self-limiting characteristics of jelly mushroom glycolipids (AM-1) use.

The levels at which jelly mushroom glycolipids would become self-limiting (i.e. due to its viscosity and possible effect on organoleptic properties of proposed beverage categories) are above the levels of its intended use as specified in this GRAS Notice.

5. EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

General recognition of safety for the notified substance, long chain glycolipids from *Dacryopinax spathularia* (i.e. jelly mushroom glycolipids), is established through scientific procedures; therefore, information regarding experience based on common use of the notified substance in food prior 1958 is not applicable.

However, the fruiting bodies of the producer organism *Dacryopinax spathularia* (syn.: *Cantharellus spathularius* or sweet osmanthus ear) are edible and evidence for their traditional use as food in many countries is documented. Details are provided in Appendix 4 and summarized below in Part 6 (Historical Consumption).

6. BASIS FOR CONCLUSION OF GRAS STATUS FOR JELLY MUSHROOM GLYCOLIPIDS (AM-1) (NARRATIVE)

This section of the GRAS Notice fulfills the requirements of 21 CFR 170.250 by providing a narrative in regard to the generally available and accepted scientific data, information, methods, or principles that are relied on to establish safety.

Introduction

The current safety evaluation of jelly mushroom glycolipids (AM-1) is based on pivotal published (Bitzer et al., 2017a,b,c), as well as unpublished but corroborative, data and information regarding the metabolism and toxicity profile of AM-1. All pivotal safety studies were conducted in accordance with U.S. Food and Drug Administration (FDA) Good Laboratory Practice (GLP) regulations (21 CFR 58; FDA, 1987) and/or the Organisation for Economic Co-operation and Development (OECD) Principles of GLP (ENV/MC/CHEM(98)17). Study protocols were designed in accordance with the applicable testing guidelines of OECD and/or U.S. FDA Redbook (FDA 1993, 2000).

In summary, AM-1 and its ultimate hydrolysis product *in vivo*, long chain fatty acids (LCFA), are poorly absorbed by the oral route and are primarily eliminated in the feces without absorption. Absorbed components appear to be almost completely metabolized to CO_2 and expired. There were no metabolites of safety concern identified for AM-1 and its ultimate hydrolysis product LCFA and no accumulation of these compounds in tissues. AM-1 has low potential for systemic toxicity with oral repeated dose (90-day) no-observed-adverse-effect level (NOAELs) of $\geq$ 1200 mg/kg bw/day in rats (oral drinking water administration) and $\geq$ 1000 mg/kg bw/day in dogs (oral capsule administration), the highest dose levels tested. AM-1 was determined to be non-genotoxic based on the results of a complete battery of *in vitro* genetic toxicity assays in bacteria as well as in mammalian cells including human lymphocytes. AM-1 is not a reproductive or developmental toxicant as confirmed in robust 2-generation reproduction toxicity and embryofetal toxicity studies in rodents (oral gavage administration).

The totality of the evidence provides a basis upon which to conclude that the uses of AM-1 described in this GRAS Notice satisfy the safety standard of Reasonable Certainty of No Harm. In addition, these data and information are known and accepted by a consensus of qualified experts in the general scientific community. Thus, this information base not only assures that the intended uses of AM-1 described in this Notice are safe, but also comprises common knowledge that AM-1 is also generally recognized as safe under its intended conditions of use.

The following subsections of this Notice provide a general description of the data and information that support the above conclusions. In addition, the conclusions reached by the Expert Panel are presented in Exhibit I and are considered to be accurate by the Notifier.

Historical Consumption

Glycolipids such as AM-1 are part of the normal human diet with the most abundant sources of glycolipids identified as eggs and dairy products, cereals, and soybeans (Leray, 2015).

The fruiting bodies of the jelly mushroom glycolipids production organism *Dacryopinax spathularia* (syn.: *Cantharellus spathularius*) are edible and evidence for their traditional use as food in many countries in Asia and Africa is documented. A field guide on foraging mushrooms in Oregon (USA) cites a recipe with *Dacryopinax spathularia* along with a description for identification of this edible mushroom (Meuninck, 2017), demonstrating that this mushroom has also been used as food in North America.

Dacryopinax spathularia is listed in the Food and Agriculture Organization of the United Nations (FAO) compendium on edible mushrooms (Boa, 2004) with proven food use documented in China (Zhishu et al., 1993), Japan (MycoBank, 2017), and Cameroon (Van Dijk et al., 2003). Additional species of the Dacryomycetaceae family (e.g. *Dacrymyces palmatus* and *Ditiola peziziformis*) are also known to be edible and confirmed in the FAO compendium. Culinary use of the fruiting bodies is further described for Malaysia (Lee et al., 2009) and India (Ao et al., 2016).

The presence of jelly mushroom glycolipids (AM-1) as constituents in collected fruiting bodies of *Dacryopinax spathularia* has been confirmed by HPLC-MS analyses. Further, AM-1 was also found in small fruiting bodies of the producer strain MUCL 53181 grown on wood substrate in the laboratory (INS; *unpublished data on file*).

Additional supporting references and discussion of the food uses of the jelly mushroom glycolipids (AM-1) production organism *Dacryopinax spathularia,* and its fruiting bodies, are provided in Appendix 4.

Safety Assessment of Jelly Mushroom Glycolipids Production Organism

The safety of the jelly mushroom glycolipids (AM-1) production organism *Dacryopinax spathularia* MUCL 53181 has been assessed utilizing scientific procedures as outlined by Pariza and Johnson (2001) for safety evaluation of microbial enzyme preparations used in food processing. Using this paradigm, the AM-1 ingredient may be considered the "test article" that is produced by a mushroom fermentation culture, which is comparable to an enzyme preparation produced by a microbial culture. Based on the outcome of the decision tree (summarized in Figure 3) including strain characterization, screening for undesirable attributes and metabolites, and experimental evidence of safety for the produced AM-1 ingredient, it was concluded that *D. spathularia* MUCL 53181 is considered safe for use in the production of AM-1 as an ingredient for human consumption. Additional details supporting the safety assessment of the AM-1 production organism *D. spathularia* MUCL 53181 are provided in Appendix 3.

Figure 3: Pariza and Johnson (2001) Decision Tree Analysis of Jelly Mushroom Glycolipids (AM-1) Production Organism, *Dacryopinax spathularia* MUCL 53181

Question 1. Strain genetically modified?

No; The identity of *Dacryopinax spathularia* strain MUCL 53181 was confirmed by morphological, molecular phylogenetic and chemotaxonomic methodology. No genetic modifications to the strain are performed.

(The evaluation proceeded to Question 6.)

Question 6. Production strain from safe strain lineage?

No; The "safe strain lineage" criteria are not met for strain MUCL 53181 because the strain is not genetically modified and, hence, there is no parental strain.

(The evaluation proceeded to Question 7.)

Question 7. Production strain non-pathogenic?

Yes; The species *Dacryopinax spathularia* is classified as Risk Group 1 by the German Federal Institute for Occupational Safety and Health (BAuA) and can therefore be considered unlikely to cause human disease. There are no reports of pathogenic effects of fungal species belonging to the Dacrymycetes family. Thus, it is concluded that *D. spathularia* MUCL 53181 does not have any pathogenic potential. Furthermore, because the finished jelly mushroom glycolipids product does not contain any live organisms, pathogenicity is not a concern with respect to consumer safety (Pariza and Johnson, 2001).

(The evaluation proceeded to Question 8.)

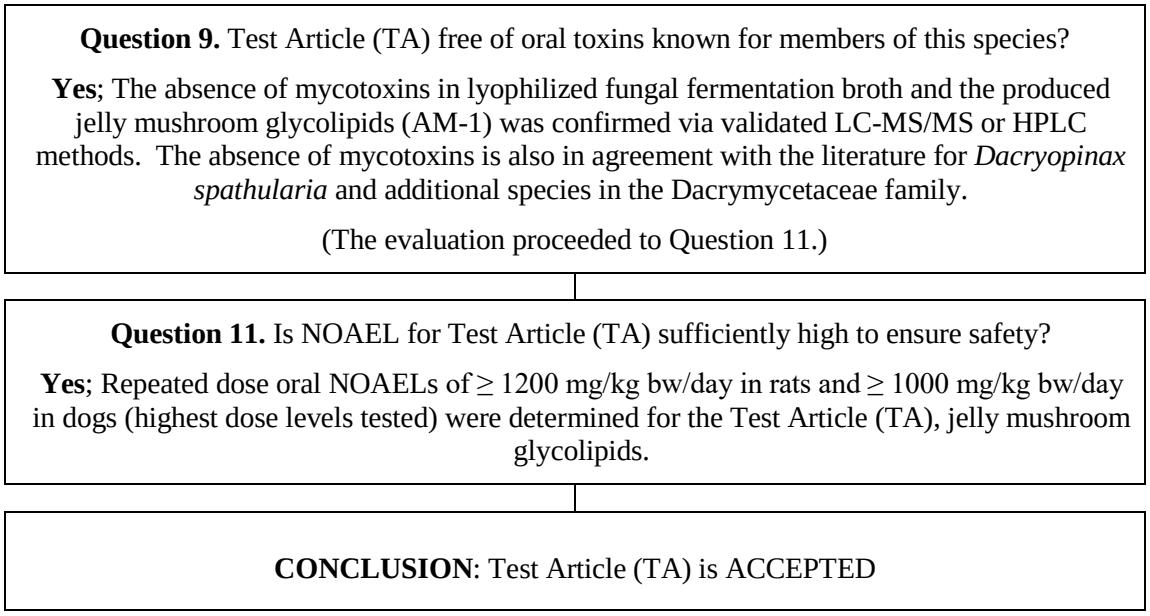
Question 8. Test Article (TA) free of antibiotics?

Yes; The absence of any medically or clinically relevant antibiotics and corresponding compound classes in the fermentation broth of strain MUCL 53181 has been confirmed via extensive HPLC analyses. These results are consistent with the literature on *Dacryopinax* species, for which the only known secondary metabolites or products are glycolipids, carotenoids, and polysaccharides (Nishida et al., 1988; Stadler et al., 2012; Vail and Lilly, 1968; Chen and Seviour, 2007)

Further, because rapid hydrolysis and elimination of jelly mushroom glycolipids occurs *in vivo* (Bitzer et al., 2017a), the antimicrobial properties of the parent material would be absent or greatly diminished in the lower intestines so there is low concern for disruption of healthy gut microflora. Further, the predominant effect of the TA is antifungal. There is no or weak activity against Gram-negative bacteria, and moderate activity against Gram-positive bacteria.

(The evaluation proceeded to Question 9.)

Figure 3: Pariza and Johnson (2001) Decision Tree Analysis of Jelly Mushroom Glycolipids (AM-1) Production Organism, *Dacryopinax spathularia* MUCL 53181 (*continued)*



Absorption, Distribution, Metabolism and Excretion (ADME) Profile of Jelly Mushroom Glycolipids (AM-1)

Methods

The pharmacokinetics, excretion balance (i.e. mass balance as a measurement of test-article equivalents in excreta samples), and tissue distribution of [^{14}C]-AM-1 and [^{14}C]-Long Chain Fatty Acid (LCFA) equivalents following single or repeated administration to Sprague Dawley rats, aged 8-10 weeks, were evaluated. The study was performed in compliance with the U.S. Food and Drug Administration (FDA) Good Laboratory Practice Regulations (21 CFR Part 58) (FDA, 1987) and the study protocol was designed in general accordance with U.S. FDA Redbook Guideline Redbook II Guidelines, Toxicological Principles for the Safety Assessment of Food Ingredients, Chapter V B, Metabolism and Pharmacokinetic Studies (FDA, 1993). Detailed study methods are reported in Bitzer et al., 2017a and summarized briefly below.

Based on the results of previous *in vitro* experiments in simulated gastric fluid and *in vivo* pharmacokinetics studies in rats with [^{13}C]-AM-1, it was hypothesized that following ingestion, AM-1 passes mostly unchanged to the lower GI tract where predominantly its ester linkages and, partially, its glycosidic linkages are prone to hydrolysis by microflora in the lower intestine to its components glucose, xylose, acetate, isovalerate, and long chain fatty acids (LCFA) molecules (Figure 4). Since it was anticipated that its minor components, glucose, xylose, acetate, and isovalerate, would be further metabolized rapidly and incorporated for normal physiological functions, the major hydrolysis component of AM-1, i.e. LCFA, was studied in parallel to AM-1. This approach allowed the pharmacokinetics, excretion balance, and tissue distribution experiments to elucidate the ultimate fate of the parent compound, AM-1, by also studying its major hydrolysis product, LCFA.

For all study phases, rats received equimolar doses of either [^{14}C]-AM-1 or [^{14}C]-LCFA via oral or intravenous (IV) administration followed by collection of biological samples at specified intervals. Because the test articles were uniformly radiolabeled with [^{14}C] and based on their respective molecular weights (MW), equimolar doses of AM-1 (average MW = 985) and LCFA (average MW = 455) were calculated for all Groups (Table 5). The radiolabeled and non-radiolabeled dose of AM-1 or LCFA to be administered was calculated on a mg/kg body weight basis using a dosage volume of 10 ml/kg for the oral route and 2 ml/kg for the IV route. Following dosing, the Excretion Balance phase animals were placed into glass metabolism cages for separate collection of expired air, urine, and feces.

Figure 4. Proposed Degradation of Jelly Mushroom Glycolipids (AM-1) to LCFA.
First (step a), sequential hydrolysis of ester groups occurs. Total hydrolysis, i.e. deglycosylation towards the free fatty acids, is only observed when heated with strong acids or by microbial transformation.

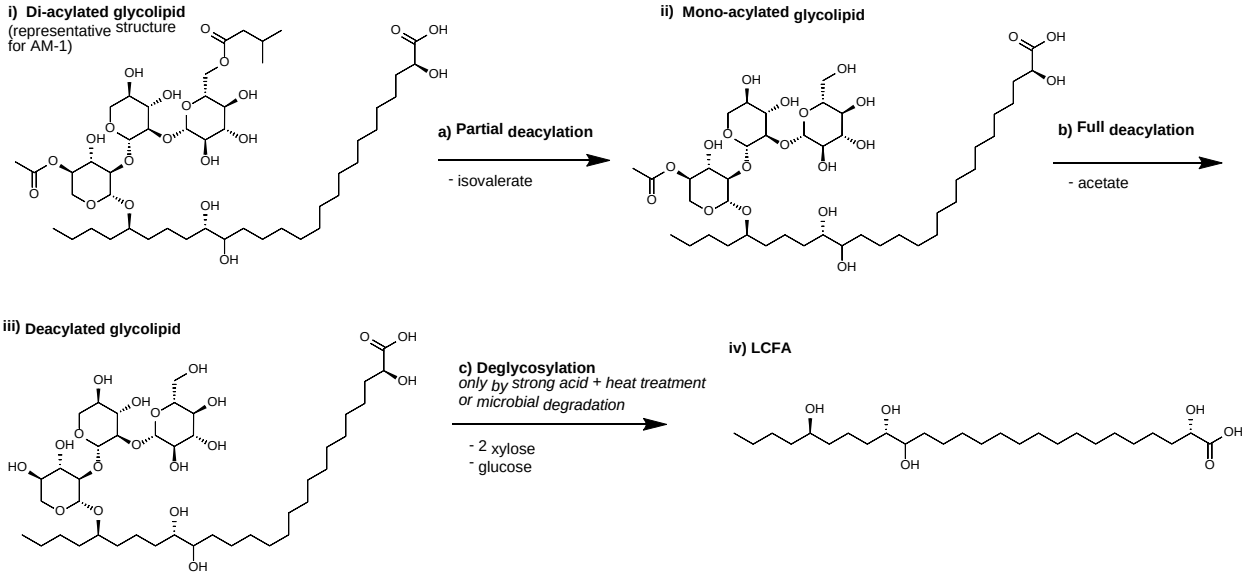


Table 5. Experimental Design and Group Assignment During a Pharmacokinetics, Excretion Balance, and Tissue Distribution Study of [^{14}C]-AM-1 and [^{14}C]-LCFA in Sprague Dawley Rats (Bitzer et al., 2017a)

Group	Route	Test Substance	MW (g/mol)	Dose Concentration (mg/mL)	Target Dosage Level (mg/kg bw)*	Target Dosage Level (mmol/kg bw)	Dosage Volume (mL/kg bw)	Approximate Radioactivity (µCi/kg)**	Number of Animals	Sample Collection
Pharmacokinetic Phase										
1	Oral	AM-1	985	10	100	0.10	10	100	9M/9F	Blood
2	Oral	LCFA	455	4.6	46	0.10	10	100	9M/9F	
3	IV	AM-1	985	5	10	0.010	2	60	9M/9F	
4	IV	LCFA	455	2.3	4.6	0.010	2	24	9M/9F	
Single Dose – Excretion Balance Phase										
5	Oral	AM-1	985	10	100	0.10	10	100	3M/3F	Urine, Feces, Expired Air
6	Oral	LCFA	455	4.6	46	0.10	10	100	3M/3F	
Single Dose – QWBA Phase										
7	Oral	AM-1	985	10	100	0.10	10	100	7M/7F	Blood, Carcass for QWBA
8	Oral	LCFA	455	4.6	46	0.10	10	100	7M/7F	
Repeated Dose – Excretion Balance Phase										
9	Oral	AM-1	985	10	100	0.10	10	100	3M/3F	Urine, Feces, Expired Air
10	Oral	LCFA	455	4.6	46	0.10	10	100	3M/3F	
Repeated Dose - QWBA Phase										
11	Oral	AM-1	985	10	100	0.10	10	100	7M/7F	Blood, Carcass for QWBA
12	Oral	LCFA	455	4.6	46	0.10	10	100	7M/7F	

QWBA = quantitative whole body autoradiography

* Actual doses administered in mass balance phase (Groups 5, 6, 9, 10) ranged from 99% to 101% of target mg/kg dosage level.

** Actual doses administered in mass balance phase (Groups 5, 6, 9, 10) ranged from 76% to 97% of target µCi/kg radioactivity.

Details regarding the dose groups, number of animals, and samples collected in each phase of the study are presented in Table 5. For the repeated dose phases, rats received a single daily oral dose of either AM-1 or LCFA for 14 consecutive days followed by a single oral dose of [^{14}C]-AM-1 or [^{14}C]-LCFA. Following dosing in the pharmacokinetic phase, blood samples were collected from 3 animals/sex rotating among 3 sub-groups at select time points through 72 h post-dosing. Blood samples were collected from the jugular vein into tubes containing K_3EDTA as the anticoagulant and processed to plasma for analysis of total radioactivity by LSC.

In the excretion balance phases, expired air, activated carbon, urine, feces, cage rinse, cage wash, and select carcass samples were analyzed for total radioactivity by LSC. At approximately 1, 2, 4, 8, 24, 48, and 168 h post-dosing, 1 animal/sex/QWBA group were anesthetized with isoflurane and a whole blood sample of approximately 4 mL was collected via cardiac puncture into tubes containing K_3EDTA as the anticoagulant. Concentrations of radioactivity were calculated in selected tissues, as follows: adrenal gland; bone (femur); bone marrow (femur); brain; cecum; eye; fat; heart; kidney; kidney (cortex); kidney (medulla); large intestine; liver; lung; muscle (femoral); ovaries (females); pancreas; pituitary gland; prostate (males); skin; small intestine; spleen; stomach; thymus; thyroid gland; testes (males); urinary bladder; uterus (females).

Results

A detailed discussion of the study results, along with additional supporting data tables and figures, is provided in Bitzer et al. (2017a). Pharmacokinetic parameters for the [^{14}C]-AM-1 and [^{14}C]-LCFA oral dose groups are presented in Table 6. Circulating radioactivity at C_{max} was equal to <0.1% (AM-1 equivalents) and 0.2% (LCFA equivalents) of the administered dose, if the approximate amount of circulating plasma in a 250 g rat is assumed to be 7.8 g. The initial phase in the PK profile, whose primary character is determined by absorption, differed between AM-1 and LCFA equivalents and T_{max} was much earlier for LCFA equivalents (Figure 5). We interpret this observation as indicating that orally administered LCFA was directly and rapidly absorbed, whereas AM-1 underwent a number of hydrolysis steps before its components were absorbed; therefore, accounting for the time delay in T_{max} for AM-1. Also, based on the IV profiles, slow absorption of AM-1 followed by hydrolysis in circulation may be occurring. There was approximately a 2-fold difference in the dose-normalized AUC_{last} between AM-1 (approximately 1.3) and LCFA (approximately 2.3) equivalents, which is proportional to the higher molecular weight of AM-1. However, there was no apparent difference in the terminal elimination phase half-life between AM-1 and LCFA equivalents, which was determined to be approximately 30 h.

Pharmacokinetic parameters for the [^{14}C]-AM-1 and [^{14}C]-LCFA IV dose groups are presented in Table 7. Higher C_0 for AM-1 versus LCFA equivalents is tentatively attributed to a transient contribution from glucose, xylose, acetate, and isovalerate, following the hydrolysis of AM-1. Owing to the short half-life of these compounds, they would be rapidly removed from the circulating blood, whereas LCFA would not, resulting in the initially higher C_0 for AM-1 compared with LCFA, followed by similar plasma concentrations for both substances. There was approximately a 2-fold difference in the dose-normalized AUC_{inf} between AM-1 (approximately 11) and LCFA (approximately 22) equivalents, which mirrors the two-fold

difference in MW of these compounds. Based on the similarity of the PK analysis and subsequent tissue distribution and excretion balance behavior, the data is interpreted to mean that an equivalent concentration of LCFA (the ultimate hydrolysis product of AM-1) was present in systemic circulation following equimolar administration of AM-1 or LCFA, suggesting that AM-1 can be hydrolyzed to LCFA in circulation following an IV dose. Because the molecule is uniformly labeled, the calculations transforming administered radioactivity necessarily assume that test article equivalents result from AM-1 (average MW = 985) following administration of AM-1, when it is likely a two-fold concentration of AM-1 derived LCFA (average MW = 455) with similar total mass. Thus, as a function of moles of test article administered, the pharmacokinetic behavior of AM-1 and LCFA equivalents following IV administration were virtually indistinguishable (Figures 6 and 7). However, there was no apparent difference in the terminal elimination phase half-life between AM-1 and LCFA equivalents, which was determined to be approximately 37 h (overall average). The similarity between AM-1 and LCFA equivalents in the terminal elimination phase following IV administration also indicates that AM-1 is at least partially hydrolyzed in circulation to LCFA.

Table 6. Pharmacokinetics of Test Article Equivalents in Group 1 and Group 2 Rats following an Oral Dose of [^{14}C]-AM-1 at 100 mg/kg or [^{14}C]-LCFA at 46 mg/kg (Bitzer et al., 2017a)

	Group 1 (AM-1)		**Group 2 (LCFA)**	
	Males	Females	Males	Females
PK Parameter (Units)				
AUC_{last} (h*µg equiv./g)	119	140	112	101
AUC_{inf} (h*µg equiv./g)	147	NC	141	131
DN AUC_{last}	1.19	1.40	2.44	2.19
AUC_{inf} Extrap (%)	19.1	NA	20.1	23.2
C_{max} (µg equiv./g)	2.46	3.06	3.39	2.88
T_{max} (h)	8	24[a]	4	2
$T_{1/2}$ (h)	27	NA	30.3	32.6
Rsq	0.998	NA	0.992	0.986
F (%)	10.7	12.7	12.6	8.98

[a] In Group 1 females the 24 h time point demonstrated an unusually high standard deviation between the three samples. This inter-animal variability and differences between groups in a standard sampling design led to a slightly higher value at 24 h post-dosing versus the 8 h Tmax observed in Group 1 males.

NC = Not Calculated; NA = Not Applicable; AUC_{last} = area under the analyte concentration versus time curve from the time of dosing to the time of the last concentration >LLOQ; AUC_{inf} = estimate of the area under the concentration versus time curve from the time of dosing to infinity; DN = Dose normalized; AUC_{inf} Extrap = percentage of AUC_{inf} due to extrapolation from T_{last} infinity; C_{max} = maximum measured concentration of the analyte in matrix; T_{max} = sampling time at which C_{max} was reached; $T_{1/2}$ = half-life for the analyte in matrix; Rsq = R-squared (coefficient of determination); F = Bioavailability.

Table 7. Pharmacokinetics of Test Article Equivalents in Group 3 and Group 4 Rats following an IV Dose of [^{14}C]-AM-1 at 10 mg/kg or [^{14}C]-LCFA at 4.6 mg/kg (Bitzer et al., 2017a)

	Group 3 (AM-1)		**Group 4 (LCFA)**	
	Males	Females	Males	Females
PK Parameter (Units)				
AUC_{last} (h*µg equiv./g)	87.6	88.4	68.4	76.5
AUC_{inf} (h*µg equiv./g)	111	110	88.6	112
AUC_{inf} Extrap (%)	20.9	19.8	22.7	31.9
DN AUC_{inf}	11.1	11.0	19.3	24.4
C_0 (µg equiv./g)	89.1	109	8.26	8.48
Cl (g/min/kg)	1.51	1.51	0.866	0.682
Vss (g/kg)	3900	3770	2470	2540
$T_{1/2}$ (h)	32.8	31.1	35.3	47.9
Rsq	0.986	0.982	0.998	0.985

AUC_{last} = area under the analyte concentration versus time curve from the time of dosing to the time of the last concentration >LLOQ; AUC_{inf} = estimate of the area under the concentration versus time curve from the time of dosing to infinity; AUC_{inf} Extrap = percentage of AUC_{inf} due to extrapolation from T_{last} infinity; DN = Dose normalized; C_0 = estimated analyte concentration in matrix immediately following intravenous administration; Cl = apparent systemic clearance for the analyte in matrix; Vss = estimate of the volume of distribution for the analyte at steady state; $T_{1/2}$ = half-life for the analyte in matrix; Rsq = R-squared (coefficient of determination).

Figure 5. Mean Concentration of Test Substance Equivalents in Rat Plasma (µg/g) following Oral Administration of [^{14}C]-AM-1 or [^{14}C]-LCFA (Bitzer et al., 2017a)

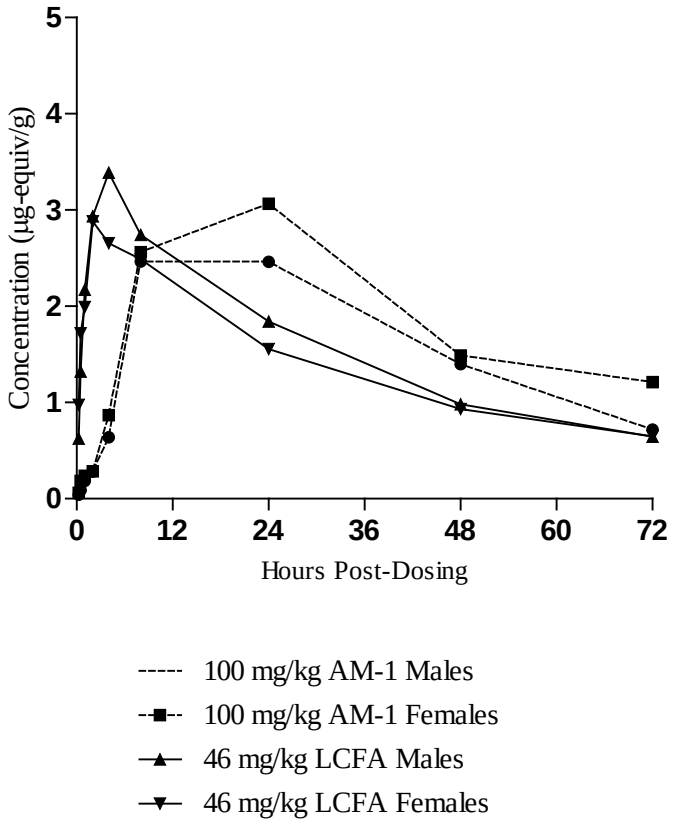


Figure 6. Mean Concentration of Test Substance Equivalents in Rat Plasma (µg/g) following IV Administration of [^{14}C]-AM-1 (Bitzer et al., 2017a)

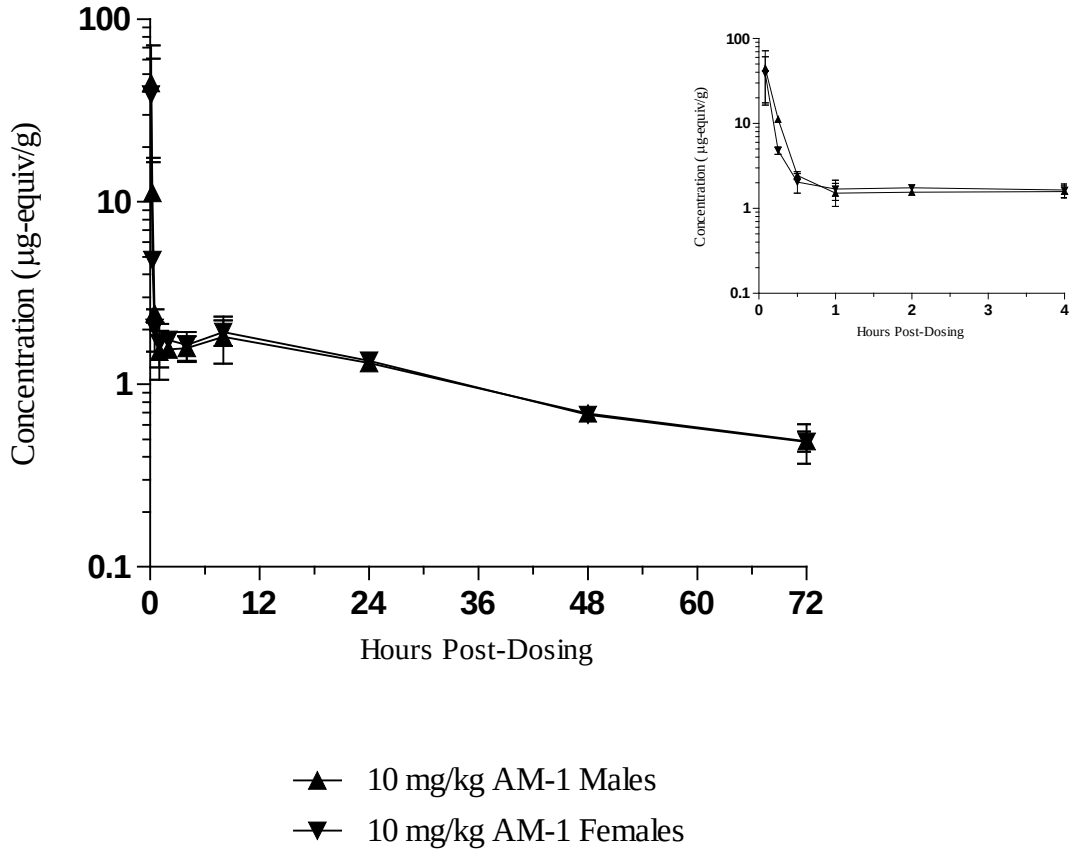
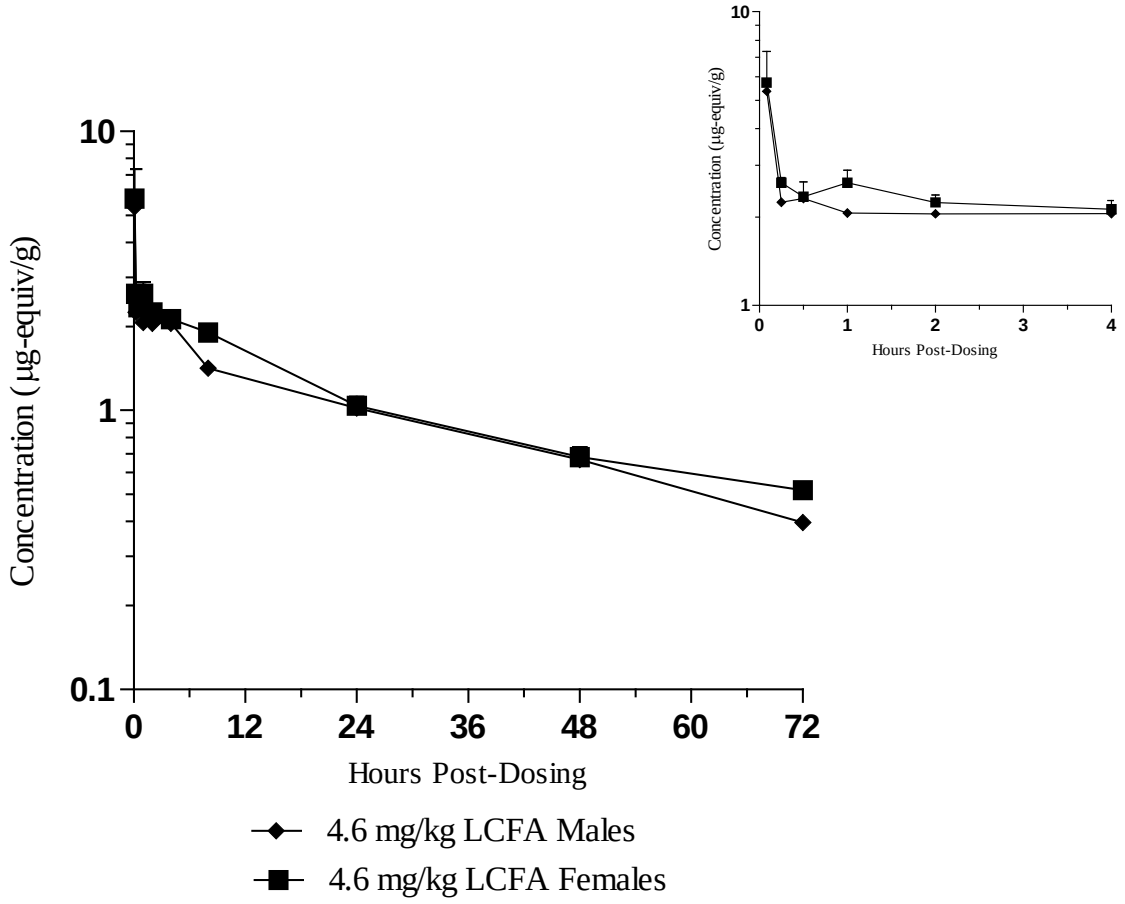


Figure 7. Mean Concentration of Test Substance Equivalents in Rat Plasma (µg/g) following IV Administration of [^{14}C]-LCFA (Bitzer et al., 2017a)



Based on the excretion data, approximately 88% to 101% of the administered dose was recovered in expired air, urine, feces, and carcass from male or female rats following single or repeated oral administration of [^{14}C]-AM-1 at 100 mg/kg or single or repeated oral administration of equimolar [^{14}C]-LCFA at 46 mg/kg (Bitzer et al., 2017a). There appeared to be no difference in the excretion of AM-1 or LCFA equivalents based on sex (males versus females) or single versus repeated exposures. Approximately 15% to 21% of the recovered radioactivity was captured in the CO_2 trap for expired air, approximately 1% to 3% of the recovered radioactivity was in the urine, and approximately 63% to 83% of the dose was eliminated in feces. Possible sources of CO_2 include intestinal metabolism of hydrolysis products by microbiota. It is likely that only a very small fraction of the AM-1 is hydrolyzed before elimination, because if a significant fraction were hydrolyzed, then a strong contribution of the metabolites (other than LCFA) on the PK and excretion balance would be seen, especially differences in the percent of dose in the urine. Approximately 2% of the recovered radioactivity was in the carcass of select animals. The majority of both test articles are eliminated in the feces without absorption.

