Appendix IV
Structural Analyses of 2'-FL and LNnT

1. Analytical Reports on the Chemical Structures of 2'-O-Fucosyllactose (2'FL) and Lacto-N-neotetraose (LNnT) Produced by Fermentation. Pages 2-57.
2. X-Ray Crystallography Data
    a. World Intellectual Property Organization, International Publication Number WO 2011/150939 A1 (08.12.2011) – Polymorphs of 2'-O-Fucosyllactose and Producing Thereof. Pages 58-106
    b. World Intellectual Property Organization, International Publication Number WO 2011/100980 A1 (25.08.2011) – A Method for the preparation of the Tetrasaccharide Lacto-N-Neotetraose (LNNT) Containing N-Acetyllactosamine. Pages 107-169.

Analytical Reports on the Chemical Structures of 2'-*O*-Fucosyllactose (2'FL) and Lacto-N-neotetraose (LNnT) Produced by Fermentation

Including comparison to chemically synthesised and to authentic-natural 2'FL and LNnT, respectively

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Glykos

NMR spectral analysis of synthetic and natural human milk oligosaccharides.

Study sponsor: Glycom A/S

Glykos Finland Ltd.

6.11.2015

Glykos Finland Oy Viikinkaari 6 FIN-00790 Helsinki Finland www.glykos.fi

Contents

Aim of the study

The aim of this study was to compare NMR spectral data between oligosaccharides originating from biotechnological process, from chemical synthesis and from natural human milk. For the present experiments, Glycom A/S provided two synthetic oligosaccharides, 2'FL and LNnT, originating from biotechnological process, and the NMR spectral data of these samples were compared to those obtained with chemical synthesis (Glycom A/S) and natural oligosaccharides provided by Glykos Finland.

Materials and methods

NMR spectra were recorded using a Bruker Avance III 600 MHz instrument equipped with a QCINP cryoprobe. Prior to NMR experiments the saccharides were lyophilized twice from D_2O and then dissolved in 600 µl D_2O. All experiments were performed at 22 °C.

In one dimensional ^1H spectra HDO signal was suppressed by 4 s volume selective presaturation. 16 scans were collected.

For NOESY spectra Bruker's pulse program NOESYGPPHPP was used. The mixing time was 1 s and matrices with 1024*256 points were collected.

Human milk derived oligosaccharides 2-fucosyllactose (2'FL) and Lacto-N-neotetraose (LNnT) were obtained from Biocarb Chemicals (Sweden).

Chemically synthesized oligosaccharides were provided by Glycom A/S: 2'FL, Batch L06112K (Compound A) and LNnT, Batch L01032K (Compound B).

Oligosaccharides produced by biotechnological process were provided by Glycom A/S: 2'FL, Batch CPN291300315 (Compound C) and LNnT, Batch 2547750801 (Compound D).

Results

The ^1H spectra of synthetic Fucα1-2Galβ1-4Glc (2'FL) samples, Compound A and Compound C, are shown in Fig 1. The major signals are identical in the spectra, and furthermore, signals are in full agreement with the natural 2'FL originating from human milk (reported in April 2015) and literature values [1]. The biotechnologically produced sample, Compound C, shows small impurity signals (Fig. 1B, inset) assigned to acetate (1.94 ppm), acetyl esters (2.11-2.16 ppm) and citric acid (2.59 and 2.73 ppm) which are not observed in Compound A (2'FL from chemical synthesis).

The NOESY spectra of Compound A and Compound C are shown in Figure 2. The same intra- and inter-residual correlations are observed, and these data fully agree with the NOESY data recorded with natural 2'FL originating from human milk (reported in April 2015). These spectra confirm that the molecules have the same stereochemical configuration as well as three-dimensional structure.

The ^1H spectra of synthetic Galβ1-4GlcNAcβ1-3Galβ1-4Glc (LNnT) samples, Compound B and Compound D, are shown in Figure 3. The spectra are identical and the chemical shifts correspond to

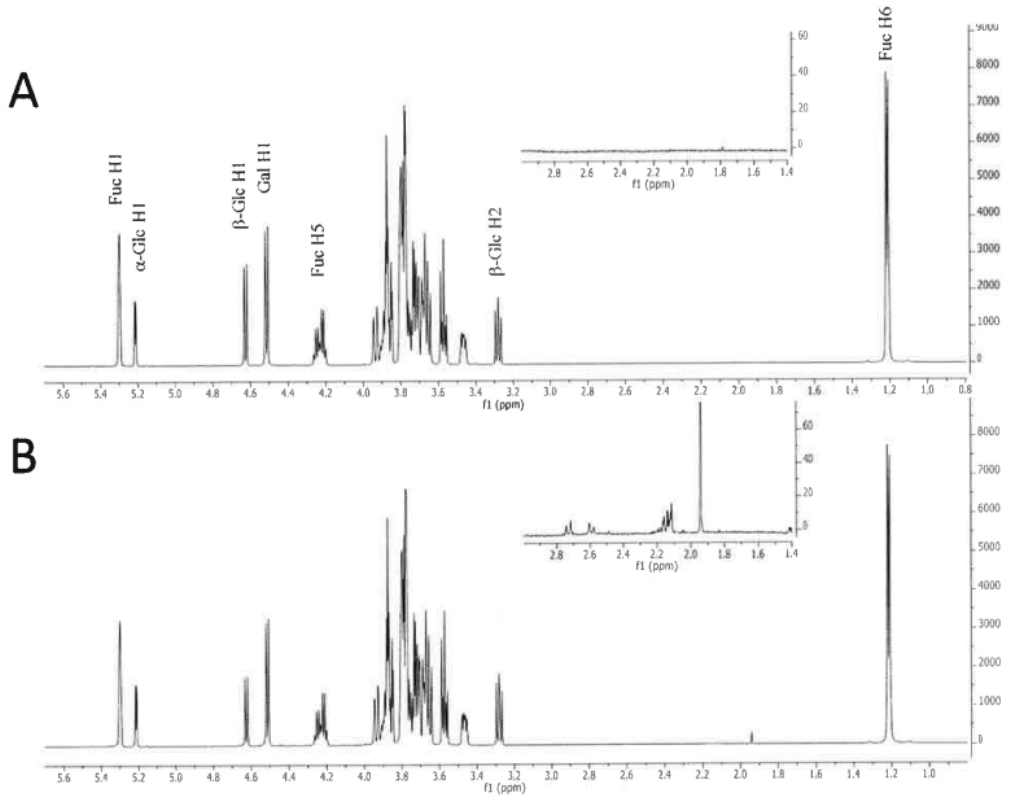

Figure 1. [1]H-spectra of synthetic 2'FL samples. (A) Compound A (chemical synthesis), (B) Compound C (biotechnological synthesis).

natural LNnT originating from human milk (reported in April 2015) and those reported in literature for Galβ1-4GlcNAcβ1-3Galβ1-4Glc [2].

NOESY spectra of Compound B and Compound D are presented in Figure 4. The same NOE correlations can be assigned in both spectra. Furthermore, the data fully agree with the NOESY data recorded with natural LNnT originating from human milk (reported in April 2015).

Minor differencies in the appearance of the NOE spectra are due to small difference in sample concentration. In addition, it should be noted that NOE intensity is intrinsically low for a tetrasaccharide in D_2O at 600 MHz field. In this situation motional properties of the molecule approach zero crossing conditions where NOE is not observable. The impact of molecular motion to NOE intensity is clearly seen on the recorded spectra. The 2'FL trisaccharide behaves as a small molecule yielding positive NOE crosspeaks (red on the spectra). In marked contrast, NOEs are negative (blue on the spectra) for Galβ1-4GlcNAcβ1-3Galβ1-4Glc, indicative of a larger molecular weight.

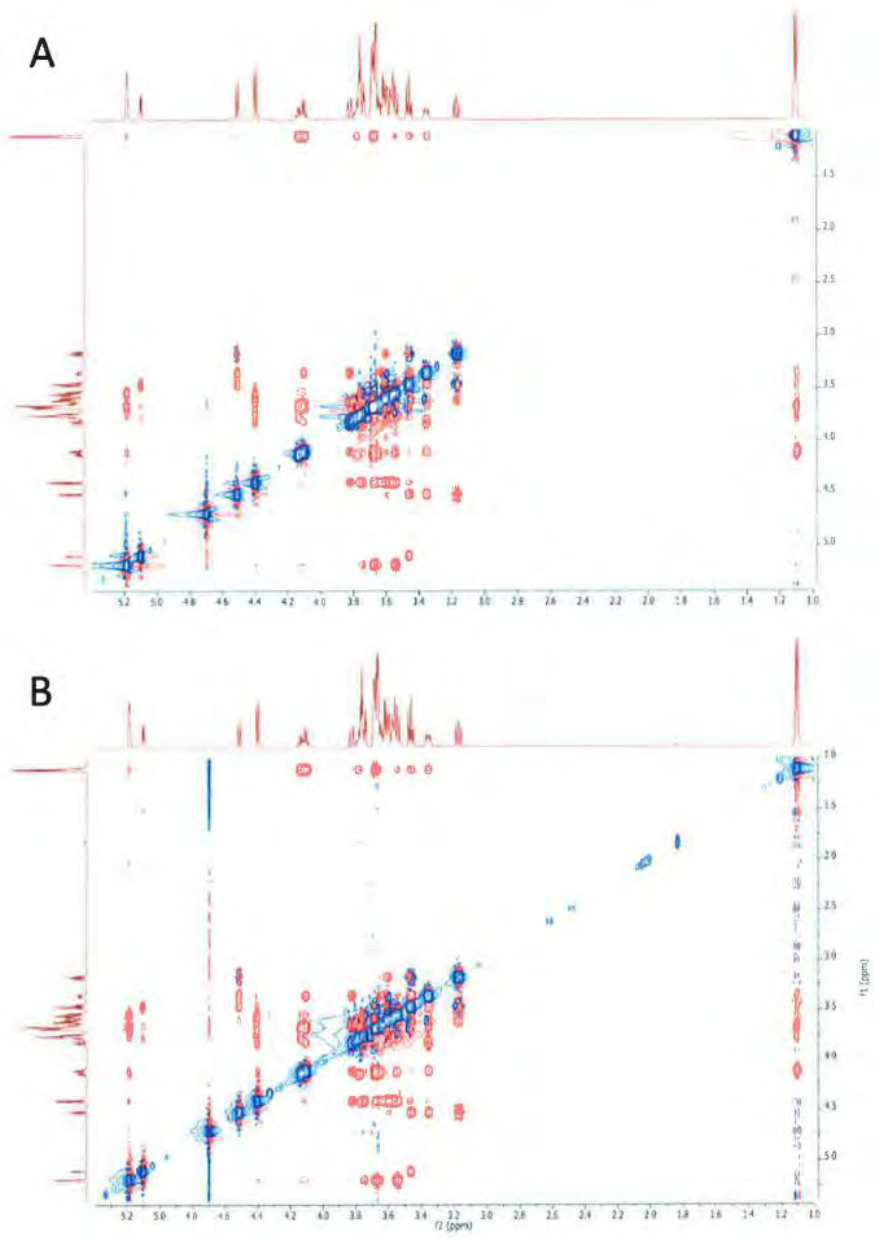

Figure 2. NOESY spectra of synthetic 2'FL samples: (A) Compound A and (B) Compound C.

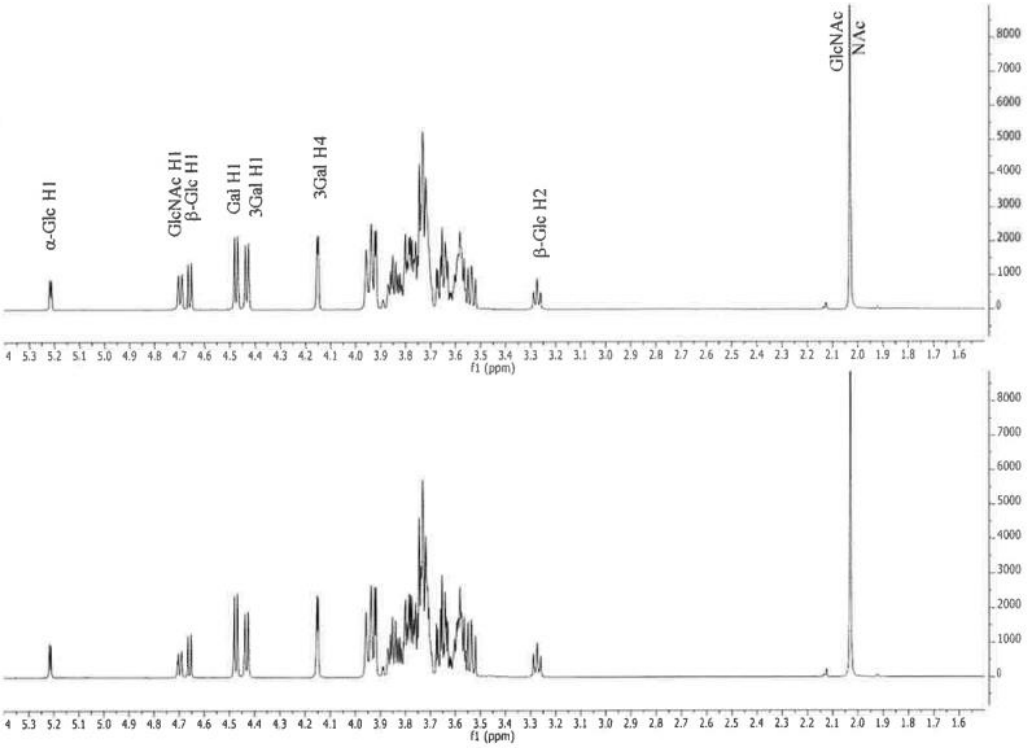

Figure 3. [1]H-spectra of synthetic LNnT samples. (A) Compound B (chemical synthesis), (B) Compound D (biotechnological synthesis).

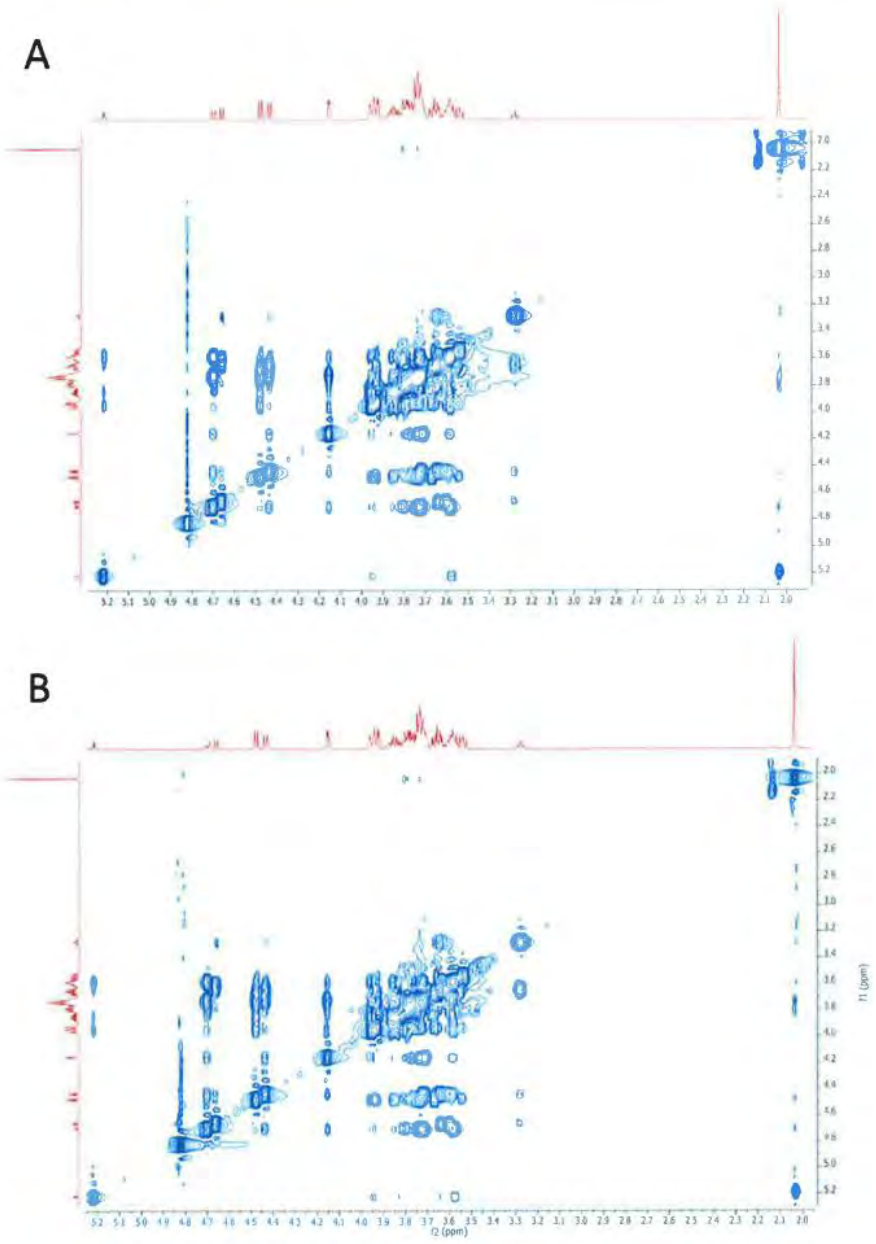

Figure 4. NOESY spectra of synthetic LNnT samples: (A) Compound B and (B) Compound D.

