Lipase-catalyzed synthesis of fatty acid esters of trisaccharides

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Abstract

Carbohydrate fatty acid esters have a broad spectrum of applications in the food, cosmetic and pharmaceutical industries. The enzyme-catalyzed acylation is significantly more selective than the chemical process and is carried out at milder conditions. Compared with mono- and disaccharides, the acylation of trisaccharides has been less studied. However, trisaccharide esters display notable bioactive properties, probably due to the higher hydrophilicity of the sugar head group. In this chapter, we describe the acylation of two trisaccharides, maltotriose and 1-kestose, catalyzed by different immobilized lipases, using vinyl esters as acyl donors. To illustrate the potential of such compounds, the antitumor activity of 6''-*O*-palmitoyl-maltotriose is shown.

Key Words

Lipases, acylation, sugar esters, transesterification, regioselectivity, surfactants, maltotriose, 1-kestose.

1. Introduction

The industrial synthesis of sugar esters is usually performed at high temperatures and catalyzed by basic compounds, which give rise to coloured side-products with low selectivity [1]. In fact, the regioselective acylation of carbohydrates is difficult to achieve due to the presence of multiple hydroxyl groups with similar reactivity [2]. In contrast with the chemical synthesis, the enzyme-catalyzed processes are notably more selective and take place at milder conditions [3,4]. Lipases and proteases are typically the most useful enzymes for this purpose [5].

A medium in which a polar reagent (the sugar) and a non-polar fatty acid donor are soluble is required for enzymatic acylation of carbohydrates. In addition, this medium must be compatible with enzyme activity. We developed a strategy based on the use of a mixture of a tertiary alcohol (2-methyl-2-butanol) and a polar solvent (DMSO), which was successfully applied to the synthesis of monosaccharide (glucose) esters [6], disaccharide (e.g. sucrose, maltose, leucrose) esters [7-10] and even trisaccharide (maltotriose) esters [11].

Most of the research on sugar ester synthesis has been focused on monosaccharides and disaccharides [1,12]. In contrast, the acylation of trisaccharides and higher oligosaccharides has been scarcely studied [13-18]. The main advantage of trisaccharide and oligosaccharide esters, regarding simple sugars derivatives, lies in their significantly higher aqueous solubility, as a consequence of the increased hydrophilicity of the sugar head group [19].

Interestingly, bioactive properties of trisaccharide esters are very promising. It is worth mentioning their antimicrobial effect against microorganisms involved in food spoilage [20], their anticariogenic effect [21]

and anti-cancer properties [11]. Due to their low toxicity, non-antigenicity, bioactivity and biodegradability, trisaccharide esters have a wide range of potential applications [1,22].

In this work, we describe the synthesis of several fatty acid esters of two trisaccharides (*see* **Fig. 1**), maltotriose [8] and 1-kestose [15], by a transesterification strategy with vinyl esters. Different immobilized lipases were employed as biocatalysts.

2. Materials

2.1. Reaction components

1. Carbohydrates: 1-Kestose and maltotriose (Sigma-Aldrich).
2. Acyl donors: Vinyl laurate, vinyl myristate, vinyl palmitate and vinyl stearate (TCI, Japan).
3. Biocatalysts:
    - Immobilized lipase from *Thermomyces lanuginosus* (formerly *Humicola lanuginosa)* immobilized on Celite (diatomaceous earth, 30-80 mesh, BDH, England) or granulated with silica (Lipozyme TL-IM, Novozymes A/S, Denmark) for maltotriose acylation.
    - Immobilized lipase from *Candida antarctica B* (Lipozyme 435, Biotecsa, Mexico) for 1-kestose reaction.
4. Solvents: Hexane, n-heptane, methanol and 2-methyl-2-butanol (Sigma-Aldrich); acetone (Scharlab); dimethyl sulfoxide (DMSO) (Merck). To assure anhydrous conditions, all solvents were dried in molecular sieves (3 Å, 1.6 mm, Sigma Aldrich) at least for 24 h.