The apparent oral bioavailability (F) of AM-1 and LCFA equivalents (approximately 11%; overall average for males and females combined) determined in the pharmacokinetic phase may reflect the absorption of degradation products of AM-1 and LCFA formed in the gut including CO_2 (which must be present systemically to be eliminated in the expired air), monosaccharides (from AM-1 only) and short chain fatty acids (SCFA). Quantitative analysis of the disposition of the radioactivity into the possible metabolic products formed prior to or after absorption is not practical due to the number of radiolabeled species and the number of absorption, distribution, and elimination processes occurring concurrently, but the existing data support the conclusion that oral bioavailability of both AM-1 and LCFA equivalents is very low with ≤0.2% of the administered dose present in the plasma at C_{max}, and less than 3.5% of the dose recovered in the urine (Bitzer et al., 2017a).

[^{14}C]-AM-1 or [^{14}C]-LCFA equivalents-derived radioactivity was detected by quantitative whole body autoradiography in all tissues measured (Bitzer et al., 2017a). C_{max} and AUC_{last} for [^{14}C]-AM-1- and [^{14}C]-LCFA-equivalents-derived radioactivity was highest in the tissues of the GI tract, as expected following oral administration. The eye, testes (males only), and brain had only very low concentrations at a small number of time points in select animals. The remaining tissues had concentrations of test article equivalents ranging from just above the lower limit of quantitation to <30,000 ng/g (excluding GI tract), which represented a low concentration relative to the administered dose, and no target tissues were identified. T_{max} was variable depending on the tissue, but generally correlated with the plasma or whole blood T_{max}, and was often earlier for LCFA than for AM-1. For the majority of the tissues, $T_{1/2}$ was considered not to be reportable or was an approximation; however, both AM-1 and LCFA concentrations were below the limit of quantification by 168 h following either single or repeated dosing suggesting tissue half-lives of approximately 24-40 h, which is consistent with the observed plasma $T_{1/2}$. Tissue:plasma ratios (calculated using AUC_{last}) indicated that test article equivalents in tissue were primarily associated with the perfusion of tissues by whole blood, and were <0.7 for poorly perfused tissues. In addition to the similarity in tissue distribution between AM-1 and LCFA, there was no notable impact of single versus repeated dosing on the tissue distribution of either test article. Variations in the concentrations of AM-1 equivalents in tissues of the GI tract are expected based on individual variability in intestinal motility depending on the

rat and when diet was ingested. The high concentrations in the various tissues of the GI tract, low concentration of circulating radioactivity, and low concentrations in the remaining tissues are consistent with low oral bioavailability, which suggests only a small portion of the administered AM-1 was absorbed into systemic circulation, as also evident in the autoradioluminograms.

Conclusion

In conclusion, AM-1 and LCFA and their GI tract metabolites are poorly absorbed by the oral route and are primarily eliminated in the feces without absorption. The pharmacokinetic, tissue distribution, and excretion balance data derived in this study (Bitzer et al., 2017a) are consistent with an interpretation that following ingestion, AM-1 is partially hydrolyzed to its components, glucose, xylose, acetate, isovalerate and LCFA. The expected small primary metabolites glucose, xylose, acetate, and isovalerate are expected to have a fast and high bioavailability but rapid clearance and thus to contribute marginally to the observed test article equivalents in blood and tissues after oral administration of AM-1. These results support an interpretation that systemic exposure to AM-1 or its metabolites would be very limited following oral ingestion. AM-1 is proposed for use as a food ingredient with antimicrobial / preservative properties without offering nutritional, taste or other technical benefits to foods or beverages. Therefore, limited absorption of AM-1 is a desirable property and serves to minimize exposure by the consumer to AM-1 or its major hydrolysis product, LCFA. In addition to limited exposure after a single dose there was no change to the pharmacokinetics, distribution, and elimination after repeated exposures, which is positive in regards to its application as a food ingredient where repeated ingestion is expected. Further, because rapid hydrolysis and elimination of jelly mushroom glycolipids occurs *in vivo*, the antimicrobial properties of the parent material would be absent or greatly diminished in the lower intestines so there is low concern for disruption of healthy gut microflora.

Toxicology Profile of Jelly Mushroom Glycolipids (AM-1)

Acute Toxicity, Irritation, and Sensitization Studies with Jelly Mushroom Glycolipids

As described in Bitzer et al. (2017b), AM-1 was demonstrated to be of low acute toxicity, via the oral and dermal routes of exposure, non-irritating to the skin and eyes, non-sensitizing.

Acute oral toxicity and LD_{50} with AM-1

The acute oral toxicity of AM-1 in female rats (WISTAR Crl: WI(Han)) was determined with the stepwise procedure (3 animals per step), according to OECD Testing Guideline No. 423 and EC 440/2008, Method B.l tris (OPPTS 870.1000, OPPTS 870.1100) (Schmid, 2012). The test item AM-1 was suspended in the vehicle (sterile water) at a concentration of 0.2 g/mL and administered via oral gavage at a dose volume of 10 mL/kg in order to achieve the desired dosage of 2,000 mg/kg bw. There were no deaths following a single oral gavage dose of AM-1 (2,000 mg/kg bw) or any signs of toxicity. The body weight gain of the test animals was within the normal range of variation for this strain (14-day observation period). The slight piloerection

observed on Day 1 in some animals, and resolved by Day 2, was not considered to be related to the test item but to the administration procedure and the possible stress induced. At necropsy, no treatment-related macroscopic findings were observed in any animal of any step.

Acute Dermal Toxicity and Irritation Studies

An *in vitro* acute skin irritation study was conducted to assess the skin irritation potential of AM-1 using 3D-tissue cultures of Human Reconstructed Epidermis (RHE 42.BIS-OECD Testing Guideline No. 439) (Ghioni, 2011a). AM-1 was dissolved in 100% dimethyl sulfoxide (DMSO) and subsequently applied as a 2% DMSO solution in saline (16 ± 0.5 µL) directly to the whole epithelium surface. DMSO (2%) in saline solution and sodium dodecyl sulfate (SDS) (5%) were used as negative and positive control articles, respectively. After an exposure period of 42min + 42h recovery, the test item did not significantly reduce the cell viability compared to the negative control. The positive control satisfied acceptance criteria as an irritant (≤50 %) whereas for the test item the mean tissue viability was >50% justifying classification as a non-irritant.

In vitro Skin Corrosion Test: Human Skin Model Test

The corrosive potential of AM-1 was assessed by means of the *in vitro* Human Skin Model Test with EpiDerm™ tissues models (OECD Testing Guideline No. 431) (Heppenheimer, 2013). Independent duplicate tissues of EpiDerm™ were exposed to the test item at 0.5% (w/w) solution or 5.0% (w/w) suspension, each in deionized water, the negative control (deionized water) or the positive control (8.0 N KOH) for 3 minutes and 1 hour, respectively. Afterwards, the test and the control items were rinsed off the tissues, and a 3 hour incubation period (37 ± 1°C, 5 ± 0.5 % CO_2) with MTT solution followed. Compared to the negative control, exposure to the positive control induced a decrease in the relative absorbance, both for the 3-minute exposure period (28.6%) and for the 1-hour exposure period (13.2%) thus confirming the validity of the test system and the specific batch of tissue models. After exposure to the test item formulations the relative absorbance value only decreased slightly to 98.5% and to 92.3% after a 3-minute exposure. After a 1-hour exposure the relative absorbance value was not reduced (110.8% and 110.6%). Since the threshold for corrosivity (50% after the 3 minutes exposure and 15% after the 1 hour exposure) was not affected, the test item was not considered to be corrosive.

Dermal Sensitization Study in Guinea Pigs (Buehler Method)

A dermal sensitization test was conducted with Hartley albino guinea pigs in 3 groups (preliminary irritation group 4; test group 20; naive control group 10) to determine the potential for AM-1 to produce sensitization after repeated topical applications (OECD Testing Guideline No. 406, Buehler Method) (Lowe, 2013). A 60% w/w mixture of the test substance in distilled water, the highest non-irritating concentration (HNIC) of AM-1, was selected for the induction application and challenge test. Very faint erythema (0.5) was noted at two of twenty test sites 24 hours after challenge and irritation cleared from the affected sites by 48 hours. Based on these findings and on the evaluation system used, AM-1 is not considered to be a contact sensitizer.

Dermal Sensitization Study; Human Repeat Insult Patch Test (HRIPT)

The contact sensitization potential of AM-1 was studied in an Institutional Review Board (IRB)-supervised repeat insult patch test in 50 human subjects (Tatsene, 2013). A series of nine consecutive 24 hour exposures was applied for three consecutive weeks followed by a challenge test applied once to a previously unexposed test site. The test material AM-1 was diluted to 0.5% in distilled water. A 0.2 mL volume of the test material was dispensed onto an occlusive, hypoallergenic patch applied directly to the skin of the right and left intrascapular regions of the back. No adverse reactions of any kind were noted during the course of this study. The test material (AM-1), when tested at a dilution of 0.5% in distilled water, is therefore considered a non-primary irritant and non-primary sensitizer to the skin.

Acute Eye Irritation Test

A study was conducted to assess the eye irritation potential of AM-1 using 3D-tissue cultures of Human Corneal Epithelium (the HCE model) (Ghioni, 2011b). The test item was dissolved in 100% DMSO and subsequently applied as a 2% DMSO solution in saline (30 µL) directly to the whole epithelium surface. DMSO (2%) in saline solution and ethanol (<10% cell viability) were used as the negative and positive control, respectively. The test was performed at the following exposure: 1h exposure + 16h recovery for test item and controls. As expected, the positive control severely reduced the cell viability under 50%. According to the prediction model, the test item did not significantly reduce the cell viability compared to the negative control. The test item was therefore not considered to be an eye irritant.

Phototoxicity Study

The phototoxicity potential of AM-1 was studied in Balb/c 3T3 cells maintained in culture for 24h for formation of monolayers (OECD Testing Guideline No. 432) (Ghioni, 2011c). Two 96-well plates per test item were pre-incubated with eight different concentrations of the test substance (up to 100 µg/mL) for 1 h. Each chemical/control was tested in 6 replicates. Thereafter one of the two plates was exposed to the highest non-cytotoxic irradiation UVA dose (5 J/cm^2) whereas the other plate was kept in the dark. Based on the validation study, a test substance with a Photo-Irritation-Factor (PIF; defined as EC50 (-UVA) / EC50 (+UVA)) < 2 or a Mean Photo Effect MPE; used in case PIF cannot be determined; calculated by comparison of the complete concentration response curves) < 0.1 predicts "no photo-toxicity", a PIF > 2 and < 5 or a MPE; > 0.1 and < 0.15 predicts "probable photo-toxicity", a PIF ≥ 5 or a MPE ≥ 0.15 predicts "photo-toxicity". The Mean Photo Effect (MPE) for the test item was calculated as 0.03; therefore, AM-1 is classified as not phototoxic.

<u>Assessment of Mutagenicity and Carcinogenicity Potential for Jelly Mushroom Glycolipids</u>

Based on the chemical structural characteristics of jelly mushroom glycolipids (AM-1), i.e. a glycolipid mixture with no reactive groups, there is low potential for carcinogenicity or mutagenicity from dietary intake of this material. In addition, as discussed above, the

metabolism profile for AM-1 is well understood (Bitzer et al., 2017a) and allows one to conclude that there are no carcinogenic or mutagenic metabolites of AM-1 formed *in vivo*.

As briefly described in Bitzer et al. (2017b), a series of three *in vitro* studies was performed to confirm the expectation that AM-1 is non-genotoxic and has low potential for carcinogenicity. The results of these studies corroborate the above conclusions based on the structure and metabolism profile for AM-1, which are generally available (Bitzer et al., 2017a). Additional details for the genotoxicity studies with AM-1 are summarized in the following paragraphs.

Genotoxicity Studies with Jelly Mushroom Glycolipids

A series of three studies was conducted on AM-1 to assess the genotoxic potential of this material. In the *in vitro* bacterial mutation assay (Ames test; OECD Testing Guideline No. 471) with AM-1, there was no evidence of mutagenic activity in *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 and TA 100, or in the *Escherichia coli* strain WP2 uvrA, in both the absence and the presence of metabolic activation (S9-mix), at any non-cytotoxic dose level. Three bacterial reverse mutation tests were performed. In the first and second test the test substance was dissolved at 50 mg/mL and in the third test at 200 mg/mL based on a purity of 99% (five concentrations were tested for all strains: 62, 185, 556, 1667 and 5000 µg/plate). Certain concentrations of the test material were toxic to strain TA 1537 at 185 µg/plate in ±S9 mix in the first test and to strain TA 100 at 5000 µg/plate in –S9 mix, in the second test as well to strain TA 1537 at 300 µg/plate in ±S9 mix. It was concluded that AM-1 is not mutagenic under the conditions employed in this study (van den Wijngaard, 2012).

In the *in vitro* human lymphocyte study (micronucleus test; OECD Testing Guideline No. 487), the test substance AM-1 was examined for its potential to induce micronuclei in cultured binucleated human lymphocytes, in both the absence and presence of a metabolic activation system (S9-mix) (Usta, 2012). The highest concentration of AM-1 tested was 5000 µg/mL. AM 1 did not show a statistically significant increase in the number of binucleated cells containing micronuclei when compared to concurrent control cultures, in the presence and absence of metabolic activation, at all time points and at any of the concentrations analyzed. From the results obtained in the *in vitro* micronucleus test it is concluded that, under the conditions used in this study, the test substance AM-1 is not clastogenic and/or aneugenic to cultured human lymphocytes (Usta, 2012).

AM-1 was tested for mutagenic potential in the *in vitro* mouse lymphoma thymidine kinase assay (MLA) according to OECD Testing Guideline No. 490 and EC No. 440/2008 (Verspeek-Rip, 2016). A maximum dose of 4000 µg/mL was selected as the upper dose limit for the preliminary toxicity test. A suspension of L5178Y mouse lymphoma cells was incubated with and without metabolic activation (±S9, prepared from rat livers) at AM-1 concentrations ranging from 52 – 4000 µg/mL. In the absence of S9-mix, AM-1 (50- 450 µg/mL exposure medium) did not induce a significant increase in the mutation frequency in the first experiment. This result was confirmed in a repeat experiment with modification in the duration of treatment with a 24-hour treatment period (150-500 µg/mL exposure medium). In the presence of S9-mix, AM-1 (100- 800 µg/mL exposure medium) did not induce a significant increase in the mutation

frequency. The numbers of small and large colonies in the AM-1 treated cultures were comparable to the numbers of small and large colonies of the solvent controls. In conclusion, AM-1 is not mutagenic in the TK mutation test system under the experimental conditions of the study (Verspeek-Rip, 2016).

Subchronic (Repeated Dose) Toxicity Studies with Jelly Mushroom Glycolipids

90-Day Oral (Drinking Water Administration) Toxicity Study in Rats

The subchronic toxicity of AM-1 was evaluated in a 90-day oral (drinking water administration) study in male and female CD® Crl:CD(SD) rats, approximately 5-6 weeks of age (Bitzer et al., 2017b). The study was performed in compliance with the Organisation for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice Regulations (OECD, 1998), which are compatible with the U.S. Food and Drug Administration (FDA) Good Laboratory Practice Regulations (21 CFR Part 58) (FDA, 1987). The study protocol was designed in general accordance with U.S. FDA Redbook Guideline IV.C.4.a. "Subchronic Toxicity Studies with Rodents" and the OECD Testing Guideline No. 408 "Repeated Dose 90-day Oral Toxicity Study in Rodents". Main study groups were comprised of 20 animals/sex/group. An additional 10 animals/sex for Groups 1 and 4 were designated as Recovery animals which were maintained on study for a 28-day observation period following cessation of test article administration.

The test article was diluted with the vehicle, drinking water (tap water), at target concentrations of 0.15%, 0.5%, and 1.5% (1.5, 5.0, and 15 mg/mL, respectively) and orally administered to the test animals *ad libitum* for 90 days. The control animals received fresh tap water on the same schedule/regimen as the treatment group animals. Dose levels were selected based on the results of a dose range-finding toxicity study in which no test article-related signs of systemic toxicity were noted in rats treated with dose levels of 0.1%, 0.5%, or 1.0% AM-1 in drinking water for 14 days.

All main study and recovery animals were randomly assigned to groups for neurological screening evaluation during pre-treatment and in study week 12. Recovery phase animals were also evaluated during study week 16. Body weights and food consumption were recorded weekly and water consumption was recorded daily. Blood samples for hematology, coagulation, and serum chemistry parameters were collected during study weeks 2 (Day 14), 6 (Day 42), and 13 (Day 91) for all main study and recovery animals, and during study week 17 (Day 119) for all recovery animals (Groups 1 and 4). Urine samples were collected over a 16-hour interval during study weeks 10 (Day 65) and 13 (Day 85) from all main study and recovery animals, and during study week 17 (Day 115) from all recovery animals. A standard listing of clinical chemistry and urinalysis parameters were analyzed in accordance with the applicable testing guidelines of OECD and FDA Redbook. On Day 91 (main study, one day after the last administration of the test article) or Day 119 (recovery phase, 28 days following cessation of test article administration), a complete gross necropsy was performed on all animals euthanized under CO_2 anesthesia and exsanguinated. The organs and tissues of all main study animals of the control and high-dose (1.5% AM-1) treatment groups and any prematurely deceased animals were

examined microscopically after preparation of paraffin sections and hematoxylin-eosin (H-E) staining. The stomach of all main study animals in the low- and mid-dose (0.15% and 0.5%) treatment groups as well as of all recovery animals was examined histologically due to observations in the stomach/forestomach of the main study animals in the high-dose (1.5% AM-1) group. Additional microscopic examinations were performed on the heart, liver, and one kidney (males and females using Oil Red O staining) and on one testis and one epididymis (males only using periodic acid-Schiff staining) from the main study animals in the control and high-dose (1.5% AM-1) treatment groups.

A detailed discussion of the study results, along with supporting data tables and figures, is provided in Bitzer et al. (2017b). There were no test article-related deaths or changes in behavior or external appearance of the study animals. The decreased drinking water consumption of male and female rats treated with 1.5% AM-1 via the drinking water, in particular during study weeks 1 and 2, and a few later intervals, was attributed to fairly high viscosity, surfactant qualities and/or a decreased palatability of the drinking water containing high concentrations of AM-1. Further, the food consumption of male and female rats treated with 1.5% AM-1 was decreased in study week 1. Hence, the body weight of the high-dose (1.5% AM-1) male animals was marginally below the body weight of the control animals from study week 1 throughout the treatment period (statistically significant during the first 6 weeks of the study only); however, bodyweights of females receiving 1.5% AM-1 were unaffected. It is well known in the scientific literature that under normal circumstances rats maintain a fairly constant ratio of food intake to water intake. Thus, reduced water intake is associated with reduced food intake and a reduction in body weight gain. This voluntary reduction in food intake and thus growth defends the constant ratio of body weight to lean body mass (Collier and Knarr, 1966; Crampton and Lloyd, 1954; Jackson and Smith, 1931). In contrast to the effects reported in this drinking water study, repeated oral administration of AM-1 via gelatin capsule administration up to 1000 mg/kg bw/day was well tolerated in Beagle dogs (Bitzer et al., 2017c; study summarized below) and only minimal differences in food consumption (not statistically significant) and cumulative body weight gain were noted in the high-dose (1000 mg AM-1/kg bw/day) females only. Therefore, the differences observed in the present drinking water study were considered to be an artifact of the route of dosing administration related to high viscosity, surfactancy, and reduced palatability, and not an indication of systemic toxicity of the test article.

The statistically significant differences noted for select clinical pathology parameters in the present 90-day rat study (Bitzer et al., 2017b; Tables 3 and 4) are also regarded as secondary stress-related effects due to decreased drinking water intake and the resulting slight dehydration of the animals and not due to a direct systemic toxic effect of the test article. Consistent patterns that would suggest biological significance were not observed for several statistically significant parameters, since the results were within the laboratory's historical control data ranges, were not present in a dose-related manner, and/or lacked correlation with histopathological findings.

No adverse test article-related effects were noted on hematological and serum chemistry parameters, urinalysis, eyes or optic region, or relative (to body weight at necropsy) and absolute organ weights at any dose levels at the end of the treatment period in the present study. Macroscopic inspection at necropsy did not reveal any test article-related changes in the organs or tissues of treated male and female rats. Microscopic examination of all scheduled organs and tissues of the high-dose (1.5% AM-1) group male and female animals, gross lesions of the high-

dose (1.5% AM-1) group animals, and the stomach of the low- and intermediate-dose (0.15% and 0.5%) rats did not reveal morphological changes that are considered to be related to the administration of the test article.

At the end of the 4-week recovery period (limited to the high-dose and control groups) mean body weights among the high-dose (1.5% AM-1) group males and females were within the range of the control group, supporting the conclusion that the slightly decreased body weights of the high-dose (1.5% AM-1) group male animals during the treatment period were associated with reduced intake of the treated drinking water. Additionally, clinical pathology parameters in the high-dose (1.5% AM-1) group animals were comparable to control group values at the end of the 4-week recovery period with the exception of select parameters in males (RBC, LUC, MCV, MCH, albumin, and Ca) or females (LUC and TPT), which appear to be spurious statistical variation and without biological relevance due to only slight differences from controls, some of which occurred due to control values that were at the high or low end of the historical control range.

In conclusion, minor variations in some parameters (i.e. body weight, select clinical chemistry) evaluated were considered incidental and secondary to reduced drinking water consumption / mild dehydration due to reduced palatability of the test article-treated drinking water. The no-observed-adverse-effect level (NOAEL) for systemic toxicity was considered to be 1.5% AM-1 in the drinking water, equivalent to 1201 and 1423 mg/kg bw/day for male and female rats, respectively. This NOAEL is corroborated by the results of a 90-day subchronic oral (capsule administration) toxicity study of AM-1 in Beagle dogs in which no adverse effects were observed at doses up to 1000 mg/kg bw/day (Bitzer et al., 2017c).

90-Day Oral (Capsule Administration) Study in Dogs

The subchronic toxicity of AM-1 was evaluated in a 90-day oral capsule study in male and female Beagle dogs, approximately 4-5 months of age (Bitzer *et al*., 2017c). The study was performed in compliance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR Part 58) (FDA, 1987). The study protocol was designed in general accordance with U.S. FDA Redbook Guideline IV.C.4.b. “Subchronic Toxicity Studies with Non-Rodents” and the OECD Testing Guideline No. 409 “Repeated Dose 90-day Oral Toxicity Study in Non-Rodents”. Study groups were comprised of 4 animals/sex/group.

The test article was administered by oral capsule at doses of 150, 500, or 1000 mg/kg bw/day for 90 days. The control animals received the same number of empty capsules equivalent to that used for the same sex in the high-dose group. Each animal received an approximate 5 to 15 mL flush of water using a graduated syringe following the daily administration of capsules.

The selected route of administration for this study was oral (capsule) because the oral route is the intended route of exposure. Although drinking water was selected as the vehicle for the previous subchronic oral toxicity in rats to better simulate the anticipated route of consumer exposure, and due to technical infeasibility of dose formulation with this viscous test material in

other vehicles (e.g. rodent diet), oral capsule administration was selected for the present study to mitigate the palatability issues observed in the rat drinking water administration study (Bitzer et al., 2017b). All animals were randomly assigned to groups for neurological screening evaluation during pre-treatment and in study weeks 11 and/or 12. Body weights were recorded weekly and food consumption was recorded daily. Blood and urine samples for clinical pathology evaluations (hematology, coagulation, serum chemistry, and urinalysis) were collected during acclimation (study week -1), during study weeks 2 and 6, and at the scheduled necropsy (study week 13). A standard listing of clinical chemistry and urinalysis parameters were analyzed in accordance with the applicable testing guidelines of OECD and FDA Redbook. A complete gross necropsy was performed on all animals. The organs and tissues of all animals of the control and high-dose treatment groups were examined microscopically after preparation of paraffin sections and hematoxylin-eosin (H-E) staining. Gross lesions were examined microscopically from all animals.

A detailed discussion of the study results, along with supporting data tables and figures, is provided in Bitzer et al. (2017c). There were no test article-related deaths or changes in behavior or external appearance of the study animals. Clinical observations attributed to the capsule administration method included emesis, clear or frothy (white) material around the mouth and salivation. Additionally, abnormal (soft or mucoid) feces and diarrhea were noted occasionally and occurred in similar incidence in the control groups; therefore, these observations were not considered test article-related. A minimal test article-related reduction in body weight compared to the control group (not statistically significant at any weekly interval) was observed in the high-dose (1000 mg AM-1/kg bw/day) treatment group females (approximately 6% by study week 12). Cumulative body weight gains in the 1000 mg/kg bw/day group females were statistically significantly lower than the control group from Study Weeks 0 to 1, 0 to 12 (approximately 27%), and 0 to 13 (approximately 30%). A minimal reduction in food consumption was also observed for most weeks of dosing for the 1000 mg/kg bw/day group females compared to the control group, although not statistically significant. One might speculate that AM-1, which has surfactant properties at high aqueous concentrations, may have made the dogs feel unwell for some time after dosing because of high concentrations in the stomach contents after release from administered capsules, resulting in slightly lessened appetite. There were no other test article-related effects on food consumption or body weights noted. Statistically significant changes were noted infrequently for some hematological, serum chemistry, or coagulation parameters; however, these differences were not considered test article-related because they were minimal and/or lacked a dose response, or values were similar to those measured during acclimation or were within the laboratories historical control ranges. Additionally, inconsistent statistically significant findings may also be related to random variation seen in studies with small group sizes. No test article-related effects were noted on the urinalysis parameters

The microscopic examination of all scheduled organs and tissues of the high-dose group male and female animals and gross lesions from all animals showed no signs of test article-related histologic alterations. Histologic changes were considered to be incidental findings or related to some aspect of experimental manipulation other than administration of the test article.

In conclusion, oral administration of AM-1 to sexually mature Beagle dogs at dose levels of 150, 500, and 1000 mg/kg bw/day for a minimum of 91 days was well tolerated at all dosages.

Test substance-related changes were limited to minimal effects on food consumption and body weights in the 1000 mg/kg bw/day group females. Therefore the no-observed-adverse-effect level (NOAEL) was considered to be 1000 mg/kg bw/day, the highest dosage level tested. This NOAEL is corroborated by the results of the 90-day oral (drinking water administration) toxicity study in rats (i.e. 15 mg/mL in drinking water, equivalent to1201 and 1423 mg AM-1/kg bw/day for male and female rats, respectively), in which observed differences in drinking water consumption, body weights, and clinical pathology parameters were considered an artifact of the route of dose administration and viscosity of the drinking water containing higher concentrations of AM-1 (Bitzer et al., 2017b).

Assessment of Reproductive and Developmental Toxicity Potential for Jelly Mushroom Glycolipids

Jelly mushroom glycolipids (AM-1) is poorly absorbed via the oral route (~11% oral bioavailability of AM-1 equivalents) and there was no accumulation of AM-1 in the reproductive organs of rats given single or repeated oral doses via gavage (Bitzer et al., 2017a). In addition, as discussed above, repeated oral administration with AM-1 had no effect on reproductive organ weights of rats or dogs, and there were no test-article related macroscopic or microscopic findings in reproductive organs (Bitzer et al., 2017b/c). Therefore, it can be concluded that AM-1 has low potential for reproductive and developmental toxicity.

As discussed in detail below, developmental and reproductive toxicity study studies were performed to confirm the expectation that AM-1 is not a reproductive or developmental toxicant (Herberth, 2017a,b). The results of these studies corroborate the above conclusions based on the ADME profile for AM-1, as well as results of systemic toxicity studies in the rat and dog, which are generally available (Bitzer et al., 2017a/b/c).

Rat Developmental Toxicity Study with Jelly Mushroom Glycolipids

AM-1 was evaluated in an oral gavage developmental toxicity study in female Crl:CD(SD) rats approximately 11-14 weeks of age (Charles River Laboratories Ashland Study No. WIL-294507; Herberth, 2017a). The study was performed in compliance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR Part 58) (FDA, 1987). The study protocol was designed in general accordance with U.S. FDA Redbook Guideline IV.C.9.b. “Guidelines for Developmental Toxicity Studies” and the OECD Testing Guideline No. 414 “Prenatal Developmental Toxicity Study”. Study groups were comprised of 24 successfully mated female rats/group.

The test article (identified as IMD AM-1; supplied as a beige powder) was diluted with the vehicle (reverse osmosis-treated water) to prepare dose formulation concentrations of 15, 50, and 100 mg/mL (adjusted based on the glycolipid content of 95%). Doses were administered at a dose volume of 10 mL/kg by oral gavage to female rats from Gestation Days 6-19 resulting in final dose levels of 150, 500, and 1000 mg/kg bw/day. Control animals received vehicle only on the same schedule/regimen as the treatment group animals. The test article formulations were confirmed to be stable under the conditions of storage during the study and met the laboratory-defined acceptance criteria for concentration and homogeneity of suspensions. The test article

was not detected in the analyzed vehicle formulation that was administered to the control group. The selected route of administration for the definitive developmental toxicity study was oral (gavage) because this is a potential route of exposure for humans and the typical route employed for this study design.

Individual clinical observations were recorded daily during Gestation Days 0–20 (prior to dose administration during the treatment period). Animals were also observed for signs of toxicity 1–2 hours following dose administration. Body weights and food consumption were recorded on Gestations Days 0 and 6-20 (daily). All rats were euthanized on Gestation Day 20 by carbon dioxide inhalation and a laparohysterectomy and macroscopic examination was conducted according to the above-referenced testing guidelines and standard laboratory procedures for developmental and reproductive toxicity studies. Fetuses were examined for external, visceral, and skeletal malformations and developmental variations (Stuckhardt and Poppe, 1984; Woo and Hoar, 1972; Wilson, 1965).

All females in the 150, 500, and 1000 mg/kg bw/day groups survived to the scheduled necropsy on Gestation Day 20. Rales were noted in a dose-dependent manner 1–2 hours after dose administration in the 150, 500, and 1000 mg/kg bw/day groups sporadically throughout the treatment period (Gestation Days 6–19). Clear and/or red material around the nose and/or mouth were noted in the 500 and 1000 mg/kg bw/day groups 1–2 hours after dose administration sporadically during Gestation Days 7–16. The rales and clear and/or red material findings were considered test article-related but not adverse because they generally did not persist to the daily examination the following day and there were no corresponding signs of systemic toxicity at any dosage level. Thus, these observations were likely an artifact of the oral gavage route of administration. There were no test article-related effects on body weights, body weight gains, gravid uterine weights, net body weights (the Gestation Day 20 body weight minus gravid uterine weight), or net body weight gains (the Gestation Day 0–20 body weight change exclusive of the gravid uterine weight) in the 150, 500, and 1000 mg/kg bw/day groups or food consumption in the 150 mg/kg bw/day group. Lower mean food consumption was noted during Gestation Days 6–9 and 15–20 for the 1000 mg/kg bw/day group and Gestation Days 15–20 for the 500 mg/kg bw/day group compared to the control group. These changes at 500 and 1000 mg/kg bw/day were considered test article-related but not adverse because the effects were transient and not of sufficient magnitude to impact mean absolute body weights. At the scheduled necropsy on Gestation Day 20, no test article-related macroscopic findings were noted for females at any dosage level. Intrauterine growth and survival and fetal morphology in all test article-treated groups were unaffected by maternal test article administration.

In conclusion, given the lack of any adverse test article-related effects on survival, clinical observations, body weights or food consumption, necropsy, intrauterine growth and survival, or fetal morphology, a dosage level of 1000 mg/kg bw/day (the highest dosage level evaluated) was considered to be the no-observed-adverse-effect level (NOAEL) for maternal toxicity and embryo/fetal developmental toxicity when AM-1 was administered orally by gavage to bred female Crl:CD(SD) rats (Herberth, 2017a).