Conclusions

Human milk type oligosaccharides 2'FL and LNnT originating from biotechnological synthesis process were provided by Glycom A/S for NMR spectral comparison to chemically synthesized and natural human milk derived counterparts.

The NOESY spectra of biotechnologically and chemically synthesized 2'FL show the same intra- and inter-residual correlations, and these data fully agree with the NOESY data recorded with natural 2'FL originating from human milk. These spectra confirm that the molecules have the same stereochemical configuration as well as three-dimensional structure.

The two synthetic 2'FL compounds show very similar 1D-^{1}H-spectra. The major signals are indistinguishable, but small impurity signals, assigned to acetate, citric acid and acetyl esters, are detected in the biotechnology compound as compared to the chemically synthesized counterpart.

The NOESY spectra of biotechnologically and chemically synthesized LNnT show the same NOE correlations, which fully agree with the NOESY data recorded with natural LNnT originating from human milk. Thus these compounds have identical stereochemical configurations. Furthermore, the 1D-^{1}H-spectra of the two synthetic LNnT samples are fully comparable.

Date November 6th, 2015

Sample preparation

Data analysis and reporting

Approved by

References

1. A ^{1}H and ^{13}C study of oligosaccharides from human milk. Application of the computer program CASPER. Hermansson K., Jansson P.-E., Kenne L., Widmalm G. and Lindh F. *Carbohydr. Res.* 235 (1992) 69-81.
2. www.glycosciences.de/database/structure LinucsID 1222

Glykos

NMR comparison of synthetic and natural human milk oligosaccharides

Study sponsor: Glycom A/S

Glykos Finland Ltd.

20.4.2015

Glykos Finland Oy Viikinkaari 6 FIN-00790 Helsinki Finland www.glykos.fi

Contents

Aim of the study

The aim of this study was to compare NMR spectral data between synthetic and human milk derived oligosaccharides. For the study, Glycom A/S provided two synthetic oligosaccharides, 2'FL and LNnT. The NMR spectral data of these samples were compared to those obtained with natural human milk oligosaccharides provided by Glykos Finland.

Materials and methods

NMR spectra were recorded using a Bruker Avance III 600 MHz instrument equipped with a QCINP cryoprobe. Prior to NMR experiments the saccharides were lyophilized twice from D_2O and then dissolved in 600 µl D_2O. All experiments were performed at 22 °C.

In one dimensional ^1H spectra HDO signal was suppressed by 4 s volume selective presaturation. 16 scans were collected.

For NOESY spectra Bruker's pulse program NOESYGPPHPP was used. The mixing time was 1 s and matrices with 1024*256 points were collected.

Human milk derived oligosaccharides 2-fucosyllactose (2'FL) and Lacto-N-neotetraose (LNnT) were obtained from Biocarb Chemicals (Sweden).

Test substances by Glycom A/S were 2'FL, Batch L06112K (Compound A) and LNnT, Batch L01032K (Compound B).

Results

Comparison of ^1H spectra of compound A and Fucα1-2Galβ1-4Glc (2'FL) extracted from human milk (Fig 1.) shows that they are identical. Chemical shifts correspond to those reported for Fucα1-2Galβ1-4Glc [1]. NOESY spectra of compound A and Fucα1-2Galβ1-4Glc extracted from human milk are shown in Figures 2 and 3 respectively. The same intra- and inter-residual correlations are visible. These spectra confirm that the molecules have the same stereochemical configuration as well as three dimensional structure i.e. compound A is Fucα1-2Galβ1-4Glc. Interestingly, the 1,2-disubstitution of galactose results in a stacked three dimensional conformation demonstrated by interresidual NOEs between fucose and reducing end glucose indicated on the spectrum.

Comparison of ^1H spectra of compound B and Galβ1-4GlcNAcβ1-3Galβ1-4Glc (LNnT) extracted from human milk is shown in figure 4. The spectra are identical and the chemical shifts correspond to those reported for Galβ1-4GlcNAcβ1-3Galβ1-4Glc [2]. NOESY spectra of compound B and Galβ1-4GlcNAcβ1-3Galβ1-4Glc extracted from human milk are presented in Figures 5 and 6. The same NOE correlations can be assigned in both spectra. As no conflicting signals could be identified it is deduced that compound B is Galβ1-4GlcNAcβ1-3Galβ1-4Glc.

Minor differencies in the appearance of the spectra are due to different sample concentration. In addition, NOE intensity is intrinsically low for a tetrasaccharide in D_2O at 600 MHz field. In this situation motional properties of the molecule approach zero crossing conditions where NOE is not

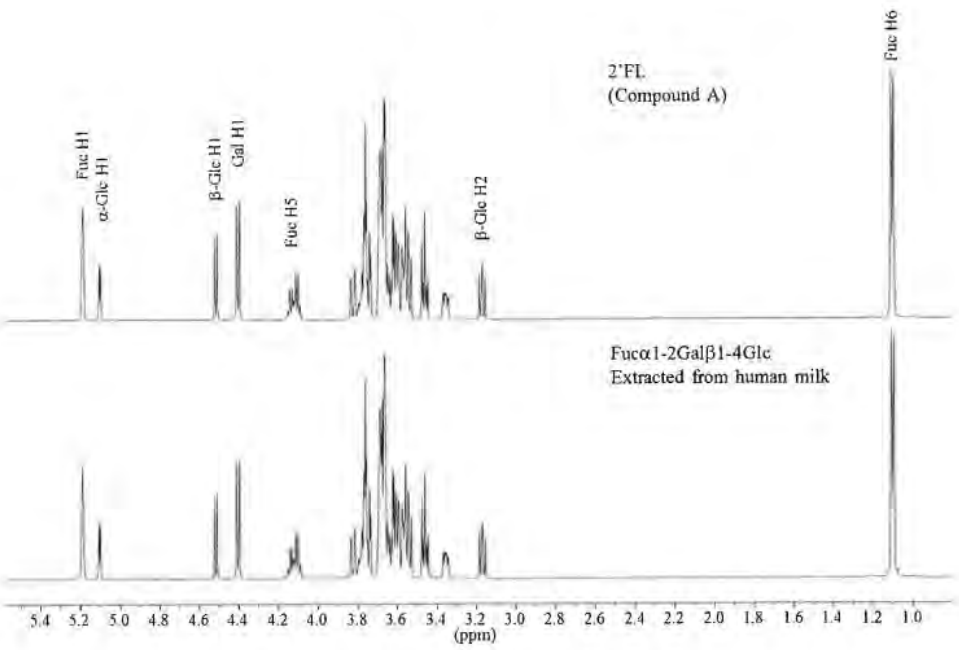

Figure 1. ^{1}H spectra of compound A and natural Fucα1-2Galβ1-4Glc extracted from human milk

observable. The impact of molecular motion to NOE intensity is clearly seen on the recorded spectra. The trisaccharide Fucα1-2Galβ1-4Glc behaves as a small molecule yielding positive NOE crosspeaks (red on the spectra). On the other hand NOEs are negative (blue on the spectra) for Galβ1-4GlcNAcβ1-3Galβ1-4Glc, indicative of a larger molecular weight.

According to one dimensional ^{1}H spectra Compounds A and B are pure and contain only Fucα1-2Galβ1-4Glc and Galβ1-4GlcNAcβ1-3Galβ1-4Glc, respectively. Impurities with concentration higher than 1% were not detected. ^{1}H coupling to ^{13}C (natural abundance 1.1%) splits signals making the identification of very low concentration impurities challenging.

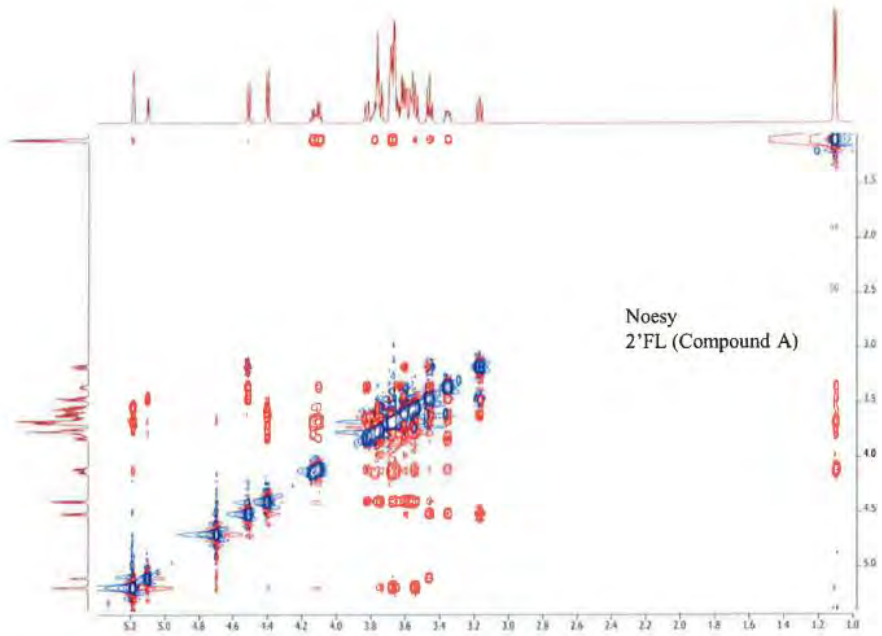

Figure 2. NOESY spectrum of compound A.

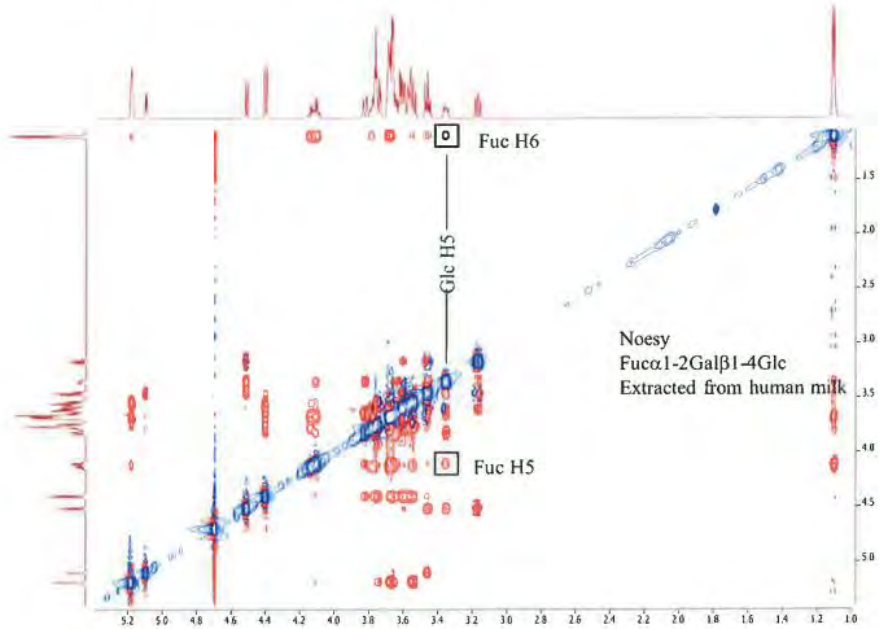

Figure 3. NOESY spectrum of Fucα1-2Galβ1-4Glc extracted from human milk.

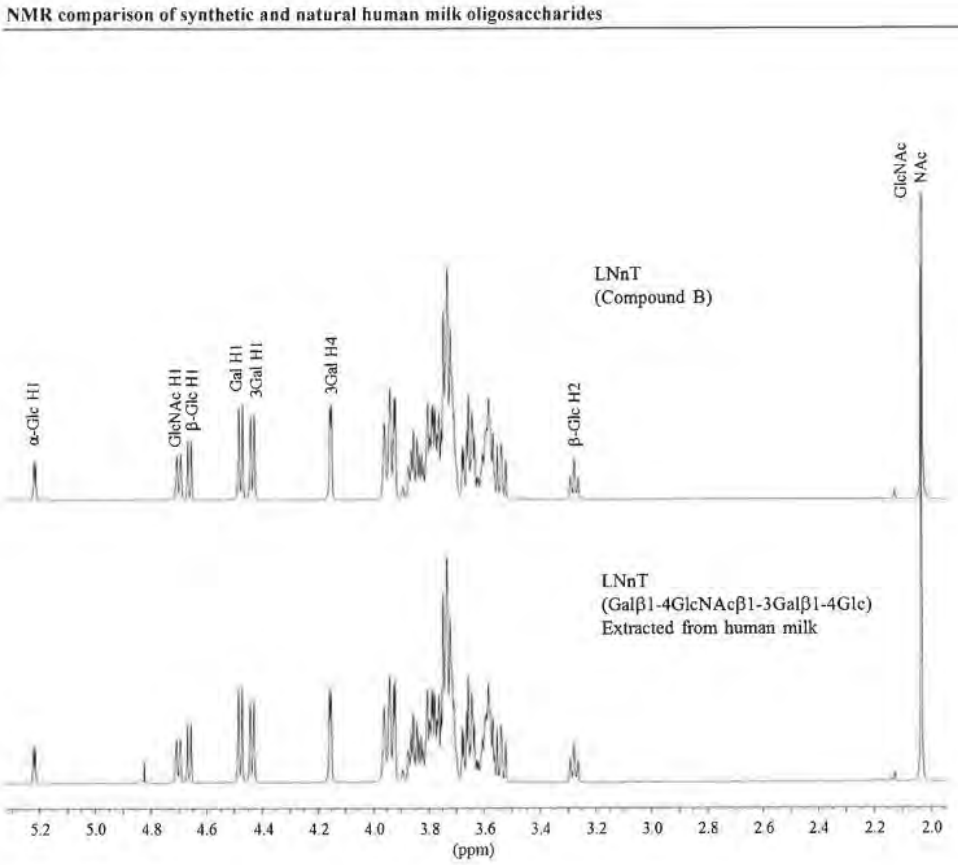

Figure 4. ^{1}H spectra of compound B and natural Galβ1-4GlcNAcβ1-3Galβ1-4Glc extracted from human milk

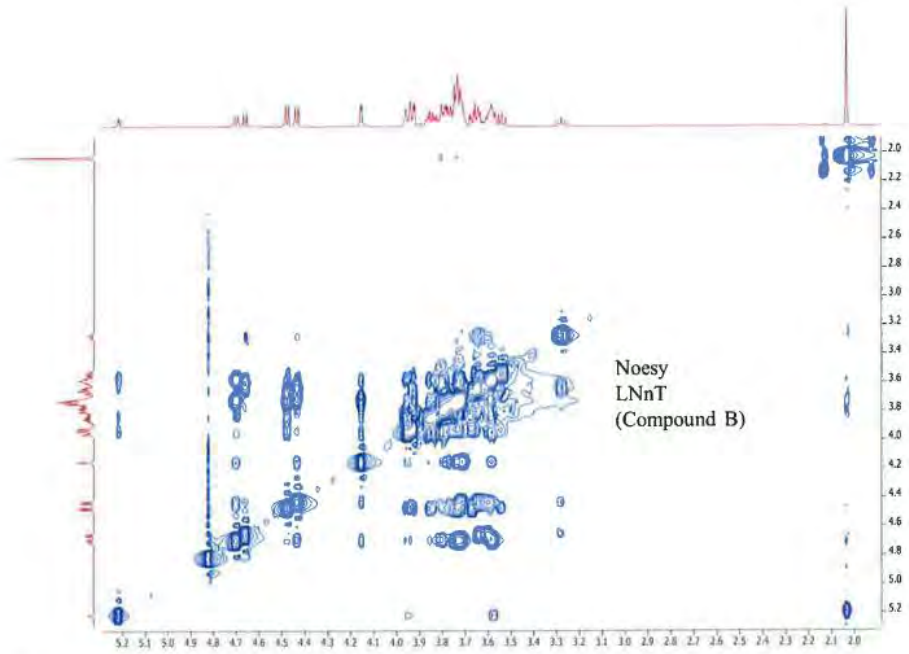

Figure 5. NOESY spectrum of compound B.