2.2. HPLC Components

1. Pump: SP8810 (Spectra-Physics) or ProStar 230 (Varian).
2. Oven: The column temperature was kept at 40 °C.
3. Column: Nucleosil 100-C18 (250 x 4.6 mm, 5 μm) for maltotriose esters and Zorbax SB-C18 (250 x 4.6 mm, 5 μm) for 1-kestose esters.
4. Detectors: Identification of trisaccharide esters was performed using a refractive index detector (Shoedex, Showa Denka K.K.) or an evaporative light-scattering detector (PL-ELS 2100, Varian). The latter was adjusted to 1.6 L/min nitrogen flow rate, 60 °C nebulization temperature, 105 °C evaporation temperature (*see* **Note 1**).
5. Software: Integration was carried out using the Varian Star 4.0 software.

2.3. Electrospray ionization (ESI) analysis

1. Equipment: Bruker micrOTOF-Q II (Bruker Daltonics, Bremen, Germany).
2. Samples preparation: Samples were dissolved in methanol and were injected directly to the spectrometer.
3. Analysis conditions: Positive ion mode [ESI+], capillary potential of -4.5 kV, dry gas temperature 200 °C and the drying gas flow 4 L/min. Total ion chromatograms from m/z 500 to 3000.
4. Software: MS data was processed using Compass TM software (Bruker Daltonics).

2.4. Silica gel chromatography

1. For the purification of maltotriose derivatives, mix silica gel 60 (0.06-0.2 mm, 70-230 mesh, Merck) with the eluent (see the corresponding section) and let humidify for 5 min (under stirring).

2. Pour the silica into the column and let the mobile phase pass through it until obtaining the desired height, leaving 2 cm of mobile phase at the column head.
3. Evaporate the solvent in the reaction mixture using a rotary evaporator. Then, add silica gel and 5 ml of mobile phase. Evaporate the mixture, again.
4. Put the dried silica with reaction mixture into the column and start adding the mobile phase.
5. Take out aliquots (5 ml) and follow the progress of the purification by Thin Layer Chromatography (TLC).

2.5. Thin Layer Chromatography

1. Cut silica plates (Silica gel/TLC cards, 10 x 20 cm, with fluorescent indicator 254 nm, layer thickness 0.2 mm) into 10 x 4 cm plates.
2. Add 10 ml of mobile phase (see corresponding section) in the TLC cuvette.
3. Mark the application points on the plate and add 1 µl of the aliquot in each well. Let it dry and introduce the plate in the TLC cuvette.
4. Let the mobile phase elute until it is almost at the end of the plate.
5. Stain the plate with Bial's reagent diluted with 4 volumes of ethanol (*see* **Note 2**).
6. Dry and heat the plate at 120ºC for 5 minutes.

3. Methods

3.1 Acylation of maltotriose

1. Dissolve maltotriose (303 mg, 0.6 mmol) in 5 mL of DMSO.

2. Add 2-methyl-2-butanol to 25 mL final volume (20% DMSO in the reaction mixture, *see* **Note 3**).
3. For immobilisation of lipase from *Thermomyces lanuginosus* in Celite, the pH of commercial Lipolase 100L solution (100 ml) was adjusted to 7.0. The support (8 g) was added and the suspension stirred for 30 min at 4°C. Then, 200 ml of cold acetone (0°C) were slowly added with stirring. The immobilised enzyme was filtered, washed with acetone, dried *in vacuo*, and stored at 0°C.
4. Weight 2.5 g of biocatalyst (*Thermomyces lanuginosus* lipase immobilised on Celite or granulated with silica) and 2.5 g of 3 Å molecular sieves to maintain anhydrous conditions (*see* **Note 4**).
5. Keep the suspension for 30 minutes at 40 ºC with orbital stirring (*see* **Note 5**).
6. Add the fatty acid vinyl ester (7.5 mmol) and incubate the mixture for 24 h at 40 ºC (*see* **Note 6**).
7. Monitor the reaction by thin layer chromatography (TLC) on silica gel 60 plates (Merck) (see section 2.5) using chloroform/methanol 4:1 (v/v) as eluent.
8. Cool, filter and wash the solid phase with 3 volumes of 2-methyl-2-butanol.
9. Precipitate maltotriose ester in the liquid phase adding between 2.5 and 10 volumes of n-heptane.
10. Filter the white solid.