Rat Two-Generation Reproduction Toxicity Study with Jelly Mushroom Glycolipids

AM-1 was evaluated in an oral gavage two-generation reproductive toxicity study in male and female Crl:CD(SD) rats (Charles River Laboratories Ashland Study No. WIL-294508; Herberth, 2017b). The study was performed in compliance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR Part 58) (FDA, 1987). The study protocol was designed in general accordance with U.S. FDA Redbook Guideline IV.C.9.a. "Guidelines for Reproduction Studies" and the OECD Testing Guideline No. 416 "Two-Generation Reproduction Toxicity Study".

Methods (Herberth, 2017a)

The experimental design for this study consisted of 3 test article-treated groups and 1 control group, composed of 25 rats/sex/group. The selected animals were approximately 6 weeks old at the initiation of test article administration. During cohabitation, the rats were paired (1 female to 1 male). Following positive evidence of mating, or at the end of the 14-day mating period, the F_0 and F_1 females were individually housed until weaning on Lactation Day 21. The weaned F_1 pups selected as parents for the next generation were housed 2–3 per cage by sex until pairing. At all times, animals were housed in appropriately sized solid-bottom cages with bedding material.

The test article (identified as IMD AM-1; supplied as a beige powder) was diluted with the vehicle (reverse osmosis-treated water) to prepare dose formulation concentrations of 15, 50, and 100 mg/mL (adjusted based on the glycolipid content of 95%). Doses were administered at a dose volume of 10 mL/kg by oral gavage resulting in final dose levels of 150, 500, and 1000 mg/kg bw/day. The test article formulations were confirmed to be stable under the conditions of storage during the study and met the laboratory-defined acceptance criteria for concentration and homogeneity of suspensions. The test article was not detected in the analyzed vehicle formulation that was administered to the control group.

F_0 and F_1 males and females were dosed once daily for a minimum of 70 consecutive days prior to mating. Dose administration for the F_0 and F_1 males continued throughout mating and through the day prior to euthanasia. The F_0 and F_1 females continued to be dosed throughout mating, gestation, and lactation, through the day prior to euthanasia. F_0 males and females were dosed for 128–133 consecutive days and F_1 males and females were directly dosed for 138–148 consecutive days. The offspring of the F_0 and F_1 generations (F_1 and F_2 litters, respectively) were potentially exposed to the test article in utero, as well as via the milk while nursing. The F_1 pups selected for mating (25 sex/group) were directly administered the test article following weaning (beginning on PND 21) and dosing continued following a similar regimen as the F_0 parental generation. Control animals received vehicle only on the same schedule/regimen as the test article treatment group animals. The selected route of administration for the definitive study was oral (gavage) because this is a potential route of human exposure.

Individual F_0 and F_1 male body weights were recorded weekly throughout the study and prior to the scheduled necropsy. Individual F_0 and F_1 female body weights were recorded weekly until evidence of copulation was observed. Once evidence of mating was observed, female body weights were recorded on Gestation Days 0, 4, 7, 11, 14, 17, and 20 and on Lactation Days 0 (when possible), 1, 4, 7, 11, 14, 17, and 21. F_0 and F_1 male and female food

consumption was measured weekly except during cohabitation. Following the breeding period, individual food consumption for males and for females with no evidence of mating was measured on a weekly basis until the scheduled necropsy. For females with evidence of a positive mating, food consumption was measured during gestation and lactation at the same intervals as body weight measurements. Vaginal lavages were performed daily and evaluated microscopically to determine the stage of the estrous cycle of each F_0 and F_1 female for 21 days prior to cohabitation and continuing until evidence of mating was observed or until the end of the mating period. The average estrous cycle length was determined for each animal. Vaginal lavages were also performed on the day of necropsy to determine the stage of the estrous cycle. The F_0 and F_1 animals were paired on a 1 male to 1 female basis within each treatment group after a minimum of 70 days of treatment. Positive evidence of mating was confirmed and was defined as Gestation Day 0. All females were allowed to deliver naturally and the day of parturition was designated postnatal day (PND) 0. Beginning on PND 0, pups were sexed and examined for gross malformations, and the numbers of stillborn and live pups were recorded. Individual gestation length and mating, fertility, copulation, and conception indices were calculated.

Intact offspring that were found dead or euthanized in extremis (by an intraperitoneal injection of sodium pentobarbital) from PND 0 to 4 were necropsied using a fresh dissection technique (Stuckhardt and Poppe, 1984). A detailed gross necropsy was performed on any pup found dead or euthanized in extremis (by an intraperitoneal injection of sodium pentobarbital) after PND 4 and prior to weaning. To reduce variability among the litters, 8 pups/litter, 4 pups/sex when possible, were randomly selected on PND 4. Each pup received a clinical observation on PND 1, 4, 7, 14, and 21 and individual sex determination was made on PND 0, 4, 14, and 21. Individual pup body weights were obtained on PND 1, 4, 7, 14, 17, and 21. A minimum of 1 male and 1 female F_1 pup/litter from each treatment group were randomly selected prior to weaning (PND 21) to comprise the F_1 generation (25 rats/sex/group). To assess the maturation of the selected F_1 pups, the following developmental landmarks were evaluated. Each F_1 male pup was observed for balanopreputial separation beginning on PND 35 (Korenbrot et al., 1977) and each F_1 female pup was observed for vaginal perforation beginning on PND 25 (Adams et al., 1985). Body weights were recorded at the age of attainment for these landmarks.

Following the completion of weaning of the F_1 and F_2 offspring, all surviving F_0 and F_1 adults received a detailed physical examination, females received a vaginal lavage to determine state of estrous and all were euthanized. Spermatogenic endpoints (sperm motility [including progressive motility], morphology, and numbers) were recorded for all F_0 and F_1 males, and ovarian primordial follicle counts were recorded for all F_1 females in the control and high dose groups and for all F_1 females suspected of reduced fertility. For females that delivered or had macroscopic evidence of implantation, the numbers of former implantation sites were recorded. The number of unaccounted-for sites was calculated and the numbers of corpora lutea and former implantation sites were also recorded for females necropsied during gestation through Lactation Day 4. The testing guideline-required list of tissues and organs were weighed and/or retained from all parental F_0 and F_1 rats. Microscopic evaluations were performed on select tissues for all parental F_0 and F_1 animals in the control and high-dose groups and for all animals found dead. Reproductive organs of the F_0 and F_1 adult rats suspected of reduced fertility were examined microscopically.

Results (Herberth, 2017a)

There were no test article-related effects on F_0 or F_1 parental survival at any dosage level. A few non-test article-related deaths occurred in the F_0 and F_1 generations which were considered the likely result of the intubation error based on macroscopic findings noted at necropsy (including dark red discoloration and/or areas of the lungs, lungs that were not fully collapsed, and/or foamy tracheal contents) and microscopic finding of inflammation, hemorrhage, and/or edema of the lungs in multiple animals that may have been related to inadvertent pulmonary aspiration of the test article during or following gavage.

Test article-related increased incidences of rales and red and/or clear material around the nose and/or mouth were noted in the 150, 500, and 1000 mg/kg/day group F_0 and F_1 males and females compared to the control group occasionally at the detailed physical examinations or daily examinations, and more frequently at 1-2 hours following dose administration. These findings were generally noted in a dose-related manner throughout the treatment period for both generations. Red and/or clear material findings around the nose and/or mouth are common following oral gavage administration and although dose-responsive, these observations only occasionally persisted to the detailed physical examinations or daily examinations, and therefore were considered non-adverse in the absence of other signs of systemic toxicity. The increased occurrence of rales 1-2 hours following dose administration was attributed to the surfactant properties of the test article combined with oral gavage dosing and was not considered adverse. Similar observations of respiratory distress related to aspiration of an irritant dosing material, especially following treatment with the more concentrated/viscous suspensions at higher doses, were reported in rat and rabbit developmental toxicity studies conducted with ethyl lauroyl arginate (i.e. LAE) via gavage (EFSA, 2007).

No test article-related effects on mean body weights and body weight gains (including body weights during gestation and lactation) were noted in the 150, 500, and 1000 mg/kg/day group F_0 males and females or the F1 females. Test article-related lower mean body weight gains were noted in the 150, 500, and 1000 mg/kg/day group F_1 males during PND 21–28, when pups were just weaned and first receiving oral gavage doses of the test article. Mean body weight gains in these groups were generally similar to the control group throughout the remainder of the study. As a result of the initial lower mean body weight gains during PND 21 28, mean body weights for F_1 males were between 4.0% and 7.1% lower than the control group throughout the study. These differences were not considered adverse because the mean body weights in these groups at termination (PND 161) were only 5.4% to 6.1% lower than the control group, demonstrating that the initial effects were ameliorated over the course of the generation. Mean food consumption evaluated as g/animal/day, and food efficiency in the 150, 500, and 1000 mg/kg/day groups for the F_0 and F_1 males and females was unaffected by test article administration throughout the study (including gestation and lactation).

No test article-related effects on F_0 and F_1 reproductive performance, including mating and fertility, male copulation and female conception indices, estrous cycle lengths, pre-coital intervals, gestation length, the process of parturition, and spermatogenesis parameters (motility, progressive motility, testicular and epididymal sperm concentration, sperm production rate, and the percentage of morphologically normal sperm), were observed at any dosage level. In addition, there were no test article-related macroscopic or microscopic findings or effects on

organ weights noted for F_0 and F_1 males and females at any dosage level, or on the F_1 female primordial follicle counts.

The mean number of pups born, live litter size, percentage of males per litter at birth, and postnatal survival through PND 21 were unaffected by administration of the test article to the F_0 and F_1 parental animals at all dosage levels. The F_1 and F_2 pups did not display any effects of test article exposure as evaluated by their general physical condition, body weights, necropsy findings, or organ weights.

Conclusion (Herberth, 2017a)

Based on the lack of effects on F_0 and F_1 reproductive performance (mating, fertility, copulation and conception indices, estrous cyclicity, and spermatogenic endpoints), the no-observed-adverse-effect level (NOAEL) for parental reproductive toxicity of AM-1 when administration orally via gavage to Crl:CD(SD) rats was 1000 mg/kg/day. There were no adverse effects on survival, clinical observations, body weight or food consumption parameters, macroscopic or microscopic findings, or organ weights for F_0 or F_1 males and females at any dosage level. Based on these results, a dosage level of 1000 mg/kg/day was considered to be the NOAEL for F_0 and F_1 parental systemic toxicity. There were no test article-related effects on F_1 and F_2 postnatal survival, development, or growth during the pre-weaning period, and therefore, the NOAEL for neonatal toxicity was considered to be 1000 mg/kg/day (Herberth, 2017a).

Safety Assessment of Jelly Mushroom Glycolipids Minor Component

As described in Appendix 1-A, the minor presence of glycolipids containing 3-hydroxy-3-methylglutarate (HMG) as an acyl unit has been noted in certain batches of jelly mushroom glycolipids (AM-1), including those batches of AM-1 tested in the 90-day rat and dog toxicity studies, rat ADME study, and rat developmental and reproductive toxicity studies (Bitzer et al., 2017a,b,c; Herberth 2017a,b). Although the existing toxicology database for AM-1 supports the safety of HMG as a potential hydrolysis product of a minor component of AM-1 (< 1% calculated average concentration), a formal safety assessment of HMG is provided below for completeness.

Background and Safety Database for HMG

3-Hydroxy-3-methylglutarate (HMG; 3-hydroxy-3-methylglutaric acid; meglutol; CAS RN 503-49-1) is a dicarboxylic acid that is an analogue of glutaric acid in which the two hydrogen atoms at position 3 are substituted by a hydroxyl and a methyl group, respectively. HMG is expected to be completely metabolized in the fatty acid pathway and tricarboxylic cycle as other dicarboxylic acids. HMG is a component of 3-hydroxy-3-methylglutaryl-coenzyme A, i.e. HMG-CoA, which is an intermediate in primary metabolism; in particular, in the mevalonate and ketogenesis pathways as well as leucine biosynthesis (see Appendix 1-A, Occurrences). Thus, HMG is considered a naturally occurring constituent of human physiology and according to guidance in the FDA Redbook (1993) Concern Level paradigm; it would be classified as Structure Category A, i.e. chemical structures of lowest concern.

The distribution of radioactivity after oral administration of ^{14}C-HMG was studied by whole-body autoradiography in male Swiss albino mice (Savoie and Lupien, 1975a). The ^{14}C-HMG test compound was promptly and almost completely absorbed. Radioactivity was seen in organs responsible for cholesterol synthesis, i.e. mainly the liver and intestinal wall, with rapid and pronounced uptake of radioactivity also seen in the kidney. The authors concluded that the kidney is not only the main excretory route of HMG but also represents an important site of distribution for the compound (Savoie and Lupien, 1975a).

Toxicological investigations of HMG have been performed in rats and mice (Savoie and Lupien, 1975b). Pregnant female Long Evans rats were administered daily intraperitoneal (i.p.) or oral gavage doses of HMG at 75, 250, and 500 mg/kg bw/day on Gestation Days (GD) 7-11. Pregnant female Swiss mice were administered i.p. (1600 mg/kg bw/day) or oral gavage (3700 mg/kg bw/day) doses of HMG on GD 8 and 10. Euthanasia and laparotomy was performed on approximately half of the pregnant rats on GD 21 while the remaining rats were allowed to deliver their offspring. All mice were sacrificed on GD 20 for performance of laparotomy. There were no effects of HMG treatment on reproductive performance of the dams, and no gross malformations of the fetuses or offspring were observed. The numbers of resorptions, percentage of live offspring after one month (evaluated in rats only), and fetal weights (reported for mice only) were not significantly different than controls (Savoie and Lupien, 1975b).

HMG was evaluated in 36 patients for its effect on plasma lipids in a double-blind human clinical trial (Lupien et al., 1979). Treatment groups were placebo, 0.750 g, 1.5 g, 2.25 g, and 3 g/day. There were no clinical or biological adverse effects due to the administration of HMG for 8 weeks. Hematologic, hepatic, renal, and metabolic function tests were clinically unaltered (Lupien et al., 1979).

Since HMG is an analogue of glutaric acid, glutaric acid provides additional information supporting the safety evaluation of HMG. When teratology studies were performed with glutaric acid in rats at 125, 400 and 1300 mg/kg and in rabbits at 40, 160 and 500 mg/kg, no adverse effects on pregnancy and no embryotoxic or teratogenic effects were observed (Bradford et al., 1984). Glutaric acid was tested *in vitro* in the Umu assay (Sakagami et al., 1989) and in a rat *in vivo* bone marrow chromosomal aberrations assay (San Sebastian, 1989) at oral gavage doses as high as 2750 mg/kg with negative results in both instances.

Finally, no adverse test-article related effects were observed in the toxicology and ADME studies (Bitzer et al., 2017a,b,c; Herberth 2017a,b) performed using AM-1 test articles containing HMG at theoretical levels of 0.88% and 1.50% (i.e. calculated maximum theoretical amounts after complete hydrolysis based on measured amounts of HMG-containing glycolipids).

Potential Consumer Exposure

Assuming the unlikely event of complete hydrolysis, the mean theoretically possible concentration of HMG from three representative batches of AM-1 may be calculated as 0.96% (Appendix 1-A). Since glycolipids constitute approximately 93% of the AM-1 product, and based on the conservative, upper estimates of consumer intake for AM-1 (see Part 3. Dietary

Exposure), potential dietary intake of HMG through consumption of formulated beverages containing AM-1 is calculated as follows:

Total U.S. population: 1.09 mg AM-1/kg bw/day (90^{th} percentile per user intake estimate for all proposed uses, maximum use levels) x 93% glycolipids x 0.96% HMG = **9.7 µg** HMG/kg bw/day

Children 1-6 years: 3.29 mg AM-1/kg bw/day (90^{th} percentile per user intake estimate for all proposed uses, maximum use levels) x 93% glycolipids x 0.96% HMG = **29.4 µg** HMG/kg bw/day.

However, excretion balance data from the rat ADME study indicates that AM-1 [^{14}C] equivalents in urine and exhaled CO_2 are less than about 20% of administered dose, i.e. following single oral administration approximately 16-20% of recovered radioactivity was captured as expired CO_2 and approximately 1% was in urine (Bitzer et al., 2017a). Therefore, potential exposure to this minor component is expected to be significantly lower than the calculation above, and more realistic, but still conservative estimates of consumer exposure may be derived as follows:

Total U.S. population: 1.09 mg AM-1/kg bw/day (90^{th} percentile per user intake estimate for all proposed uses, maximum use levels) x 93% glycolipids x 0.96% HMG x 20% administered dose = **1.9 µg** HMG/kg bw/day

Children 1-6 years: 3.29 mg AM-1/kg bw/day (90^{th} percentile per user intake estimate for all proposed uses, maximum use levels) x 93% glycolipids x 0.96% HMG x 20% administered dose = **5.8 µg** HMG/kg bw/day.

Even the most conservative assumptions and calculated intake values for HMG are below 50 µg/kg bw/day. Whereas more realistic but still conservative assumptions result in calculated intake values for the Total U.S. population and Children 1-6 years (1.9 and 5.8 µg HMG/kg bw/day) that bracket the threshold of 2.5 µg/kg bw/day for a Structure Category A chemical to be considered Concern Level I, i.e. the lowest concern in the FDA Redbook paradigm (FDA, 2000).

Conclusion

In conclusion,

- HMG may be present as an acyl group on a minor portion of the AM-1 glycolipids
- As an acyl group, HMG theoretically may be freed via hydrolysis following intake of AM-1 in the diet, with an average calculated theoretical concentration of < 1% in representative AM-1 batches
- HMG is a naturally occurring constituent of human physiology and diet as a component of HMG-CoA
- The existing toxicology database for AM-1 supports the safety of HMG as a potential hydrolysis product of a minor component of AM-1, since it was contained in test samples used in those studies (up to 1.5% calculated theoretical concentration)

- The results of an organ distribution study, reproduction and developmental toxicity studies in two species, and a human clinical study with HMG, as well as supporting teratology and genetic toxicity study data on glutaric acid (a structural analogue of HMG) support the safety of HMG as a potential minor component of AM-1

- Dietary intake calculations using realistic, but still conservative assumptions result in potential exposures to HMG from use of AM-1 in non-alcoholic beverages as being approximately the same as the FDA Redbook (1993) exposure threshold for Concern Level I (lowest concern)

- Considering all of these factors, the presence of HMG as a potential hydrolysis product of a minor component of AM-1 is not a safety concern

Summary and Discussion of ADME and Toxicology Database for Jelly Mushroom Glycolipids

A battery of *in vitro* genetic toxicity studies with AM-1 were all negative, confirming the expectation that AM-1, a glycolipid mixture with no structural alerts for genotoxicity and no genotoxic metabolites, has low potential for mutagenicity or carcinogenicity. The ADME study demonstrated that jelly mushroom glycolipids (AM-1) are poorly absorbed following oral intake, and the AM-1 which is absorbed appears to be rapidly hydrolyzed to non-toxic compounds that are easily excreted. AM-1 hydrolysis products glucose, xylose, acetate, isovalerate and long-chain fatty acids are naturally occurring in the human diet, and if absorbed they may be ultimately metabolized to CO_2. Subchronic toxicity, developmental toxicity, and multi-generation reproduction toxicity studies established high NOAELs with only minor non-adverse effects observed that were related to the surfactant / unpalatable properties of AM-1 and/or an artifact of the oral gavage route of administration. Based on the well-defined ADME and PK profile of AM-1 in rodents (Bitzer et al., 2017a), as well as the results of oral subchronic toxicity studies with AM-1 in rats and dogs (Bitzer et al., 2017b,c), it can also be concluded that chronic toxicity and carcinogenicity are not endpoints of safety concern for AM-1.

Allergenicity

Glycolipids are not identified as food allergens or mediators of food allergy in comprehensive scientific opinions by authoritative bodies (e.g. NIAID, 2011; EFSA, 2014). A food allergy occurs when an allergen within food (i.e. usually a specific protein in a food) sets off a chain of reproducible reactions involving the immune system (NIAID, 2011; Sicherer and Sampson, 2014). As such, safety assessment of biotechnology products for potential risk of food allergy follows a weight-of-evidence approach, involving consideration of the source of the introduced protein and comparison to know allergens (Selgrade et al., 2009). Jelly mushroom glycolipids (AM-1) may contain up to 3% total protein content (calculated based on the nitrogen content, measured using the Kjehldahl method, multiplied by the generally accepted factor of 6.25) which is associated with non-viable cell debris originating from the production organism, *Dacryopinax spathularia* (refer to *Safety Assessment of Jelly Mushroom Glycolipids Production*

Organism presented above). Therefore, AM-1 does not contain any other introduced protein that would be expected to result in an allergic reaction.

While exposure to red mammalian meat containing the oligosaccharide galactose-α-1,3-galactose (α-Gal) expressed in glycoproteins or glycolipids has caused allergic reactions among individuals who have become sensitized to α-Gal after tick bites (Wolver et al., 2013; Steinke, 2015), α-Gal or similar carbohydrates are not present in the AM-1 glycolipid mixture.

The jelly mushroom glycolipids (AM-1) product does not contain any of the major food allergens identified by the U.S. Food Allergen and Consumer Protection Act (FALCPA) of 2004 [i.e., eggs, fish (e.g., bass, flounder, cod), Crustacean shellfish (e.g., crab, lobster, shrimp), tree nuts (e.g., almonds, walnuts, pecans), peanuts, wheat, or soybeans]. The presence of other potential food allergens as identified in the European Regulation (EC) 1169/2011 and the Chinese Regulation GB23779-2009 is also excluded [i.e., celery, lupine, milk and lactose, mollusks, mustard, sesame seed, sulfur dioxide or sulfite]. Furthermore, the equipment used to manufacture jelly mushroom glycolipids (AM-1) is not used to process any of the above-listed known food allergens.

In addition, to the best of our knowledge, food types listed as major food allergens do not contain glycolipids similar to AM-1. Although the presence of glycolipid type compounds in some of these foods is reported, e.g. for milk, eggs, and soybeans (Newberg and Chaturvedi, 1992; Fujino et al., 1970, Jyonouchi et al., 2011; Leray, 2015), the particular glycolipid molecules described, such as sphingomyelin, belong to the class of sphingolipids, which are more complex than AM-1 (i.e. a trisaccharide with long-chain fatty acid backbone; see Figure 1), having a polar head oligosaccharide group and two non-polar tail groups consisting of a long-chain fatty acid and a long-chain amino alcohol. Thus, these molecules are very different in their chemical structures and molecular properties from AM-1. The ability for milk-derived sphingomyelin to engage invariant natural killer T cell (iNKT) T cell receptor (TCR) has been reported, although the role of iNKTs in food allergy is still not fully understood (Jyonouchi et al., 2011). In addition, a single investigation regarding the potential for peanut glycolipid antigens to activate immune mechanisms related to food allergy was identified in the NIH Research Portfolio Online Reporting Tool (RePORT) (Umetsu, 2010); however, the results of this research have not been reported and the peanut "lipid and lipid-carbohydrate complexes" described in the grant Abstract are not considered to be similar to AM-1 glycolipids.

Finally, AM-1 is not well absorbed via the oral route (Bitzer et al., 2017a), and there were no test article-related effects on any hematological parameters related to immune system responses (e.g. white blood cell count, absolute and relative (% of total leukocytes) differential blood counts) in the repeat-dose oral toxicity studies in rats and dogs administered AM-1 at extremely high doses (Bitzer et al., 2017b,c). AM-1 was also non-sensitizing in dermal sensitization studies in animals and humans (details presented above); however, the relevance of dermal sensitization models to distinguish allergenic from non-allergenic food extracts is still questionable (Selgrade et al., 2009).

In consideration of the above information, it can be concluded that there is negligible potential for jelly mushroom glycolipids (AM-1) to induce symptoms of food allergy.

Summary of Safety Assessment and GRAS Conclusion

The safety and GRAS status of jelly mushroom glycolipids (AM-1) is based on the totality-of-the-evidence of published data and information regarding the AM-1 chemical structural characteristics, production organism with historical food uses, ADME profile, subchronic toxicity profile in multiple species, and low potential for carcinogenicity, mutagenicity, or developmental and reproductive toxicity. Unpublished data and information is also available as supplemental evidence regarding the safety of AM-1, which corroborate the conclusions that can be made based on generally available and accepted scientific data, information, or methods.

In summary, jelly mushroom glycolipids (AM-1) and its ultimate hydrolysis product LCFA are poorly absorbed by the oral route and are primarily eliminated in the feces without absorption. Absorbed components appear to be almost completely metabolized to CO_2 and expired. There were no metabolites of safety concern identified for AM-1 and its ultimate hydrolysis product LCFA and no accumulation of these compounds in tissues. Due to the rapid hydrolysis and elimination of AM-1 that occurs *in vivo*, the antimicrobial properties of the parent material would be absent or greatly diminished in the lower intestines so there is low concern for disruption of healthy gut microflora. AM-1 has low potential for systemic toxicity with oral repeated dose (90-day) NOAELs of ≥ 1200 mg/kg bw/day in rats (oral drinking water administration) and ≥ 1000 mg/kg bw/day in dogs (oral capsule administration), the highest dose levels tested, demonstrating that chronic toxicity and carcinogenicity are not endpoints of safety concern for AM-1. AM-1 was determined to be non-genotoxic based on the results of a complete battery of *in vitro* genetic toxicity assays in bacteria as well as in mammalian cells including human lymphocytes. AM-1 is not a reproductive or developmental toxicant as confirmed in robust 2-generation reproduction toxicity and embryofetal toxicity studies in rodents (oral gavage administration). The NOAELs of ≥ 1000 mg/kg bw/day determined in the rodent two-generation reproduction toxicity and developmental toxicity studies are consistent with the NOAELs of the 90-day oral toxicity studies, providing additional confidence that there are no toxicological endpoints of concern.

Acceptable Daily Intake (ADI) for Jelly Mushroom Glycolipids (AM-1)

The pivotal toxicology safety studies with jelly mushroom glycolipids (AM-1) are the 90-day rat oral toxicity study (Bitzer et al., 2017b) and the 90-day dog oral toxicity study (Bitzer et al., 2017c) which determined NOAELs of ≥ 1200 mg/kg bw/day in rats and ≥ 1000 mg/kg bw/day in dogs (highest dose levels tested). To be conservative, the lowest NOAEL determined in these two studies is utilized for derivation of an Acceptable Daily Intake (ADI) for jelly mushroom glycolipids as presented below.

The default safety factor typically used in estimating the acceptable daily intake from toxicological data as outlined in this GRAS Notice is 100-fold. This factor is based on an interspecies factor of 10 and an intra-species factor of 10. As discussed above, chronic toxicity and carcinogenicity are not endpoints of concern for AM-1 because of its lack of chemical structural alerts, low oral bioavailability, simple and common hydrolysis products, and rapid and

complete elimination of any metabolites; therefore, an additional safety factor was not applied in the calculation of the ADI. Based on the NOAEL of ≥ 1000 mg/kg bw/day in the 90-day oral toxicity study in Beagle dogs (Bitzer et al., 2017c), an ADI of ≥ 10 mg/kg bw/day can be calculated for jelly mushroom glycolipids (AM-1) (see Table 8).

Table 8. Acceptable Daily Intake for Jelly Mushroom Glycolipids (AM-1) from Totality-of-Evidence

Basis of ADI (Reference)	Key Data	Safety Factor	ADI for AM-1
90-day oral toxicity study in Beagle dogs (Bitzer et al., 2017c)	NOAEL ≥ 1000 mg/kg bw/day	100x	10 mg/kg bw/day

Comparison of ADI and EDI for Jelly Mushroom Glycolipids (AM-1)

As noted above, the Estimated Daily Intake (EDI) of jelly mushroom glycolipids (AM-1) was calculated using the most recent dietary survey results from the National Health and Nutrition Examination Survey (NHANES). AM-1 intake was determined by multiplying each NHANES respondents' two-day average beverage intake by the maximum proposed use level for each category. The EDI of AM-1 from all proposed uses (and assuming the maximum proposed use level for each beverage category) is less than or equal to 0.51 mg/kg bw/day at the mean and 1.09 mg/kg bw/day at the 90th percentile of intake among users in the U.S. population. A user is defined as having consumed a food/beverage that would contain AM-1 during the two days of the NHANES survey. Consideration of only two days of food/ beverage consumption data results in a conservative upper estimate of potential AM-1 intake. As noted above (see Part 3. Dietary Exposure), the highest per user mean and 90th percentile estimates of intake on a bodyweight basis when separated by population group is among children 1-6 years at 1.52 mg/kg bw/day and 3.29 mg/kg bw/day, respectively (from all proposed uses and assuming the maximum proposed use level for each beverage category). However, even this conservative upper estimate of intake is sufficiently below (approximately 3-fold lower) the ADI of 10 mg/kg bw/day determined for jelly mushroom glycolipids (AM-1). Further, owing to the low oral bioavailability of AM-1 demonstrated in the *in vivo* ADME study (Bitzer et al., 2017a), it can be concluded that actual consumer exposure to jelly mushroom glycolipids (AM-1) from the proposed uses in beverages may be as much as 10-fold lower.

In conclusion, the totality of the evidence of available information relevant to the safety of jelly mushroom glycolipids (AM-1), including identity, specifications, manufacturing process, analytical characterization, stability, safety of production organism, probable consumer exposure, and toxicology profile, provides a basis upon which to conclude that there is a reasonable certainty that jelly mushroom glycolipids (AM-1), produced in accordance with current good manufacturing practices and FSMA principles, is not harmful under its intended conditions of use.

General Recognition of the Safety of Jelly Mushroom Glycolipids (AM-1)

The current safety evaluation and GRAS conclusion for jelly mushroom glycolipids (AM-1) is based on published data and information which establish the general recognition of the safety of AM-1 for its proposed use. Unpublished data and information is also available as supplemental evidence regarding the safety of AM-1, which corroborate the conclusions that can be made based on generally available and accepted scientific data, information, or methods.

In order to assure that the common knowledge about the safety of AM-1 is generally accepted by a consensus of qualified experts, the Notifier has convened an independent review of this document by prominent experts in the field of food and food ingredient safety. The individuals comprising this Panel are qualified by scientific training and experience to evaluate the safety of substances intended to be added to food. They have critically evaluated the available information summarized in this document and have individually and collectively concluded that AM-1, produced consistent with current Good Manufacturing Practice and meeting the specifications described herein, is safe under its intended conditions of use. The Panel further concluded that these uses of AM-1 satisfy the safety standard of reasonable certainty of no harm. The Panel's GRAS opinion is included as an attachment to this document (See Exhibit I).

The Notifier is not aware of information that would be inconsistent with a conclusion that the proposed uses of AM-1, meeting appropriate specifications and used according to current Good Manufacturing Practice, are GRAS.

None of the data or information included in this GRAS Notification has been claimed as exempt from disclosure under FOIA.

7. LIST OF SUPPORTING DATA AND INFORMATION

The following references are considered generally available, unless otherwise noted. Appendix 1 (CoAs and other Technical Data for AM-1), Appendix 2 (Consumer Exposure Assessment Report for AM-1), Appendix 3 (Supplemental Report on Safety Evaluation of AM-1 Production Organism), Appendix 4 (Supplemental Report on Food Use of AM-1 Production Organism), and Exhibit I (signed Expert Panel report) are not generally available but are attached for reference.

References

Adams, J., Buelke-Sam, J., Kimmel, C.A., et al. 1985. Collaborative behavioral teratology study: protocol design and testing procedure. *Neurobehav. Toxicol. Teratol*. 7, 579–586. As cited in Herberth, 2017b (Study No. WIL-294508).

Ao, T., Deb, C.R., and Khruomo, N. 2016. Wild edible mushrooms of Nagaland, India: a potential food resource. *Journal of Experimental Biology and Agricultural Sciences* 4, 59-65.

Boa, E. 2004. Wild Edible Fungi: A Global Overview Of Their Use And Importance To People (Non-Wood Forest Products). Food & Agriculture Organization of the UN, p. 134. ISBN 92-5-105157-7.

Bitzer, J., Henkel, T., Nikiforov, A.I., Rihner, M.O., and Thomas, J.A. 2017a. Pharmacokinetics, Excretion Balance, and Tissue Distribution of [^{14}C]-Labeled Glycolipids and Long Chain Fatty Acids ([^{14}C]-LCFA) from *Dacryopinax spathularia* in Rats. *Food Chem. Toxicol*. 109, 552-568.

Bitzer, J., Henkel, T., Nikiforov, A.I., Rihner, M.O., and Leuschner, J. 2017b. A 90-day oral toxicity study of glycolipids from *Dacryopinax spathularia* in CD® rats. *Food Chem. Toxicol*. 109, 569-584.

Bitzer, J, Henkel, T., Nikiforov, A.I., Rihner, M.O., and Henderson, K.A. 2017c. A 90-day oral toxicity study of glycolipids from *Dacryopinax spathularia* in Beagle dogs. *Food Chem. Toxicol*. 109, 544-551.

Bradford, J.C., Brown, G.L., Caldwell, J.A. and Drobeck, H.P. 1984. Teratology and mutagenicity studies with glutaric acid. *Teratology*, 29(2), 19A.

Chen, J. and Seviour, R. 2007. Medicinal importance of fungal β-(1→3),(1→5)-glucans. *Mycol. Res.* 111, 635-652.

Collier, G. and Knarr, G. 1966. Defense of water balance in the rat. *Journal of Comparative and Physiological Psychology* 61, 5-10.

Crampton, E.W. and Lloyd, L.E. 1954. The effect of water restriction on the food intake and food efficiency of growing rats. *Journal of Nutrition* 54, 221-224.