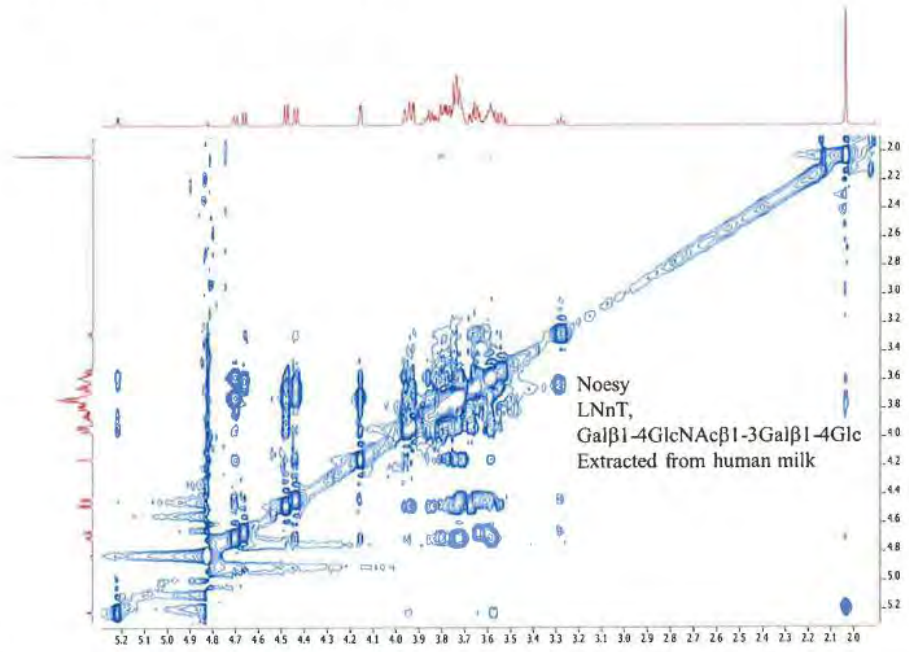

Figure 6. NOESY spectrum of Galβ1-4GlcNAcβ1-3Galβ1-4Glc extracted from human milk.

Conclusions

Synthetic human milk type oligosaccharides 2'FL and LNnT provided by Glycom A/S yielded identical one-dimensional proton NMR spectra as compared to their natural counterparts. The NOESY spectra of synthetic and natural oligosaccharides were also similar, minor spectral differences arise from sample concentration variations.

In conclusion, based on data presented in this report, the synthetic oligosaccharides 2'FL and LNnT are pure and identical to natural human milk derived 2'-fucosyllactose and Lacto-N-neotetraose.

Date April 20th, 2015

Sample preparation

Data analysis and reporting

Approved by

References

1. A ^{1}H and ^{13}C study of oligosaccharides from human milk. Application of the computer program CASPER. Hermansson K., Jansson P.-E., Kenne L., Widmalm G. and Lindh F. *Carbohydr. Res.* 235 (1992) 69-81.
2. www.glycosciences.de/database/structure LinucsID 1222

Template 1.2

ANALYTICAL REPORT A-GH-2014-001

ANALYTICAL REPORT ON CHEMICAL STRUCTURE

Glycom Compound Code	Common Short Name	Cost Center
2C4	2FL	335/1240/600

Date of Request	02.12.2013	**To:**	István Bajza, Christoph Röhrig, Gyula Dékány, Györgyi Osztrovszky
Request by		**cc:**	
Aim of Study	To summarize the available analytical data connected to structure assignment of synthesized 2'-O-Fucosyllactose (2'-FL). To demonstrate the chemical identity between synthesized 2'-FL by Glycom and naturally occurring 2'-FL based on comparison to reported data in the literature.		

Author	**January 8, 2014**
Reviewed by	**January 14, 2014**

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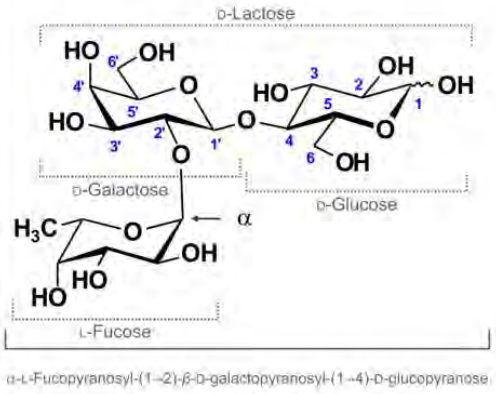
Template 1.2

ANALYTICAL REPORT A-GH-2014-001

I. Compound details

Product name	2'-O-Fucosyllactose
IUPAC name	α-L-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
Abbreviations	2'-FL, 2'FL, 2-FL, 2FL
Molecular formula	$C_{18}H_{32}O_{15}$
Molecular weight	488.44 (mono-isotopic mass: 488.1741)
CAS number	41263-94-9
Chemical Structure	

II. Introduction

2'-O-Fucosyllactose (2'-FL) is a naturally occurring trisaccharide found in mammalian milk with the highest concentrations present in *human* milk, and is therefore typically referred to as a *human* milk oligosaccharide (HMO). 2'-FL is a trisaccharide consisting of L-fucose, D-galactose and D-glucose. Alternatively, the structure can be described as consisting of the monosaccharide L-fucose and the disaccharide D-lactose, which are linked by a α(1→2) bond to form the trisaccharide. An oligosaccharide is a generic term for a saccharide polymer with a typical degree of polymerization below 20 (~3-20). Whilst human milk contains a combination of oligosaccharides (HMOs), it is important at this stage to emphasize that 2'-FL is a clearly defined trisaccharide.

The molecular structure of 2'-FL has been elucidated by Richard Kuhn in 1955 (1), and since then detailed structure characterization by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) techniques was also reported (2). Our aim is to confirm without any doubt that the manufactured 2'-FL is fully identical to the naturally occurring 2'-FL that is present in human breast milk based on ^{1}H- and ^{13}C-NMR and mass spectrometry (MS) data.

This report summarizes the available analytical data connected to structural assignment. The NMR and MS data are reported on batch L06112K of chemically synthesized 2'-FL

III. NMR data

The ^{1}H and ^{13}C NMR spectra were acquired on a Varian VNMR SYSTEM™ spectrometer using a triple resonance Z-gradient probe at 600 and 150 MHz, respectively. 25 mg of 2'-FL was dissolved in 0.6 mL D_2O for the tests. The measurements were performed at 25 °C. Chemical shifts are referenced to DSS (δ_H: 0.00 ppm, δ_C: 0.00 ppm). The full and the expanded ^{1}H NMR spectrum, and the ^{13}C NMR spectrum are shown in Figures 1, 2 and 3, respectively.

Resonance assignments of the spectra were based on series of one dimensional selective TOCSY measurements (Figures 4) and two dimensional ^{1}H-^{13}C correlation experiments (gHSQCAD, gHMBCAD Figures 5, 6 and 7). These measurements were performed on batch 2FL-1000g. The one dimensional ^{1}H and ^{13}C spectra of 2'FL batches L06112K and 2FL-1000g are identical within experimental error.

Dissolved in D_2O the glucose ring exists as a mixture of α and β forms, with isomeric ratio $\alpha/\beta \approx 1/1$. The assignments of ^{1}H and ^{13}C resonances are given in Table 1.

The assigned ^{1}H and ^{13}C NMR spectra and the 2D NMR spectra (HSQC and HMBC) are in full agreement with the structure of 2'-FL. Furthermore the differences between the measured ^{1}H and ^{13}C NMR chemical shifts of the chemically synthesized 2'-FL (batch L06112K) and the reported chemical shifts of 2'-FL from authentic source, Seikagu Co (2) are less than 0.2 ppm. These differences are due to the different concentration of the solution used for the measurements, 85 mMol for the synthetic 2'-FL and 3-15.4 mMol for authentic 2'-FL. The good agreement between the two sets of chemical shifts proves the identity of the synthetic 2'-FL with authentic 2'-FL.

Table 1. ^{1}H and ^{13}C resonance assignments of **2'-FL (batch L06112K)**

Ring	proton	δ (ppm)	multiplicity	J (Hz)	carbon	δ (ppm)
α-D glucose	H-1	5.22	d	4.0	C-1	94.5
	H-2	3.59	dd	9.2, 4.0	C-2	74.0
	H-3	3.80	dd	9.6, 9.2	C-3	74.0
	H-4	3.71	dd	9.6, 9.0	C-4*	78.6
	H-5	3.91	m		C-5	73.1
	H-6x	3.90	m		C-6	62.7
	H-6y	3.80	m			
β-D glucose	H-1	4.63	d	8.1	C-1	98.6
	H-2	3.29	dd	9.3, 8.1	C-2	76.6
	H-3	3.58	dd	9.6, 9.3	C-3	77.0
	H-4	3.72	dd	9.8, 9.6	C-4*	78.5
	H-5	3.47	ddd	9.8, 5.2, 1.8	C-5	78.0
	H-6x	3.94	dd	11.8, 1.8	C-6	62.9
	H-6y	3.76	dd	11.8, 5.2		
β-D galactose	H-1	4.52	d	7.8	C-1	103.0#
	H-2	3.66	dd	9.0, 7.8	C-2	79.0
	H-3	3.88	m		C-3	77.9
	H-4	3.90	m		C-4	71.9
	H-5	3.81	m		C-5	76.3
	H-6x	3.81	m		C-6	63.8#
	H-6y	3.74	m			
α-L-fucose	H-1	5.30	d	2.0	C-1	102.0
	H-2	3.80	m		C-2	70.9
	H-3	3.80	m		C-3	72.3#
	H-4	3.82	d	1	C-4	74.4
	H-5	4.22, 4.25	qd	6, 1	C-5	69.6#
	CH_3	1.22	d	6	CH_3	18.0#

* :reversed assignment is also possible

#: averaged chemical shift of split peak due to presence of α and β forms of the glucose ring

Template 1.2 **ANALYTICAL REPORT A-GH-2014-001**

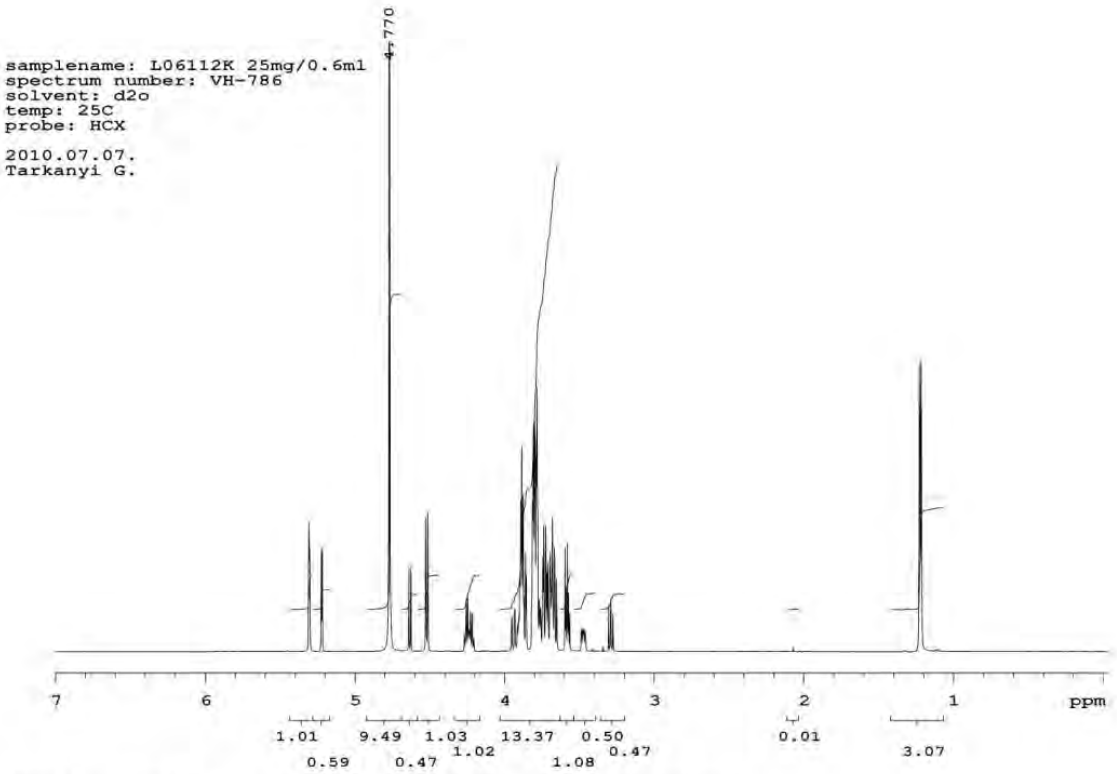


Figure 1. ^{1}H NMR spectrum of **2'-FL (batch L06112K)** in D2O at 25 °C, 600 MHz

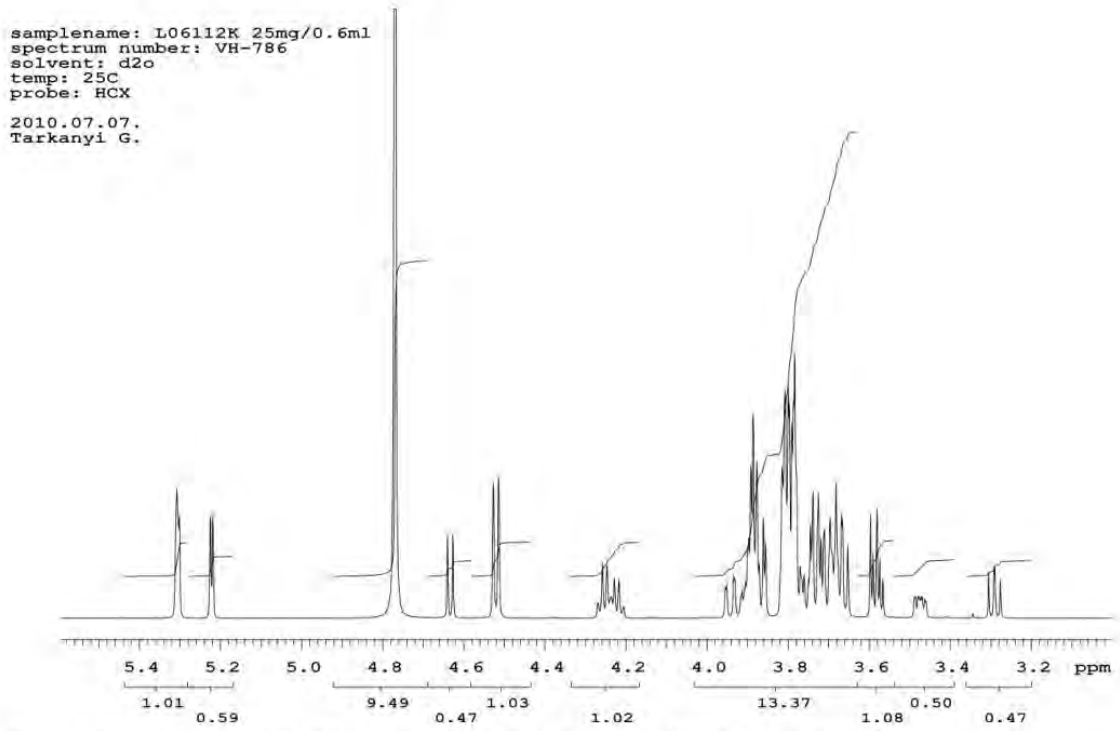


Figure 2. Expanded part of the ^{1}H NMR spectrum of **2'-FL (batch L06112K)** in D2O at 25 °C, 600 MHz

Template 1.2

ANALYTICAL REPORT A-GH-2014-001

samplename: L06112K 25mg/0.6ml
spectrum number: VC-279
solvent: d2o
temp: 25C
probe: HCX