11. Recrystallize the reaction product in acetone and dry the product *in vacuo*. Calculate the yield based on the weight of isolated ester (*see* **Table 1**).
12. Analyze the conversion (referred to the initial amount of maltotriose) and the ratio monoester/diester (*see* **Table 1**) by reverse-phase HPLC (see section 2.2). The specific conditions of mobile phase and flow rate are:

- Mobile phase 85:15 (v/v) methanol / H_2O and flow rate 1.5 mL/min for esters of lauric and myristic acids.
- Mobile phase 95:5 (v/v) methanol / H_2O and flow rate of 1.1 mL/min for esters of palmitic and stearic acids.

13. The isolated products were fully characterized by spectroscopic techniques (NMR, FTIR and high resolution MS)
14. NMR analyses determined that the acylation took place in the 6-OH of the glucose moiety at the non-reducing end (*see* **Fig. 2** for reaction scheme of maltotriose monolaurate synthesis).
15. To determine whether these compounds could exert a potential pharmacological effect, their cytotoxic activities against two human cancer cell lines, Hep-G2 and HeLa, were studied by means of the colorimetric MTT test [23] (*see* **Note 7**). 6''-*O*-palmitoyl-maltotriose treatment resulted in a dose-dependent decrease of cell viability of both Hep-G2 and HeLa cells (*see* **Fig. 3**). Curve analysis allowed graphic estimation of the concentration causing 50% cell growth inhibition (IC50). The IC50 values for 6''-O-palmitoylmaltotriose against the growth of Hep-G2 and HeLa cancer cell lines were 2.3 µM and 3.6 µM, respectively.

3.2 Acylation of 1-kestose

1. Weigh 1-kestose (320 mg, 0.63 mmol) and add vinyl laurate (1.040 ml, 4 mmol) to a volume of 10 ml of hexane (in 15 mL thermostated-capped cylindrical glass tube) with 10% (w/v) of activated molecular sieves and 10% (w/v) of Lipozyme 435 B from *Candida antarctica* (*see* **Note 8**).
2. Put the glass tube under orbital agitation at 60 ºC.
3. Follow the progress of the reaction for 96 h with analytical HPLC (see section 2.2). The mobile phase was 90:10 (v/v) methanol / H_2O and the flow rate 0.6 mL/min.
4. Perform a liquid-liquid extraction to isolate the product. The carbohydrate fatty acid ester will be in the organic phase.
5. Remove the solvent by speed-vac evaporation.
6. Dissolve the sample in methanol and inject it directly to the spectrometer ESI-MS. The sugar molecules usually form singly-charged ions by adding Na^+. For example, 1-kestose without acylation produces the peak $[M+Na]^+$ at m/z of 527.2 (*see* **Fig. 4A**). The enzymatic acylation reaction of 1-kestose produced a major adduct peak at m/z of 709.5, corresponding to monolaurate (*see* **Note 9**), and a second adduct peak at m/z 891.5, corresponding to dilaurate (*see* **Fig. 4B**).

4. Notes

1. The chromatographic detection of vinyl esters can be performed with an absorbance or photodiode array detector. Quantification can be carried out at 216 nm.

2. Bial's reagent is prepared mixing 0.4 g orcinol, 200 ml of concentrated hydrochloric acid and 0.5 ml of a 10% ferric chloride (w/v) solution.
3. Although the use of 5% DMSO instead of 20% DMSO in the acylation process gives rise to a higher conversion (about 3.5-fold compared with 20% DMSO, *see* **Table 1**), the presence of 10% diesters in the final product must be considered if the objective is to obtain a pure monoester.
4. The presence of water during the reaction decreases the reaction yield. This is due to the capability of the enzyme to use water as acceptor and hydrolyze the acyl donor (releasing the corresponding acid) and the obtained product (reversing the reaction). Most of the water in the reaction mixture comes from the biocatalyst and the solvents. Thus, it is advisable to dry the solvents using 3 Å molecular sieves and also to include molecular sieves in the reaction mixture. It is also possible to increase the reaction yield by dehydration of the biocatalyst in a vacuum desiccator overnight.
5. Avoid the use of magnetic stirrers. Enzymes are unstable in the presence of this kind of stirring. It is preferable to use orbital shakers to carry out the reaction.
6. Vinyl esters were chosen as acyl donors since the equilibrium can be shifted towards the ester formation (the resulting vinyl alcohol tautomerizes towards low-boiling-point acetaldehyde). Using ethyl esters as acyl donors, both the conversion and reaction rate are one order of magnitude lower than that obtained with the corresponding vinyl ester.
7. HepG2 is a cell line derived from a human hepatoma and is representative of a differentiated tumor cell. HepG2 shows a limited, but measurable, hepatic functionality. HeLa, on the other hand, is a rather undifferentiated

cell line with notable cell growth capability. Results are the average of three different experiments and are expressed as the percentage of cell growth inhibition compared with a control in absence of ester.