EFSA (European Food Safety Authority). 2007. Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on a request from the Commission related to an application on the use of ethyl lauroyl arginate as a food additive. *The EFSA Journal* 511, 1-27.

EFSA (European Food Safety Authority). 2014. Scientific Opinion on the evaluation of allergenic foods and food ingredients for labelling purposes. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). *EFSA Journal*, 12(11), 3894.

FDA (U.S. Food and Drug Administration). 1987. Good Laboratory Practice Regulations. Final Rule. 21 Code of Federal Regulations (CFR) Part 58. Food and Drug Administration, U.S. (FDA) Department of Health and Human Services, Washington, DC.

FDA (U.S. Food and Drug Administration). 1993. Toxicological Principles for the Safety Assessment of Food Ingredients: Redbook II, Draft Guidance. Chapter V B, Metabolism and Pharmacokinetic Studies. Food and Drug Administration, U.S. (FDA), Center for Food Safety and Applied Nutrition (CFSAN), Washington, DC.

FDA (U.S. Food and Drug Administration). 2000. Toxicological Principles for the Safety Assessment of Food Ingredients: Redbook 2000. Food and Drug Administration, U.S. (FDA), Center for Food Safety and Applied Nutrition (CFSAN), Washington, DC. Updated 2007.

Fujino, Y., Nakano, M., and Saeki, T. 1970. The chemical structure of glycolipids of Bovine milk. *Agricultural and Biological Chemistry* 34(3), 442-447.

Ghioni, A. 2011a. In vitro Acute Skin Irritation RHE 42.BIS-OECD 439. VitroScreen SRL, Milano, Italy. Unpublished Report submitted to InterMed Discovery GmbH. Study No. VS. 52-11B. Data as published in Bitzer et al. 2017b.

Ghioni, A. 2011b. Eye Irritation Potential on In vitro Reconstructed Human Corneal Epithelium. VitroScreen SRL, Milano, Italy. Unpublished Report submitted to InterMed Discovery GmbH. Study No. VS. 51-11B. Data as published in Bitzer et al. 2017b.

Ghioni, A. 2011c. BALB 3T3-NRU Phototoxicity Assay: OECD TG 432. VitroScreen SRL, Milano, Italy. Unpublished Report submitted to InterMed Discovery GmbH. Study No. VS. 54-11B. Data as published in Bitzer et al. 2017b.

Heppenheimer, A. 2013. IMD AM-1: *In vitro* Skin Corrosion Test: Human Skin Model Test. Harlan Cytotest Cell Research GmbH. Unpublished Report submitted to IMD Natural Solutions GmbH (INS). Study No. 1561500. Data as published in Bitzer et al. 2017b.

Herberth, M.T. 2017a. An Oral (Gavage) Developmental Toxicity Study of IMD AM-1 in Rats. Charles River Laboratories Ashland, LLC, Ashland, OH, USA. Unpublished Final Report submitted to IMD Natural Solutions GmbH (INS). Study No. WIL-294507.

Herberth, M.T. 2017b. A Two-Generation Reproductive Toxicity Study of IMD AM-1 Administered Orally by Gavage in Rats. Charles River Laboratories Ashland, LLC, Ashland, OH, USA. Unpublished DRAFT Report submitted to IMD Natural Solutions GmbH (INS). Study No. WIL-294508.

Jackson, C.M. and Smith, V.D.E. 1931. The effects of deficient water intake on the growth of the rat. *American Journal of Physiology* 97, 146-153.

Jyonouchi, S., Abraham, V., Orange, J.S., Spergel, J.M., Gober, L., Dudek, E., Saltzman, R., Nichols, K.E., and Cianferoni, A. 2011. Invariant natural killer T cells from food allergic versus non-allergic children exhibit differential responsiveness to milk-derived sphingomyelin. *J Allergy Clin Immunol*. 128(1), 102-109.e13.

Korenbrot, C.C., Huhtaniemi, I.T., and Weiner, R.W. 1977. Preputial separation as an external sign of pubertal development in the male rat. *Biol. Reprod*. 17, 298–303. As cited in Herberth, 2017b (Study No. WIL-294508).

Lambe, J., Kearney, J., Leclercq, C., Zunft, H., De Henauw, S., Labmerg-Allardt, C., Dunne, A., and Gibney, M. 2000. The influence of survey duration on estimates of food intakes and its relevance for public health nutrition and food safety issues. *Eur J Clin Nutr*. 54, 166-173.

Lee , S.S., Chang, Y.S., and Noraswati, M.N.R. 2009. Utilization of macrofungi by some indigenous communities for food and medicine in Peninsular Malaysia. *Forest Ecology and Management* 257, 2062-2065.

Leray, C. 2015. Lipids: Nutrition and Health. CRC Press, Taylor & Francis Group. Boca Raton,FL, pp.154-155. ISBN 978-1-4822-4231-7.

Lowe, C. 2013. Dermal Sensitization Study in Guinea Pigs (Buehler Method). Unpublished Report submitted to IMD Natural Solutions GmbH. Product Safety Labs Study No. 35537. Data as published in Bitzer et al. 2017b.

Lupien, P.J., Moorjani, S., Brun, D., and Bielmann, P. 1979. Effects of 3-hydroxy-3-methylglutaric acid on plasma and low density lipoprotein cholesterol levels in familial hypercholesterolemia. *J. Clin. Pharmacol*., 19(Feb-Mar), 120-126.

Martin, G.W. 1948. New or noteworthy tropical fungi. IV. Lloydia 11, 116.

Meuninck, J. 2017. Foraging Mushrooms Oregon. Finding, Identifying, and Preparing Edible Wild Mushrooms. Falcon Guides, Rowman / Littlefield, pp. 74-75, ISBN 978-1-4930-2669-2.

MycoBank. 2017. Online database provided by the International Mycological Association. Retrieved June 6, 2107 from http://www.mycobank.org/

Newburg, D.S. and Chaturvedi, P. 1992. Neutral glycolipids of human and bovine milk. *Lipids*. 27, 923-927.

NCHS (National Center for Health Statistics). 2012. National Health and Nutrition Examination Survey Data 2011-2012. Hyattsville, MD: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Available from: http://wwwn.cdc.gov/Nchs/Nhanes/Search/nhanes11_12.aspx.

NCHS (National Center for Health Statistics). 2014. National Health and Nutrition Examination Survey Data 2009-2010. Hyattsville, MD: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Available from: http://wwwn.cdc.gov/Nchs/Nhanes/Search/nhanes09_10.aspx.

NIAID (National Institute of Allergy and Infectious Diseases). 2011. Guidelines for the Diagnosis and Management of Food Allergy in the United States. U.S. Department of Health and Human Services, National Institutes of Health (NIH) Publication No. 11-7699.

Nishida, F., Mori, Y., Isobe, S., Furuse, T., and Suzuki, M. 1988. Structures of deacetyl glykenins-1, A, and C, glycosidic antibiotics from Basidiomycetes sp. *Tetrahedron Lett*. 29, 5287-5290.

Pariza, M.W. and Foster, E. M. 1983. Determining the safety of enzymes used in food processing. *J. Food Protect.* 46, 453-463.

Pariza, M.W. and Johnson, E.A. 2001. Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. *Regul. Toxicol. Pharmacol*. 33, 173-186.

Sakagami, Y., Yamasaki, H., Ogasawara, N., Yokoyama, H., Ose, Y., and Sato, T. 1989. Evaluation of genotoxic activities of disinfectants and their metabolites by the umu test. *Mutat. Res*. 216(6), 373.

San Sebastian, J.R. 1989. *In vivo* bone marrow cytogenetics rat metaphase analysis. Submitted by Monsanto Co. (1989) with cover letter dated 8/14/92. U.S. EPA Doc # 88-920007732; Microfiche #OTS0538652.

Savoie, L.L. and Lupien, P.-J. 1975a. Organ distribution of 3-hydroxy-3methylglutaric acid, a potential anticholesterolemic agent. *Can. J. Physiol*. 53(4), 638-643.

Savoie, L.L. and Lupien, P.-J. 1975b. Preliminary toxicological investigations of 3-hydroxy-3-methylglutaric acid (HMG). I: Study of acute toxicity and of teratogenic activity in rats and mice. *Arzneimittelforschung. (Drug Res.)* 25(8), 1284-1286.

Schmid, S. 2012. Acute Oral Toxicity (Acute Toxic Class Method) with IM-11. Unpublished Report; BSL BIOSERVICE Study No. 115763. Data as published in Bitzer et al. 2017b.

Selgrade, M. K., Bowman, C. C., Ladics, G. S., Privalle, L., and Laessig, S. A. 2009. Safety assessment of biotechnology products for potential risk of food allergy: implications of new research. *Toxicological Sciences* 110(1), 31-39.

Sicherer, S.H. and Sampson, H.A. 2014. Food allergy: Epidemiology, pathogenesis, diagnosis, and treatment. *J Allergy Clin Immunol*. 133, 291-307.

Stadler, M., Bitzer, J., Köpcke, B., Reinhardt, K., and Moldenhauer, J. 2012. Long chain glycolipids useful to avoid perishing or microbial contamination of materials. WO2012/167920A1.

Steinke, J.W., Platts-Mills, T.A.E., and Commins, S.P. 2015. The alpha gal story: Lessons learned from connecting the dots. *J Allergy Clin Immunol*. 135(3), 589-597.

Stuckhardt, J.L. and Poppe, S.M. 1984. Fresh visceral examination of rat and rabbit fetuses used in teratogenicity testing. *Teratog. Carcinog. Mutagen*. 4, 181–188. As cited in Herberth, 2017a (Study No. WIL-294507).

Tatsene, M. 2013. 50 Human Subject Repeat Insult Patch Test Skin Irritation/Sensitization Evaluation (Occlusive Patch). Unpublished Report submitted to IMD Natural Solutions GmbH (INS). AMA Laboratories, Inc. Study No. MS13.RIPT.M8804O.50.INS. Data as published in Bitzer et al. 2017b.

Umetsu, D. 2010. Peanut Glycolipid Antigens Activate Natural Killer T Cells Causing Severe Allergy. Project Summary for Grant awarded to the Boston Children's Hospital by the National Institute of Allergy and Infectious Diseases. Project Number: 1R21AI087951-01. NIH Research Portfolio Online Reporting Tool (RePORT); Accessed September 2017. https://projectreporter.nih.gov/project_info_description.cfm?aid=7877661&icde=32887234

Usta, B. 2012. In vitro Micronucleus Test (Including a Preliminary Dose Range Finding Toxicity Test) with IM 11 Grade A in Cultured Human Lymphocytes. TNO Triskelion Study Code 9917/05. Unpublished Report submitted to InterMed Discovery GmbH. Data as published in Bitzer et al. 2017b.

Vail, W.J., and Lilly, V.G. 1968. The Location of Carotenoid Pigments and Thickness of the Cell Wall in Light- and Dark-Grown Cells of Dacryopinax spathularia. *Mycologia* 60, 902-907.

van den Wijngaard, M.J.M. 2012. Bacterial Reverse Mutation Test with IM-11 grade A. Unpublished Report submitted to InterMed Discovery GmbH. TNO Triskelion, Study Code: 9905/07. Data as published in Bitzer et al. 2017b.

van Dijk, H, Onguene, N.A., and Kuyper, T.W. 2003. Knowledge and Utilization of Edible Mushrooms by Local Populations of the Rain Forest of South Cameroon. *Ambio* 32, 19–23.

Verspeek-Rip, C.M. 2016. Evaluation of the Mutagenic Activity of IMD AM-1 in an In Vitro Mammalian Cell Gene Mutation Test with L5178Y Mouse Lymphoma Cells. WIL Research

Europe B.V., Hambakenwetering 7, The Netherlands. Unpublished Report submitted to IMD Natural Solutions GmbH. Study No. 510377. Data as published in Bitzer et al. 2017b.

Wolver, S.E., Sun, D.R., Commins, S.P., and Schwartz, L.B. 2012. A peculiar cause of anaphylaxis: No more steak? The journey to discovery of a newly recognized allergy to Galactose-alpha-1,3-galactose found in mammalian meat. *J Gen Intern Med*. 28(2), 322-325.

Woo, D.C and Hoar, R.M. 1972. Apparent hydronephrosis as a normal aspect of renal development in late gestation of rats: the effects of methyl salicylate. *Teratology* 6, 191–196. As cited in Herberth, 2017a (Study No. WIL-294507).

Wilson. J.G. 1965. Embryological considerations in teratology. In: Wilson JG, Warkany J, eds. Teratology: Principles and Techniques. Chicago, IL: University of Chicago Press; pp. 251–277. As cited in Herberth 2017a (Study No. WIL-294507). Also published in *Annals of the New York Academy of Sciences* 123, 219–227, March 1965.

Zhishu, B., Zheng, G., and Taihui, L. 1993. The macrofungus flora of China's Guangdong province, The Chinese University Press of Hong Kong, pp. 51-52, ISBN 962-201-556-5.

APPENDIX 1.

TECHNICAL DOCUMENTATION FOR JELLY MUSHROOM GLYCOLIPIDS (AM-1)

1-A: 3-Hydroxy-3-Methylglutarate (HMG) Summary Report

1-B: AM-1 Certificates of Analysis

1-C: AM-1 Bulk Powder Stability Study Data

1-D: AM-1 Stability in Beverage Applications

1-E: AM-1 Efficacy and Applications Data

IMD Natural Solutions GmbH
Otto-Hahn-Str. 15
44227 Dortmund
Germany

AM-1 glycolipids containing 3-hydroxy-3-methylglutarate (HMG) as acyl unit

Date: 2017-10-10
Document Type: Report
Number of Pages: 7

Summary

A minor portion of jelly mushroom glycolipids (AM-1) contain 3-hydroxy-3-methylglutarate (HMG) as acyl unit attached to the glucose of the trisaccharide moiety.

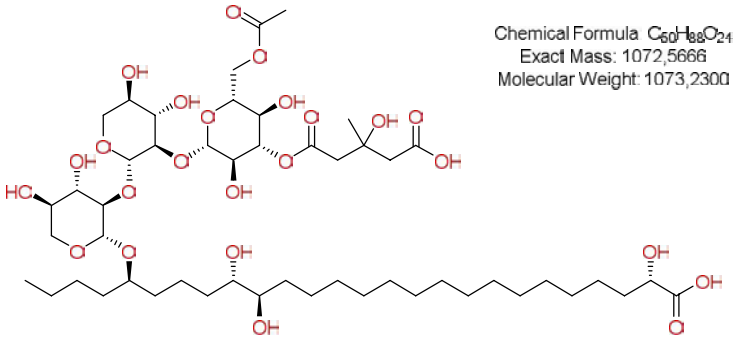
The maximum ratio of HMG-bearing glycolipids is fixed as <12% by the internal specification of AM-1, referring to a maximum of <1.78% (w/w) HMG that could be released theoretically by hydrolysis. HMG as acyl unit is present in various plant metabolites, some of which occurring in edible plant parts. HMG-CoA (HMG bound as thioester to coenzyme A) is a primary metabolite in terpene biosynthesis, ketogenesis, and leucine degradation pathways.

1. AM-1 glycolipids containing HMG as acyl unit

3-hydroxy-3-methylglutarate (HMG) has recently been identified by INS as an acyl substituent occurring in a minor part of the glycolipids in the AM-1 glycolipid mixture. In order to guarantee batch-to-batch consistency, the maximum ratio of HMG-containing congeners within the total AM-1 glycolipid mixture has been fixed in an internal specification as <12%. This is verified analytically by HPLC-MS analysis of each batch.

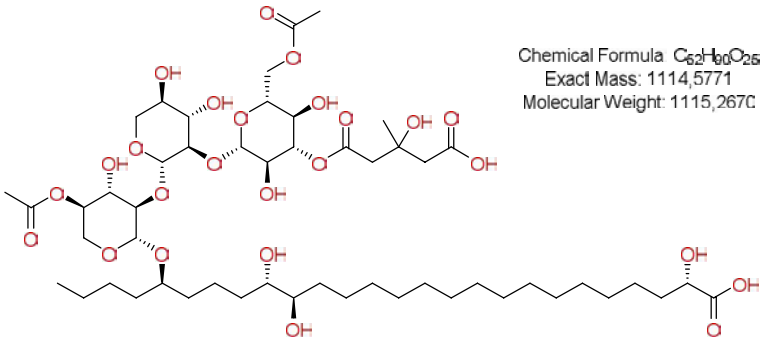
Two groups of HMG-containing glycolipid congeners can be distinguished based on their molecular weight and are presented below.

- **Group I, representative chemical structure:**



HMG after complete hydrolysis: 15.11% of initial weight of Group I glycolipids.

- **Group II, representative chemical structure:**



HMG after complete hydrolysis 14.54% of initial weight of Group II glycolipids.

Maximum HMG amounts in percent by weight are calculated *assuming a worst-case-scenario* of 100% hydrolysis of the acyl units, a maximum amount of HMG-bearing glycolipids being present as fixed in the internal specification of AM-1, and a hypothetic glycolipid content of 100% in the product (i.e., water, protein, fat and sodium chloride content are not considered, although their presence actually lowers total HMG content).

Internal specification of AM-1: <12% (sum of HMG-containing glycolipids Group I + II)

→ **Calculated theoretical maximum amount of HMG obtained from AM-1:** <1.78% (w/w)

2. Data of representative batches

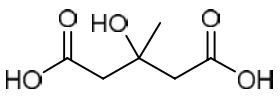
Based on HPLC-MS analysis data of the glycolipid composition of each individual batch, the hypothetic maximum amount of HMG after complete hydrolysis can be calculated based on the data presented in the previous Section. Results are given in percent by weight.

a) Lot No. AM1-00023-05-01 (*used for 90-day oral toxicity study in rats and rat ADME study*): HMG max. 0.88%

b) Lot No. S160401 (*used for 90-day oral toxicity in dogs, reproductive toxicity study and developmental toxicity study in rats*): HMG max. 1.50%

c) Lot No. S160701 (*currently used lot from pilot production*): HMG max. 0.49%

3. Chemical structure and identifiers of HMG

IUPAC name:	3-Hydroxy-3-methylpentanedioic acid
Chemical name:	3-Hydroxy-3-methylglutaric acid (HMG)
Synonyms:	Dicrotalic acid, Mevalon, Medroglutaric acid
INN:	Meglutol
CAS RN:	503-49-1
RTECS:	MA3753000
Chemical structure:	 Chemical Formula: C6H10O5 Exact Mass: 162,0528 Molecular Weight: 162,1410

4. Occurrence of HMG in nature

a) In free form

The free form of HMG (i.e., the dicarboxylic acid) is known from certain plants:

- *Crotolaria dura* and *Crotalaria globifera*, South-African plants belonging to the Fabaceae family (Harbone et al, 1998)
- *Tillandsia usneoides*, also known as "Spanish Moss" (Witherup et al, 1995)

b) Bound in other metabolites (except HMG-CoA)

HMG is known as acyl substituent in various metabolites from plants and fungi (Dictionary of Natural Products, 2017). More than 230 substances are known, some of which are present in edible sources.

Examples for metabolites from edible plants having HMG as an acyl unit are Lablaboside D from hyacinth beans (*Dolichos lablab*) and Licoagroside B found in *Glycyrrhiza glabra* (licorice).

HMG-esterified flavonoids in various Citrus species including *Citrus aurantifolia* (lime) and *Citrus sudachi* (a Japanese citrus fruit used for food flavoring in place of lemon) are well described. Also the fruits from *Rubus* spp., e.g. blackberries, and lingonberries (*Vaccinium vitis-ideae*) are known to contain flavonoids acylated with HMG.

Ginger (*Zingiber officinale* rhizome) has been described to contain glycosylated camphene mono-terpenoids bearing HMG as acyl group at the glucose unit just like in AM-1 glycolipids. Similarly, leek (*Allium porrum*) contains various terpenoids bearing a glucose unit esterified with HMG.

In flax seeds (*Linum usitatissimum*) dimers, trimers and oligomers formed of HMG and flaxseed lignin are present and part of the edible oil.

As a conclusion, it can be stated that plant components containing HMG as an acyl group are part of the human diet.

c) As thioester with coenzyme A (HMG-CoA)

The coenzyme A thioester of HMG (3-hydroxy-3-methylglutaryl-coenzyme A, "**HMG-CoA**") is an intermediate in primary metabolism in humans (see standard biochemistry textbooks, e.g. Nelson and Cox, 2017). It is an intermediate in the mevalonate pathway, the ketogenesis pathway, and in the degradation of leucine. Free 3-hydroxy-3-methylpentanedioic acid (**HMG**), however, is neither a substrate nor a product in any known human biosynthetic pathway.

In the **mevalonate pathway** towards biosynthesis of terpenoids, HMG-CoA is formed from acetyl-CoA and acetoacetyl-CoA by the HMG-CoA synthase (see **Figure 1**). In the next step, HMG-CoA is converted to mevalonate by the HMG-CoA reductase. This enzyme is known in particular for its inhibition by certain drugs, e.g. the statins, because it is the rate limiting step in cholesterol biosynthesis.

In the **ketogenesis pathway**, acetyl-CoA derived from oxidative degradation of fatty acids or ketogenic amino acids can be converted into ketone bodies (i.e. acetone, beta-hydroxybutyrate, and acetoacetate) serving as transportable acetyl-CoA equivalents when glucose levels are low. Ketogenesis is primarily taking place in the mitochondria of the liver cells. In the biosynthetic pathway, two acetyl-CoA units are converted to acetoacetyl-CoA. A third CoA unit is added by the HMG-CoA synthase (like in the mevalonate pathway) to form HMG-CoA (see **Figure 2**). Release of acetyl-CoA again by the HMG-CoA lyase leads to acetoacetate as first ketone body, which may be converted into acetone by decarboxylation or beta-hydroxybutyrate by dehydrogenation.

Degradation of leucine, a ketogenic amino acid, involves HMG-CoA as intermediate (see **Figure 3**) which can either be subjected to the ketogenesis pathway to form acetyl-CoA or acetoacetate, or be utilized in terpenoid biosynthesis. Both pathways have been described before. The main degradation pathway of leucine leads in four steps via isovalery-CoA to 3-methylglutaconyl-CoA, which is hydrated enzymatically to HMG-CoA.

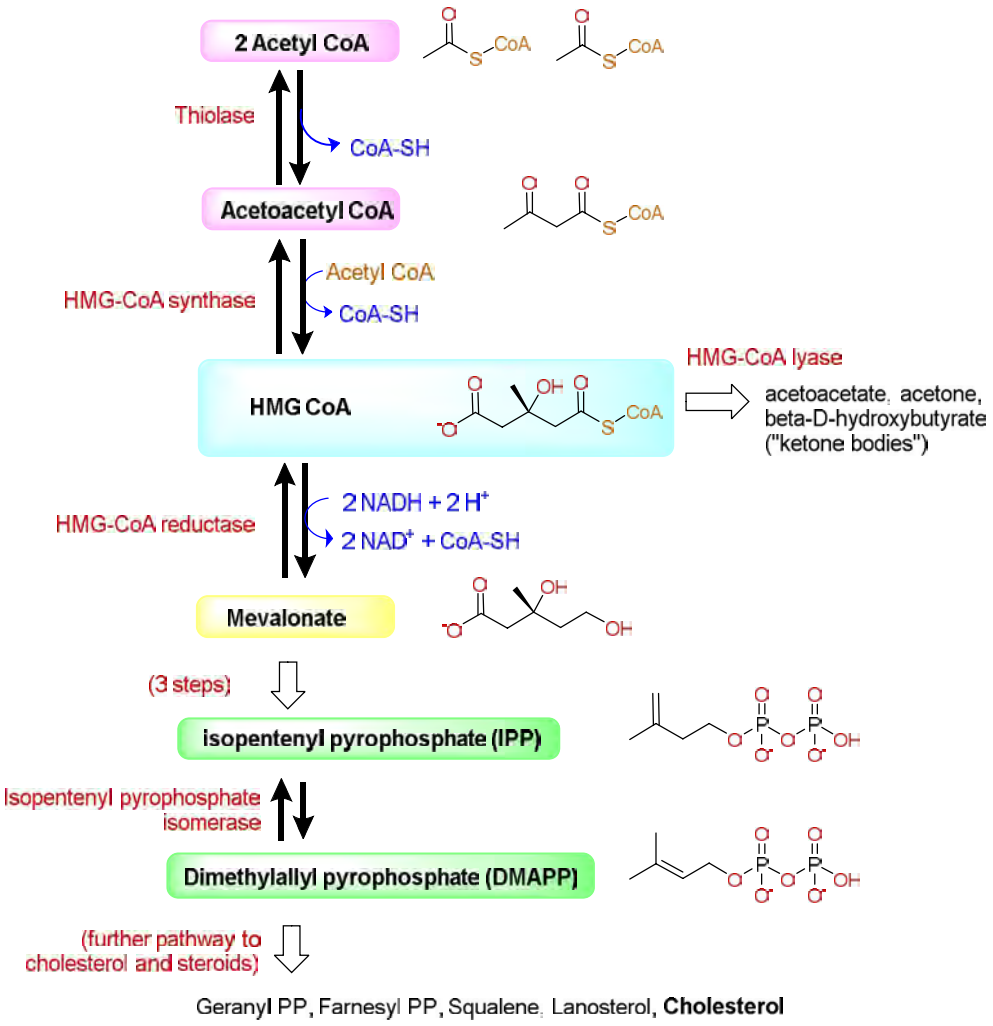


Figure 1: Mevalonate pathway.

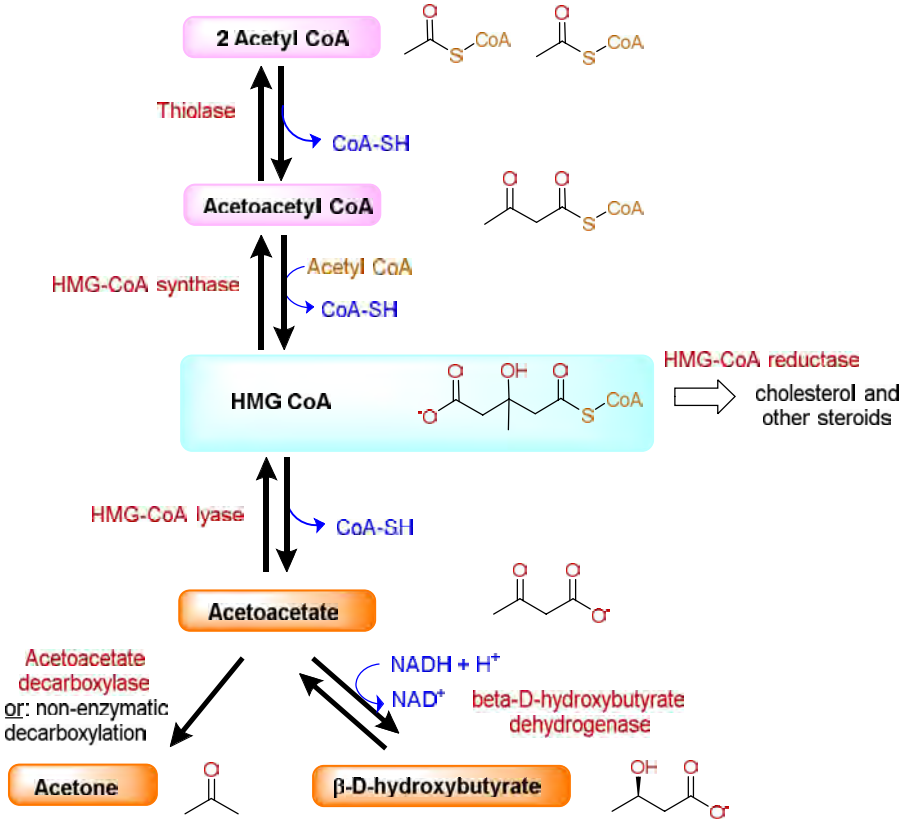


Figure 2: Ketogenesis pathway. The biosynthesis of ketone bodies (orange background) from acetyl CoA is done via HMG-CoA as intermediate. The formation of HMG-CoA from acetyl-CoA is identical with the mevalonate pathway.

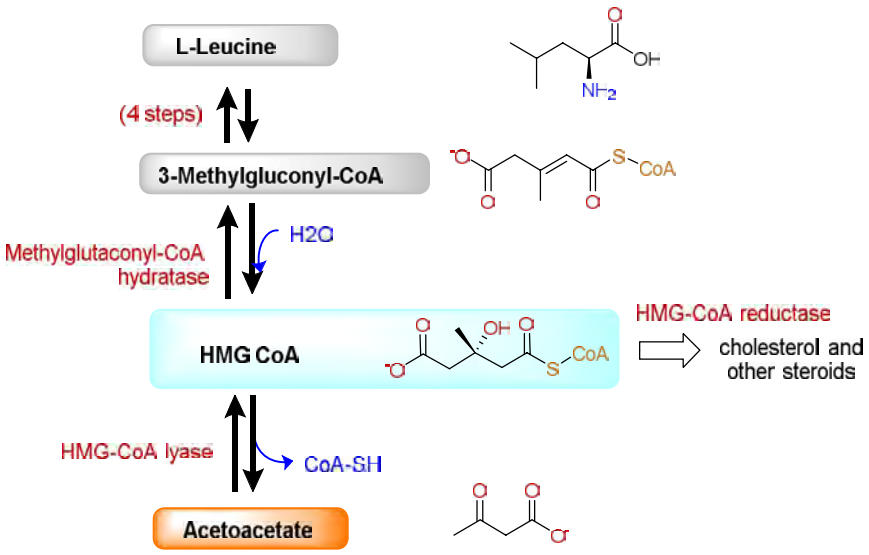


Figure 3: Leucine metabolism (main pathway).

5. References

Dictionary of Natural Products, 2017: Version 26.1, CRC Press, Taylor & Francis Group; online access via http://dnp.chemnetbase.com on 2017-10-06.

Harborne, J.B., Baxter, H., Moss, G.P. 1998: Phytochemical Dictionary: A Handbook of Bioactive Compounds from Plants, Second Edition, Taylor & Francis, Chapter 5.

Nelson, D.L., and Cox, M.M. 2017: Lehninger Principles of Biochemistry, 7th edition, W.H. Freeman and Co., ISBN 978-1464126116.

Witherup, K.M., McLaughlin, J.L., Judd, R.L., Ziegler, M.H., Medon, P.J., and Keller, W.J. 1995: Identification of 3-Hydroxy-3-methylglutaric Acid (HMG) as a Hypoglycemic Principle of Spanish Moss (*Tillandsia usneoides*), *J. Nat. Prod.* 58, 1285–1290. doi: 10.1021/np50122a023

IMD Natural Solutions GmbH
Otto-Hahn-Str. 15 | 44227 Dortmund | Germany
info@imd-natural-solutions.com

Certificate of Analysis

Identification

Product name:	**AM-1**
Chemical structure:	Glycolipid mixture
Synonyms:	Jelly mushroom glycolipids
Lot-No:	S160401
Production date:	2016-07-14
Expiry date	2019-07-14

Analysis results

Analysis	Acceptance	Result
a) Properties		
Appearance	Powder	Conforms
Color	Light ivory, ivory or beige	Light ivory (RAL 1015)
Solubility (>20 g/l)	Clear solution	Conforms
Turbidity (0.1% in water)	<8 NTU	2.3 NTU
pH (0.1% in water)	5.0 – 7.0	5.6
Water content	<5.0%	1.5%
Total protein (N x 6.25)	<3.0%	1.7%
Total fat	<2.0%	1.5%
Sodium	1.7 – 3.3%	2.0%
Total glycolipids (based on dry weight)	≥93%	95%
b) Identity		
Identity by HPLC-MS	Conform to chromatogram and mass spectra of standard	Conforms
c) Heavy Metals (ICP-MS)		
Arsenic (As)	<1 ppm	0.4 ppm
Cadmium (Cd)	<1 ppm	<0.1 ppm
Lead (Pb)	<2 ppm	0.2 ppm
Mercury(Hg)	<1 ppm	<0.1 ppm
Nickel (Ni)	<2 ppm	0.6 ppm
d) Microbiological Purity		
Total aerobic microbial count (TAMC)	≤100 CFU/g	<10 CFU/g
Total yeast and mold count (TYMC)	≤10 CFU/g	<10 CFU/g
Coliforms	≤3 MPN/g	<3 MPN/g
Escherichia coli	≤3 MPN /g	<3 MPN/g
Salmonella spec.	Absent in 25 g	Absent

Remarks

The results comply with the specification.

Date:	Name:	Signature:
2016-08-22	Dr. Jens Bitzer Director, Product Development & Analytics	(b) (6)

IMD Natural Solutions GmbH
Otto-Hahn-Str. 15 | 44227 Dortmund | Germany
info@imd-natural-solutions.com

Certificate of Analysis

Identification

Product name:	**AM-1**
Chemical structure:	Glycolipid mixture
Synonyms:	Jelly mushroom glycolipids
Lot-No:	S160701
Production date:	2016-08-22
Expiry date	2019-08-22

Analysis results

Analysis	Acceptance	Result
a) Properties		
Appearance	Powder	Conforms
Color	Light ivory, ivory or beige	Light ivory (RAL 1015)
Solubility (>20 g/l)	Clear solution	Conforms
Turbidity (0.1% in water)	<8 NTU	1.6 NTU
pH (0.1% in water)	5.0 – 7.0	6.5
Water content	<5.0%	1.9%
Total protein (N x 6.25)	<3.0%	1.7%
Total fat	<2.0%	0.6%
Sodium	1.7 – 3.3%	1.7%
Total glycolipids (based on dry weight)	≥93%	97%
b) Identity		
Identity by HPLC-MS	Conform to chromatogram and mass spectra of standard	Conforms
c) Heavy Metals (ICP-MS)		
Arsenic (As)	<1 ppm	0.4 ppm
Cadmium (Cd)	<1 ppm	<0.1 ppm
Lead (Pb)	<2 ppm	0.4 ppm
Mercury(Hg)	<1 ppm	<0.1 ppm
Nickel (Ni)	<2 ppm	0.5 ppm
d) Microbiological Purity		
Total aerobic microbial count (TAMC)	≤100 CFU/g	<10 CFU/g
Total yeast and mold count (TYMC)	≤10 CFU/g	<10 CFU/g
Coliforms	≤3 MPN/g	<3 MPN/g
Escherichia coli	≤3 MPN /g	<3 MPN/g
Salmonella spec.	Absent in 25 g	Absent

Remarks

The results comply with the specification.