2010.07.07.
Tarkanyi G.

INDEX	FREQUENCY	PPM	HEIGHT
1	15528.832	102.978	23.3
2	15521.007	102.926	27.0
3	15387.982	102.044	42.1
4	14869.574	98.606	23.4
5	14254.332	94.526	27.0
6	11911.717	78.991	42.3
7	11856.942	78.628	27.9
8	11839.335	78.511	21.5
9	11764.998	78.018	19.3
10	11752.282	77.934	52.0
11	11612.410	77.006	21.3
12	11556.656	76.637	18.8
13	11508.728	76.319	30.6
14	11216.268	74.379	44.9
15	11161.493	74.016	30.5
16	11158.559	73.997	31.4
17	11021.621	73.089	31.5
18	10912.070	72.362	27.6
19	10907.180	72.330	23.3
20	10839.689	71.882	26.9
21	10835.776	71.856	22.1
22	10688.079	70.877	42.2
23	10502.235	69.644	30.0
24	10498.322	69.618	20.0
25	9627.789	63.846	28.2
26	9623.876	63.820	22.1
27	9482.048	62.879	17.4
28	9462.485	62.749	21.4
29	2710.471	17.974	26.3
30	2705.581	17.942	34.5

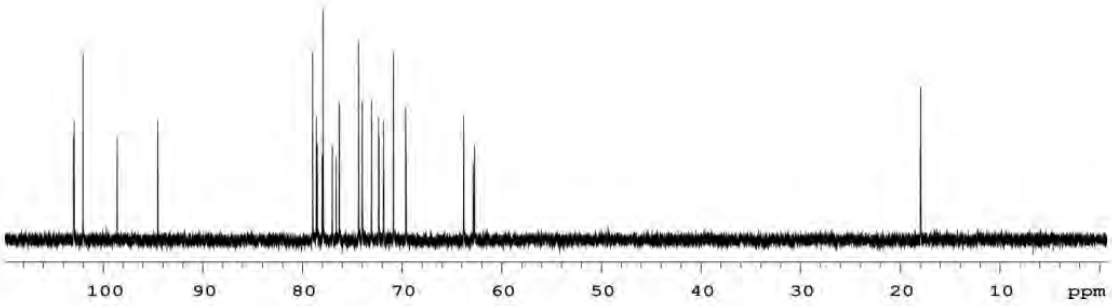


Figure 3. ^{13}C NMR spectrum of **2'-FL (batch L06112K)** in D_2O at 25°C, 150 MHz

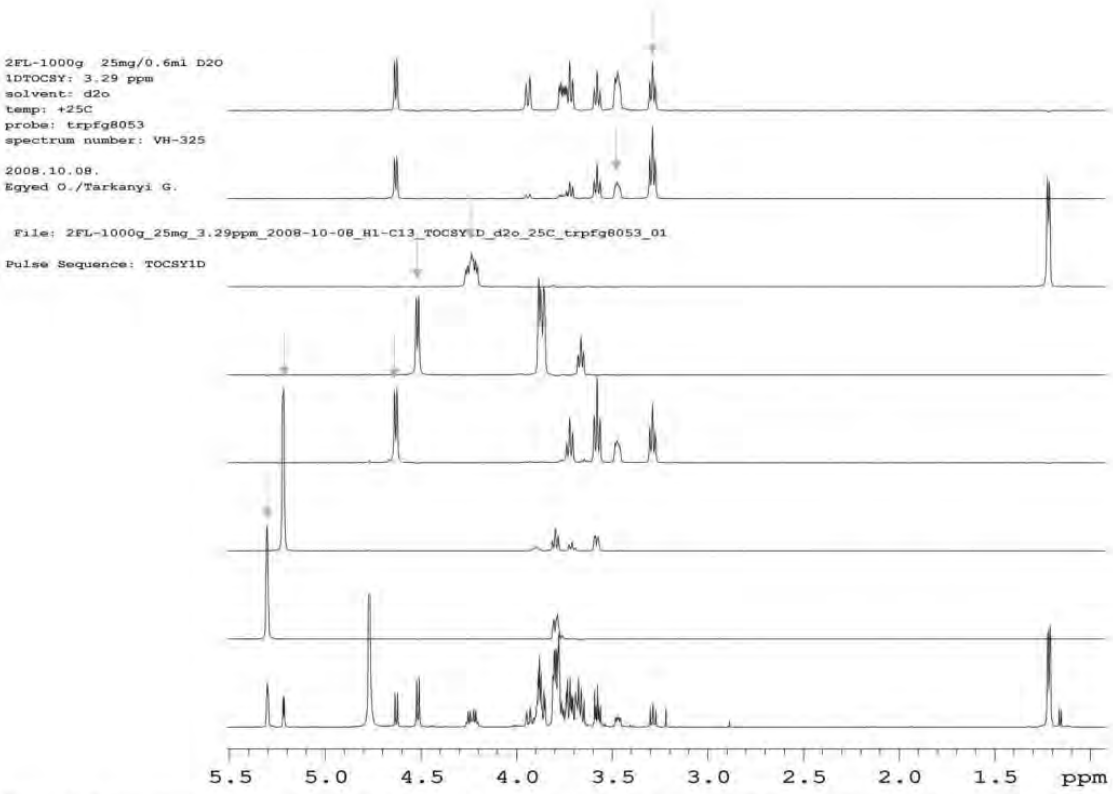


Figure 4. Series of selective one dimensional TOCSY spectra of **2'-FL (batch 2FL-1000g)** in D2O at 25 °C, 600 MHz

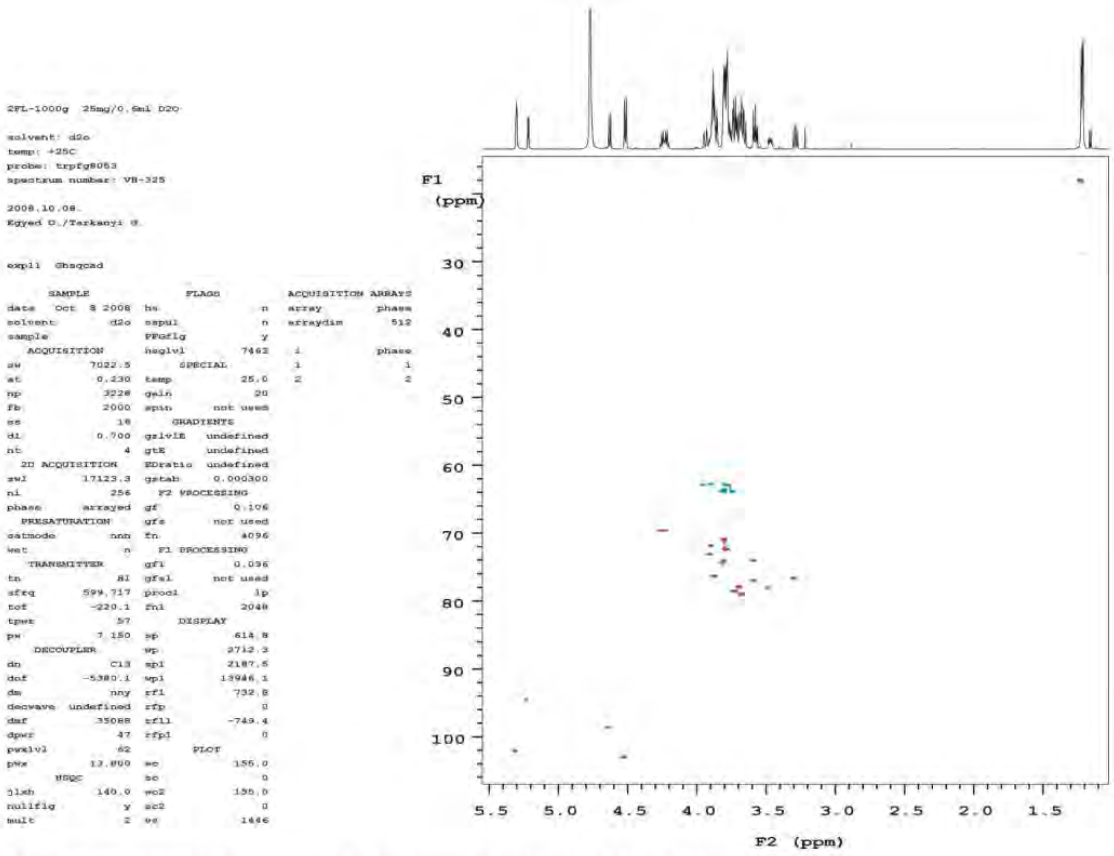


Figure 5. ^{1}H – ^{13}C HSQC spectrum of **2'-FL (batch 2FL-1000g)** in D2O at 25 °C, 600 MHz

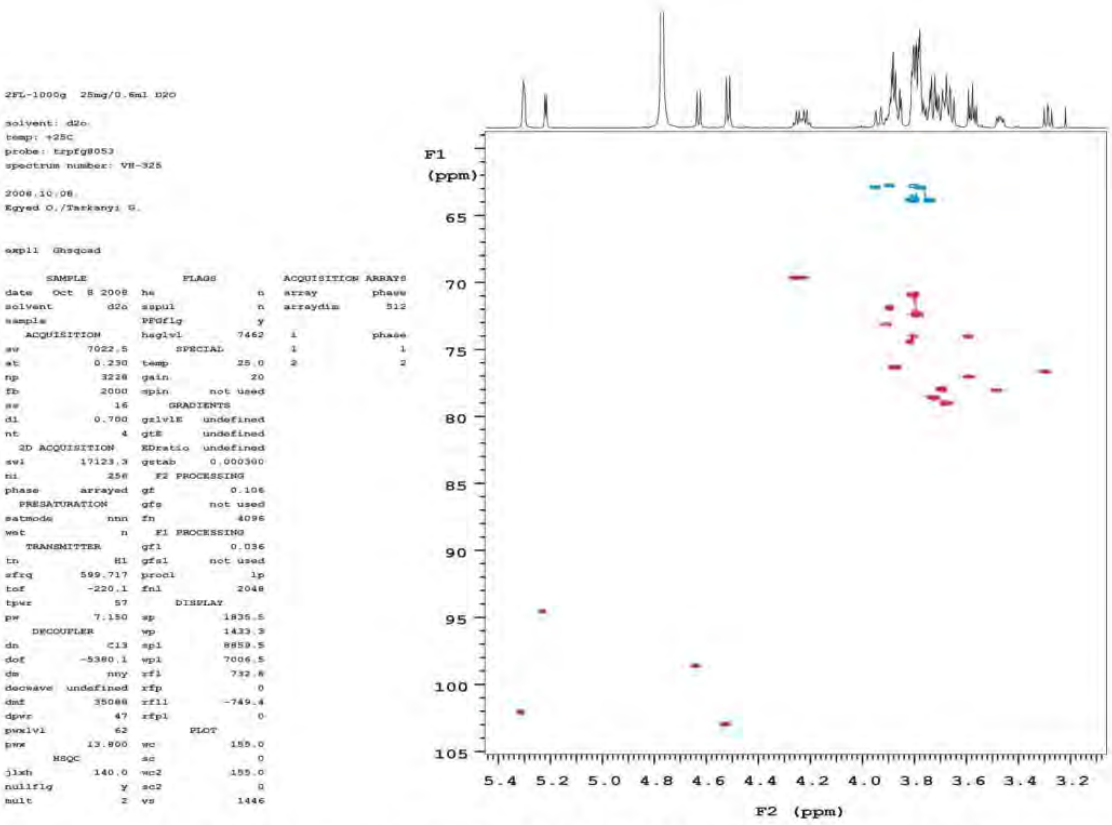


Figure 6. Expanded part of the $^{1}H - ^{13}C$ HSQC spectrum of **2'-FL (batch L06112K)** in D2O at 25 °C, 600 MHz

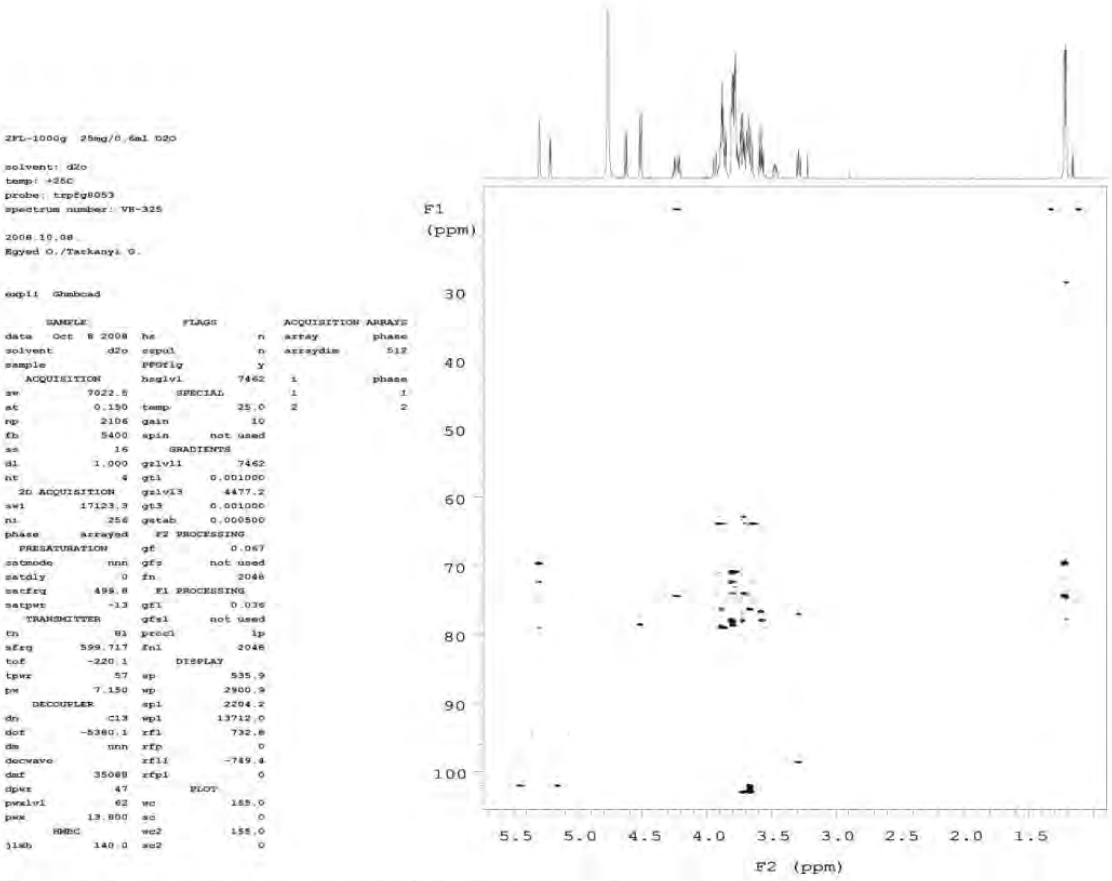


Figure 7. $^{1}H - ^{13}C$ HMBC spectrum of **2'-FL (batch 2FL-1000g)** in D2O at 25 °C, 600 MHz

IV. MS-data

The following data has been collected according to the instrumental parameters listed below:

Instrument:	Bruker micrOTOF-Q II
Sample preparation:	Sample was dissolved in ACN : H_2O = 1 : 1 (0.1% HOAc)
Flow rate:	Direct infusion 3µL/min
Ionization:	ESI negative
Dry temperature:	180°C
Mode:	Full scan MS and CID MS/MS (collision energy 15eV)
Calibration:	with Na-formate cluster solution

The full scan MS spectrum is shown in Figure 8. In the MS spectrum of 2'-FL (batch L06112K) the most intense ion is at m/z 487.1696 Dalton, corresponding to M-H. The exact mass confirmed the $C_{18}H_{32}O_{15}$ molecular formula of 2'-FL since the prediction software proposed the molecular formula of 2'-FL-H as best score (Figure 9).

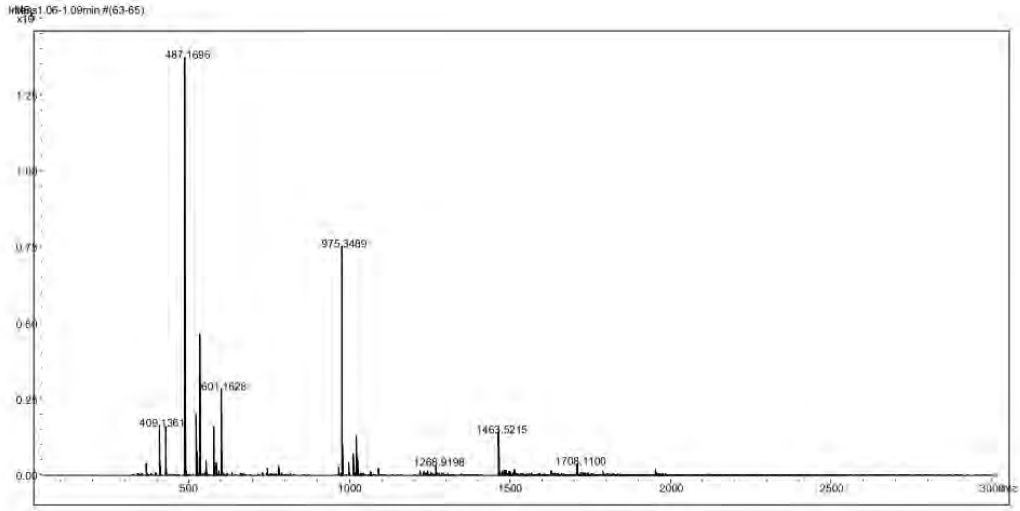


Figure 8. Full scan MS spectrum of **2'-FL (batch L06112K)**

SmartFormula Manually

Min: C13

Max:

C 13-n

Generate

Help

Note: for m < 2000 the elements C, H, N, and O are considered implicitly.