8. Lipozyme 435 B displays a hydrolytic activity towards tripropionin around 1700 U/g. It is advisable to check this activity before starting the synthesis. The hydrolytic activity is measured titrimetrically at pH 8.0 and 30 °C using a pH-stat (Mettler, Model DL 50). The reaction mixture contains tripropionin (0.4 ml, final concentration 105 mM), acetonitrile (0.6 ml) and buffer (19 ml, Tris-HCl 1 mM, NaCl 0.1 M, pH 8.0). The pH is automatically kept at 8.0 with 0.1 N NaOH as titrant. One enzyme unit (U) corresponds to the formation of 1 µmol of fatty acid per min.
9. The monolaurate synthesized in this work is expected to be acylated at the terminal fructose moiety of 1-kestose, due to less steric hindrance in the active site of the lipase. It has been previously reported that *C. antarctica* lipase B displays a notable regiospecificity to acylate the 6-OH of fructose in contrast with the other primary hydroxyl 1-OH [4].

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Table 1. Acylacion of maltotriose with vinyl esters of different chain length in mixtures 2-methyl-2-butanol/DMSO catalyzed by *T. lanuginosus* lipase immobilized in Celite. Table adapted with permission from reference [8].

Acyl donor	**Percentage of DMSO**	**Conversion (%)[a] Monoester / Diester**	**Yield (%)[b]**
Vinyl laurate	5	88 / 10	74
Vinyl laurate	20	25 / <1	21
Vinyl myristate	20	32 / <1	26
Vinyl palmitate	20	33 / <1	28
Vinyl stearate	20	38 / <1	27

[a] Determined by HPLC (referred to the initial concentration of maltotriose)

[b] Referred to the weight of isolated product

Figure legends

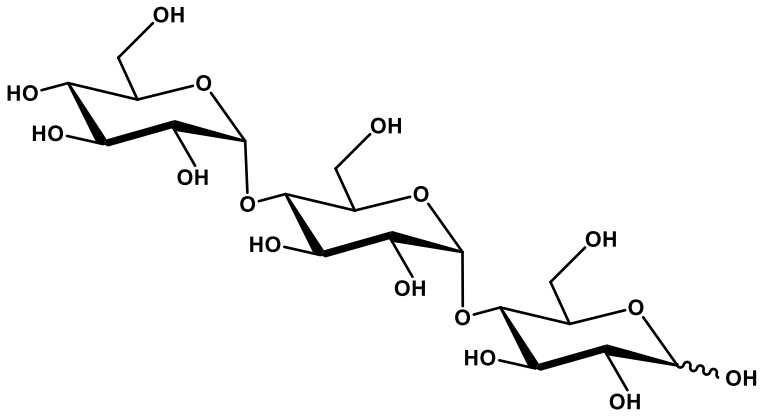
Fig. 1. Structure of maltotriose and 1-kestose.

Fig. 2. Reaction scheme for the synthesis of 6''-*O*-palmitoyl-maltotriose catalyzed by *Thermomyces lanuginosus* lipase.

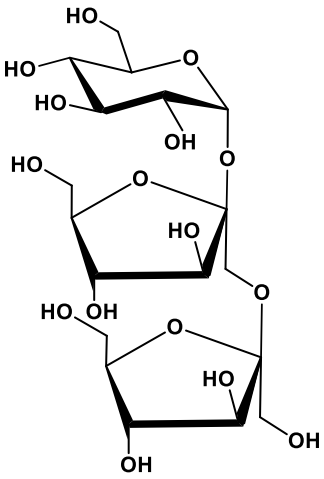
Fig. 3. Cell viability of several cellular models after exposure to 6''-*O*-palmitoyl-maltotriose. Hep-G2 (●) and HeLa (○) cell lines were exposed to monoester for 24 h, and cell viability was measured by the MTT test after a further 24 h treatment. Each point represents the average of the data derived from three independent experiments ± standard deviation. Figure adapted with permission from reference [11].