Date:	Name:	Signature:
2016-12-16	Dr. Jens Bitzer Director, Product Development & Analytics	(b) (6)

IMD Natural Solutions GmbH
Otto-Hahn-Str. 15 | 44227 Dortmund | Germany
info@imd-natural-solutions.com

Certificate of Analysis

Identification

Product name:	**AM-1**
Chemical structure:	Glycolipid mixture
Synonyms:	Jelly mushroom glycolipids
Lot-No:	170102
Production date:	2017-02-03
Expiry date	2020-02-03

Analysis results

Analysis	Acceptance	Result
a) Properties		
Appearance	Powder	Conforms
Color	Light ivory, ivory or beige	Light ivory (RAL 1015)
Solubility (>20 g/l)	Clear solution	Conforms
Turbidity (0.1% in water)	<8 NTU	3.8 NTU
pH (0.1% in water)	5.0 – 7.0	6.6
Water content	<5.0%	3.8%
Total protein (N x 6.25)	<3.0%	1.7%
Total fat	<2.0%	0.7%
Sodium	1.7 – 3.3%	3.0%
Total glycolipids (based on dry weight)	≥93%	95%
b) Identity		
Identity by HPLC-MS	Conform to chromatogram and mass spectra of standard	Conforms
c) Heavy Metals (ICP-MS)		
Arsenic (As)	<1 ppm	0.9 ppm
Cadmium (Cd)	<1 ppm	<0.1 ppm
Lead (Pb)	<2 ppm	0.5 ppm
Mercury(Hg)	<1 ppm	<0.1 ppm
Nickel (Ni)	<2 ppm	0.3 ppm
d) Microbiological Purity		
Total aerobic microbial count (TAMC)	≤100 CFU/g	<10 CFU/g
Total yeast and mold count (TYMC)	≤10 CFU/g	<10 CFU/g
Coliforms	≤3 MPN/g	<3 MPN/g
Escherichia coli	≤3 MPN /g	<3 MPN/g
Salmonella spec.	Absent in 25 g	Absent

Remarks

The results comply with the specification.

Date:	Name:	Signature:
2017-06-06	Dr. Jens Bitzer Director, Product Development & Analytics	(b) (6)

IMD Natural Solutions GmbH
Otto-Hahn-Str. 15 | 44227 Dortmund | Germany
info@imd-natural-solutions.com

Stability data

Product

AM-1

Composition

Jelly Mushroom Glycolipids

Conditions

Lot No.: AM-1-00184-01-01

Methods: LC-MS analysis of glycolipid content and composition,
Visual appraisal for color and appearance

Storage conditions: 20 °C (ambient) and 40 °C

Packaging: Glass vials

Results

Storage [months]	20 °C (ambient)		40 °C	
	Recovery [%]	Appearance	Recovery [%]	Appearance
AM-1 as dry powder:				
0	95.1	Solid powder, light ivory	95.3	Solid powder, light ivory
6	94.7	Solid powder, light ivory	96.5	Solid powder, light ivory
12	95.6	Solid powder, light ivory	94.7	Solid powder, light ivory
24	94.8	Solid powder, light ivory	95.8	Solid powder, light ivory
36	94.9	Solid powder, light ivory	97.1	Solid powder, light ivory

Conclusion

The product is stable as a dry powder at temperatures of 40 °C or below.

IMD Natural Solutions GmbH

Date issued: 04.08.2017

IMD Natural Solutions GmbH
Otto-Hahn-Str. 15
44227 Dortmund
Germany

Stability of Jelly Mushroom Glycolipids (AM-1) in Beverage Applications

Date:	2017-09-28
Document Type:	Report
Number of Pages:	4

Summary

Jelly mushroom glycolipids (herein referred to as "**AM-1**") is stable in beverage applications under typical storage conditions.

Dependent on the type of beverage and the pH value (particularly carbonated soft drinks and acidic), a shift within the glycolipid mixture towards non-acylated glycolipids is found, caused by hydrolysis of ester moieties. The non-esterified glycolipids maintain the desired antimicrobial activity, although a bit lower than the initial mixture. This hydrolysis mechanism is also expected to be the first step of degradation following ingestion in the human gastrointestinal tract. Hydrolysis products are acetate and isovalerate.

1. Stability of AM-1 in beverages

AM-1 is stable in beverage applications.

Stability of AM-1 in beverages was tested in 110 commercially obtained beverages. The beverages were chosen from different categories, including carbonated soft drinks (CSD), fruit drinks (FD), Enhanced waters, sport drinks, energy drinks, syrups and various ready-to-drink teas. Storage conditions were 3 months at ambient temperature. Analysis was done by high performance liquid chromatography coupled with mass spectrometry (LCMS), being capable of differentiating the individual glycolipid congeners of the jelly mushroom glycolipid mixture.

Degradation of the glycolipids to its principal components glucose, xylose and long-chain fatty acids (LCFA) did not occur in any beverage.

Hydrolysis of ester moieties of the glycolipids was found, to a degree dependent on the pH value and cloudiness of the individual beverage (**Figure 1**). For instance, carbonated soft drinks (CSD) with low pH generally showed a higher degree of ester hydrolysis compared to cloudy fruit juices. Further, beverages with pH >3.5 showed only low degrees of ester hydrolysis.

The result of this hydrolysis process is a higher ratio of deacylated glycolipids being present in the glycolipid mixture than initially. The preserving properties of this partially deacylated glycolipid mixture are still very good, although slightly reduced compared to the initial material as shown by antimicrobial tests. Hydrolysis products are acetate and isovalerate (**Figure 2**).

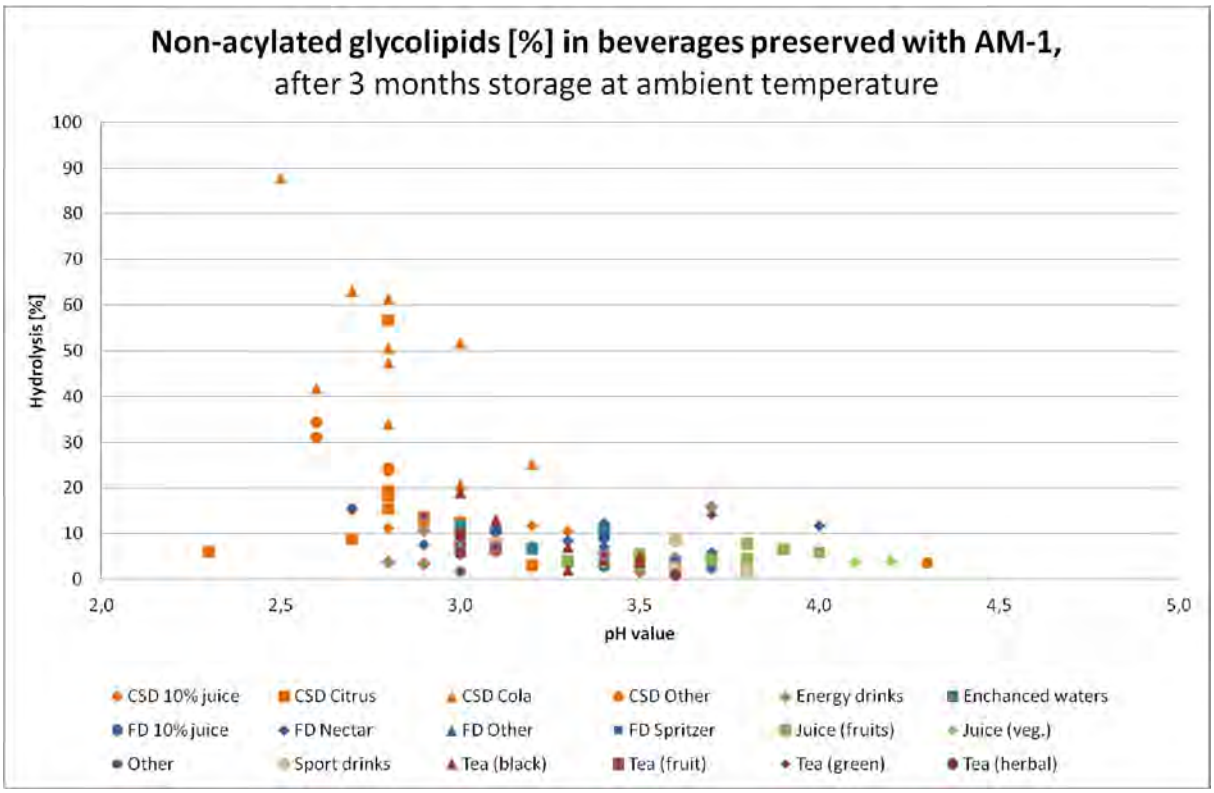


Figure 1: Percent of non-acylated (i.e. hydrolyzed) glycolipids versus pH value for AM-1 in various beverages preserved with AM-1, after 3 months storage at ambient temperature. [CSD = carbonated soft drinks, FD = fruit drinks].

2. Mechanism of AM-1 hydrolysis in aqueous solution

AM-1 is stable in aqueous solution.

Long-term experiments of AM-1 in pure aqueous solution at different concentrations confirmed that the glycolipids remain intact for at least 36 months under refrigerated conditions. Degradation to its principal components LCFA (long chain fatty acids), xylose and glucose does not occur.

Minor hydrolysis of ester moieties is found in a time-, pH- and temperature-dependent manner, leading to a shift in the glycolipid mixture towards deacylated glycolipids (see **Figure 2**). Longer incubation times, lower pH values (<3.5) and higher temperatures (≥40°C) favor the hydrolysis of acetyl and isovaleryl ester moieties of the AM-1 glycolipids. The result of this process is a higher ratio of deacylated glycolipids being present than initially. The preserving properties of this partially deacylated glycolipid mixture are still very good, although slightly reduced compared to the initial material. Hydrolysis products are acetate and isovalerate.

This hydrolysis mechanism and resulting products is also expected to be the first step of degradation in the gastrointestinal tract following ingestion of AM-1 and is consistent with experimental results in simulated gastric / intestinal fluid and in rodents (Bitzer et al, 2017).

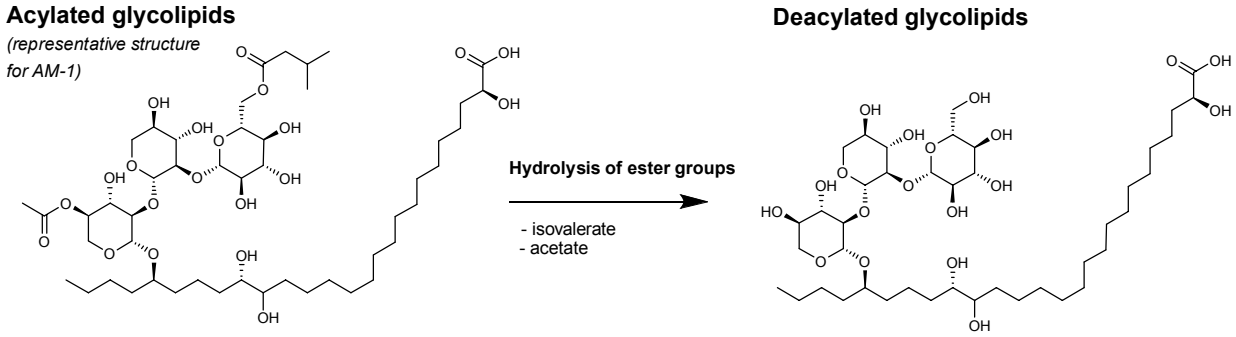


Figure 2: Hydrolysis mechanism for AM-1 glycolipids.

3. References

Bitzer, J., Henkel, T., Nikiforov, A.I., Rihner, M.O., and Thomas, J.A. **2017**. Pharmacokinetics, Excretion Balance, and Tissue Distribution of [^{14}C]-Labeled Glycolipids and Long Chain Fatty Acids ([^{14}C]-LCFA) from *Dacryopinax spathularia* in Rats. *Food Chem. Toxicol*. 2017 Aug 26. pii: S0278-6915(17)30489-1. doi: 10.1016/j.fct.2017.08.038. *In press*. [Epub ahead of print].

IMD Natural Solutions GmbH
Otto-Hahn-Str. 15
44227 Dortmund
Germany

Efficacy data for Jelly Mushroom Glycolipids (AM-1)

Date:	2017-09-15
Document Type:	Report
Number of Pages:	8

1. Introduction

Jelly mushroom glycolipids from *Dacryopinax spathularia* (hereinafter referred to as "**AM-1**") are a mixture of glycolipids with antimicrobial properties. Its prominent antifungal effect makes AM-1 in particular useful as a **naturally derived preserving agent to prevent spoilage of beverages by common yeasts and molds.** Thus, AM-1 can substitute for frequently used chemical preservatives like benzoic acid, sorbic acid, or sulfites.

2. Microbial spoilage of beverages

A number of organisms are responsible for spoiling a variety of beverages materials, including cold-filled beverages. Most beverages have an acidic pH value, favoring the growth of fungi including yeasts and molds.

Typical spoilage yeasts in beverages include the genera *Saccharomyces*, *Zygosaccharomyces*, *Candida* and *Dekkera*. In particular yeasts of the genus *Zygosaccharomyces* have had a long history as spoilage yeasts within the food industry. This is due mainly to the fact that these species can grow in the presence of high sucrose, ethanol, acetic acid, sorbic acid, benzoic acid, and sulfur dioxide concentrations (which are some of the commonly used preservatives).

Typical spoilage molds in beverages include the genera *Penicillium*, *Aspergillus* and *Brettanomyces.* Further, heat resistant mold spores of *Byssochlamys*, its anamorphic (asexual) stages *Paecilomyces*, and *Neosartorya* spp. can survive pasteurization and may spoil hot-filled products, including carbonated beverages, sport drinks, and teas.

Also certain pathogenic bacteria are known to spoil beverages, e.g. *Bacillus cereus* or certain lactic acid bacteria. These are known to tolerate the acidic environment found in the beverage. Their growth is sometimes difficult to prevent, requiring the use of (ultra) high temperature treatment or other physical methods in combination with chemical preservative systems.

3. Limitations of currently used chemical preservatives

Current preservation systems for acidic, shelf-stable, carbonated and non-carbonated beverages, e.g. soft drinks, generally rely on weak acid preservatives (i.e., benzoic and/or sorbic acid and their salts). Benzoic and sorbic acids inhibit growth of yeasts, molds, and bacteria with some exceptions and limitations.

The weak acids in beverages exist in equilibrium between their dissociated and non-dissociated forms, which is dependent on the dissociation constant of the acid (pKa) and the beverage's pH. The pKa for benzoic acid is 4.19 and the pKa of sorbic acid is 4.76. A beverage pH below the pKa of the involved acid pushes the equilibrium towards the non-dissociated form, which is the antimicrobially active form. Therefore, weak acid preservatives are only effective in the low pH range.

Further, genetic adaptation of microorganisms is of growing concern also in the beverage industry (Piper, 2001; Stratford, 2013). Certain yeast strains identified as *Zygosaccharomyces bailii*, *Z. bisporus*, *Candida krusei*, and *Saccharomyces cerevisiae* have developed specific genes that enable them to resist the weak acid preservatives and grow (Mira, 2013). The levels of weak acids necessary

to overcome this adaptation are often far beyond regulatory limits on use levels. Therefore, spoilage of preserved teas, juice-containing beverages, and carbonated beverages is due to preservative-adapted microorganisms.

Another intrinsic disadvantage of benzoic and sorbic acid is the fact that these organic acids have a taste impact on the beverage, as their typical use level is rather high (100 ppm and above). This off-taste is usually undesired and needs to be considered during development and refinement of the beverage composition, e.g. by masking this taste effect with added flavors.

4. Minimum Inhibitory Concentrations (MICs) of AM-1 against spoilage organisms

AM-1 exhibits excellent antimicrobial activity against relevant spoilage organisms, as shown by the Minimum Inhibitory Concentrations (MICs). MIC values for AM-1 are much lower than those of the benchmarks sorbic acid and benzoic acid tested in parallel, allowing for low use levels in finished beverages to achieve the desired technical effect.

Generally, AM-1 has

- Very potent activity against yeast and mold
- Good activity against Gram-positive bacteria
- Weak or no activity against Gram-negative bacteria

MIC values were obtained using the micro-dilution method according to DIN 58940-8. Incubation was done in clear flat-bottom 96-well plates at 28 °C. MIC values were determined as the minimum concentration without visible growth (or detectable turbidity using a photometer). Inoculum concentration was adjusted to ca. 1 x 10^5 cells/ml as determined by viable cell count. For comparison, sodium benzoate and potassium sorbate were tested in parallel to AM-1. Concentrations in [mg/l] for the benchmarks are corrected to the corresponding free carboxylic acids.

	MIC [mg/l]			
Organism	**AM-1**	**Sorbic acid**	**Benzoic acid**	**Conditions**
Saccharomyces cerevisiae	3.1	250	250	SDB medium, pH 5.6, 28°C, 72 h
Aspergillus niger	3.1	250	250	
Zygosaccharomyces bailii	<3.9	>1000	>1000	Clear apple juice medium, pH 3.3, 28°C, 4 weeks
Dekkera bruxeliensis	<3.9	1000	250	
Aspergillus niger	7.8	1000	>1000	
Byssochlamys fulva	<3.9	500	500	

SDB = Sabouraud Dextrose Broth

MIC values of jelly mushroom glycolipids (AM-1) against further spoilage bacteria, yeasts, and molds:

Category	Taxonomy	MIC [mg/l]
Bacteria	*Bacillus cereus* (ATCC11778)	12.5
	Bacillus subtilis (ATCC6633)	1.6
	Propionibacterium acnes (ATCC6919)	60
	Clostridium perfringens (ATCC13124)	60
	Clostridium sporogenes (ATCC3584)	50
	Enterococcus faecalis (ATCC19433)	50
	Listeria welshimeri (DSM15452)	25
	Listeria monocytogenes (ATCC19111)	50
	Lactobacillus plantarum (DSM12028)	25
	Leuconostoc mesenteroides (ATCC 8293)	6.3
	Staphylococcus aureus (ATCC6538)	25
Molds	*Aspergillus fumigatus (ATCC1028)*	20
	Aspergillus niger (ATCC16404)	6.3
	Byssochlamys fulva (DSM62097)	3.1
	Mucor plumbeus (MUCL49355)	6.3
	Talaromyces luteus (CBS348.51)	<3.9
	Dekkera bruxellensis (DSM70726)	6.3
	Dekkera naardenensis (DSM70743)	12.5
Yeasts	*Saccharomyces cerevisiae (MUCL 53497)*	12.5
	Zygosaccharomyces bailii (DSM70492)	3.1
	Zygosaccharomyces bailii (ATCC 60484)	25
	Zygosaccharomyces bisporus (ATCC 52407)	3.1
	Zygosaccharomyces bisporus (DSM70415)	12.5
	Zygosaccharomyces florentinus (DSM70506)	6.3
	Zygosaccharomyces rouxii (NCYC381)	6.3
	Candida albicans (ATCC10231)	12.5

5. Application data for AM-1 in beverages

Application data for AM-1 were successfully determined in more than 150 commercially available beverages, proving the applicability of AM-1 in different beverage matrices.

Tests were designed as antimicrobial challenge tests, using the following test organisms:

Yeasts

Saccharomyces cerevisiae
Zygosaccharomyces rouxii
Zygosaccharomyces bailii

Molds

Aspergillus niger
Byssochlamys nivea
Penicillium roqueforti

AM-1 was added to the non-preserved beverages, usually as certain volume of a 10 mg/l stock solution in water. The beverage was mixed thoroughly to assure homogenous dissolution of AM-1. Then, the test organisms depicted above were added either as yeast mixture or mold mixture. In both cases, the initial inoculum was ca. 100 cfu/ml as proven by viable cell count at test start. Incubation was done at ambient temperature for three months and included benchmark controls (preserved with either benzoic or sorbic acid) and growth controls (i.e. the non-preserved beverage).

In case of flasks, tests were carried out in the original container without protection from light. In case of cans, Tetra-Pak® or other non-closable containers, the beverages were filled into sterile glass bottles before start of the test and protected against light during the test, as would have been the case in the original container.

Readout was done visually on a regular basis and – after three months – by determination of the viable cell count (cfu/ml) using the spread or pour plate method with SDB or OSA agar.

Results show that the minimum effective concentration of AM-1 depends on the nature of the beverage and can be summarized for each beverage category as follows:

Beverage category	Typical AM-1 use level [mg/l]
Carbonated soft drinks (CSD)	Cola or citrus-type, incl. concentrates: 3 – 10 10% juice, turbid: 10 – 25
Fruit drinks	Clear fruit drinks: 3 – 5 10% juice, turbid: 5 – 25 spritzer, nectar, other turbid fruit drinks: 25 – 80
Teas	Clear 3 – 10 With juice content: 10 – 50

Enhanced waters	2 – 25
Energy drinks	10 – 50
Sport drinks	3 – 50
Juice	Clear juices: 5 – 10 turbid, apple: 25 turbid, other: 50 – 100

Within a specific beverage category, the needed amount of AM-1 depends on pH value (low pH beverages need less AM-1), turbidity (low turbidity beverages need less AM-1) and carbonization (non-carbonated beverages need slightly higher AM-1 levels).

For comparison: under identical test conditions, required concentrations of benzoate and sorbate are above regulatory limits to pass the test (ca. 600 – 1200 mg/l for benzoic acid; ca. 600 – 900 mg/l for sorbic acid).

Example:

AM-1 was added to commercially obtained fruit drink “Capri Sun Multivitamin” containing 12% juice and no declared preservative. AM-1 was applied at 10 mg/l use level. Without AM-1, rapid spoilage was observed within a few days, while AM-1 treated beverages were without microbial growth until the end of the test after 3 months. Sorbate and benzoate treated comparisons needed 1000 ppm each to pass the test while 500 ppm samples failed.

Example:

Preservation of 6 citrus-type carbonated soft drinks

AM-1 was applied to 6 different citrus-type carbonated soft drinks at **3 (clear) to 10 (turbid)** ppm use level. Despite inoculation with mold or yeast mixtures, no microbial growth occurred during the three month test period. Without preservative, yeasts and molds grew visibly within 7- 14 days.

6. Comparison of antimicrobial efficacy of AM-1 with sorbate and benzoate

Efficacy against benzoate- and/or sorbate-tolerant spoilage yeasts:

Test of specific microorganisms (isolates from the beverage industry) with resistance/adaptation against sorbate and benzoate showed that, most likely due to a unique mode of action, no cross-resistance to existing preservative solutions exists for AM-1.

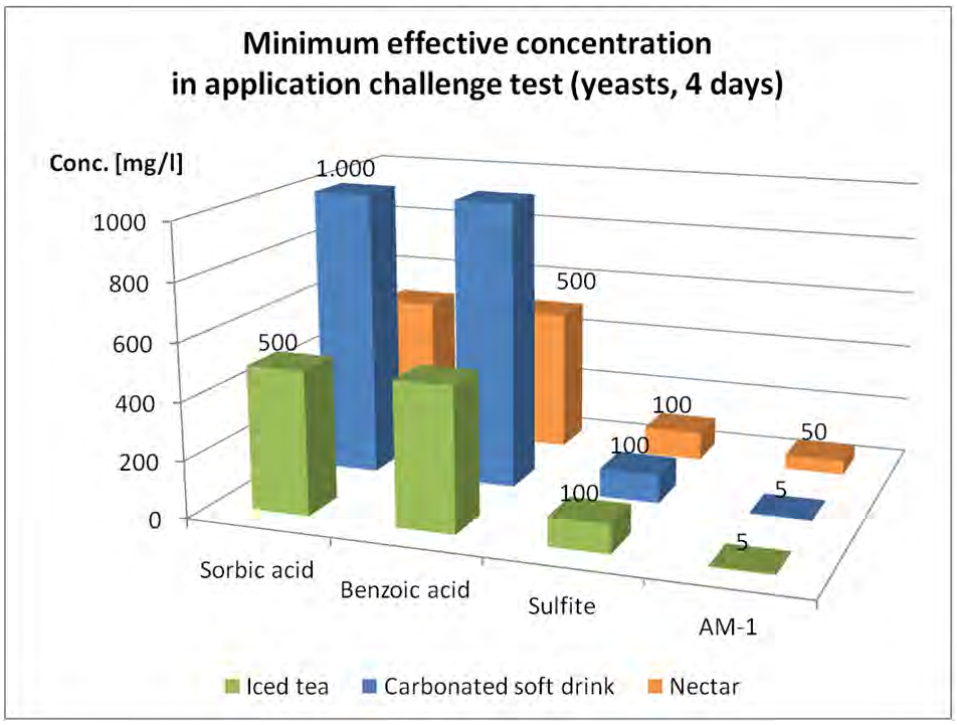
Example: *Saccharomyces cerevisiae* strain FU74037 was isolated as a sorbate- and benzoate-tolerant strain from a commercial carbonated soft drink. The MIC value at pH 3.6 against AM-1 was found to be **3.1 mg/l**, while 200 mg/l benzoate or benzoate could not completely inhibit growth of this organism.

Comparison of 4-day MIC data in selected beverages:

Four selected commercial beverages (bought from hot fill in Germany, i.e. non-preserved) were subjected to an antimicrobial challenge test as outlined in Section 5, but – deviating from the described protocol – with incubation at 28 °C for four days. Then, viable cell counts were determined using the streaking plate method on SDB agar plates.

Table: MIC values after 4 days in [mg/l] in selected beverages

	Carbonated soft drink		Nectar		Orange juice		Iced tea	
Preservative	yeasts	molds	yeasts	molds	yeasts	molds	yeasts	molds
Sorbic acid	1000	250	500	250	500	250	500	250
Benzoic acid	1000	100	500	100	2000	100	500	500
Metabisulfite	100	100	100	100	500	100	100	100
AM-1	5	5	50	25	100	50	5	3



7. References

Mira, N.P., Teixeira, M.C. (**2013**) Microbial mechanisms of tolerance to weak acid stress, *Front. Microbiol*. 4, 416

Piper, P., Ortiz Calderon, C., Hatzixanthis, K., Mollapour, M. (**2001**) Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives, *Microbiology* 147, 2635-2642.

Stratford, M., Steels, H., Nebe-von-Caron, G., Novodvorska, M., Hayer, K., Archer, D.B. (**2013**) Extreme resistance to weak-acid preservatives in the spoilage yeast Zygosaccharomyces bailii. *Int. J. Food Microbiol*. 166, 126-134.

Stadler, M., Bitzer, J., Köpcke, B., Reinhardt, K., Moldenhauer, J. (**2012**) Long chain glycolipids useful to avoid perishing or microbial contamination of materials. WO2012/167920A1.

APPENDIX 2.

Exposure Assessment for AM-1 in Select Beverages (Exponent, 2016)

Exponent®

Center for Chemical Regulation and Food Safety

Exposure Assessment for AM-1 in Select Beverages

Exposure Assessment for AM-1 in Select Beverages

Prepared for

Dr. Thomas Henkel
Managing Director
IMD Natural Solutions GmbH
Otto-Hahn-Str. 15
44227 Dortmund
Germany

Prepared by

Exponent, Inc.
1150 Connecticut Ave, NW
Suite 1100
Washington, DC 20036

April 14, 2016

1507390.000 - 9989

Contents

List of Tables

List of Acronyms

bw	Body weight
DHHS	U.S. Department of Health and Human Services
EDI	Estimated Daily Intake
FARE®	Foods and Residues Evaluation Program®
FDA	U.S. Food and Drug Administration
FNDDS	Food and Nutrient Database for Dietary Studies
g	Grams
mo	Months
NCHS	National Center for Health Statistics
NFS	Not Further Specified
NHANES	National Health and Nutrition Examination Survey
NS	Not Specified
RTD	Ready-to-drink
U.S.	United States
USDA	U.S. Department of Agriculture
VIF	Variance inflation factor
y	Years

Introduction

At the request of IMD Natural Solutions GmbH (INS), Exponent conducted an exposure assessment to estimate the total daily intake of a new material, AM-1, proposed to be added to seven beverage types. The estimated daily intake (EDI) of AM-1 was calculated following the U.S. Food and Drug Administration's (FDA's) guidance to industry[1] using food consumption data from the 2009-2012 National Health and Examination Survey (NHANES) and is provided for the total U.S. population and 5 subpopulations including: 1.) infants, ages 0 to 11 months (mo), 2.) children, ages 1 to 6 years (y), 3.) children, ages 7 to 12 y, 4.) adolescents, ages 13 to 19 y, and 5.) adults, ages 20 y and older. The data and methods used to conduct the intake assessment and results are summarized in this report.

[1]http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm074725.htm

Data and Methods

Proposed Use and Levels

AM-1 is proposed for use in seven beverage types within the following three broad food categories: beverages and beverage bases, nonalcoholic; coffee and tea; and processed fruits and fruit juices. The maximum proposed use levels of AM-1 range from 25 ppm to 100 ppm in the finished product. Table 1 lists the proposed use beverage categories as well as the corresponding maximum use level of AM-1 for each beverage type.

Table 1. Proposed uses and levels for AM-1 in beverages

Beverage category	Beverage type	Maximum AM-1 Use Level (ppm)
Beverages and beverage bases, nonalcoholic	Carbonated soft drinks	25
	Fruit drinks	80
	Sport drinks	50
	Energy drinks	50
	Enhanced waters	25
Coffee and tea	Tea, RTD	50
Processed fruits and fruit juices	Juice	100

RTD = ready-to-drink

Consumption Data

Intakes of AM-1 from proposed beverage types were derived using the What We Eat in America (WWEIA) dietary component of the National Health and Nutrition Examination Surveys (NHANES) 2009-2012. This continuous survey uses a complex multistage probability sample designed to be representative of the civilian U.S. population (NCHS 2012, 2014). The NHANES datasets provide nationally representative nutrition and health data and prevalence estimates for nutrition and health status measures in the United States. As part of the examination, trained dietary interviewers collected detailed information on all foods and beverages consumed by respondents in the previous 24 hour time period (midnight to midnight). A second dietary recall was administered by telephone three to ten days after the first dietary

interview, but not on the same day of the week as the first interview. The dietary component of the survey is conducted as a partnership between the U.S. Department of Agriculture (USDA) and the U.S. Department of Health and Human Services (DHHS). DHHS is responsible for the sample design and data collection, and USDA is responsible for the survey's dietary data collection methodology, maintenance of the databases used to code and process the data, and data review and processing. A total of 16,011 individuals in the survey period 2009-2012 provided two complete days of dietary recalls.

Analysis

Using the WWEIA consumption data, Exponent estimated the 2-day average daily intake of AM-1 on a "per capita" and "per user" basis. In this analysis, a user was anyone who reported consuming any of the proposed beverages on either of the survey days (USDA's user definition). We identified each participant who reported consuming a proposed beverage on either of the survey days, and we used that individual's responses for both survey days. Zero consumption days are included in calculating that individual's average daily intake. For example, if someone reported consuming 240 grams (g) of juice on day 1 and 120 g of juice on day 2, his/her 2-day average juice consumption would be 180 g ([240+120]/2). The analysis was limited to individuals who provided two complete and reliable dietary recalls as determined by NCHS. The 2-day average intakes by each individual were estimated using Exponent's Foods and Residues Evaluation Program (FARE® version 11.14) software. Exponent uses the statistically weighted values from the survey in its analyses following the analytical guidelines provided by NHANES (NCHS 1996). The statistical weights compensate for variable probabilities of selection, adjusted for non-response, and provide intake estimates that are representative of the U.S. population. Intake estimates for AM-1 were calculated in units of milligrams per day (mg/day), as well as normalized by bodyweight (mg/kg-bw/day) based on each NHANES individual's bodyweight.

In the analysis, the 2-day average intake of AM-1 was estimated by multiplying the reported intake of beverages from the 24-hr recall with the proposed maximum use level (see Table 1)

and the cumulative sum over the two 24-hr recalls was divided by two. Estimates of intake that may be less statistically reliable are flagged based on guidance from NCHS (NCHS 1996). Specifically, estimated mean intakes are flagged when based on a sample size of less than 30 times the variance inflation factor (VIF) (30 x VIF) and estimates of 90th percentile of intakes are flagged when based on a sample size of less than 8 times the VIF divided by 0.10 (8 x VIF/0.10). VIF estimates of 2.04 and 2.5 were estimated by USDA for the NHANES periods 2009-2010 and 2011-12 (USDA 2012; USDA 2014b). We are not aware of a published VIF estimate for the combined NHANES 2009-2012. Using a VIF of 2.5, estimated per user mean intakes are flagged when based on a sample size of less than 75 (30 x 2.5). Similarly, using a VIF of 2.5, estimated per user 90th percentile intakes are flagged when based on a sample size of less than 200 (8 x 2.5/0.10).