Measured m/z: 487.1696 Tolerance: 4 mDa Charge: -1

Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
487.1696	1	C 18 H 31 O 15	23.64	487.1668	-2.8	-5.7	5.0	3.5	even	ok
	2	C 19 H 27 N 4 O 11	63.85	487.1682	-1.4	-2.9	7.9	8.5	even	ok
	3	C 16 H 19 N 14 O 5	19.49	487.1668	-2.8	-5.7	14.9	14.5	even	ok
	4	C 20 H 23 N 8 O 7	100.00	487.1695	-0.1	-0.2	20.8	13.5	even	ok
	5	C 17 H 15 N 18 O	42.49	487.1682	-1.4	-2.9	27.2	19.5	even	ok
	6	C 24 H 27 N 2 O 9	17.11	487.1722	2.6	5.3	27.5	12.5	even	ok
	7	C 21 H 19 N 12 O 3	38.76	487.1709	1.3	2.6	35.3	18.5	even	ok
	8	C 25 H 23 N 6 O 5	2.62	487.1735	3.9	8.1	46.5	17.5	even	ok
	9	C 31 H 23 N 2 O 4	2.97	487.1663	-3.3	-6.7	65.4	21.5	even	ok
	10	C 32 H 19 N 6	6.00	487.1677	-1.9	-4.0	78.2	26.5	even	ok
	11	C 36 H 23 O 2	7.98	487.1704	0.8	1.5	89.3	25.5	even	ok

Automatically locate monoisotopic peak Maximum number of formulas: 500

Check rings plus double bonds Minimum: -0.5 Maximum: 40

Electron configuration: even

Filter H/C element ratio Minimum H/C: 0 Maximum H/C: 3

Estimate carbon number Generate immediately

Show Pattern

Figure 9. Confirmation of the formula of M-H ion

CID MS/MS (collision energy 15eV) of the M-H ion of 2'-FL (batch L06112K) is shown in Figure 10. The assignment of the observed fragment ions is summarized in Figure 11. The fragmentation pattern is in full agreement with the structure of 2'-FL. These data further confirm that the synthesized 2'-FL (batch L06112K) is identical with 2'-FL of human milk.

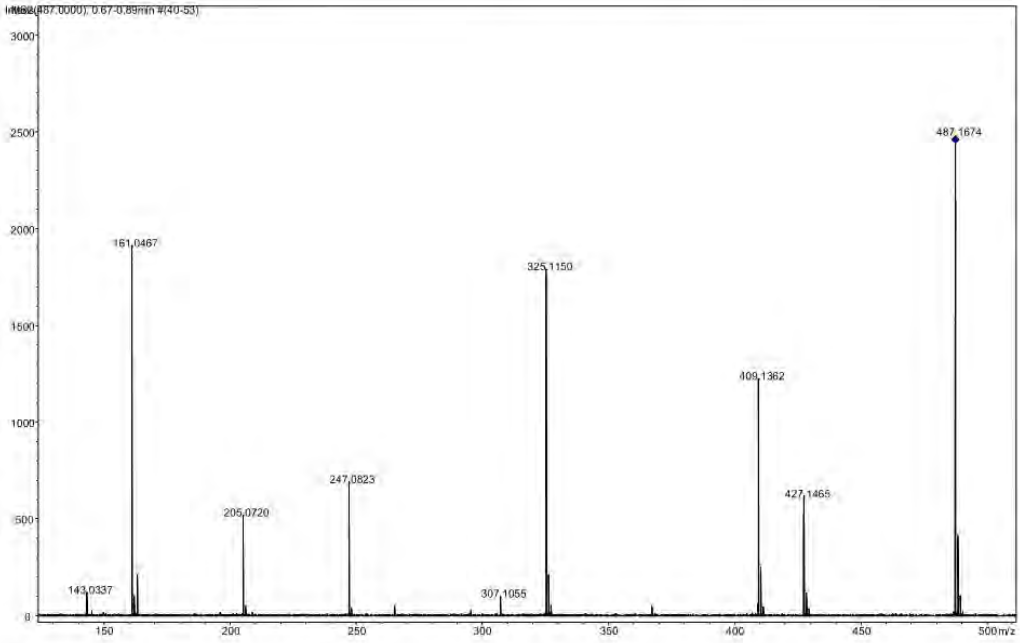
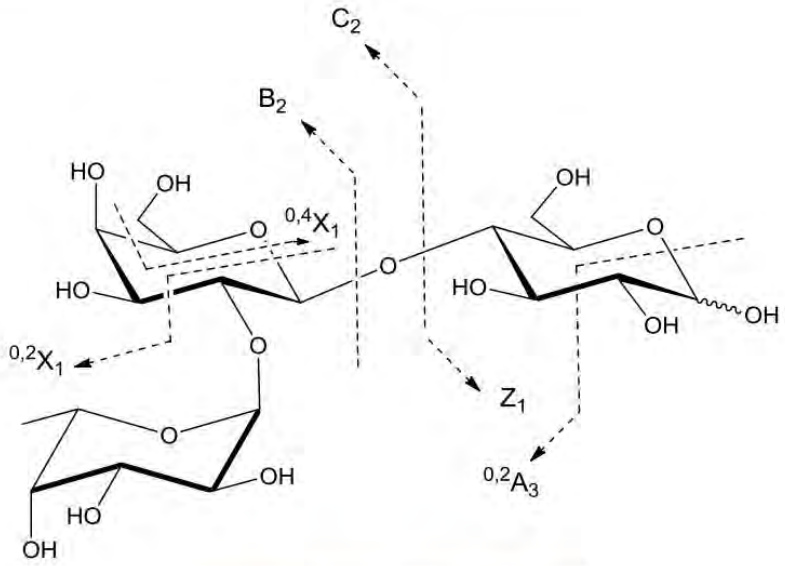


Figure 10. CID MS/MS (collision energy 15eV) MS spectrum of the M-H ion of **2'-FL (batch L06112K)**



Fragment ions	Assignment
487	$[M-H]^-$
427	$^{0,2}A_3$
409	$^{0,2}A_3$-H_2O
325	C_2
307	B_2
247	$^{0,4}X_1$-H_2O/C_2
205	$^{0,2}X_1$/C_2
161	Z_1

Figure 11. Assignment of the fragment ions of the CID MS/MS (collision energy 15eV) spectrum of the M-H ion of 2'-FL (batch L06112K)

V. References

[1] KUHN, R., BAER, H. H. & GAUHE, A. **1955**. Fucosido-lactose, das Trisaccharid der Frauenmilch. *Chem. Ber., 88*, 1135-1146.
[2] SHIZUKA, Y., NEMOTO, T., FUJIWARA, M., FUJITA, K.-I. & NAKANISHI, H. **1999**. Three-dimensional structure of fucosyllactoses in an aqueous solution. *J. Carbohydr. Chem., 18*, 523-533.

Template 1.2

ANALYTICAL REPORT A-GH-2013-110

ANALYTICAL REPORT ON CHEMICAL STRUCTURE

Glycom Compound Code	Common Short Name	Cost Center
1C6	LNnT	410/1190/600

Date of Request	08.08.2013	**To:**	
Request by		**cc:**	
Aim of Study	To summarize the available analytical data connected to structure assignment of synthesized Lacto-N-neotetraose. To demonstrate the chemical identity between synthesized LNnT by Glycom and naturally occurring LNnT based on comparison to reported data in the literature.		

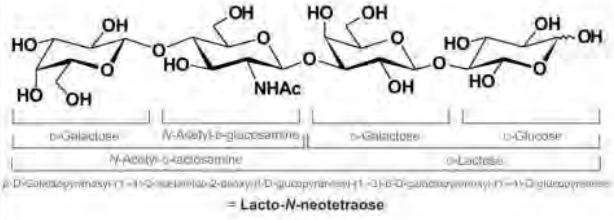
Author		**October 31, 2013**
Reviewed by		**October 31, 2013**

Table of Contents

Template 1.2

ANALYTICAL REPORT A-GH-2013-110

I. Compound details

Product name	Lacto-*N*-neotetraose
IUPAC name	β-D-Galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
Abbreviations	LNnT
Molecular formula	$C_{26}H_{45}NO_{21}$
Molecular weight	707.63
CAS number	13007-32-4
Chemical Structure	 = Lacto-*N*-neotetraose

II. Introduction

Lacto-*N*-neotetraose (LNnT) is a naturally occurring tetrasaccharide found in mammalian milk with the highest concentrations present in *human* milk, and is therefore typically referred to as a *human* milk oligosaccharide (HMO). LNnT is a linear tetrasaccharide consisting of D-galactose, *N*-acetylglucosamine, D-galactose and D-glucose. An oligosaccharide is a generic term for a saccharide polymer with a typical degree of polymerization below 20 (~3-20). Whilst human milk contains a combination of oligosaccharides (HMOs), it is important at this stage to emphasize that LNnT is a clearly defined tetrasaccharide.

The molecular structure of LNnT has been elucidated by Richard Kuhn in 1962 (1), and since then detailed structure characterization by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) techniques was also reported (2). Our aim is to confirm without any doubt that the manufactured LNnT is fully identical to the naturally occurring LNnT that is present in human breast milk based on ^{1}H- and ^{13}C-NMR and mass spectrometry (MS) data.

This report summarizes the available analytical data connected to structural assignment. All data were collected on batch L01032K of chemically synthesized LNnT

III. NMR data

The ^{1}H and ^{13}C NMR spectra were acquired on a Varian VNMR SYSTEM™ spectrometer using a triple resonance Z-gradient probe at 600 and 150 MHz, respectively. 16 mg of LNnT was dissolved in 0.7 mL D_2O:CD_3OD 3:2 v/v for the tests. The measurements were performed at 5 °C. Chemical shifts are referenced to the residual methanol signals (δ_H: 3.29 ppm, δ_C: 49.15 ppm). The full and the expanded ^{1}H NMR spectrum, and the ^{13}C NMR spectrum are shown in Figures 1, 2 and 3, respectively.

Resonance assignments were based on two-dimensional ^{1}H-^{1}H (gDQCOSY, TOCSY, Figures 4 and 5) and ^{1}H-^{13}C correlation experiments (gHSQCAD, gHMBCAD Figures 6, 7, 8 and 9).

Dissolved in D_2O:CD_3OD = 3:2 v/v the glucose ring exists as a mixture of α and β forms, with isomeric ratio α/β = 55/45. The assignments of ^{1}H and ^{13}C resonances are given in Table 1.

The assigned ^{1}H and ^{13}C NMR spectra and the 2D NMR spectra (COSY, TOCSY, HSQC and HMBC) are in full agreement with the structure of LNnT. Furthermore the differences between the measured ^{1}H and ^{13}C NMR chemical shifts of the chemically synthesized LNnT (batch L01032K) and the reported chemical shifts of LNnT isolated from human milk (2) are less than 0.1 and 0.3 ppm, respectively. These differences are due to the different measurement conditions, in D_2O:CD_3OD 3:2 v/v at 5 °C for the synthetic LNnT and in D_2O at 27 °C for isolated LNnT. The good agreement between the two sets of chemical shifts proves the identity of the synthetic LNnT with isolated LNnT.

Table 1. ^{1}H and ^{13}C resonance assignments of **LNnT (batch L01032K)**

Ring	proton	δ (ppm)	multiplicity	J (Hz)	carbon	δ (ppm)
Gl (α)	H-1	5.16	d	3.6	C-1	93.2
	H-2	3.51	dd	9.9, 3.6	C-2	72.5
	H-3	3.78	dd	9.9, 8.5	C-3	72.7
	H-4	3.58	dd	10.0, 8.5	C-4	79.5
	H-5	3.89	m		C-5	71.3
	H-6x	3.82	m		C-6	61.2
	H-6y	3.82	m			
Gl (β)	H-1	4.59	d	8.0	C-1	97.2
	H-2	3.22	dd	8.1, 8.0	C-2	75.2
	H-3	3.57	dd	9.2, 8.1	C-3	75.7
	H-4	3.59	dd	9.2, 8.0	C-4	79.5
	H-5	3.53	m		C-5	76.1
	H-6x	3.77	m		C-6	61.3
	H-6y	3.91	m			
Gal(i)	H-1	4.38	d	8.1	C-1	104.3
	H-2	3.56	dd	10.0, 8.1	C-2	71.4
	H-3	3.65	dd	10.0, 3.2	C-3	83.1
	H-4	4.10	dd	3.2, 1.0	C-4	69.7
	H-5*	3.65	m		C-5*	76.2
	H-6x	3.79	m		C-6	62.3
	H-6y	3.65	m			
GlNAc	H-1	4.65	d	8.2	C-1	104.1
	H-2	3.77	dd	9.5, 8.2	C-2	56.4
	H-3	3.67	m		C-3	73.4
	H-4	3.68	m		C-4	79.2
	H-5	3.51	m		C-5	75.8
	H-6x	3.83	m		C-6	61.0
	H-6y	3.90	m			
	NH-CO-$\underline{C}H_3$	2.0	s		NH-CO-$\underline{C}H_3$	23.3
					NH-$\underline{C}O$-CH_3	175.7
Gal(e)	H-1	4.42	d	7.5	C-1	104.2
	H-2	3.50	dd	9.6, 7.5	C-2	72.3
	H-3	3.60	dd	9.6, 3.0	C-3	73.9
	H-4	3.86	dd	3.0, 1.5	C-4	69.9
	H-5*	3.66	m		C-5*	76.6
	H-6x	3.79	m		C-6	62.3
	H-6y	3.65	m			

* reversed assignment is also possible

Template 1.2

ANALYTICAL REPORT A-GH-2013-110

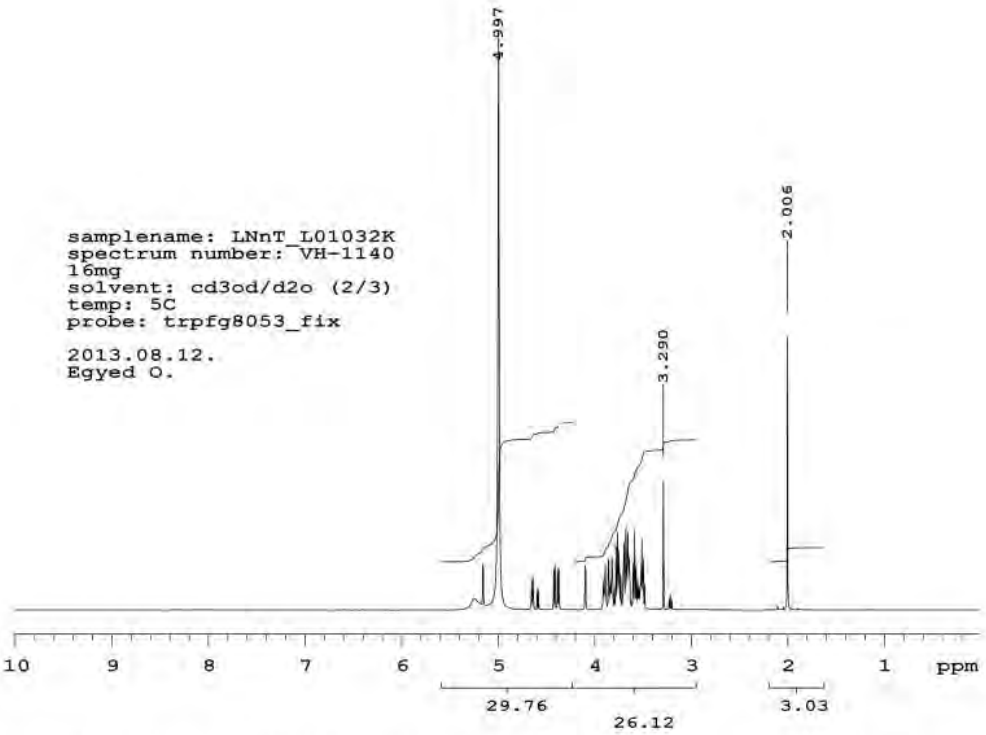


Figure 1. ^{1}H NMR spectrum of **LNnT (batch L01032K)** in D_2O:CD_3OD = 3:2 v/v at 5°C, 600 MHz