Fig 4. (A) ESI-MS in positive mode of 1-kestose (m/z value of 527.2) and (B) mono and diacylated 1-kestose with vinyl laurate (m/z values of 709.4 and 891.5, respectively). Figure adapted with permission from reference [15].

Fig. 1

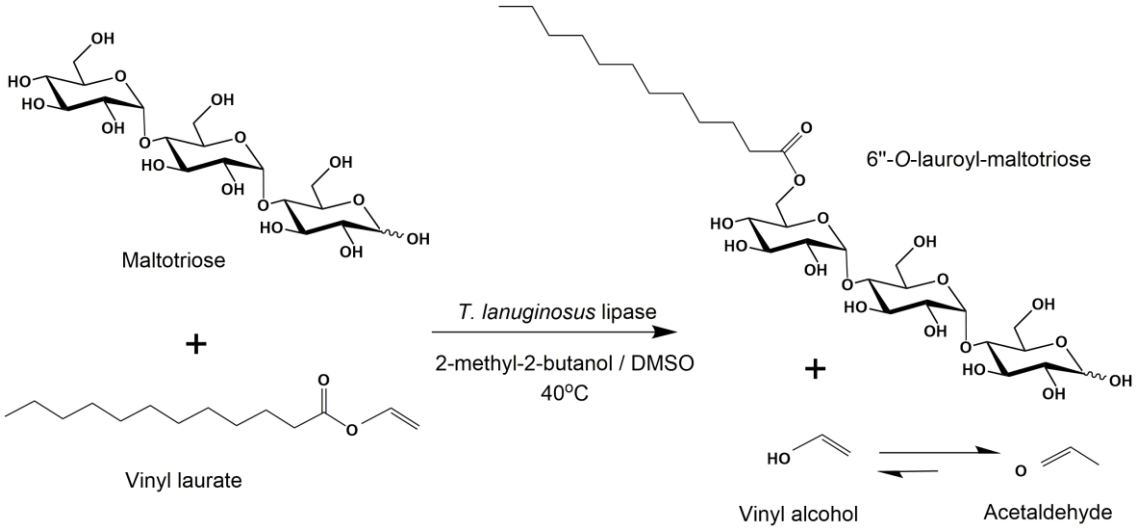


Maltotriose



1-Kestose

Fig. 2


Maltotriose
Vinyl laurate
T. lanuginosus lipase
2-methyl-2-butanol / DMSO
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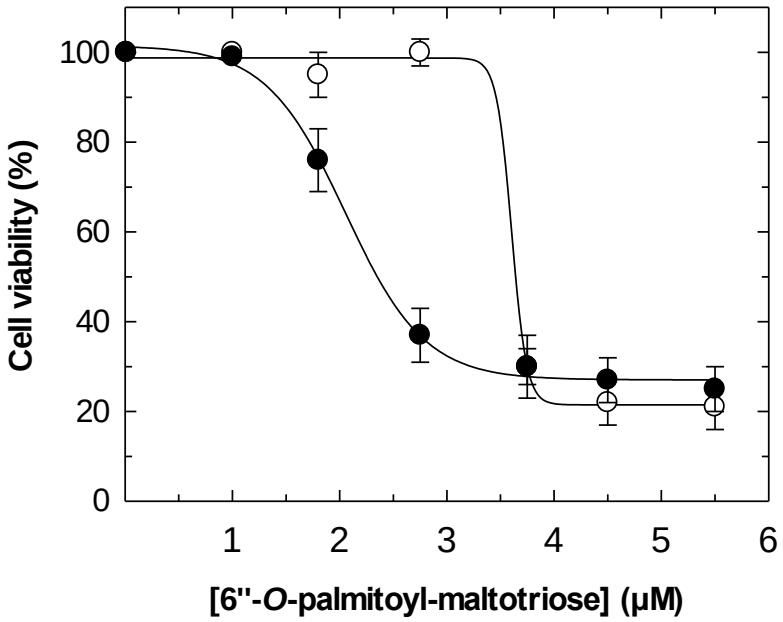
6''-O-lauroyl-maltotriose
Vinyl alcohol
Acetaldehyde