Selection of NHANES Food Codes

Exponent identified foods reported consumed in the WWEIA, NHANES 2009-12 that correspond to any of the seven beverage types with proposed uses of AM-1. The list of food codes (and their description) that were included in the intake assessment is provided in Appendix I. Consumption data in the NHANES survey are reported on an "as consumed basis". That is, if a survey participant consumed a burrito, the consumption amount reported in the survey for that subject would be for the total amount of the burrito consumed, and not for the ingredients (cheese, salsa, tortilla, rice, and ground beef) used to make that burrito. In cases where only a component of the food is intended for the proposed use of AM-1, Exponent utilized USDA's Food and Nutrient Database for Dietary Studies (FNDDS), version 2011-2012 (USDA, 2014a), that translates the food as consumed into its corresponding ingredients (and gram amounts) or recipes. In the current assessment, USDA recipes were used to determine the contribution of soda, fruit drink, and/or juice to alcoholic mixed drinks (i.e., Bloody Mary, Long Island iced tea, Seven and Seven).

Powders for teas and beverages made from powder were excluded from the assessment as were baby products.

Results

Two-day average intake estimates of AM-1 from the proposed use in seven beverage types were calculated based on food consumption data collected in NHANES 2009-2012 and corresponding maximum AM-1 use levels. Both the *per capita* and *per user* mean and 90th percentile results for the total U.S. population, infants age 0-11 mo; children 1-6 y, 7-12 y; adolescents 13-19 y; and adults 20+ y in units of mg/day and mg/kg-bw/day are provided in Table 2 by each proposed beverage type. Table 3 summarizes the estimated cumulative intake of AM-1 from all proposed foods by population group in units of mg/day and mg/kg-bw/day.

Table 2. Mean 2-day average estimated daily intake of AM-1 for proposed use by beverage type among the total US population and subpopulations; NHANES 2009-2012

				Per Capita		Per User		Per Capita		Per User	
		Un-wtd	%	Mean	90th	Mean	90th	Mean	90th	Mean	90th
Main category	Sub-category	N[1]	User	- - - - mg/day - - - -				- - - - mg/kg-bw/day - - - -			
TOTAL US POPULATION											
Beverages and beverage bases, nonalcoholic	Carbonated soft drinks	8202	55.7	6.74	18.69	12.09	25.44	0.09	0.26	0.17	0.34
	Energy drinks	267	2.5	0.32	0	12.89	24.02	<0.005	0	0.17	0.33
	Enhanced waters	235	1.9	0.17	0	8.76	14.86	<0.005	0	0.13	0.24
	Fruit drinks	4470	23.4	4.68	17.36	20.01	39.61	0.09	0.30	0.40	0.83
	Sport drinks	1005	6.7	1.35	0	20.19	39.72	0.02	0	0.31	0.63
Coffee and tea	Tea, RTD	2258	15.9	2.95	10.68	18.59	38.62	0.04	0.14	0.25	0.50
Processed fruits and fruit juices	Juice	6767	38.3	8.55	26.51	22.30	43.38	0.18	0.50	0.48	1.01
INFANTS 0-11 MO											
Beverages and beverage bases, nonalcoholic	Carbonated soft drinks*	11	1.1	0.01	0	0.63	0.84	<0.005	0	0.07	0.10
	Energy drinks*	0	0	0	NA	0	NA	0	NA	0	NA
	Enhanced waters*	0	0	0	NA	0	NA	0	NA	0	NA
	Fruit drinks*	36	3.8	0.27	0	7.13	11.06	0.03	0	0.78	1.39
	Sport drinks*	11	1.3	0.05	0	4.19	7.72	0.01	0	0.51	1.13
Coffee and tea	Tea, RTD*	9	1.7	0.06	NA	3.36	NA	0.01	NA	0.35	NA
Processed fruits and fruit juices	Juice**	97	12.2	1.19	2.90	9.75	22.57	0.13	0.31	1.03	2.20

Main category	Sub-category	Un-wtd N[1]	% User	Per Capita Mean	Per Capita 90th	Per User Mean	Per User 90th	Per Capita Mean	Per Capita 90th	Per User Mean	Per User 90th
				- - - - mg/day - - - -				- - - - mg/kg-bw/day - - - -			
CHILDREN 1-6 Y											
Beverages and beverage bases, nonalcoholic	Carbonated soft drinks	659	31.2	1.11	4.25	3.57	7.62	0.06	0.22	0.19	0.37
	Energy drinks*	0	0	0	NA	0	NA	0	NA	0	NA
	Enhanced waters*	13	0.6	0.03	0	4.52	6.42	<0.005	0	0.25	0.31
	Fruit drinks	952	40.8	6.33	19.89	15.51	30.95	0.37	1.17	0.90	1.74
	Sport drinks**	125	6.0	0.56	0	9.37	19.48	0.03	0	0.55	1.09
Coffee and tea	Tea, RTD**	116	4.3	0.30	0	6.95	15.61	0.02	0	0.41	0.86
Processed fruits and fruit juices	Juice	1535	67.6	13.89	35.14	20.55	43.20	0.88	2.25	1.30	2.90
CHILDREN 7-12 Y											
Beverages and beverage bases, nonalcoholic	Carbonated soft drinks	1050	57.0	3.82	10.79	6.71	13.87	0.10	0.27	0.18	0.35
	Energy drinks*	5	0.1	0.01	NA	3.49	NA	<0.005	NA	0.04	NA
	Enhanced waters*	30	2.0	0.14	0	6.69	22.11	<0.005	0	0.16	0.27
	Fruit drinks	907	43.5	6.95	21.45	15.97	30.02	0.20	0.60	0.45	0.86
	Sport drinks**	186	11.1	1.58	3.78	14.25	33.47	0.04	0.08	0.37	0.71
Coffee and tea	Tea, RTD**	142	7.0	0.77	0	11.04	21.40	0.02	0	0.28	0.51
Processed fruits and fruit juices	Juice	1019	50.1	8.53	24.29	17.04	31.26	0.24	0.66	0.48	0.91
ADOLESCENTS 13-19 Y											
Beverages and beverage bases, nonalcoholic	Carbonated soft drinks	1154	64.4	7.61	19.77	11.81	23.07	0.11	0.28	0.18	0.35
	Energy drinks*	39	3.7	0.55	0	14.81	27.09	0.01	0	0.19	0.33
	Enhanced waters*	54	3.7	0.28	0	7.46	11.80	<0.005	0	0.12	0.19
	Fruit drinks	669	30.4	6.52	21.67	21.46	47.64	0.10	0.36	0.34	0.71
	Sport drinks	240	14.2	2.70	11.16	19.09	35.24	0.04	0.16	0.28	0.52
Coffee and tea	Tea, RTD	320	18.8	3.12	12.07	16.60	31.38	0.05	0.17	0.25	0.43
Processed fruits and fruit juices	Juice	785	39.2	9.26	27.46	23.61	44.98	0.15	0.44	0.37	0.73

Main category	Sub-category	Un-wtd N[1]	% User	Per Capita Mean (mg/day)	Per Capita 90th (mg/day)	Per User Mean (mg/day)	Per User 90th (mg/day)	Per Capita Mean (mg/kg-bw/day)	Per Capita 90th (mg/kg-bw/day)	Per User Mean (mg/kg-bw/day)	Per User 90th (mg/kg-bw/day)
ADULTS 20+ Y											
Beverages and beverage bases, nonalcoholic	Carbonated soft drinks	5328	58.2	7.71	21.36	13.23	27.46	0.09	0.26	0.16	0.33
	Energy drinks	223	2.9	0.36	0	12.62	23.94	<0.005	0	0.16	0.30
	Enhanced waters**	138	1.9	0.18	0	9.52	15.44	<0.005	0	0.13	0.24
	Fruit drinks	1906	18.7	4.09	14.83	21.89	43.34	0.05	0.19	0.27	0.56
	Sport drinks	443	5.4	1.26	0	23.34	48.55	0.02	0	0.28	0.56
Coffee and tea	Tea, RTD	1671	18.1	3.53	12.39	19.52	39.27	0.04	0.16	0.24	0.49
Processed fruits and fruit juices	Juice	3332	34.3	8.03	25.44	23.44	44.67	0.11	0.34	0.31	0.57

[1] Unweighted number of users; %user, per capita and per user estimates for NHANES derived using the statistical weights provided by the NCHS.

* Sample size inadequate to provide reliable estimates at the *per user* mean and 90th percentile of intake.

**Sample size inadequate to provide reliable estimates at the *per user* 90th percentile of intake.

NA = Not available; estimate not calculated due to small sample size.

Table 3. Mean 2-day average estimated daily intake of AM-1 for all proposed beverage types combined among the total US population and subpopulations; NHANES 2009-2012

			Per Capita		Per User		Per Capita		Per User	
	Un-wtd		Mean	90th	Mean	90th	Mean	90th	Mean	90th
Population	N	% User	- - - - mg/day - - - -				- - - - mg/kg-bw/day - - - -			
Total US	13504	85.4	24.76	55.36	28.99	58.56	0.43	0.98	0.51	1.09
Infants 0-11 mo	132	16.6	1.58	5.07	9.53	20.68	0.17	0.55	1.02	2.26
Children 1-6 y	1995	89.1	22.21	47.34	24.92	49.35	1.36	3.06	1.52	3.29
Children 7-12 y	1783	93.1	21.79	43.88	23.41	44.44	0.60	1.24	0.64	1.27
Adolescents 13-19 y	1713	92.2	30.04	62.58	32.57	64.02	0.46	0.98	0.50	1.03
Adults 20+ y	7882	84.6	25.15	56.64	29.71	60.76	0.32	0.71	0.37	0.75

[1] Unweighted number of users; %user, per capita and per user estimates for NHANES derived using the statistical weights provided by the NCHS.

References

National Center for Health Statistics (NCHS). 2014. National Health and Nutrition Examination Survey Data 2009-2010. Hyattsville, MD: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Available from: http://wwwn.cdc.gov/Nchs/Nhanes/Search/nhanes09_10.aspx.

National Center for Health Statistics (NCHS). 2012. National Health and Nutrition Examination Survey Data 2011-2012. Hyattsville, MD: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Available from: http://wwwn.cdc.gov/Nchs/Nhanes/Search/nhanes11_12.aspx.

National Center for Health Statistics (NCHS). 1996. Analytic and Reporting Guidelines: The Third National Health and Nutrition Examination Survey, NHANES III (1988-94). Hyattsville, MD: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Available at: http://www.cdc.gov/nchs/data/nhanes/nhanes3/nh3gui.pdf.

U.S. Department of Agriculture, Agricultural Research Service. 2014a. USDA Food and Nutrient Database for Dietary Studies 2011-2012. Food Surveys Research Group Home Page, http://www.ars.usda.gov/ba/bhnrc/fsrg.

U.S. Department of Agriculture, Agricultural Research Service. 2014b. Nutrient Intakes from Food and Beverages: Mean Amounts Consumed per Individual, by Gender and Age, What We Eat in America, NHANES 2011-2012. Available at: http://www.ars.usda.gov/SP2UserFiles/Place/80400530/pdf/1112/Table_1_NIN_GEN_11.pdf

U.S. Department of Agriculture, Agricultural Research Service. 2012. Energy Intakes: Percentages of Energy from Protein, Carbohydrate, Fat, and Alcohol, by Race/Ethnicity and Age, What We Eat in America, NHANES 2009-2010. Available at: http://www.ars.usda.gov/SP2UserFiles/Place/80400530/pdf/0910/Table_6_EIN_RAC_09.pdf.

Appendix I: Foods Included in Analysis

	Food code	Food description
Beverages and beverage bases, nonalcoholic		
Carbonated Soft Drinks	92400000	Soft drink, NFS
	92400100	Soft drink, NFS, sugar-free
	92410310	Soft drink, cola-type
	92410315	Soft drink, cola type, reduced sugar
	92410320	Soft drink, cola-type, sugar-free
	92410330	Soft drink, cola-type, with higher caffeine
	92410340	Soft drink, cola-type, decaffeinated
	92410350	Soft drink, cola-type, decaffeinated, sugar-free
	92410360	Soft drink, pepper-type
	92410370	Soft drink, pepper-type, sugar-free
	92410390	Soft drink, pepper-type, decaffeinated
	92410400	Soft drink, pepper-type, decaffeinated, sugar-free
	92410410	Cream soda
	92410420	Cream soda, sugar-free
	92410510	Soft drink, fruit-flavored, caffeine free
	92410520	Soft drink, fruit-flavored, sugar free, caffeine free
	92410550	Soft drink, fruit flavored, caffeine containing
	92410560	Soft drink, fruit flavored, caffeine containing, sugar-free
	92410610	Ginger ale
	92410620	Ginger ale, sugar-free
	92410710	Root beer
	92410720	Root beer, sugar-free
	92411510	Cola with fruit or vanilla flavor
	92411610	Cola with fruit or vanilla flavor, sugar-free
	92416010	Mavi drink
	92431000	Carbonated juice drink, NS as to type of juice
	92432000	Carbonated citrus juice drink
	92433000	Carbonated noncitrus juice drink
	92804000	Shirley Temple*
	93301000	Cocktail, NFS*
	93301142	Seven and Seven*
	93301190	Rum and cola*
	93301270	Fruit punch, alcoholic*
	93301360	Long Island iced tea*
Energy drinks	92650000	Red Bull Energy Drink
	92650005	Red Bull Energy Drink, sugar-free
	92650200	Monster Energy Drink
	92650205	Mountain Dew AMP Energy Drink
	92650210	Mountain Dew AMP Energy Drink, sugar-free
	92650700	Rockstar Energy Drink
	92650705	Rockstar Energy Drink, sugar-free

	Food code	Food description
	92650800	Vault Energy Drink
	92650805	Vault Zero Energy drink
	92651000	Energy drink
	95310200	Full Throttle Energy Drink
	95310400	Monster Energy Drink
	95310500	Mountain Dew AMP Energy Drink
	95310560	NOS Energy Drink
	95310600	Red Bull Energy Drink
	95310700	Rockstar Energy Drink
	95310750	SoBe Energize Energy Juice Drink
	95310800	Vault Energy Drink
	95311000	Energy Drink
	95312400	Monster Energy Drink, Lo Carb
	95312500	Mountain Dew AMP Energy Drink, sugar-free
	95312550	No Fear Energy Drink, sugar-free
	95312600	Red Bull Energy Drink, sugar-free
	95312700	Rockstar Energy Drink, sugar-free
	95312800	Vault Zero Energy Drink
	95312900	XS Energy Drink
Enhanced water	94210100	Propel Water
	94210200	Glaceau Water
	94210300	SoBe Lifewater
	94220200	Glaceau Water, low calorie
Fruit drinks	11552200	Orange Julius
	42403010	Coconut water (liquid from coconuts)
	42404010	Coconut water, canned or bottled
	64200100	Fruit nectar, NFS
	64201010	Apricot nectar
	64202010	Cantaloupe nectar
	64203020	Guava nectar
	64204010	Mango nectar
	64205010	Peach nectar
	64210010	Papaya nectar
	64213010	Passion fruit nectar
	64215010	Pear nectar
	64221010	Soursop (Guanabana) nectar
	92510610	Fruit juice drink
	92510650	Tamarind drink, Puerto Rican (Refresco de tamarindo)
	92510720	Fruit punch, made with fruit juice and soda
	92510730	Fruit punch, made with soda, fruit juice, and sherbet or ice cream
	92511010	Fruit flavored drink (formerly lemonade)
	92511250	Citrus fruit juice drink, containing 40-50% juice
	92512040	Frozen daiquiri mix, frozen concentrate, not reconstituted
	92512050	Frozen daiquiri mix, from frozen concentrate, reconstituted
	92512090	Pina Colada, nonalcoholic
	92512110	Margarita mix, nonalcoholic

	Food code	Food description
	92513000	Fruit flavored frozen drink
	92530410	Fruit flavored drink, with high vitamin C
	92530510	Cranberry juice drink or cocktail, with high vitamin C
	92530610	Fruit juice drink, with high vitamin C
	92530950	Vegetable and fruit juice drink, with high vitamin C
	92531030	Fruit juice drink, with thiamin (vitamin B1) and high vitamin C
	92550030	Fruit juice drink, low calorie, with high vitamin C
	92550040	Fruit juice drink, low calorie
	92550110	Cranberry juice drink or cocktail, low calorie, with high vitamin C
	92550350	Light orange juice beverage, 40-50% juice, lower sugar and calories, with artificial sweetener
	92550400	Vegetable and fruit juice drink, low calorie, with high vitamin C
	92550405	Vegetable and fruit juice drink, low calorie, with high vitamin C plus added vitamin E and vitamin A
	92550610	Fruit flavored drink, low calorie, with high vitamin C
	92550620	Fruit flavored drink, low calorie
	92552020	Fruit juice drink, reduced sugar, with thiamin (vitamin B1) and high vitamin C
	92552030	Fruit juice drink, reduced sugar, with vitamin E
	92560000	Fruit-flavored thirst quencher beverage
	92582100	Fruit juice drink, with high vitamin C, plus added calcium
	92582110	Fruit juice drink, with thiamin (vitamin B1) and high vitamin C plus calcium
	92582120	Fruit flavored drink, reduced sugar, with high vitamin C, plus added calcium
	93301032	Cape Cod*
	93301141	Seabreeze*
Sport drinks	92560100	Gatorade Thirst Quencher sports drink
	92560200	Powerade sports drink
	92565000	Fruit-flavored sports drink or thirst quencher beverage, low calorie
	92565100	Gatorade G2 thirst quencher sports drink, low calorie
	92565200	Powerade Zero sports drink, low calorie
	95320200	Gatorade Thirst Quencher sports drink
	95320500	Powerade sports drink
	95321000	Fruit-flavored thirst quencher beverage
	95322200	Gatorade G2 Thirst Quencher sports drink, low calorie
	95323000	Fruit-flavored sports drink or thirst quencher beverage, low calorie
	95330100	Fluid replacement, electrolyte solution
	95330500	Fluid replacement, 5% glucose in water
Coffee and tea		
Tea RTD	92301000	Tea, NS as to type, unsweetened
	92301060	Tea, NS as to type, presweetened with sugar
	92301080	Tea, NS as to type, presweetened with low calorie sweetener
	92301100	Tea, NS as to type, decaffeinated, unsweetened
	92301130	Tea, NS as to type, presweetened, NS as to sweetener
	92301160	Tea, NS as to type, decaffeinated, presweetened with sugar
	92301180	Tea, NS as to type, decaffeinated, presweetened with low calorie

	Food code	Food description
		sweetener
	92301190	Tea, NS as to type, decaffeinated, presweetened, NS as to sweetener
	92304000	Tea, made from frozen concentrate, unsweetened
	92306000	Tea, herbal
	92306020	Tea, herbal, presweetened with sugar
	92306030	Tea, herbal, presweetened with low calorie sweetener
	92306040	Tea, herbal, presweetened, NS as to sweetener
	92307500	Half and Half beverage, half iced tea and half fruit juice drink (lemonade)
	92307510	Half and Half beverage, half iced tea and half fruit juice drink (lemonade), low calorie
Processed fruits and fruit juices		
Juice	11553000	Fruit smoothie drink, made with fruit or fruit juice and dairy products*
	11553100	Fruit smoothie drink, NFS*
	61201010	Grapefruit juice, freshly squeezed
	61201020	Grapefruit juice, NS as to form
	61201220	Grapefruit juice, canned, bottled or in a carton
	61201620	Grapefruit juice, frozen (reconstituted with water)
	61210000	Orange juice, NFS
	61210010	Orange juice, freshly squeezed
	61210220	Orange juice, canned, bottled or in a carton
	61210250	Orange juice, with calcium added, canned, bottled or in a carton
	61210620	Orange juice, frozen (reconstituted with water)
	61210820	Orange juice, frozen, with calcium added (reconstituted with water)
	61213000	Tangerine juice, NFS
	61213220	Tangerine juice, canned
	61213620	Tangerine juice, frozen (reconstituted with water)
	61213800	Fruit juice blend, including citrus, 100% juice
	61213900	Fruit juice blend, including citrus, 100% juice, with calcium added
	64100100	Fruit juice, NFS
	64100110	Fruit juice blend, 100% juice
	64100200	Fruit juice blend, with cranberry, 100% juice
	64101010	Apple cider
	64104010	Apple juice
	64104600	Blackberry juice
	64105400	Cranberry juice, 100%, not a blend
	64116020	Grape juice
	64120010	Papaya juice
	64121000	Passion fruit juice
	64124020	Pineapple juice
	64126000	Pomegranate juice
	64132010	Prune juice
	64132500	Strawberry juice
	64133100	Watermelon juice
	64134000	Fruit smoothie drink, made with fruit or fruit juice only (no dairy products)
	73105010	Carrot juice

Food code	Food description
74301100	Tomato juice
74301150	Tomato juice, low sodium
74302000	Tomato juice cocktail
74303000	Tomato and vegetable juice, mostly tomato
74303100	Tomato and vegetable juice, mostly tomato, low sodium
74304000	Tomato juice with clam or beef juice
75132000	Mixed vegetable juice (vegetables other than tomato)
75132100	Celery juice
75200700	Aloe vera juice
78101000	Vegetable and fruit juice blend, 100% juice, with high vitamin C plus added vitamin E and vitamin A
93301030	Bloody Mary*
93301115	Mimosa*
93301139	Salty Dog*
93301140	Screwdriver*
93301141	Seabreeze*
93301200	Pina Colada*
93301320	Tequila Sunrise*
93404500	Sangria*
95342000	MonaVie acai blend beverage

*Only ingredient component proposed for new material use is included in the assessment

APPENDIX 3.

Safety Evaluation of AM-1 Production Organism

IMD Natural Solutions GmbH
Otto-Hahn-Str. 15
44227 Dortmund
Germany

Characterization and Safety Evaluation - of *Dacryopinax spathularia* MUCL 53181 -

Date:	2017-09-11
Document Type:	Report
Number of Pages:	11

Summary

The basidiomycete *Dacryopinax spathularia* MUCL 53181 is the producer organism of the glycolipid mixture AM-1 (jelly mushroom glycolipids). The fungal strain has been thoroughly characterized by classical and molecular means. Species, genus, and family are known to produce edible fruiting bodies, and credible evidence for their traditional use as food is given. The organism does not have any toxigenic nor pathogenic potential. It is not genetically modified.

Based on a detailed safety evaluation we conclude that *Dacryopinax spathularia* MUCL 53181 is safe for the production of food-grade glycolipids.

Safety Evaluation

1. Introduction

The edible mushroom *Dacryopinax spathularia* strain MUCL 53181 (internal identifier: FU50088) is utilized for production **Jelly Mushroom Glycolipids** (hereinafter referred to as **AM-1**). The glycolipids are recovered in high purity from the culture broth and are proposed for use as a food additive. The glycolipid mixture has antimicrobial effects and, thus, can be used to preserve various foods and beverages against spoilage.

2. Taxonomical classification and information on the fungal species

The taxonomical information was obtained from MycoBank under ID 285971 (MycoBank, 2017).

Accepted name:	Dacryopinax spathularia (Schwein.) G.W. Martin, Lloydia 11 (2): 116 (1948)
Synonyms:	≡Merulius spathularius Schwein., Schriften der Naturforschenden Gesellschaft zu Leipzig 1: 92 (1822) ≡Guepinia spathularia (Schwein.) Fr., Elenchus Fungorum 2: 32 (1828) [MB#179747] ≡Cantharellus spathularius (Schwein.) Schwein., Transactions of the American Philosophical Society 4 (2): 153 (1832) [MB#490821] ≡Guepiniopsis spathularia (Schwein.) Pat., Essai taxonomique sur les familles et les genres des Hyménomycètes: 30 (1900) [MB#499382]
Classification:	Fungi (kingdom), Basidiomycota (division), Basidiomycotina (subdivision), Dacrymycetes (class), Dacrymycetales (order), Dacrymycetaceae (family), Dacryopinax (genus)
Basionym:	Merulius spathularius Schwein., Schriften der Naturforschenden Gesellschaft zu Leipzig 1: 92 (1822)
Literature:	Martin, G.W. 1948. New or noteworthy tropical fungi - IV. Lloydia. 11(2):111-122

Above data are in full agreement with the respective entries at the NCBI Taxonomy Browser (Taxonomy ID 139277; http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=139277) and Index Fungorum (http://www.indexfungorum.org/Names/NamesRecord.asp?RecordID=285971). Access of all databases was on 2017-06-08.

Members of the class Dacrymycetes are characterized by their unique basidial morphology with two equidiametrous epibasidia, thus shaping the basidium like a tuning fork. In addition, they have dolipores with continuous parenthesomes. These common morphological and ultrastructural features were supported very well in various molecular phylogenetic studies (Hibbett, 2006, and references cited therein). The species of the Dacrymycetes belong to order Dacrymycetales, family Dacrymycetaceae. The latter family comprises eight genera, which have been traditionally separated on the basis of macroscopic (primarily relating to the basidiocarp habit) and microscopic (e.g., the wall thickness of marginal hyphae in the sterile parts of basidiocarps) morphological characters of the fruiting bodies (i.e., basidiocarps). However, this classification is not unequivocal and therefore gave rise to various alternative taxonomic concepts over the past decades. For instance, McNabb (McNabb 1965) gave the first comprehensive treatment of *Dacryopinax* and described its basidiocarp habit as follows; “Fructifications extremely variable in shape, stipitate with a spathulate, petaloid, flabellate, cupulate, obliquely cupulate, inversely cupulate, foliose, or occasionally lobed and somewhat morchelloid pileus”. This circumscription, which is still valid today, suggests that *Dacryopinax* is a complex genus. In addition, recent molecular phylogenetic studies (Shirouzu et al., 2007 and 2009) suggested that convergent evolutionary developments in the Dacrymycetales and Dacrymycetaceae might have given rise to development of similar morphological features, hence the basidiocarps that are characteristic of *Dacryopinax* and other genera of Dacrymycetaceae might have evolved independently more than once.

The fungal genus *Dacryopinax* G.W. Martin was erected by (Martin, 1948) and currently comprises 23 accepted taxa, including 22 species and one variety (MycoBank, 2017). The currently accepted type species of *Dacryopinax* is *Dacryopinax elegans*. However, by far the most cited species in the literature is *Dacryopinax spathularia* (Schwein Fr.) G.W. Martin. This species was first described from South Carolina, USA, and had been treated under different names (i.e., *Merulius spathularius*, *Guepinia spathularia*) before Martin proposed the genus *Dacryopinax*. Under *Guepinia spathularia* (which is an invalid, later synonym of a plant genus name and therefore had to be abandoned), this fungus was already reported to occur in various tropical and subtropical regions of the world, including Northern Australia, New Zealand, Asia and North America (Saccardo, 1888) and has since then been reported from numerous other countries of the world, including Malaysia (Lee, 2009) and Indonesia (Ellen, 2008). *Dacryopinax spathularia* possesses an unusual geographical distribution. According to (McNabb, 1965) it is widely distributed throughout both hemispheres, but has never been found in Europe, though it occurs in North Africa and eastern Russia. *Dacryopinax spathularia* is capable of producing edible, comparably large, typically spathulate basidiocarps. Further, the species is characterised by uniseptate spores, and thick-walled, cylindrical abhymenial hairs.

There are no reports in the literature of any poisonous species produced by the fungal family Dacrymycetaceae, even though the basidiocarps of most species are rather inconspicuous and/or have a tough, rubbery consistence that prevents their culinary use. Interestingly, the cultures of certain *Dacrymyces* species, which can be regarded as closely related to *Dacryopinax*, have been patented for their utility in production of carotene pigments (Farrow and Tabenkin, 1961).

Carotenoids are also apparently the only secondary metabolites that were so far reported from a species of *Dacryopinax*, and their production in the cultures of *D. spathularia* have been studied in detail (Vail and Lilly, 1986).

3. Characterization of the producer strain MUCL 53181

Dacryopinax spathularia MUCL 53181 was characterized by IMD Natural Solutions by morphological methodology, using phase contrast microscopy of cultures grown on solid YMG medium, and by molecular phylogenetic methods. Since the LSU or 28S/5.8S nuc-rDNA had recently been reported to be informative for the phylogenetic assessments of the Dacrymycetes (Shirouzu et al, 2009) and numerous reliable reference sequence data were available, this region of the DNA was chosen for molecular identification.

Strain MUCL 53181 was isolated from the sporocarp of an unidentified basidiomycete growing on wood in French Guiana by Sergej Buchet in 2002. On YMG agar at 23 °C, the culture attained about 10 mm diameter after 10 days of incubation. The mycelium at first appeared velvety and white, but soon attained a strong yellowish color. The occasional presence of clamp connections revealed that the fungus belongs to the Basidiomycota. After 5 days of incubation, conidiogenous cells appeared in abundance on the vegetative hyphae, showing polyblastic, sympodial conidiogenesis, producing subglobose hyaline conidia, averaging 5-6 × 2.5-3 µm in size. These characteristics were found to be largely in agreement with the data reported by Shirouzu et al. (2009).

DNA for PCR was isolated from YMG cultures. The 28S/5.8S nuc-rDNA regions were then amplified using primers LR7 and 5.8SR (Vilgalys Lab, Duke University, Durham, USA, http://www.biology.duke.edu/fungi/mycolab/primers.htm), using the PCR Taq PCR Core Kit (Qiagen), and applying a standard thermal profile with an annealing temperature of 53 °C. Amplification products were purified using SigmaSpin Post-Reaction Clean-Up columns (Sigma-Aldrich), using the protocol supplied by the manufacturer. Nucleotide sequences were obtained by cycle sequencing using a DNA Cycle Sequencing Kit (Jena Bioscience, Jena, Germany) and 5‘ IRD700-labelled primer LR0R (Vilgalys Lab). Labelled primers were custom synthesized by Eurofins MWG Operon, Ebersberg, Germany). The cycle sequencing products were then analysed using a LI-COR 4200 (Li-Cor Bioscience, Lincoln, NB) genetic analyser. The LSU nucrDNA sequence of strain MUCL 53181 is:

```
cccctagtaa ctgcgagtga agcgggaaaa gctcaaattt aaaatccctt cggggagttg      60
taatctagag acgtgttttc ggtcgttgcc tcggacaagt cccttggaac agggcgtcat     120
agagggtgag aatcccgtac ttgccgagct cccaatgact atgtgataca cgttcgaaga     180
gtcgagttgt ttgggaatcg agctcaaaat gggtgtgaaa ctccatctaa agctaaatat     240
tggcgagaga ccgata                                                    256
```

Strain MUCL 53181 was studied in comparison with an authentic strain of *Dacryopinax spathularia*, CBS 197.63, originating from Africa, which was obtained from the Centrallbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. Its morphological characteristics as well as its secondary metabolite production were in accordance with that of MUCL 53181. The conidia were subglobose to ovoid, measuring 3-6.5(-8) × 2.5-4 µm. Furthermore, a high degree of homology was observed between the 5.8S/ITS nrDNA and 28S nrDNA sequences of the two strains and reference DNA sequence data that had been published on the Internet by specialists in the taxonomy and phylogeny of Basidiomycota, under the name of *Dacryopinax spathularia* or synonyms thereof.

The LSU nucrDNA sequence of the type strain CBS 197.63 is presented below and has 98% identity to the sequence of MUCL 53181:

```
cccctagtaa ctgcgagtga agcgggaaaa gctcaaattt aaaatccctt ccgggagttg       60
taatctagag acgtgttttc ggtcgttgcc tcggacaagt cccttggaat agggcgtcat      120
agagggtgag aatcccgtac ttgccgagct cccaatgact atgtgataca cgttcgaaga      180
gtcgagttgt ttgggaatgc agctcaaaat gggtggtaaa ctccatctaa agctaaatat      240
tggcgagaga ccgata                                                      256
```

Figure 1 shows a phylogenetic tree derived from the LSU nucrDNA sequences of MUCL 53181, type strain CBS 197.63 and various other sequences of Dacrymycetaceae from public databases.

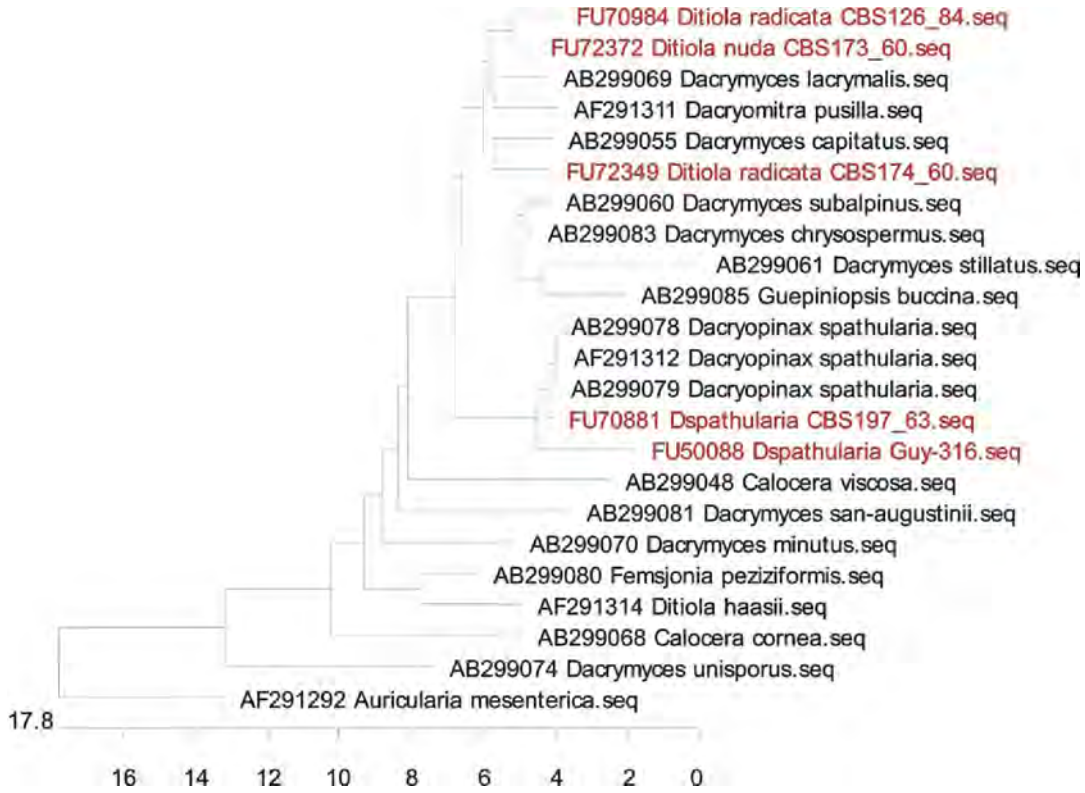


Figure 1: Phylogenetic tree. Strain MUCL 53181 is listed under the internal code "FU50088 Dspathularia Guy-316".