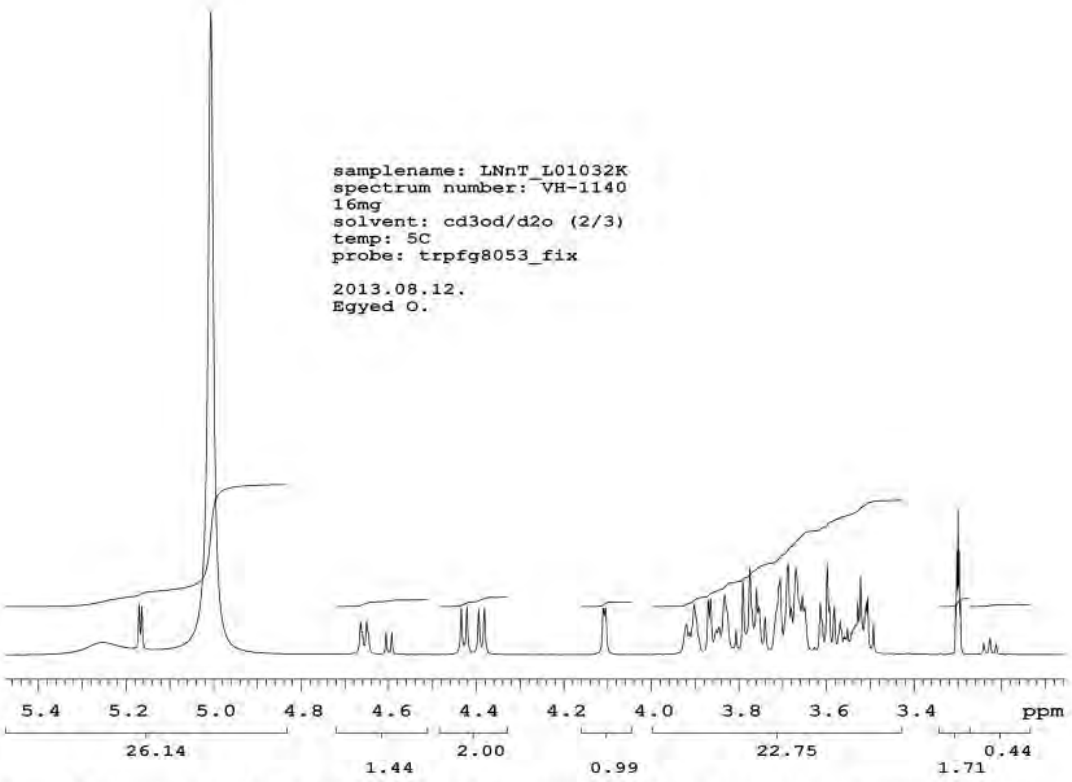


Figure 2. Expanded part of the ^{1}H NMR spectrum of **LNnT (batch L01032K)** in D_2O:CD_3OD = 3:2 v/v at 5°C, 600 MHz

Template 1.2

ANALYTICAL REPORT A-GH-2013-110

INDEX	FREQUENCY	PPM	HEIGHT
1	26494.8	175.697	47.0
2	15708.7	104.170	56.8
3	15697.0	104.092	34.0
4	14650.4	97.152	14.1
5	14044.7	93.135	21.1
6	12531.0	83.098	26.9
7	11976.4	79.420	24.5
8	11953.4	79.268	12.4
9	11938.0	79.165	27.7
10	11556.8	76.637	46.0
11	11487.4	76.177	34.6
12	11475.4	76.097	16.8
13	11433.8	75.822	36.1
14	11413.3	75.686	15.8
15	11329.9	75.133	16.3
16	11133.3	73.829	41.1
17	11069.5	73.406	36.1
18	10966.0	72.720	31.5
19	10932.8	72.499	31.2
20	10897.5	72.266	44.5
21	10758.7	71.345	34.9
22	10755.2	71.322	48.8
23	10533.9	69.854	34.1
24	10516.8	69.741	25.0
25	9397.8	62.320	55.2
26	9217.4	61.124	18.8
27	9188.0	60.929	22.3
28	8504.1	56.394	33.5
29	7476.5	49.580	98.9
30	7455.0	49.437	298.5
31	7433.3	49.293	618.6
32	7411.7	49.150	738.0
34	7368.5	48.863	340.6
35	7346.7	48.719	119.5
36	3510.0	23.276	65.9

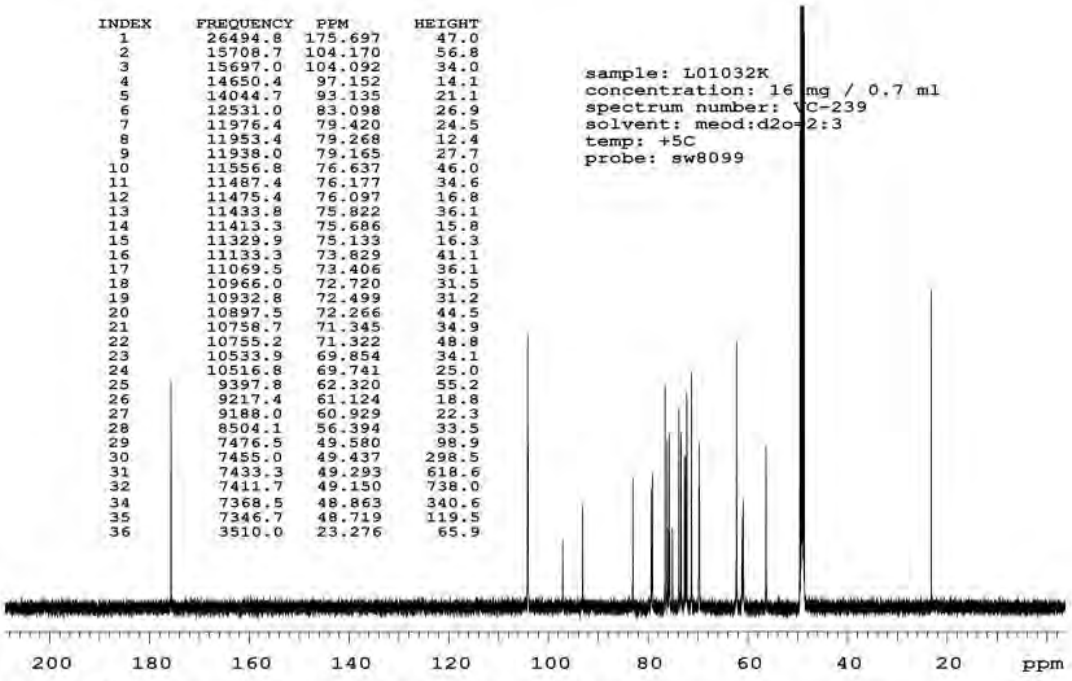


Figure 3. ^{13}C NMR spectrum of **LNnT (batch L01032K)** in D_2O:CD_3OD = 3:2 v/v at 5°C, 150 MHz.

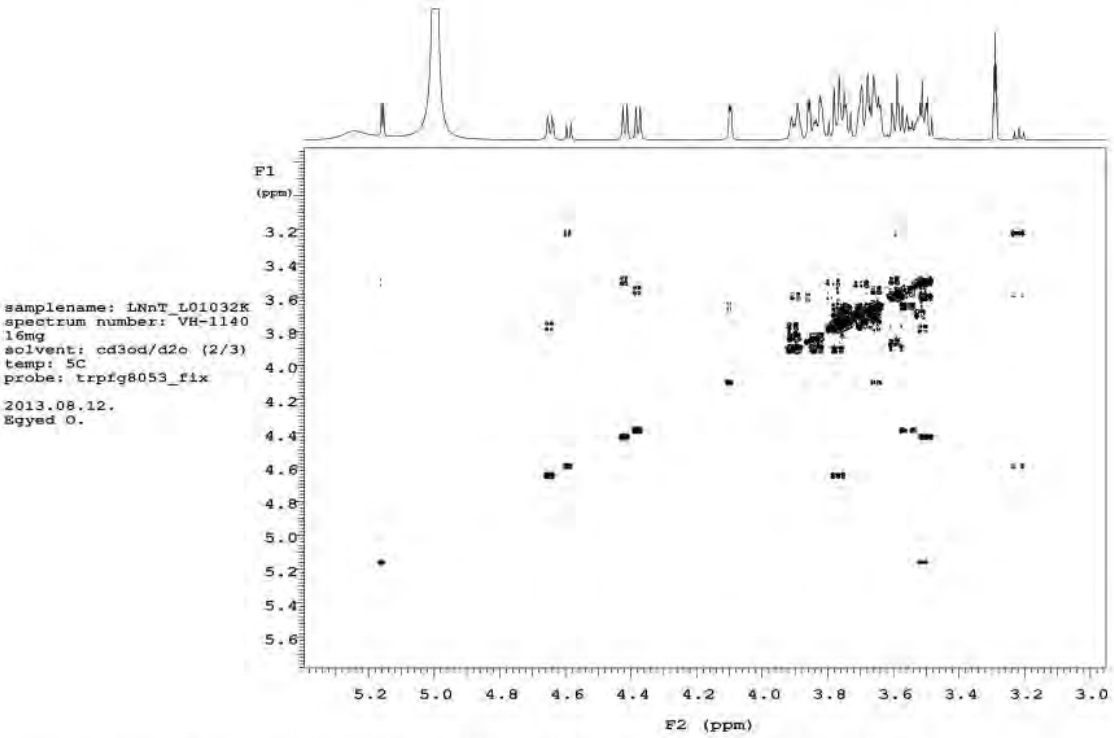


Figure 4. gDQCOSY spectrum of **LNnT (batch L01032K)** in D_2O:CD_3OD = 3:2 v/v at 5°C, 600 MHz

Template 1.2 **ANALYTICAL REPORT A-GH-2013-110**

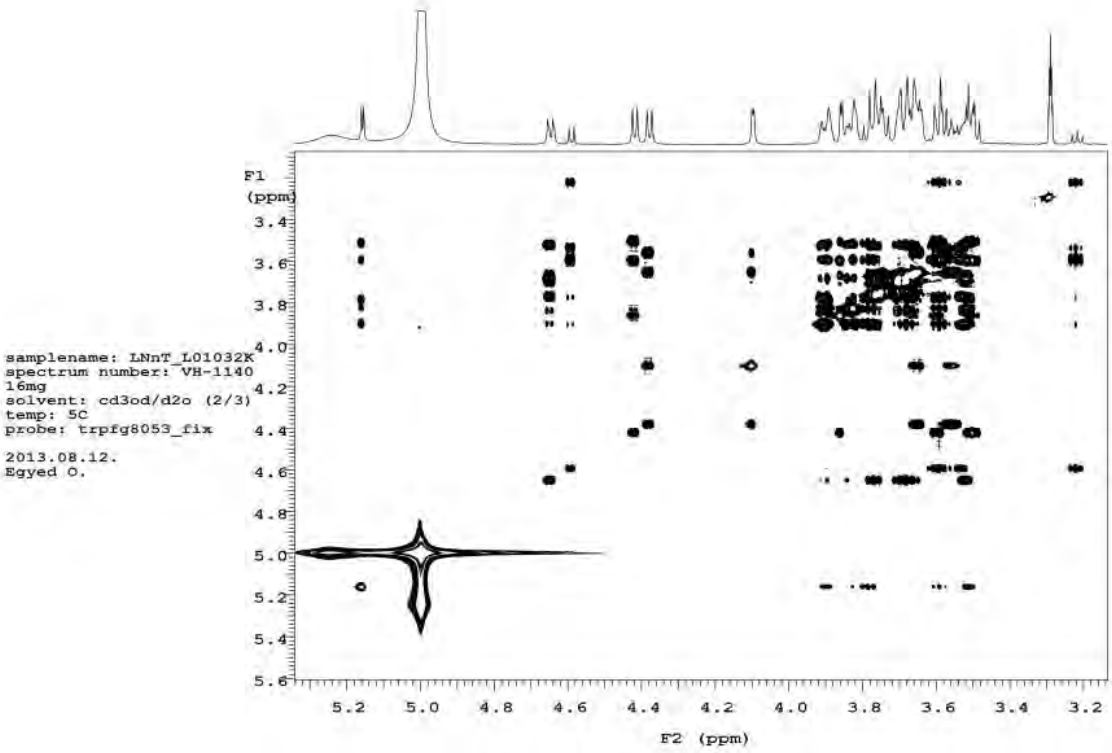


Figure 5. 2D-TOCSY spectrum of **LNnT(batch L01032K)** in D_2O:CD_3OD = 3:2 v/v at 5°C, 600 MHz

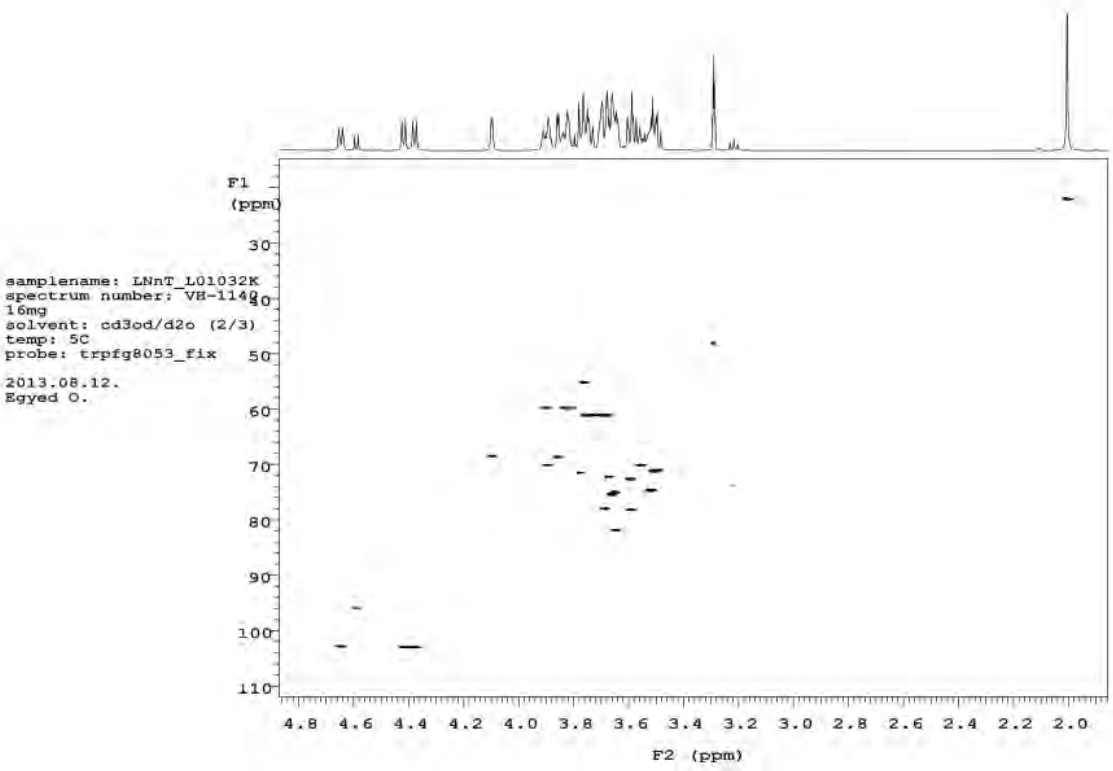


Figure 6. $^{1}H - ^{13}C$ HSQC spectrum of **LNnT (batch L01032K)** in D_2O:CD_3OD = 3:2 v/v at 5°C, 600 MHz