Fig. 3


Cell viability (%)
100
80
60
40
20
0
1
2
3
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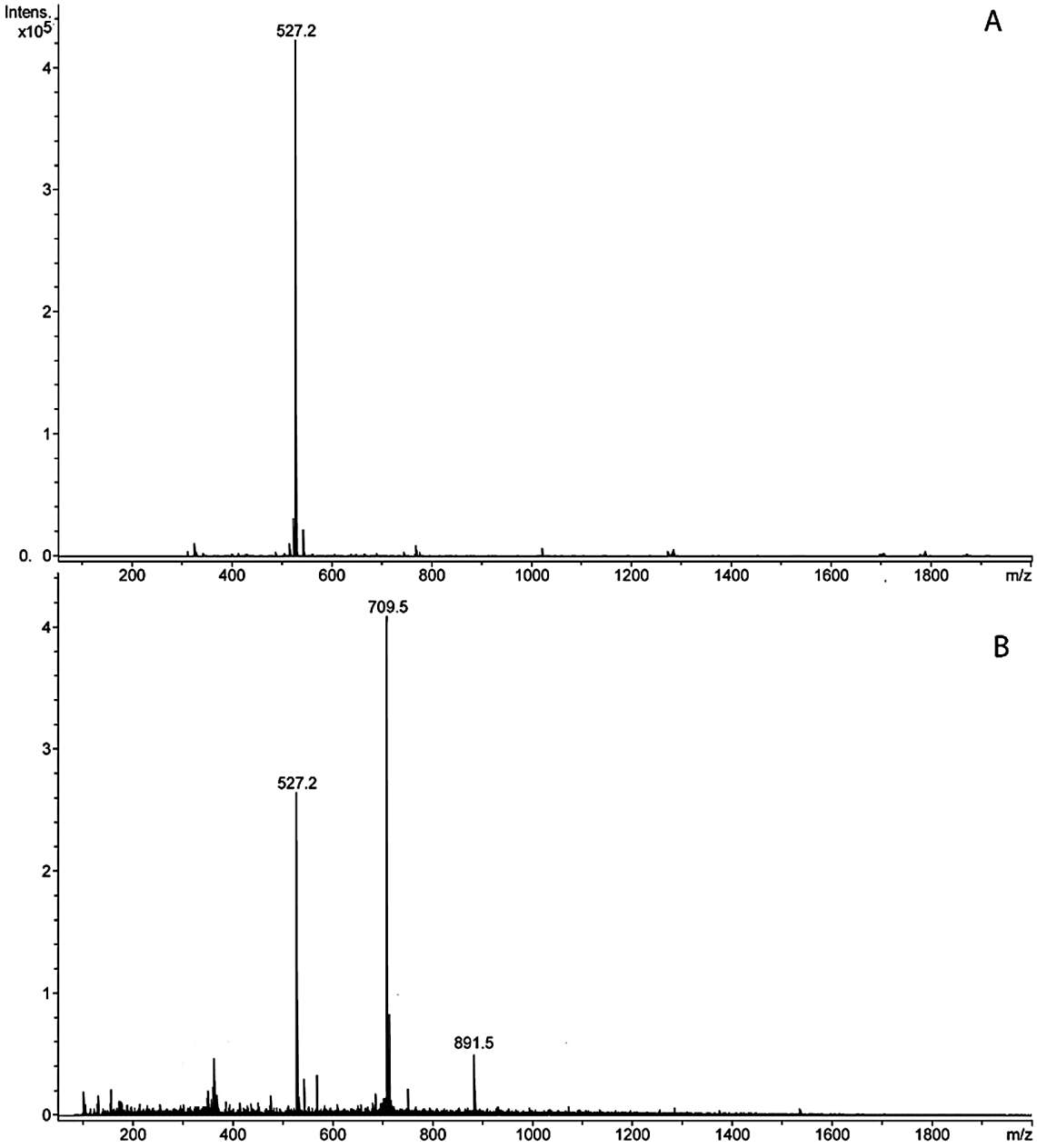
5
6
[6''-O-palmitoyl-maltotriose] (µM)

Fig. 4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