Dacryopinax spathularia MUCL 53181 is internally registered as FU50088 and has been deposited under the Budapest Treaty at BCCM/MUCL, Mycothèque de l'Université catholique de Louvain, Place Croix du Sud 3, B-1348 Louvain-la-Neuve, Belgium, under the designation number MUCL 53181 on 11.10.2010.

In summary, strain MUCL 53181 was unequivocally identified by INS to belong to the species *Dacryopinax spathularia* by a combination of morphological and molecular phylogenetic methods.

4. Toxigenic potential

Neither for the species *Dacryopinax spathularia* nor for any other species in the Dacrymycetaceae family has production of mycotoxins or other toxic compounds has been described. In contrast, many members of this fungal family are known as edible. The species *Dacryopinax spathularia* produces edible yellowish fruiting bodies. The Food and Agriculture Organization of the United Nations (FAO) lists in its compendium on edible mushrooms (Boa, 2004) proven food use for **China** (also documented by Zhishu, 1993), **Japan** (also documented by MycoBank, 2017), and by the indigenous population of **Cameroon** (also documented by van Dijk, 2003). Culinary use of the fruiting bodies is further described for **Malaysia** (Lee, 2009) and **India** (Ao, 2016). A field guide on foraging mushrooms in **Oregon (USA)** cites a recipe with *Dacryopinax spathularia* (Meuninck, 2017), next to a description for identification of this edible mushroom.

Also basidiocarps of some other members of the Dacrymycetaceae family are eaten, for example *Dacrymyces palmatus* in China (Boa, 2004) and North America (Wikipedia, 2017).

Furthermore, lyophilized fungal fermentation broth (two independent batches) and the glycolipid product AM-1 (two independent batches) were analyzed for the presence of mycotoxins. No mycotoxins were detected. The analyses were carried out compliant to European Commission (EC) Regulation No. 1881/2006 (consolidated version as of 03 Dec 2012) with regard to analysis items and EC Regulation No. 401/2006 (consolidated version as of 13 Mar 2010) which defines analytical methods performance criteria and sampling schemes. **Table 1** lists analysis items, the analytical method, and the detection limits.

In-house LCMS-based analysis using our proprietary analytical database containing the data of >15'000 pure natural reference compounds (Bitzer et al, 2007) also did not find any component with molecular weight <2000 Da except for the glycolipids to be present.

Table 1: Mycotoxins whose presence was excluded analytically.

Compound	Method	Detection limit [µg/kg]
Aflatoxin B1	LC-MS/MS	0.05
Aflatoxin B2	LC-MS/MS	0.03
Aflatoxin G1	LC-MS/MS	0.05
Aflatoxin G2	LC-MS/MS	0.03
Ochratoxin A	LC-MS/MS	0.02
Zearalenon	HPLC	10
Fumonisin B1	LC-MS/MS	50
Fumonisin B2	LC-MS/MS	50
Deoxynivalenol	LC-MS/MS	40
Nivalenol	LC-MS/MS	60
T2 Toxin	LC-MS/MS	10
HT2 Toxin	LC-MS/MS	50

The absence of any mycotoxins is in agreement with the literature and the reports on edibility and food use of *Dacryopinax spathularia*.

In conclusion, the organism does not to have any toxigenic potential.

5. Pathogenic potential

The species *Dacryopinax spathularia* is classified as Risk Group 1 by the German Federal Institute for Occupational Safety and Health (BAuA). This is consistent with WHO risk group 1, meaning that no or low individual and community risk is present and that the microorganism is unlikely to cause human disease or animal disease. WHO risk group 1 is the lowest risk group in the WHO classification system and used for other edible mushrooms as well, like e.g. *Agaricus* spp..

The risk group classification is further consistent CDC/NIH Biosafety Level 1 / Risk Group 1 for agents "not associated with disease in healthy adult humans" (CDC, 2009) and, hence, of minimal potential hazard to laboratory personnel and the environment, and with the European Group 1 (Directive 2000/54/EC and Directive 90/679/EEC) for "biological agents unlikely to cause human disease".

Not a single literature report was found on pathogenic effects of a fungal species belonging to the Dacrymycetes family.

It is concluded that *Dacryopinax spathularia* MUCL 53181 does not have any pathogenic potential. Furthermore, because the finished AM-1 product does not contain any live organisms, pathogenicity is not a concern with respect to consumer safety (Pariza and Johnson, 2001).

6. Presence of antibiotics

The only known secondary metabolites from *Dacryopinax* species are glycolipids (Nishida et al, 1988; Stadler et al, 2012) and carotenoids (Vail and Lilly, 1968). Furthermore, the production of polysaccharides has been described (Chen and Seviour, 2007).

The presence of antibiotics (i.e. potent anti-bacterial compounds such as Penicillin G) can be excluded based on extensive literature research and the results of our in-house analytics. We have searched extensively for small molecular weight components other than glycolipids and carotenoids produced by strain MUCL 53181 but did not find any traces by means of HPLC-MS, HPLC-UV and HPLC-ELSD.

Furthermore, our anti-bacterial bioassays only determined moderate anti-bacterial activity in the presence of the glycolipids. Removal of the glycolipids from the fermentation broth (e.g. by acidic precipitation) also removed the anti-bacterial activity.

All credible evidence available supports the absence of any medically or clinically relevant antibiotics in the fermentation broth of strain MUCL 53181. Because rapid hydrolysis and elimination of AM-1 occurs *in vivo* (Bitzer et al., 2017), the antimicrobial properties of the parent material would be absent or greatly diminished in the lower intestines so there is low concern for disruption of healthy gut microflora.

7. Decision tree

The strategy of our safety evaluation follows conceptually a decision tree proposed by (Pariza and Johnson, 2001) and the International Food Biotechnology Council (IFBC, 1990).

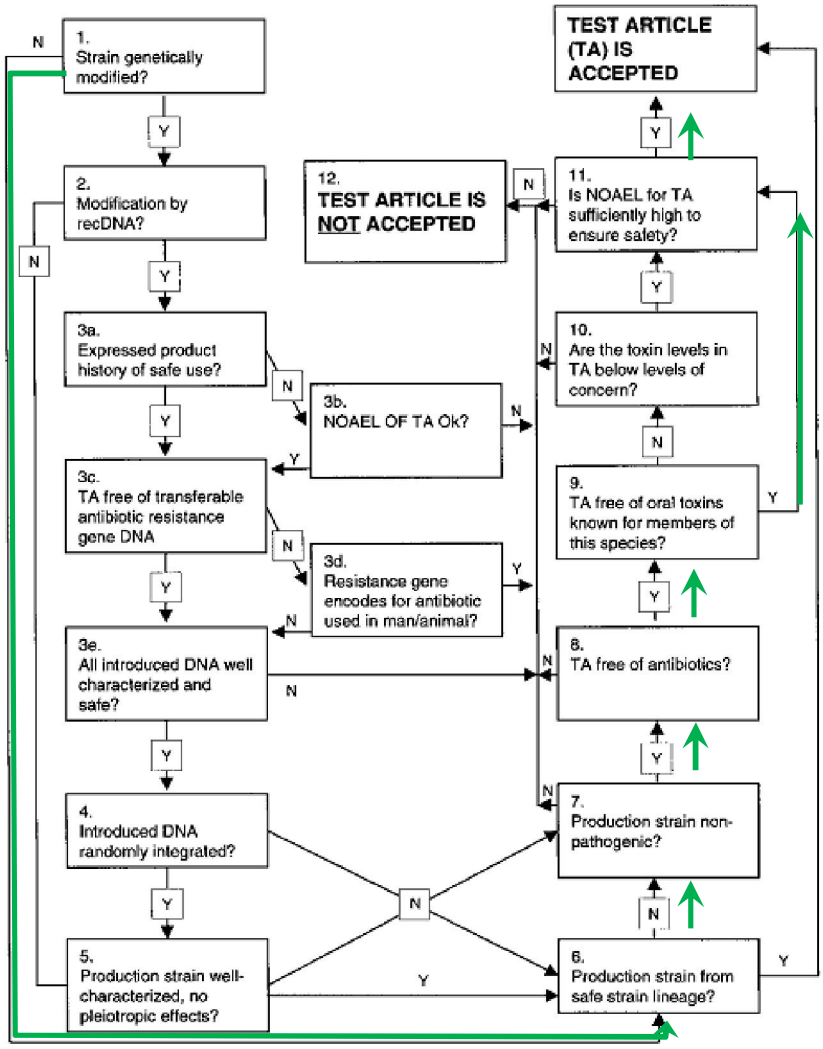


Figure 1: Decision tree proposed by (Pariza and Johnson, 2001). The green arrows follow the reasoning used in this document.

Remark: The term "safe strain lineage" refers to a cluster of related strains that have all been derived by genetic modification from a single isolate ("parental strain") that was thoroughly characterized and shown to be non-toxigenic and non-pathogenic before the modifications to improve enzyme function were initiated. It is understood that different strains of this lineage have a history of safe commercial use and products produced by strains within this lineage have been approved by authorities on the basis of toxicological tests.

The "safe strain lineage" concept does not apply for strain MUCL 53181 because the strain is not genetically modified and, hence, there is no parental strain.

8. Conclusion

The producer organism *Dacryopinax spathularia* MUCL 53181 has been thoroughly characterized by classical and molecular means.

The species is known to produce edible fruiting bodies, and credible evidence for their traditional use as food is given.

It was found that the organism does not have any toxigenic potential. This reasoning is supported by the documented edibility of the fruiting body and the absence of (myco-)toxins, both in the scientific literature on the species and in our analyses of the fermentation broth.

Furthermore, the organism does not have any pathogenic potential nor is it expected to be present in the finished glycolipid material that would be added to food.

Furthermore, the production of medically or clinically relevant antibiotics by the organism can be excluded.

Overall, we conclude that the producer organism *Dacryopinax spathularia* MUCL 53181 is safe for production of food-grade glycolipids.

References

Ao, T., Deb, C. R., Khruomo, N. (**2016**). Wild edible mushrooms of Nagaland, India: a potential food resource, *Journal of Experimental Biology and Agricultural Sciences* 4, 59-65.

Boa, E. R. (**2004**). Wild edible fungi. A global overview on their use and importance. Food and Agriculture Organization of the United Nations, ISBN 92-5-105157-7, p. 134.

Bitzer, J., Köpcke, B., Stadler, M., Hellwig, V., Ju, Y.-M-, Seip, S., Henkel, T. (**2007**). Accelerated Dereplication of Natural Products Supported by Reference Libraries. *Chimia* 61, 332-338.

Bitzer, J., Henkel, T., Nikiforov, A.I., Rihner, M.O., and Thomas, J.A. (**2017**). Pharmacokinetics, Excretion Balance, and Tissue Distribution of [^{14}C]-Labeled Glycolipids and Long Chain Fatty Acids ([^{14}C]-LCFA) from *Dacryopinax spathularia* in Rats. *Food Chem Toxicol*. 2017 Aug 26. pii: S0278-6915(17)30489-1. doi: 10.1016/j.fct.2017.08.038. In press. [Epub ahead of print].

Centers for Disease Control and Prevention (CDC), National Institutes of Health (**2009**). Biosafety in Microbiological and Biomedical Laboratories, 5th Edition, HHS Publication No. (CDC) 21-1112.

Chen, J., Seviour, R. (**2007**). Medicinal importance of fungal β-(1→3),(1→5)-glucans. *Mycol. Res.* 111, 635-652.

van Dijk, H., Onguene, N. A., Kuyper, T. W. (**2003**). Knowledge and Utilization of Edible Mushrooms by Local Populations of the Rain Forest of South Cameroon. *Ambio* 32, 19–23.

Ellen, R. (**2008**). Ethnomycology Amopn the Nuaulu of the Moluccas: Putting Berlin's "General Principles" of Ethnobiological Classification to the Test. *Economic Botany* 62, 483-496.

Farrow, W. M., Tabenkin, B. (**1961**). Microbiological production of carotenoids. US 2974044 A

Hibbett, D. S. (**2006**). A phylogenetic overview of the Agaricomycotina. *Mycologia* 98, 917-925.

International Food Biotechnology Council (IFBC). (**1990**). Safety evaluation of foods and food ingredients derived from microorganisms in biotechnologies and food: Assuring the Safety of Foods produced by Genetic modification. *Regul. Toxicol. Pharmacol*. 12, S1-S196.

Lee, S.S., Chang, Y.S., Noraswati, M.N.R. (**2009**). Utilization of macrofungi by some indigenous communities for food and medicine in Peninsular Malaysia. *Forest Ecology and Management* 257, 2062-2065.

Martin, G. W. (**1948**). New or noteworthy tropical fungi - IV. *Lloydia* 11, 111–122.

Meuninck, J. (**2017**). Foraging Mushrooms Oregon. Finding, Identifying, and Preparing Edible Wild Mushrooms. Falcon Guides, Rowman / Littlefield, pp. 74-75, ISBN 978-1-4930-2669-2.

McNabb, R. F. R. (**1965**): Taxonomic Studies on the Dacrymycetaceae. III. Dacryopinax Martin. *N. Z. J. Bot.* 3, 59-72,

MycoBank (**2017**). Online database provided the International Mycological Association, http://www.mycobank.org. Access on 2017-06-08.

Nishida, F., Mori, Y., Isobe, S., Furuse, T., Suzuki, M. (**1988**). Structures of deacetyl glykenins-1, A, and C, glycosidic antibiotics from Basidiomycetes sp. *Tetrahedron Lett*. 29, 5287-5290.

Pariza, M. W., Johnson, E. A. (**2001**). Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century. *Reg. Toxicol. Pharmacol*. 33, 173-186.

Saccardo, P.A. (**1888**). *Sylloge Fungorum* 6, 807-808.

Shirouzu, T., Hirose, D., Tokumasu, S. (**2007**). Sequence analyses of the 28S rRNA gene D1/D2 region suggest Dacrymyces (Heterobasidiomycetes, Dacrymycetales) is polyphyletic. *Mycoscience* 48, 388-394.

Shirouzu, T., Hirose, D., Tokumasu, S. (**2009**). Taxonomic study of the Japanese Dacrymycetes. *Persoonia* 23, 16-34.

Stadler, M., Bitzer, J., Köpcke, B., Reinhardt, K., Moldenhauer, J. (**2012**). Long chain glycolipids useful to avoid perishing or microbial contamination of materials. WO2012/167920A1.

Vail, W. J., and Lilly, V. G. (**1968**). The Location of Carotenoid Pigments and Thickness of the Cell Wall in Light- and Dark-Grown Cells of Dacryopinax spathularia. *Mycologia* 60, 902-907.

Wikipedia (**2017**): Dacrymyces palmatus. Last revision Nov 20, 2011. Retrieved Sept 11, 2017, from http://en.wikipedia.org/wiki/ Dacrymyces_palmatus.

Zhishu, B., Zheng, G., Taihui, L. (**1993**). The macrofungus flora of China's Guangdong province, The Chinese University Press of Hong Kong, pp. 51-52, ISBN 962-201-556-5.

APPENDIX 4.

Food Use of AM-1 Production Organism

IMD Natural Solutions GmbH
Otto-Hahn-Str. 15
44227 Dortmund
Germany

Food use of the edible mushroom - *Dacryopinax spathularia*

Date:	2017-09-11
Document Type:	Report
Number of Pages:	4

Food use of the edible mushroom *Dacryopinax spathularia*

1. Identification of genus and species

Accepted name: ***Dacryopinax spathularia*** (Schwein.) G.W. Martin, Lloydia 11 (2): 116 (1948)

Synonym: ***Cantharellus spathularius*** (Schwein.) Schwein., Transactions of the American Philosophical Society 4 (2): 153 (1832) [MB#490821]

Classification: Fungi (kingdom), Basidiomycota (division), Basidiomycotina (subdivision), Dacrymycetes (class), Dacrymycetales (order), Dacrymycetaceae (family)

(Mycobank, 2017)

The mushroom strain MUCL53181 used by IMD Natural Solutions has been collected in French Guiana in 2002. Identity and purity of the culture have been verified by classical methods (comprising macroscopic and microscopic investigation of morphology) and molecular phylogenetic methods (comprising amplification of genetic marker regions, LSU and ITS, and comparison with sequences obtained from authentic reference strains (e.g. *Dacryopinax spathularia* CBS197.63) (Stadler, 2012).

2. Credible evidence for food use

***Dacryopinax spathularia* is an edible jelly fungus**. The fruiting bodies are yellow to orange in color. In Chinese culture it is called *guihua er* ('sweet osmanthus ear').

The mushroom is also known under various synonyms (see Section 1), whereof ***Dacryopinax spathularia*** is the currently accepted name. However, the synonym ***Cantharellus spathularius*** better reflects its edibility and may be easier to understand for consumers.

The species was first described from South Carolina, USA, and had been treated under different names (i.e., *Merulius spathularius*, *Guepinia spathularia*) before Martin proposed the genus *Dacryopinax*. It occurs in various tropical and subtropical regions of the world, including Northern Australia, New Zealand, Asia, Africa, and North America (Saccardo, 1888; McNabb, 1965).

Cantharellus spathularius is also **native to Indonesia** (Ellen, 2008).

The culinary use of this mushroom is recorded for many countries. For instance, the mushroom is known as ingredient in a vegetarian dish called Buddha's delight or Lo han Jai, traditionally enjoyed by Buddhist monks (Wikipedia, 2017).

A field guide on foraging mushrooms in **Oregon (USA)** cites a recipe with *Dacryopinax spathularia* (Meuninck, 2017), next to a description for identification of this edible mushroom.

Food and Agriculture Organization of the United Nations

The Food and Agriculture Organization of the United Nations (FAO) lists in its compendium on edible mushrooms (Boa, 2004) proven food use of *Cantharellus spathularius* for **China** (also documented by Zhishu, 1993), **Japan** (also documented by MycoBank, 2017), and **Cameroon** (also documented by Van Dijk, 2003).

Like *C. spathularius*, also other species of the Dacryomycetaceae family are known as edible and confirmed in the FAO compendium, like for example *Dacrymyces palmatus* and *Ditiola peziziformis*.

Culinary use of the fruiting bodies is further described for **Malaysia** (Lee, 2009) and **India** (Ao, 2016).

Dacryopinax spathularia extract is commercially available in China for food use (Faces Biochemistry, 2017).

3. Absence of any toxigenic or pathogenic potential

The species *Dacryopinax spathularia* is classified as Risk Group 1 by the German Federal Institute for Occupational Safety and Health (BAuA). This is consistent with WHO risk group 1, meaning that no or low individual and community risk is present and that the microorganism is unlikely to cause human disease or animal disease. WHO risk group 1 is the lowest risk group in the WHO classification system and used for other edible mushrooms as well, like e.g. *Agaricus* spp.. Not a single literature report was found on pathogenic effects of a fungal species belonging to the Dacrymycetes family.

No member of the fungal family Dacryomycetaceae has the ability to produce mycotoxins, medicinal antibiotics or any other toxic metabolites. In contrast, many members of this fungal family are known as edible as depicted in **Section 2.** The only known secondary metabolites from *Dacryopinax spathularia* are glycolipids (Stadler et al, 2012) and carotenoids (Vail and Lilly, 1968). Furthermore, the production of polysaccharides has been described (Chen and Seviour, 2007).

Therefore, it is concluded that *Dacryopinax spathularia* strain MUCL 53181 does not have any pathogenic nor toxigenic potential.

4. References

Ao, T., Deb, C. R., Khruomo, N. (**2016**). Wild edible mushrooms of Nagaland, India: a potential food resource, *Journal of Experimental Biology and Agricultural Sciences* 4, 59-65.

Boa, E. (**2004**). Wild Edible Fungi: A Global Overview Of Their Use And Importance To People (Non-Wood Forest Products). Food & Agriculture Organization of the UN, p. 134. ISBN 92-5-105157-7.

Chen, J., Seviour, R. (**2007**). Medicinal importance of fungal β-(1→3),(1→5)-glucans. *Mycol. Res.* 111, 635-652.

Ellen, R. (**2008**). Ethnomycology Amopn the Nuaulu of the Moluccas: Putting Berlin's "General Principles" of Ethnobiological Classification to the Test. *Economic Botany* 62, 483-496.

Faces Biochemistry (**2017**). Product datasheet for Dacryopinax spathularia extract. Retrieved on July 3, 2017, from http://www.chemfaces.com.

Lee, S.S., Chang, Y.S., Noraswati, M.N.R. (**2009**). Utilization of macrofungi by some indigenous communities for food and medicine in Peninsular Malaysia. *Forest Ecology and Management* 257, 2062-2065.

McNabb, R. F. R. (**1965**). Taxonomic Studies on the Dacrymycetaceae. III. Dacryopinax Martin. *N. Z. J. Bot.* 3, 59-72.

Meuninck, J. **(2017).** Foraging Mushrooms Oregon. Finding, Identifying, and Preparing Edible Wild Mushrooms. Falcon Guides, Rowman / Littlefield, pp. 74-75, ISBN 978-1-4930-2669-2.

MycoBank (**2017**). Online database provided by the International Mycological Association. Retrieved June 6, 2107 from http://www.mycobank.org/

Saccardo, P.A. (**1888**). *Sylloge Fungorum* 6, 807-808.

Stadler, M., Bitzer, J., Köpcke, B., Reinhardt, K., Moldenhauer, J. (**2012**). Long chain glycolipids useful to avoid perishing or microbial contamination of materials. WO2012/167920A1.

Vail, W. J., Lilly, V. G. (**1968**). The Location of Carotenoid Pigments and Thickness of the Cell Wall in Light- and Dark-Grown Cells of Dacryopinax spathularia. *Mycologia* 60, 902-907.

van Dijk, H, Onguene, N. A., Kuyper, T. W. (**2003**). Knowledge and Utilization of Edible Mushrooms by Local Populations of the Rain Forest of South Cameroon. *Ambio* 32, 19–23.

Wikipedia (**2017**). Dacryopinax spathularia. Last revision Feb 4, 2017. Retrieved June 6, 2017, from http://en.wikipedia.org/wiki/Dacryopinax_spathularia.

Zhishu, B., Zheng, G., Taihui, L. (**1993**). The macrofungus flora of China's Guangdong province, The Chinese University Press of Hong Kong, pp. 51-52, ISBN 962-201-556-5.

EXHIBIT I.

EXPERT PANEL OPINION

Expert Panel Report on the Generally Recognized As Safe (GRAS) Determination for Long-Chain Glycolipids from *Dacryopinax spathularia*

October 19, 2017

Introduction

IMD Natural Solutions GmbH (INS) intends to use the ingredient, long-chain glycolipids from *Dacryopinax spathularia*, herein also referred to as "jelly mushroom glycolipids" or "AM-1", as an antimicrobial preservative in select non-alcoholic beverages. A panel of independent experts (the "Expert Panel"), qualified by their scientific training and relevant national and international experience to evaluate the safety of food ingredients, was convened to conduct an independent, critical and comprehensive evaluation of the available technical and safety information on jelly mushroom glycolipids (AM-1) and to determine if the proposed uses of AM-1 as a food ingredient are safe and suitable and can be considered Generally Recognized As Safe (GRAS) based on scientific procedures. The Expert Panel consisted of Professor John A. Thomas, Ph.D. (University of Indiana School of Medicine), Michael Carakostas, DVM, Ph.D. (MC Scientific Consulting, LLC), and Professor Michael W. Pariza, Ph.D. (University of Wisconsin-Madison, Department of Food Science).

A technical dossier, "Safety Evaluation Dossier Supporting a Generally Recognized As Safe (GRAS) Conclusion for the Use of Long-Chain Glycolipids from *Dacryopinax spathularia* in Non-alcoholic Beverages" (issued for Expert Panel review on 15 September 2017; revised version issued 11 October 2017), was prepared by Toxicology Regulatory Services, Inc. (TRS) and made available to the Expert Panel. The dossier contained information on the AM-1 product specifications, method of manufacture, safety and historical food use of the production organism, characterization, stability, proposed levels of use and technical effect, consumer exposure estimates, and pivotal published safety data supporting a GRAS conclusion for AM-1. The Expert Panel, independently and collectively, critically evaluated this document and the associated reference documents cited therein, and convened by telephone and videoconference with Dr. Andrey I. Nikiforov (TRS, Inc.) and Ms. Marisa O. Rihner (TRS, Inc.) on 2 October 2017.

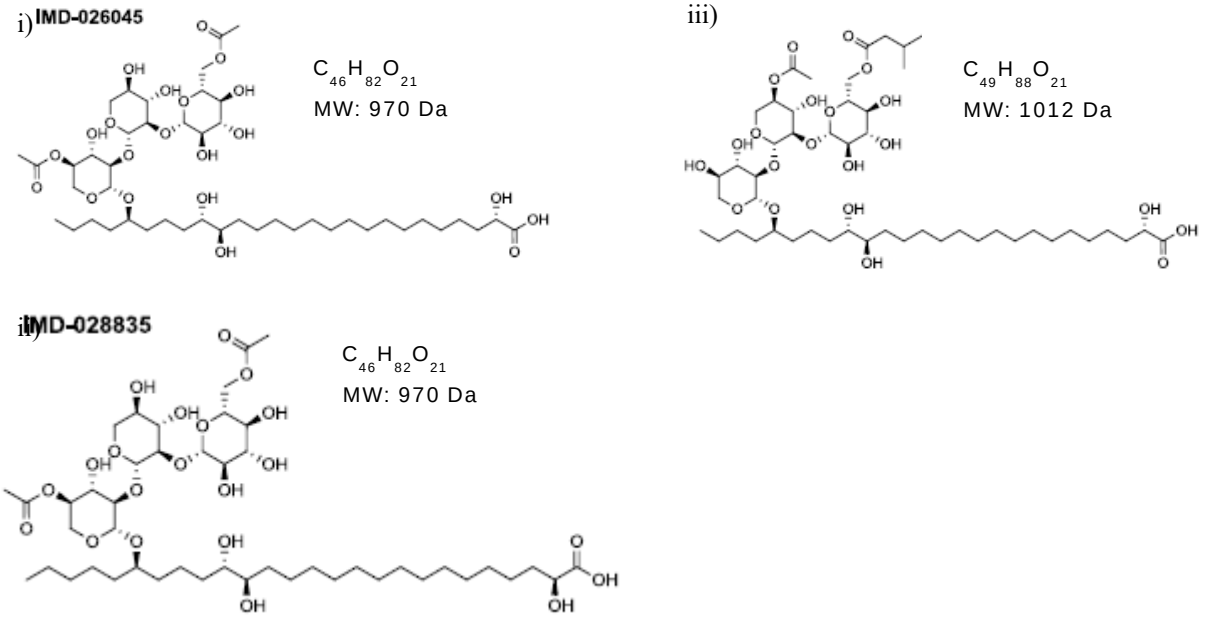
The Expert Panel unanimously concluded that the proposed uses of jelly mushroom glycolipids (AM-1), manufactured consistent with current Good Manufacturing Practices (cGMPs) and meeting appropriate food-grade specifications, are safe and suitable and GRAS based on scientific procedures. A summary of the basis for this conclusion appears below, including select details from the technical dossier, "Safety Evaluation Dossier Supporting a Generally Recognized As Safe (GRAS) Conclusion for the Use of Long-Chain Glycolipids from *Dacryopinax spathularia* in Non-alcoholic Beverages".

Basis for GRAS Determination of the Proposed Uses of Jelly Mushroom Glycolipids

Characterization

Jelly mushroom glycolipids (AM-1) is a natural glycolipid mixture with representative structures as depicted in Figure 1. The major components of jelly mushroom glycolipids (AM-1) are three structurally-related glycolipid congeners (Figure 1) all sharing a long chain fatty acid (LCFA) backbone and the same trisaccharide moiety. The acyl groups are usually acetate and isovalerate. The remaining glycolipid components are congeners of the major components. The presence of glycolipids containing the minor component 3-hydroxy-3-methylglutarate (HMG) as an acyl unit instead of acetate and isovalerate has also been noted in certain batches of AM-1 (average calculated concentration < 1.0%), as further discussed below (Safety Evaluation).

Figure 1. Representative Structures for Jelly Mushroom Glycolipids (AM-1)



Method of Manufacture

Jelly mushroom glycolipids (AM-1) is obtained via fermentation of glucose by the edible jelly fungus *Dacryopinax spathularia* strain MUCL 53181 in aerobic submerged culture. The AM-1 material is recovered from the ferment by a food grade and solvent-free process using filtration and subsequent precipitation and washing steps. There is no chemical reaction or modification of the glycolipids.

Spray or freeze drying leads to the final product as an off-white, water soluble powder. The absence of any remaining intact fungal cells from the source organism *Dacryopinax spathularia* is technically excluded by the design of the microfiltration step and has been confirmed by viable fungal cell count and microscopic control of representative batches.

The AM-1 production process follows current Good Manufacturing Practices (cGMP) as defined in 21 CFR §110 and quality management compliant with Food Safety System Certification (FSSC) 22000, including a Hazard Analysis and Risk-based Preventive Controls (HARPC) plan as per the FDA Food Safety Modernization Act (FSMA).

Conformance to proposed specifications and consistency in the manufacturing process of jelly mushroom glycolipids (AM-1) has been demonstrated by the analyses of three non-consecutive lots of commercially representative AM-1. On a dry weight basis (DWB), AM-1 has a total glycolipids content of ≥ 93% (calculated as sodium salt). The remaining ≤ 7% of dry weight is comprised of protein, fat, and sodium chloride.

Stability

AM-1 was shown to be stable for at least three years when stored as a dry powder in a closed container at ≤ 40°C. In aqueous solution, AM-1 is stable at room temperature for at least six months and under refrigerated (4-8°C) conditions for up to 1 year. AM-1 is also stable in beverage applications of various pH under typical storage conditions, with hydrolysis of the ester moieties of the glycolipids possible at low pH, elevated temperature and extended time, resulting in a higher ratio of deacylated glycolipids compared to the original mixture (INS; *unpublished data cited in AM-1 GRAS Dossier*).

There are no degradation products of safety concern associated with AM-1, as a bulk material ingredient or when formulated in low-pH beverage applications, under typical storage and use conditions. Consistent with the hydrolysis pathway of AM-1 in the gastrointestinal tract determined in experimental studies (Bitzer et al., 2017a), initial hydrolysis products are acetate and isovalerate (INS; *unpublished data cited in AM-1 GRAS Dossier*).

Physical or Technical Effect

Jelly mushroom glycolipids (AM-1) is intended for addition to beverages as an antimicrobial agent used to preserve food by preventing growth of microorganisms, particularly common yeasts and molds, and subsequent spoilage. AM-1 is reported to have good activity against Gram-positive bacteria (including spoilage organisms like

Bacillus cereus or *Listeria* spp.) but weak or no activity against Gram-negative bacteria (INS; *unpublished data cited in AM-1 GRAS Dossier*).

Compared to current chemical preservatives in beverages (e.g. sorbic acid, benzoic acid or sulfites), AM-1 was determined to have lower Minimum Inhibitory Concentrations (MICs) against *Aspergillus niger* in representative beverage matrices under identical test conditions (method DIN 58940-8) (INS; *unpublished data cited in AM-1 GRAS Dossier*).

Intended Use and Consumer Exposure

Jelly mushroom glycolipids (AM-1) is proposed for use as an antimicrobial preservative in select non-alcoholic beverages at use levels ranging from 2 to 100 ppm. Based on these use levels and daily estimates derived from the NHANES 2009-2010 and 2011-2012 database (NCHS, 2012, 2014), i.e. using calculated 2-day average intakes, the conservative estimated daily intake (EDI) of AM-1 from all proposed uses (and assuming the maximum proposed use level for each beverage category) is less than or equal to 0.51 mg/kg bw/day at the mean and 1.09 mg/kg bw/day at the 90th percentile of intake among users in the total U.S. population. On a bodyweight basis, the highest per user mean and 90th percentile intake estimates for AM-1 among population subgroups are 1.52 mg/kg bw/day and 3.29 mg/kg bw/day, respectively, for children 1-6 years old. However, the results of the *in vivo* absorption, distribution, metabolism and excretion (ADME) study with AM-1 (Bitzer et al., 2017a) support a conclusion of limited absorption of AM-1 or its major hydrolysis product, LCFA; therefore, actual consumer exposure to AM-1 may be much lower.

Safety Evaluation

The safety and GRAS status of jelly mushroom glycolipids (AM-1) is based on the totality-of-the-evidence of published data, information, and methods regarding the AM-1 chemical structural characteristics, safety and historical food use of the production organism, ADME profile, subchronic toxicity profile in multiple species, and low potential for carcinogenicity, mutagenicity, or developmental and reproductive toxicity. Unpublished data and information is also available as supplemental evidence regarding the safety of AM-1, which corroborate the conclusions that can be made based on generally available and accepted scientific data, information, or methods. These data and information are fully presented in the AM-1 GRAS Technical Dossier and select details are summarized briefly herein.