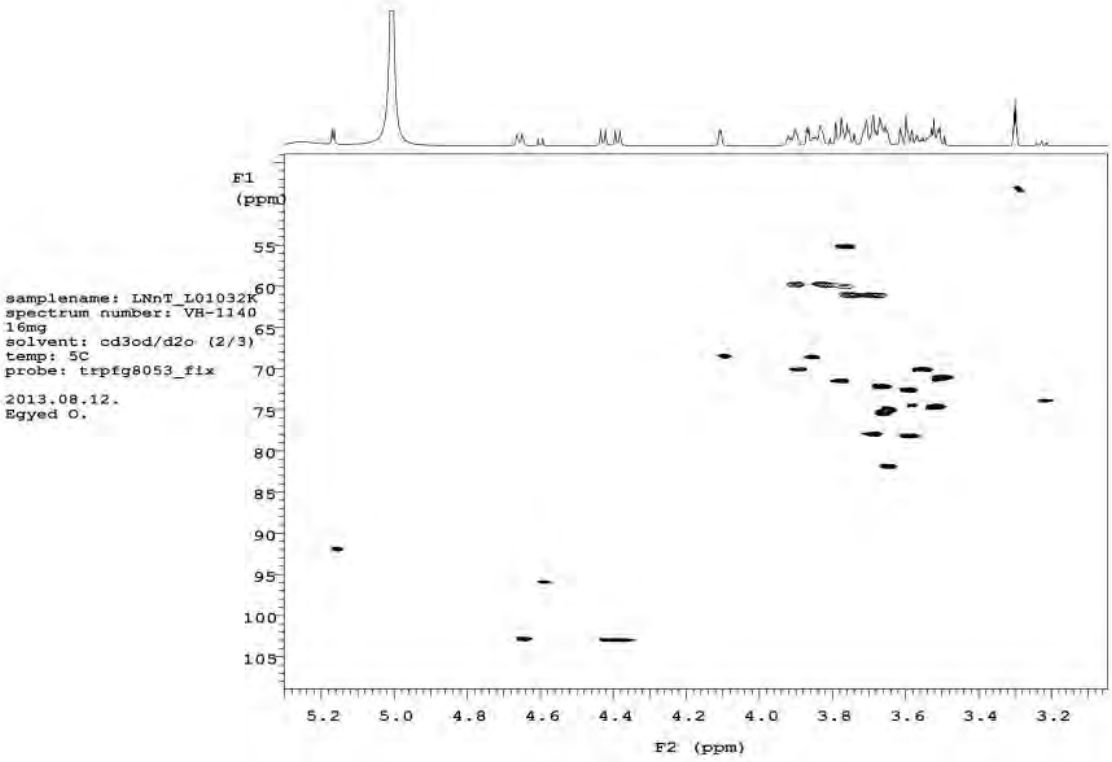


Figure 7. Expanded part of the $^{1}H - ^{13}C$ HSQC spectrum of **LNnT (batch L01032K)** in $D_2O:CD_3OD$ = 3:2 v/v at 5°C, 600 MHz

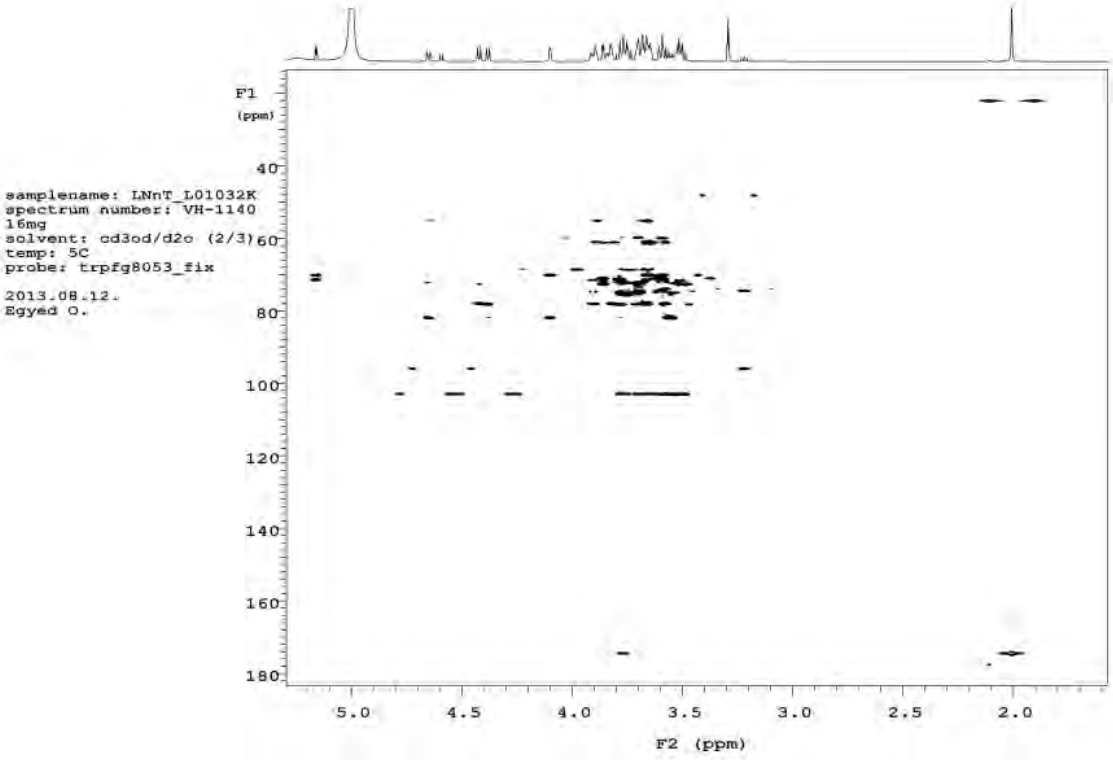


Figure 8. $^{1}H - ^{13}C$ HMBC spectrum of **LNnT (batch L01032K)** in D_2O:CD_3OD = 3:2 v/v at 5°C, 600 MHz

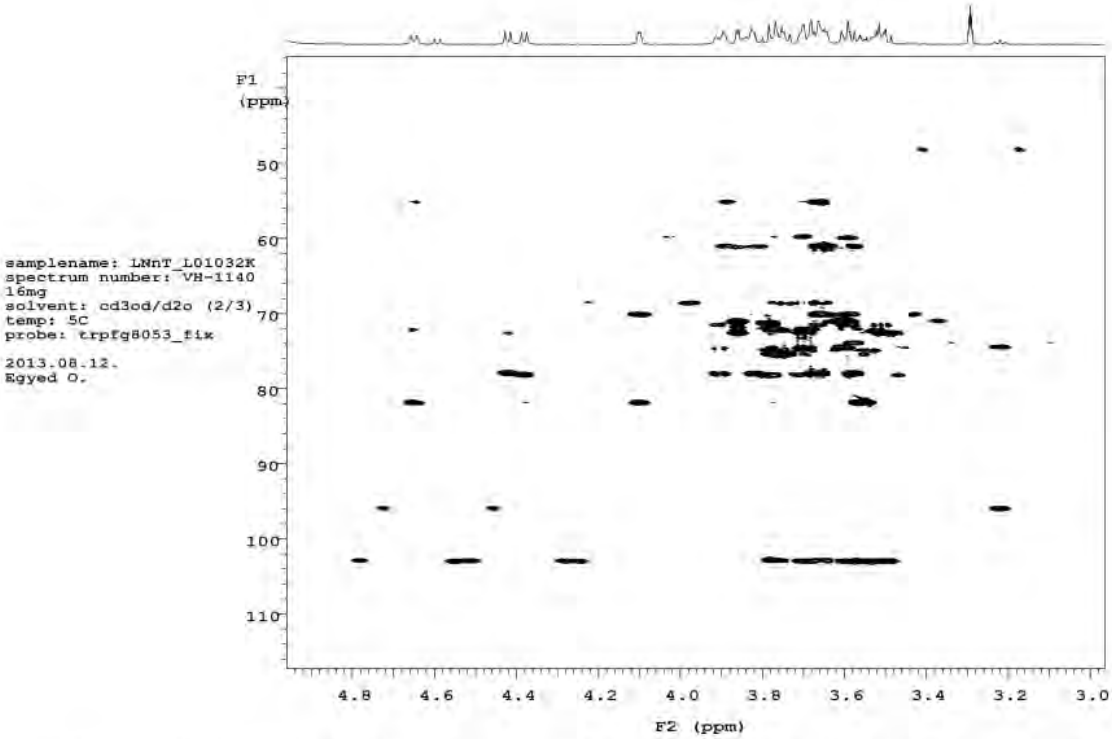


Figure 9. Expanded part of the $^{1}H - ^{13}C$ HMBC spectrum of **LNnT (batch L01032K)** in D_2O:CD_3OD = 3:2 v/v at 5°C, 600 MHz

IV. MS-data

The following data has been collected according to the instrumental parameters listed below:

Instrument:	Bruker micrOTOF-Q II
Sample preparation:	Sample was dissolved in ACN : H_2O = 1 : 1 (0.1% HOAc)
Flow rate:	Direct infusion 3µL/min
Ionisation:	ESI negative
Dry temperature:	180°C
Mode:	Full scan MS and MRM (17eV)
Calibration:	with Na-formate cluster solution

The full scan MS spectrum is shown in Figure 10. In the MS spectrum of LNnT (batch L01032K) the most intense ion is at m/z 706.2388 Dalton, corresponding to M-H. The exact mass confirmed the $C_{26}H_{45}NO_{21}$ molecular formula of LNnT since the prediction software, proposed the molecular formula of LNnT-H as best score (Figure 11).

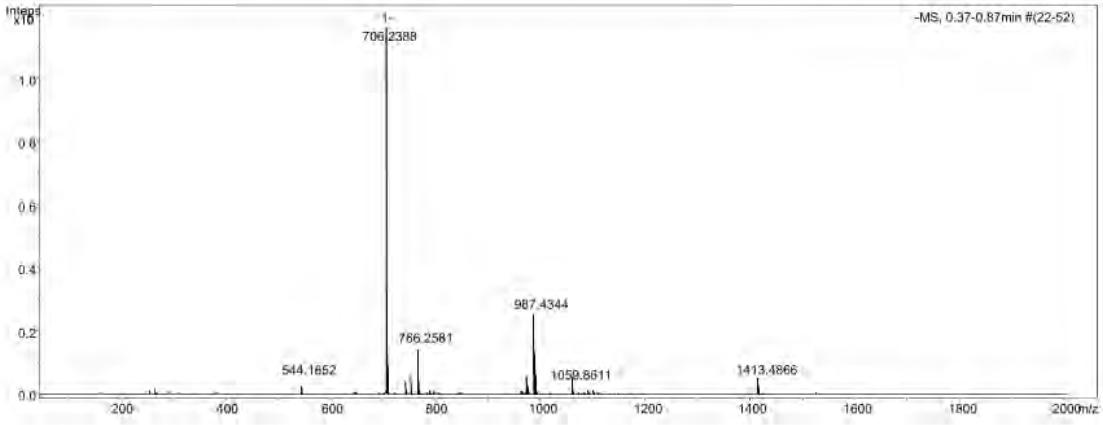


Figure 10. Full scan MS spectrum of LNnT (batch L01032K)

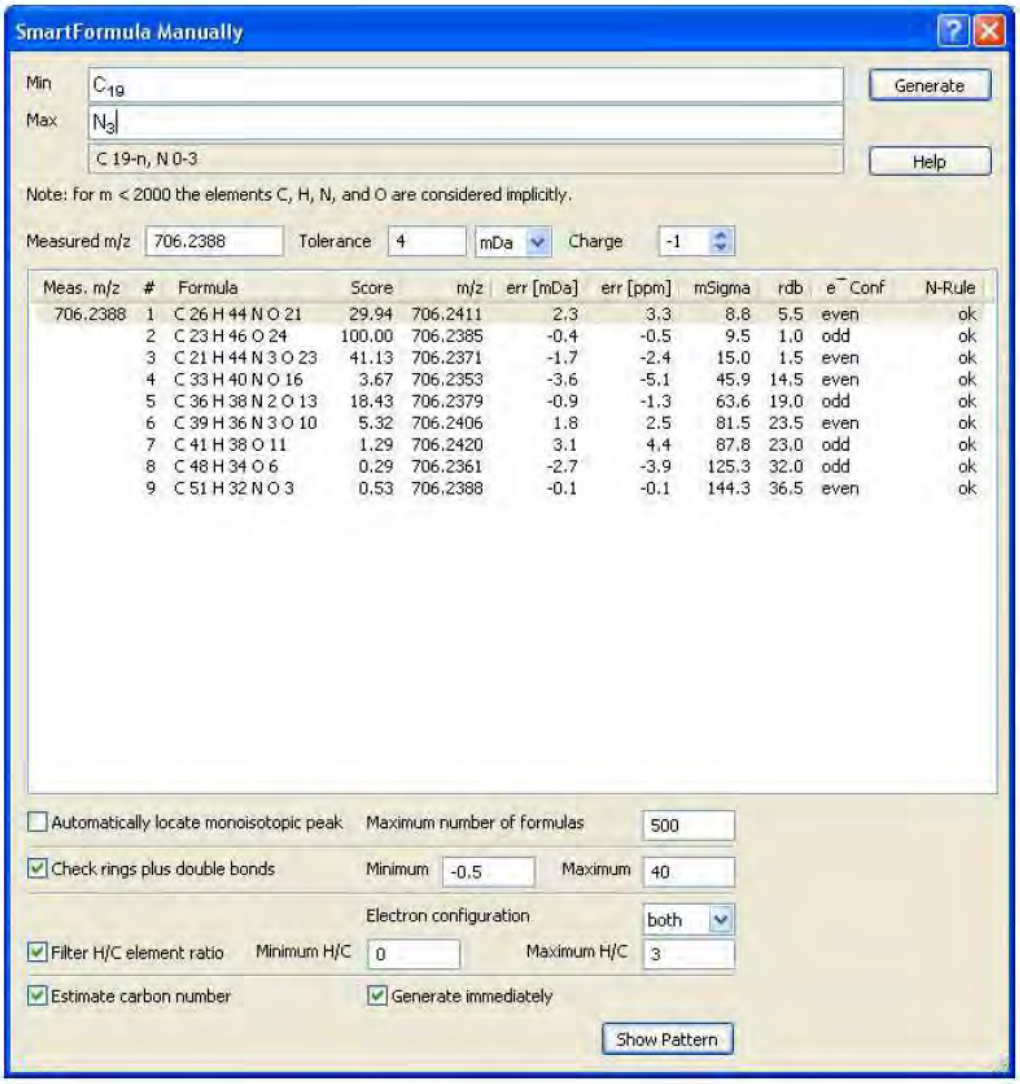


Figure 11. Confirmation of the formula of M-H ion

MRM (17eV) MS spectrum of the M-H ion of LNnT (batch L01032K) is shown in Figure 12. The assignment of the observed fragment ions is summarized in Figure 13. The fragmentation pattern is in full agreement with the structure of LNnT. All fragment ions were observed which were reported for the MS/MS spectrum of M-H ion formed by collision induced dissociation of authentic LNnT (Dextra Laboratories, Reading, UK) (3) and of LNnT isolated from human milk (4). These data unambiguously confirms that the synthesized LNnT (batch L01032K) is identical with LNnT isolated from human milk.