Historical Consumption

Glycolipids such as AM-1 are part of the normal human diet with the most abundant sources of glycolipids identified as eggs and dairy products, cereals, and soybeans (Leray, 2015).

The fruiting bodies of the jelly mushroom glycolipids production organism *Dacryopinax spathularia* (syn.: *Cantharellus spathularius*) are edible and evidence for their traditional use as food in many countries in Asia and Africa is documented. A field guide on foraging mushrooms in Oregon (USA) cites a recipe with *Dacryopinax spathularia* along with a description for identification of this edible mushroom (Meuninck, 2017), demonstrating that this mushroom has also been used as food in North America.

Dacryopinax spathularia is listed in the Food and Agriculture Organization of the United Nations (FAO) compendium on edible mushrooms (Boa, 2004) with proven food use documented in China (Zhishu et al., 1993), Japan (MycoBank, 2017), and Cameroon (Van Dijk et al., 2003). Additional species of the Dacryomycetaceae family (e.g. *Dacrymyces palmatus* and *Ditiola peziziformis*) are also known to be edible and confirmed in the FAO compendium. Culinary use of the fruiting bodies is further described for Malaysia (Lee et al., 2009) and India (Ao et al., 2016).

The presence of jelly mushroom glycolipids (AM-1) as constituents in collected fruiting bodies of *Dacryopinax spathularia* has been confirmed by HPLC-MS analyses; however, concentrations have not been determined (INS; *unpublished data on file*).

Safety Assessment of Jelly Mushroom Glycolipids Production Organism

The safety of the jelly mushroom glycolipids (AM-1) production organism *Dacryopinax spathularia* MUCL 53181 was assessed utilizing scientific procedures as outlined by Pariza and Johnson (2001) for safety evaluation of microbial enzyme preparations used in food processing. Using this paradigm, the AM-1 ingredient was considered the "test article" that is produced by a mushroom fermentation culture, which is comparable to an enzyme preparation produced by a microbial culture. Based on the outcome of the decision tree (*AM-1 GRAS Technical Dossier, Part 6*) including strain characterization, screening for undesirable attributes and metabolites, and experimental evidence of safety for the produced AM-1 ingredient, it was concluded that *D. spathularia* MUCL 53181 is considered safe for use in the production of AM-1 as an ingredient for human consumption.

Overview of ADME and Toxicology Database for Jelly Mushroom Glycolipids

ADME

The pharmacokinetics, excretion balance (i.e. mass balance as a measurement of test-article equivalents in excreta samples), and tissue distribution of [^{14}C]-AM-1 and [^{14}C]-Long Chain Fatty Acid (LCFA) equivalents following single or repeated

administration to Sprague Dawley rats, aged 8-10 weeks, were evaluated (Bitzer et al., 2017a). The study was performed in compliance with the U.S. Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Regulations (21 CFR Part 58) (FDA, 1987) and the study protocol was designed in general accordance with U.S. FDA Redbook II Guidelines (FDA, 1993). For all study phases, rats received equimolar doses of either [^{14}C]-AM-1 or [^{14}C]-LCFA via oral or intravenous (IV) administration followed by collection of biological samples (blood, urine, feces, and expired air) at specified intervals. The carcasses of select animals were also retained for processing by quantitative whole body autoradiography (QWBA).

Approximately 88% to 101% of the administered dose was recovered in expired air, urine, feces, and carcass following single or repeated oral administration of [^{14}C]-AM-1 at 100 mg/kg or equimolar doses of [^{14}C]-LCFA at 46 mg/kg (Bitzer et al., 2017a). There appeared to be no difference in the excretion of AM-1 or LCFA equivalents based on sex (males versus females) or single versus repeated exposures. C_{max} and AUC_{last} for [^{14}C]-AM-1- and [^{14}C]-LCFA-equivalents-derived radioactivity detected by quantitative whole body autoradiography was highest in the tissues of the GI tract, as expected following oral administration. The remaining tissues had low concentrations of test article equivalents relative to the administered dose and no target tissues for residence or accumulation were identified. Oral bioavailability of both AM-1 and LCFA including their metabolites was low at approximately 11% (overall average for males and females combined). The pharmacokinetic, tissue distribution, and excretion balance data derived in this study (Bitzer et al., 2017a) are consistent with an interpretation that following ingestion, AM-1 is partially hydrolyzed to its components, glucose, xylose, acetate, isovalerate and LCFA. The expected small primary metabolites glucose, xylose, acetate, and isovalerate are expected to have a fast and high bioavailability but rapid clearance and thus to contribute marginally to the observed test article equivalents in blood and tissues after oral administration of AM-1. In conclusion, AM-1 and LCFA are poorly absorbed by the oral route and are primarily eliminated in the feces without absorption. These results support an interpretation that systemic exposure to AM-1 or its metabolites would be very limited following oral ingestion (Bitzer et al., 2017a).

Genetic Toxicity and Carcinogenicity

Based on the chemical structural characteristics of jelly mushroom glycolipids (AM-1), i.e. a glycolipid mixture with no reactive groups, there is low potential for carcinogenicity or mutagenicity from dietary intake of this material. In addition, as discussed above, the metabolism profile for AM-1 is well understood (Bitzer et al., 2017a) and allows one to conclude that there are no carcinogenic or mutagenic metabolites of AM-1 formed *in vivo*.

To confirm and corroborate the conclusion that AM-1 is non-genotoxic and has low potential for carcinogenicity, a series of three *in vitro* genetic toxicity assays was performed with AM-1. In the *in vitro* bacterial mutation assay (Ames test; OECD Testing Guideline No. 471), there was no evidence of mutagenic activity in *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 and TA 100, or in *Escherichia coli* strain WP2 *uvr*A, in both the absence and the presence of metabolic activation (S9-mix), at any non-cytotoxic dose level (van den Wijngaard, 2012). AM-1 was also negative in the *in vitro* human lymphocyte study (micronucleus test; OECD Testing Guideline No. 487), and the *in vitro* mouse lymphoma thymidine kinase assay (MLA; OECD Testing Guideline No. 490 and EC No. 440/2008) in both the absence and presence of metabolic activation (Usta, 2012; Verspeek-Rip, 2016).

Subchronic (Repeated Dose) Toxicity

The subchronic toxicity of AM-1 was evaluated in a 90-day oral (drinking water administration) study in male and female CD® Crl:CD(SD) rats, approximately 5-6 weeks of age (Bitzer et al., 2017b). The study was performed in compliance with the Organisation for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice Regulations (ENV/MC/CHEM(98)17), which are compatible with the FDA GLP Regulations (21 CFR Part 58) (FDA, 1987). The study protocol was designed in general accordance with U.S. FDA Redbook 2000 Guideline IV.C.4.a. "Subchronic Toxicity Studies with Rodents" and the OECD Testing Guideline No. 408 "Repeated Dose 90-day Oral Toxicity Study in Rodents". Main study groups were comprised of 20 animals/sex/group. An additional 10 animals/sex for Groups 1 and 4 were designated as Recovery animals which were maintained on study for a 28-day observation period following cessation of test article administration. The test article was diluted with the vehicle, drinking water (tap water), at target concentrations of 0.15%, 0.5%, and 1.5% (1.5, 5.0, and 15 mg/mL, respectively) and orally administered to the test animals *ad libitum* for 90 days.

Oral administration of AM-1 to rats via the drinking water at target concentrations of 0.15%, 0.5%, and 1.5% for 90 days was not associated with any adverse test-article related effects. Minor variations in some endpoints evaluated (i.e. body weight, select clinical chemistry parameters) were considered incidental and secondary to reduced drinking water consumption / mild dehydration due to reduced palatability of the test article-treated drinking water. The no-observed-adverse-effect level (NOAEL) for systemic toxicity was considered to be 1.5% AM-1 in the drinking water, equivalent to 1201 and 1423 mg/kg bw/day for male and female rats, respectively (Bitzer et al., 2017b).

The subchronic toxicity of AM-1 was evaluated in a 90-day oral capsule study in male and female Beagle dogs, approximately 4-5 months of age (Bitzer et al., 2017c). The study was performed in compliance with the U.S. FDA GLP Regulations (21 CFR Part 58) (FDA, 1987). The study protocol was designed in general accordance with U.S. FDA Redbook 2000 Guideline IV.C.4.b. "Subchronic Toxicity Studies with Non-Rodents" and the OECD Testing Guideline No. 409 "Repeated Dose 90-day Oral Toxicity Study in Non-Rodents". Study groups were comprised of 4 animals/sex/group. The test article was administered by oral capsule at doses of 150, 500, or 1000 mg/kg bw/day for 90 days.

Oral administration of AM-1 to sexually mature Beagle dogs at dose levels of 150, 500, and 1000 mg/kg bw/day for a minimum of 91 days was well tolerated at all dosages. Non-adverse, test article-related changes were limited to minimal effects on food consumption and body weights in the 1000 mg/kg bw/day group females. Therefore, the NOAEL was considered to be 1000 mg/kg bw/day, the highest dosage level tested (Bitzer et al., 2017c).

Reproduction and Developmental Toxicity

AM-1 has no reactive groups or chemical structural alerts for reproductive or developmental toxicity (Bitzer et al., 2017a). AM-1 is poorly absorbed via the oral route and there was no accumulation of AM-1 in the reproductive organs of rats after repeated oral doses (Bitzer et al., 2017a). Additionally, oral administration of AM-1 had no effect on reproductive organ weights of rats or dogs and no macroscopic or microscopic findings in reproductive organs (Bitzer et al., 2017b,c). Therefore, based on the pivotal published data which are generally available (Bitzer et al., 2017a,b,c), it may be concluded that AM-1 has low potential for reproductive or developmental toxicity.

To corroborate the expectation that AM-1 is not a reproductive or developmental toxicant, developmental and reproductive toxicity study studies were performed in Crl:CD(SD) rats administered oral gavage doses of AM-1 at dose levels of 150, 500, and 1000 mg/kg bw/day (Herberth, 2017a,b). The studies were performed in compliance with the U.S. FDA GLP Regulations (21 CFR Part 58) (FDA, 1987) and the study protocols were designed in general accordance with the applicable U.S. FDA Redbook 2000 and OECD Testing Guidelines. Given the lack of any adverse test article-related effects on survival, clinical observations, body weights or food consumption, necropsy, intrauterine growth and survival, or fetal morphology, a dosage level of 1000 mg/kg bw/day (the highest dosage level evaluated) was considered to be the NOAEL for maternal toxicity and embryo/fetal developmental toxicity when AM-1 was administered orally by gavage to bred female Crl:CD(SD) rats (Herberth, 2017a). Similarly, based on the lack of effects on F_0 and F_1 reproductive performance (mating, fertility, copulation

and conception indices, estrous cyclicity, and spermatogenic endpoints), the NOAEL for parental reproductive toxicity of AM-1 when administered orally via gavage to Crl:CD(SD) rats was 1000 mg/kg/day. There were no adverse effects on survival, clinical observations, body weight or food consumption parameters, macroscopic or microscopic findings, or organ weights for F_0 or F_1 males and females at any dosage level. Based on these results, a dosage level of 1000 mg/kg/day was considered to be the NOAEL for F_0 and F_1 parental systemic toxicity. There were no test article-related effects on F_1 and F_2 postnatal survival, development, or growth during the pre-weaning period, and therefore, the NOAEL for neonatal toxicity was considered to be 1000 mg/kg/day (Herberth, 2017b).

Summary of ADME and Toxicology Database for Jelly Mushroom Glycolipids

In summary, a battery of *in vitro* genetic toxicity studies with jelly mushroom glycolipids (AM-1) were all negative, confirming the expectation that AM-1, a glycolipid mixture with no structural alerts for genotoxicity and no genotoxic metabolites, has low potential for mutagenicity or carcinogenicity. The *in vivo* ADME study demonstrated that AM-1 and its ultimate hydrolysis product LCFA are poorly absorbed following oral intake, and are primarily eliminated in the feces without absorption. Absorbed components appear to be almost completely metabolized to CO_2 and expired. There were no metabolites of safety concern identified for AM-1 and its ultimate hydrolysis product LCFA and no accumulation of these compounds in tissues. AM-1 hydrolysis products glucose, xylose, acetate, isovalerate and long-chain fatty acids are naturally occurring in the human diet, and if absorbed they may be ultimately metabolized to CO_2. Due to the rapid hydrolysis and elimination of AM-1 that occurs *in vivo*, the antimicrobial properties of the parent material would be absent or greatly diminished in the lower intestines so there is low concern for disruption of healthy gut microflora.

AM-1 has low potential for systemic toxicity with oral repeated dose (90-day) NOAELs of $\geq$ 1200 mg/kg bw/day in rats (oral drinking water administration) and $\geq$ 1000 mg/kg bw/day in dogs (oral capsule administration), the highest dose levels tested. AM-1 is not a reproductive or developmental toxicant as confirmed in robust 2-generation reproduction toxicity and embryofetal toxicity studies in rodents (oral gavage administration) with NOAELs of $\geq$ 1000 mg/kg bw/day (the highest dose levels tested). Only minor, non-adverse treatment-related effects were observed in the subchronic toxicity, developmental toxicity, and multi-generation reproduction toxicity studies that were related to the surfactant / unpalatable properties of AM-1 and/or an artifact of the oral gavage route of administration. Based on the well-defined ADME and PK profile of AM-1 in rodents (Bitzer et al., 2017a), as well as the results of oral

subchronic toxicity studies with AM-1 in rats and dogs (Bitzer et al., 2017b,c), it can also be concluded that chronic toxicity and carcinogenicity are not endpoints of safety concern for AM-1.

Safety Assessment of Potential Jelly Mushroom Glycolipids Minor Component

The minor presence of glycolipids containing 3-hydroxy-3-methylglutarate (HMG) as an acyl unit instead of acetate or isovalerate has been noted in certain batches of jelly mushroom glycolipids (AM-1), including those batches of AM-1 tested in the ADME and repeated dose oral toxicity studies (Bitzer et al., 2017a,b,c; Herberth 2017a,b) described above. As an acyl group, HMG theoretically may be freed via hydrolysis following intake of AM-1 in the diet, with average and maximum calculated theoretical concentrations of < 1% and 1.5% HMG, respectively, in representative AM-1 batches (i.e. calculated theoretical amounts after complete hydrolysis based on measured amounts of HMG-containing glycolipids). HMG is a naturally occurring constituent of human physiology and diet as a component of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA). In addition, the results of an organ distribution study, reproduction and developmental toxicity studies in two species, and a human clinical study with HMG (Savoie and Lupien, 1975a,b; Lupien et al., 1979), as well as supporting teratology and genetic toxicity study data on glutaric acid (Bradford et al., 1984; Sakagami et al., 1989; San Sebastian, 1989), a structural analogue of HMG, support the safety of HMG as a potential minor component of AM-1.

Evaluation of Allergenicity Potential

Glycolipids are not identified as food allergens or mediators of food allergy in comprehensive scientific opinions by authoritative bodies (e.g. NIAID, 2011; EFSA, 2014) and jelly mushroom glycolipids (AM-1) does not contain any introduced protein that would be expected to result in an allergic reaction. The AM-1 product does not contain any of the major food allergens identified by the U.S. Food Allergen and Consumer Protection Act (FALCPA) of 2004 nor other potential food allergens as identified in the European Regulation (EC) 1169/2011 and the Chinese Regulation GB23779-2009. Furthermore, the equipment used to manufacture AM-1 is not used to process any of these known food allergens.

The food types listed as major food allergens are not known to contain glycolipids similar to AM-1. Although the presence of glycolipid type compounds in some of these foods is reported, e.g. for milk, eggs, and soybeans (Newberg and Chaturvedi, 1992; Fujino et al., 1970, Jyonouchi et al., 2011; Leray, 2015), the particular glycolipid molecules described, such as sphingomyelin, belong to the class of sphingolipids, which

are very different in their chemical structures and molecular properties compared to AM-1.

In consideration of the above information and supporting details discussed in the AM-1 GRAS Technical Dossier, it can be concluded that there is negligible potential for jelly mushroom glycolipids (AM-1) to induce symptoms of food allergy.

Acceptable Daily Intake

The pivotal toxicology safety studies with jelly mushroom glycolipids (AM-1) are the 90-day rat oral toxicity study (Bitzer et al., 2017b) and the 90-day dog oral toxicity study (Bitzer et al., 2017c) which determined NOAELs of ≥ 1200 mg/kg bw/day in rats and ≥ 1000 mg/kg bw/day in dogs (highest dose levels tested). To be conservative, the lowest NOAEL determined in these two studies was utilized for derivation of an Acceptable Daily Intake (ADI) for jelly mushroom glycolipids as discussed below.

The safety factor used to estimate the ADI for jelly mushroom glycolipids (AM-1) from toxicological data as outlined in the AM-1 GRAS Technical Dossier is 100-fold, based on an interspecies factor of 10 and an intra-species factor of 10. As discussed above, chronic toxicity and carcinogenicity are not endpoints of concern for AM-1 because of its lack of chemical structural alerts, low oral bioavailability, simple and common hydrolysis products, and rapid and complete elimination of any metabolites; therefore, an additional safety factor was not applied in the calculation of the ADI. Based on the NOAEL of ≥ 1000 mg/kg bw/day in the 90-day oral toxicity study in Beagle dogs (Bitzer et al., 2017c), an ADI of ≥ 10 mg/kg bw/day was calculated for jelly mushroom glycolipids (AM-1).

Comparison of the Estimated Daily Intake to the Acceptable Daily Intake

Considering the worst-case, conservative upper estimates of intake for jelly mushroom glycolipids (i.e., 1.09 mg/kg bw/day for the Total U.S. population and 3.29 mg/kg bw/day for children 1-6 years at the 90th percentile of intake for all proposed uses, based on maximum proposed use levels), consumer intakes of jelly mushroom glycolipids from the proposed uses are sufficiently below the ADI of ≥ 10 mg/kg bw/day derived from pivotal toxicology study NOAELs. Because consumers are not likely to consume all products containing jelly mushroom glycolipids at the 90th percentile of intake, even larger margins of safety would be achieved based on more typical consumption patterns. Finally, owing to the low oral bioavailability (~11%) of AM-1 demonstrated in the *in vivo* ADME study (Bitzer et al., 2017a), it can be concluded that actual consumer exposure to jelly mushroom glycolipids (AM-1) from the proposed uses in beverages may be as much as 10-fold lower than the calculated EDIs.

Conclusion

We, the independent qualified members of the Expert Panel, have individually and collectively critically evaluated the data and information summarized above regarding the safety of long-chain glycolipids from *Dacryopinax spathularia*, herein also referred to as "jelly mushroom glycolipids" or "AM-1" for the proposed uses as an antimicrobial preservative. We unanimously conclude that the proposed uses of jelly mushroom glycolipids, produced consistent with current good manufacturing practices (cGMPs) and meeting appropriate food grade specifications, are safe and suitable and Generally Recognized As Safe (GRAS) based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

John A. Thomas, Ph.D.
University of Indiana School of Medicine

Date

(b) (6)

Michael Carakostas, DVM, Ph.D.
MC Scientific Consulting, LLC

10/20/17
Date

Michael W. Pariza, Ph.D.
University of Wisconsin-Madison,
Department of Food Science

Date

Conclusion

We, the independent qualified members of the Expert Panel, have individually and collectively critically evaluated the data and information summarized above regarding the safety of long-chain glycolipids from *Dacryopinax spathularia*, herein also referred to as "jelly mushroom glycolipids" or "AM-1" for the proposed uses as an antimicrobial preservative. We unanimously conclude that the proposed uses of jelly mushroom glycolipids, produced consistent with current good manufacturing practices (cGMPs) and meeting appropriate food grade specifications, are safe and suitable and Generally Recognized As Safe (GRAS) based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

(b) (6)

John A. Thomas, Ph.D.
University of Indiana School of Medicine

10/21/17
Date

Michael Carakostas, DVM, Ph.D.
MC Scientific Consulting, LLC

Date

Michael W. Pariza, Ph.D.
University of Wisconsin-Madison,
Department of Food Science

Date

Conclusion

We, the independent qualified members of the Expert Panel, have individually and collectively critically evaluated the data and information summarized above regarding the safety of long-chain glycolipids from *Dacryopinax spathularia*, herein also referred to as "jelly mushroom glycolipids" or "AM-1" for the proposed uses as an antimicrobial preservative. We unanimously conclude that the proposed uses of jelly mushroom glycolipids, produced consistent with current good manufacturing practices (cGMPs) and meeting appropriate food grade specifications, are safe and suitable and Generally Recognized As Safe (GRAS) based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

John A. Thomas, Ph.D.
University of Indiana School of Medicine

Date

Michael Carakostas, DVM, Ph.D.
MC Scientific Consulting, LLC

Date

(b) (6)

23 October 2017

Michael W. Pariza, Ph.D.
University of Wisconsin-Madison,
Department of Food Science

Date

References

Ao, T., Deb, C.R., and Khruomo, N. 2016. Wild edible mushrooms of Nagaland, India: a potential food resource. *Journal of Experimental Biology and Agricultural Sciences* 4, 59-65.

Boa, E. 2004. Wild Edible Fungi: A Global Overview Of Their Use And Importance To People (Non-Wood Forest Products). Food & Agriculture Organization of the UN, p. 134. ISBN 92-5-105157-7.

Bitzer, J., Henkel, T., Nikiforov, A.I., Rihner, M.O., and Thomas, J.A. 2017a. Pharmacokinetics, Excretion Balance, and Tissue Distribution of [^{14}C]-Labeled Glycolipids and Long Chain Fatty Acids ([^{14}C]-LCFA) from *Dacryopinax spathularia* in Rats. *Food Chem. Toxicol*. 2017 Aug 26. pii: S0278-6915(17)30489-1. doi: 10.1016/j.fct.2017.08.038. *In press*. [Epub ahead of print].

Bitzer, J., Henkel, T., Nikiforov, A.I., Rihner, M.O., and Leuschner, J. 2017b. A 90-day oral toxicity study of glycolipids from *Dacryopinax spathularia* in CD® rats. *Food Chem. Toxicol*. 2017 Jun 27. pii: S0278-6915(17)30358-7. doi: 10.1016/j.fct.2017.06.040. *In press*. [Epub ahead of print].

Bitzer, J, Henkel, T., Nikiforov, A.I., Rihner, M.O., and Henderson, K.A. 2017c. A 90-day oral toxicity study of glycolipids from *Dacryopinax spathularia* in Beagle dogs. *Food Chem. Toxicol*. 2017 Jul 13. pii: S0278-6915(17)30398-8. doi: 10.1016/j.fct.2017.07.026. *In press*. [Epub ahead of print].

Bradford, J.C., Brown, G.L., Caldwell, J.A., and Drobeck, H.P. 1984. Teratology and mutagenicity studies with glutaric acid. *Teratology*, 29(2), 19A.

EFSA (European Food Safety Authority). 2014. Scientific Opinion on the evaluation of allergenic foods and food ingredients for labelling purposes. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). EFSA Journal, 12(11), 3894.

FDA (U.S. Food and Drug Administration). 1987. Good Laboratory Practice Regulations. Final Rule. 21 Code of Federal Regulations (CFR) Part 58. Food and Drug Administration, U.S. (FDA) Department of Health and Human Services, Washington, DC.

FDA (U.S. Food and Drug Administration). 1993. Toxicological Principles for the Safety Assessment of Food Ingredients: Redbook II, Draft Guidance. Chapter V B, Metabolism and Pharmacokinetic Studies. Food and Drug Administration, U.S. (FDA), Center for Food Safety and Applied Nutrition (CFSAN), Washington, DC.

FDA (U.S. Food and Drug Administration). 2000. Toxicological Principles for the Safety Assessment of Food Ingredients: Redbook 2000. Food and Drug Administration, U.S. (FDA), Center for Food Safety and Applied Nutrition (CFSAN), Washington, DC. Updated 2007.

Fujino, Y., Nakano, M., and Saeki, T. 1970. The chemical structure of glycolipids of Bovine milk. *Agricultural and Biological Chemistry* 34(3), 442-447.

Herberth, M.T. 2017a. An Oral (Gavage) Developmental Toxicity Study of IMD AM-1 in Rats. Charles River Laboratories Ashland, LLC, Ashland, OH, USA. Unpublished Final Report submitted to IMD Natural Solutions GmbH (INS). Study No. WIL-294507.

Herberth, M.T. 2017b. A Two-Generation Reproductive Toxicity Study of IMD AM-1 Administered Orally by Gavage in Rats. Charles River Laboratories Ashland, LLC, Ashland, OH, USA. Unpublished DRAFT Report submitted to IMD Natural Solutions GmbH (INS). Study No. WIL-294508.

Jyonouchi, S., Abraham, V., Orange, J.S., Spergel, J.M., Gober, L., Dudek, E., Saltzman, R., Nichols, K.E., and Cianferoni, A. 2011. Invariant natural killer T cells from food allergic versus non-allergic children exhibit differential responsiveness to milk-derived sphingomyelin. *J Allergy Clin Immunol*. 128(1), 102-109.e13.

Lee , S.S., Chang, Y.S., and Noraswati, M.N.R. 2009. Utilization of macrofungi by some indigenous communities for food and medicine in Peninsular Malaysia. *Forest Ecology and Management* 257, 2062-2065.

Leray, C. 2015. Lipids: Nutrition and Health. CRC Press, Taylor & Francis Group. Boca Raton,FL, pp.154-155. ISBN 978-1-4822-4231-7.

Lupien, P.J., Moorjani, S., Brun, D., and Bielmann, P. 1979. Effects of 3-hydroxy-3-methylglutaric acid on plasma and low density lipoprotein cholesterol levels in familial hypercholesterolemia. *J. Clin. Pharmacol*., 19(Feb-Mar), 120-126.

Meuninck, J. 2017. Foraging Mushrooms Oregon. Finding, Identifying, and Preparing Edible Wild Mushrooms. Falcon Guides, Rowman / Littlefield, pp. 74-75, ISBN 978-1-4930-2669-2.

MycoBank. 2017. Online database provided by the International Mycological Association. Retrieved June 6, 2107 from http://www.mycobank.org/

Newburg, D.S. and Chaturvedi, P. 1992. Neutral glycolipids of human and bovine milk. *Lipids*. 27, 923-927.

NCHS (National Center for Health Statistics). 2012. National Health and Nutrition Examination Survey Data 2011-2012. Hyattsville, MD: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Available from: http://wwwn.cdc.gov/Nchs/Nhanes/Search/nhanes11_12.aspx.

NCHS (National Center for Health Statistics). 2014. National Health and Nutrition Examination Survey Data 2009-2010. Hyattsville, MD: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Available from: http://wwwn.cdc.gov/Nchs/Nhanes/Search/nhanes09_10.aspx.

NIAID (National Institute of Allergy and Infectious Diseases). 2011. Guidelines for the Diagnosis and Management of Food Allergy in the United States. U.S. Department of Health and Human Services, National Institutes of Health (NIH) Publication No. 11-7699.

Pariza, M.W. and Johnson, E.A. 2001. Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. *Regul. Toxicol. Pharmacol*. 33, 173-186.

Sakagami, Y., Yamasaki, H., Ogasawara, N., Yokoyama, H., Ose, Y., and Sato, T. 1989. Evaluation of genotoxic activities of disinfectants and their metabolites by the umu test. *Mutat. Res*. 216(6), 373.

San Sebastian, J.R. 1989. *In vivo* bone marrow cytogenetics rat metaphase analysis. Submitted by Monsanto Co. (1989) with cover letter dated 8/14/92. U.S. EPA Doc # 88-920007732; Microfiche #OTS0538652.

Savoie, L.L. and Lupien, P.-J. 1975a. Organ distribution of 3-hydroxy-3methylglutaric acid, a potential anticholesterolemic agent. *Can. J. Physiol*. 53(4), 638-643.

Savoie, L.L. and Lupien, P.-J. 1975b. Preliminary toxicological investigations of 3-hydroxy-3-methylglutaric acid (HMG). I: Study of acute toxicity and of teratogenic activity in rats and mice. *Arzneimittelforschung. (Drug Res.)* 25(8), 1284-1286.

Usta, B. 2012. In vitro Micronucleus Test (Including a Preliminary Dose Range Finding Toxicity Test) with IM 11 Grade A in Cultured Human Lymphocytes. TNO Triskelion Study Code 9917/05. Unpublished Report submitted to InterMed Discovery GmbH. Data as published in Bitzer et al. 2017b.

van den Wijngaard, M.J.M. 2012. Bacterial Reverse Mutation Test with IM-11 grade A. Unpublished Report submitted to InterMed Discovery GmbH. TNO Triskelion, Study Code: 9905/07. Data as published in Bitzer et al. 2017b.

van Dijk, H, Onguene, N.A., and Kuyper, T.W. 2003. Knowledge and Utilization of Edible Mushrooms by Local Populations of the Rain Forest of South Cameroon. *Ambio* 32, 19–23.

Verspeek-Rip, C.M. 2016. Evaluation of the Mutagenic Activity of IMD AM-1 in an In Vitro Mammalian Cell Gene Mutation Test with L5178Y Mouse Lymphoma Cells. WIL Research Europe B.V., Hambakenwetering 7, The Netherlands. Unpublished Report submitted to IMD Natural Solutions GmbH. Study No. 510377. Data as published in Bitzer et al. 2017b.

Zhishu, B., Zheng, G., and Taihui, L. 1993. The macrofungus flora of China's Guangdong province, The Chinese University Press of Hong Kong, pp. 51-52, ISBN 962-201-556-5.

From:	Andrey Nikiforov
To:	Anderson, Ellen
Cc:	Marisa O"Grady Rihner
Subject:	RE: Questions on GRAS Notification Dossier for LONG-CHAIN GLYCOLIPIDS FROM DACRYOPINAX SPATHULARIA
Date:	Tuesday, February 20, 2018 1:30:39 PM
Attachments:	image004.png image006.png image008.png
Importance:	High

Dear Ellen,

Thank you for your follow up email. It is correct that jelly mushroom glycolipids (AM-1) is proposed for use as an antimicrobial preservative in select non-alcoholic beverages at use levels ranging from **2 ppm to 100 ppm**, with 2 ppm being the minimum efficacious concentration for certain beverage matrices. However, the maximum proposed use levels of AM-1 for the various beverage categories range from **25 ppm to 100 ppm**, as listed in Table 2 on page 11 of 58 and in Table 1 of Appendix 2. To be conservative, these maximum proposed use levels for each beverage category were used to estimate dietary exposure of AM-1.

I hope this response provides sufficient clarification. Thank you and feel free to contact me at my email address (anikiforov@toxregserv.com) with any additional questions.

Best regards,

Andrey

TRS
TOXICOLOGY REGULATORY SERVICES
A SafeBridge Consultants Company

Andrey I. Nikiforov, Ph.D.
Scientific Director
154 Hansen Road, Suite 201
Charlottesville, VA 22911 USA
Tel: 434.977.5957 | Fax: 434.977.1856
anikiforov@toxregserv.com | www.toxregserv.com

From: Anderson, Ellen [mailto:Ellen.Anderson@fda.hhs.gov]
Sent: Tuesday, February 20, 2018 12:20 PM
To: Andrey Nikiforov
Cc: Marisa O'Grady Rihner
Subject: RE: Questions on GRAS Notification Dossier for LONG-CHAIN GLYCOLIPIDS FROM DACRYOPINAX SPATHULARIA

Hello Andrey,

I'm sorry the emails never made it to you. I sent them to 'trs@toxregserv.com' which is the email address listed on the GRAS notice. The first email was sent February 1 and is copied below. The second follow-up email was sent February 9. Thank you for your attention to this matter.

Sincerely,
Ellen

Dear Dr. Nikiforov,

We would like to clarify the maximum use levels of jelly mushroom glycolipids (AM-1) being proposed in the subject GRAS notice. We note that different use level ranges are referred to throughout the notice, probably due to a typographical error. In Table 2 on page 11 of 58 and in Table 1 of Appendix 2, the maximum proposed use levels of AM-1 are listed as 25 ppm to 100 ppm. On page 6 of 58 and in the Expert Panel report (page 5 of 16), the use levels are listed as 2 ppm to 100 ppm.

At your earliest convenience, could you please confirm the correct maximum proposed use level range?

Thank you,

Ellen

Ellen Anderson
Consumer Safety Officer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 240-402-1309
ellen.anderson@fda.hhs.gov

FDA U.S. FOOD & DRUG ADMINISTRATION

From: Andrey Nikiforov [mailto:ANikiforov@toxregserv.com]
Sent: Tuesday, February 20, 2018 12:09 PM
To: Anderson, Ellen <Ellen.Anderson@fda.hhs.gov>
Cc: Marisa O'Grady Rihner <mrihner@toxregserv.com>
Subject: Questions on GRAS Notification Dossier for LONG-CHAIN GLYCOLIPIDS FROM DACRYOPINAX SPATHULARIA
Importance: High

Dear Ellen,

I received your voice message this morning regarding the subject GRAS Notification Dossier. I searched my email records, including spam and junk mail, and have found no indication of receiving emails from you. Could you please cut & paste my email address above and resend those emails

today? We would be glad to address your questions promptly.

Thank you and please call with any questions.

Best regards,

Andrey

Andrey I. Nikiforov, Ph.D.
Scientific Director
154 Hansen Road, Suite 201
Charlottesville, VA 22911 USA
Tel: 434.977.5957 | Fax: 434.977.1856
anikiforov@toxregserv.com | www.toxregserv.com