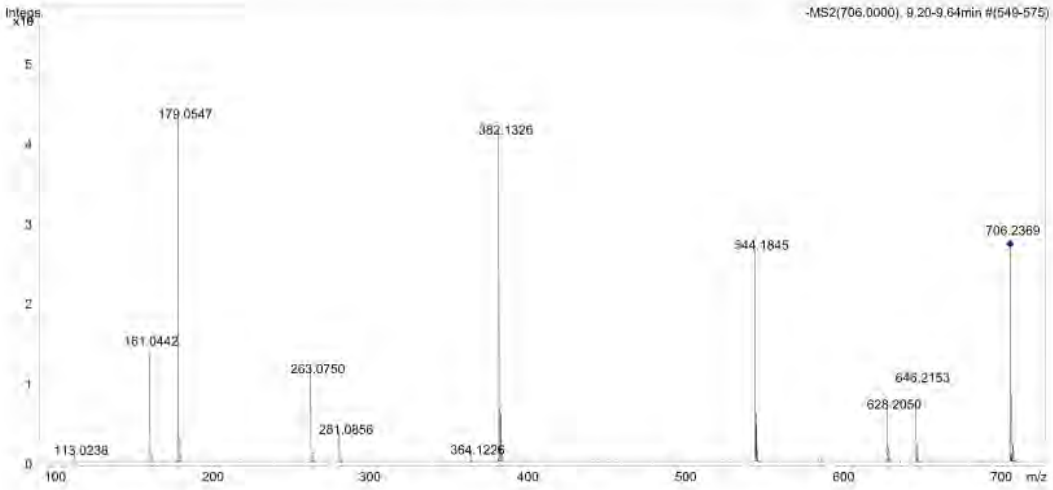
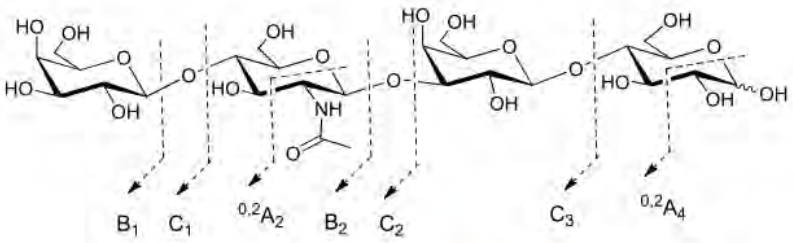


Figure 12. MRM (17eV) MS spectrum of the M-H ion of LNnT (batch L01032K)



Fragment ions	Assignment
706	$[M-H]^-$
646	$^{0,2}A_4$
628	$^{0,2}A_4$-H_2O
544	C_3
382	C_2
364	B_2
281	$^{0,2}A_2$
263	$^{0,2}A_2$-H_2O
179	C_1
161	B_1

Figure 13. Assignment of the fragment ions of the MRM(17 eV) spectrum of the M-H ion of LNnT (batch L01032K)

V. References

[1] KUHN, R. & GAUHE, A. **1962**. Die Konstitution der Lacto-N-neotetraose. *Chem. Ber., 95*, 518-522
[2] STRECKER, G., WIERUSZESKI, J. M., MICHALSKI, J. C. & MONTREUIL, J. **1989**. Assignment of the 1H- and 13C-NMR spectra of eight oligosaccharides of the lacto-*N*-tetraose and neotetraose series. *Glycoconj. J., 6*, 67-83
[3] CHAI, W., Piskarev, V., & Lawson, A.M. **2001**. Negative-ion electrospray mass spectrometry of neutral underivatized oligosaccharides. *Analytical Chemistry,73*, 651-657
[4] PFENNINGER, A., KARAS, M., FINKE, B. & STAHL, B. **2002**. Structural analysis of underivatized neutral human milk oligosaccharides int he negative ion mode by nano-electrospray MS^n. *J. Am. Soc. Mass Spectrom. 13*, 1331-1340

Source: IsoSep.com: http://www.isosep.com/leaflets/3508.pdf

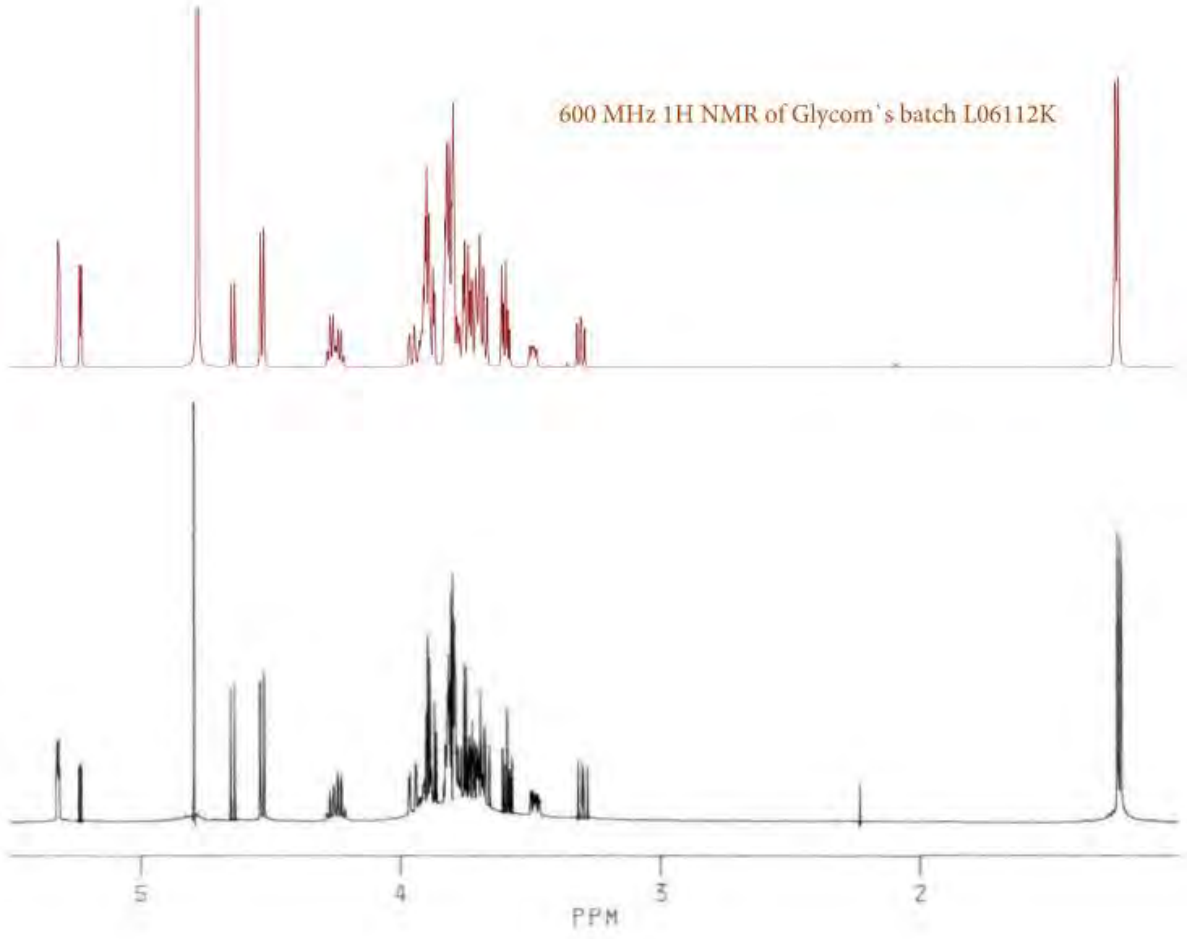
Product number

35/08

Fucα1-2Galβ1-4Glc

2'-Fucosyllactose

[41263-94-9] $C_{18}H_{32}O_{15}$
Source: Human milk
MW: 488.4
NMS: 488.174
Purity: >95% (by HPLC)
Storage: 0-5°C
Reference: Kuhn R, Baer HH, Gauhe A (1956) Chem Ber 89:2513



Source: IsoSep.com: http://www.isosep.com/leaflets/4508.pdf

Product number

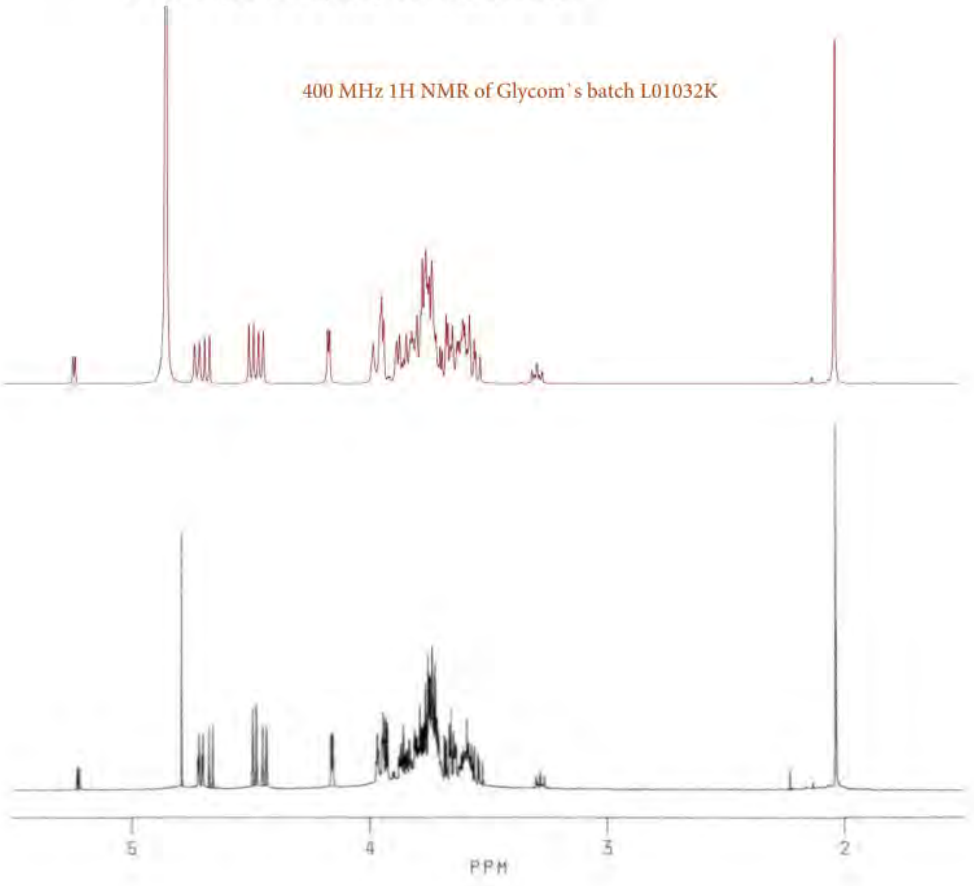
45/08

Galβ1-4GlcNAcβ1-3Galβ1-4Glc

nLcOse$_4$
Lacto-*N*-neotetraose
LNnT

[13007-32-4] $C_{26}H_{45}NO_{21}$
Source: Human milk
MW: 707.6
NMS: 707.248
Purity: >95% (by HPLC)
Storage: 0-5°C

References: 1 Kuhn R, Gauhe A (1962) Chem Ber 95:518
2 Dua VK, Bush CA (1982) Anal Biochem 133:1



X-Ray Crystallography

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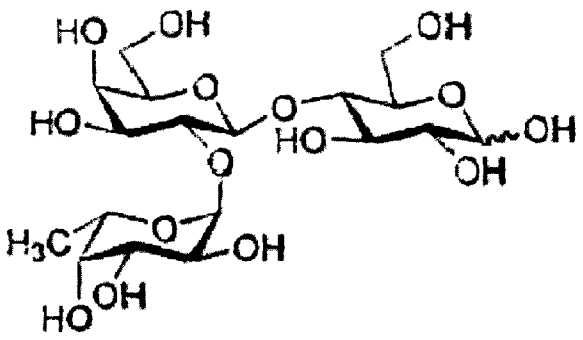
(54) Title: POLYMORPHS OF 2'-O-FUCOSYLLACTOSE AND PRODUCING THEREOF



(1)

(57) Abstract: The present invention relates to novel polymorphs of the trisaccharide 2'-0-fucosyllactose (2-FL) of formula (1), methods for producing said polymorphs and their use in pharmaceutical or nutritional compositions.

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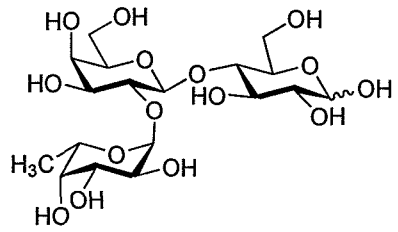
POLYMORPHS OF 2'-O-FUCOSYLLACTOSE AND PRODUCING THEREOF

FIELD OF THE INVENTION

The present invention provides novel polymorphs of the trisaccharide 2-FL, producing thereof and formulations containing the same.

BACKGROUND OF THE INVENTION

In the present years commercialization efforts for the synthesis of complex carbohydrates including secreted oligosaccharides have increased significantly due to their roles in numerous biological processes occurring in living organisms. Secreted oligosaccharides such as human milk oligosaccharides are becoming important commercial targets for nutrition and therapeutic industries. However, the syntheses and purification of these oligosaccharides and their intermediates remained a challenging task for science. One of the most important human milk oligosaccharides is 2'-*O*-fucosyllactose (2-FL, see Scheme 1) found in the highest concentration in mother's milk.



Scheme 1

Several biological roles of 2'-*O*-fucosyllactose have been suggested including but not limited to its prebiotic, antibacterial, antiviral, immune system enhancing, brain development enhancing, etc. effects making it an attractive target for large scale production/isolation/purification for nutritional and therapeutic industries.

The first mention of 2'-*O*-fucosyllactose in the literature appeared in the 1950's by Kuhn et al. (*Chem. Ber.* **1955,** *88*, 1135; *ibid.* **1956,** *89*, 2513). According to the method by Kuhn syrupy or amorphous 2-FL isolated from mother's milk was dissolved in hot 75 % methanol and abs. ethanol was gradually added in the presence of seed crystals. The seed crystals were produced in two ways: after "prolonged" storage some small crystals precipitated by the wall of the flask containing syrupy 2-FL, or 2-FL precipitated from a solution consisting of

aqueous methanol, n-butanol and n-hexanol at 4 °C after "several" weeks. The crystalline 2-FL thus obtained had the melting point of 230-231 °C (decomposed), contained no constitutional water and was supposed to be the α-form.

At those times specific human milk oligosaccharides were isolated from human milk by using sophisticated chromatographic protocols (mainly paper chromatography). However, the purities of such early isolated samples are rather uncertain due to the high number of human milk oligosaccharide isomers present in mother's milk and due to lack of availability of high performance chromatography techniques which are nowadays usual in the investigation and resolution of such complex tasks. For example, 2'-*O*-fucosyllactose and 3-*O*-fucosyllactose are both present in human milk and their chromatographic separation have been solved decades later. Though 2-FL was reported as a crystalline compound by Kuhn in 1956, because of the considerations mentioned above the purity of the isolated sample is rather ambiguous. Furthermore, since that publication no other evidence, reference or indication on the crystalline existence or occurrence of 2-FL could have been found in the art, thus 2-FL is generally available and used as amorphous (lyophilized) solid.

Crystallization or recrystallization is one of the simplest and cheapest methods to separate a product from contaminations and obtain pure substance. In addition, providing one or more crystalline modifications (polymorphs) of a solid is an important factor in product development, because the different crystalline forms affect the compound's properties - for example thermodynamic stability, solubility, density, hygroscopicity, electrical properties (such as dielectric constant, conductivity), mechanical properties (such as friability, hardness, breaking strength, elasticity), optical properties (such as colour, transparency, refraction), etc. - diversely. It enlarges the repertoire of materials that a scientist has available for improving the product's characteristics. With respect of 2-FL there is still a need for crystalline product which may simplify isolation, purification and formulation problems so far envisaged.

SUMMARY OF THE INVENTION

The present invention provides crystalline 2'-*O*-fucosyllactose polymorphs and methodologies suitable for large scale purification of 2'-*O*-fucosyllactose. Thus, the crystalline products provided by the present invention are responsible for the development of high purity 2'-*O*-fucosyllactose for nutritional and pharmaceutical industries.

BRIEF DESCRIPTION OF THE FIGURES

The invention will be described in further detail hereinafter with reference to the accompanying figures, in which:

Figure 1 shows the X-ray powder diffraction pattern of crystalline 2'-*O*-fucosyllactose polymorph I according to example A, item 1.

Figure 2 shows the X-ray powder diffraction pattern of crystalline 2'-*O*-fucosyllactose polymorph I according to example C.

Figure 3 shows the X-ray powder diffraction pattern of crystalline 2'-*O*-fucosyllactose polymorph I according to example D.

Figure 4 shows the calculated X-ray powder diffraction pattern from the single crystal structure of 2'-*O*-fucosyllactose polymorph I for CuKα radiation.

Figure 5 shows the comparison of X-ray powder diffraction patterns of different crystalline 2'-*O*-fucosyllactose polymorph I samples. 1: Example A, item 1; 2: calculated diffractogram from polymorph I single crystal; 3: Example D; 4: Example C.

Figure 6 shows the single crystal structure of 2'-*O*-fucosyllactose polymorph I.

Figure 7 shows the IR spectrum of crystalline 2'-*O*-fucosyllactose polymorph I.

Figure 8 shows the DSC thermogram of crystalline 2'-*O*-fucosyllactose polymorph I according to example A, item 1.

Figure 9 shows the DSC thermogram of crystalline 2'-*O*-fucosyllactose polymorph I according to example C.

Figure 10 shows the solid-state ^{13}C-NMR spectrum of 2'-*O*-fucosyllactose polymorph I according to example A, item 1.

Figure 11 shows the solid-state ^{13}C-NMR spectrum of 2'-*O*-fucosyllactose polymorph I according to example C.

Figure 12 shows the X-ray powder diffraction pattern of 2'-*O*-fucosyllactose polymorph II according to example E.

Figure 13 shows the X-ray powder diffraction pattern of 2'-*O*-fucosyllactose polymorph II according to example F.

Figure 14 shows X-ray powder diffraction pattern of 2'-*O*-fucosyllactose polymorph II according to example G.

Figure 15 shows the comparison of X-ray powder diffraction patterns of different crystalline 2'-*O*-fucosyllactose polymorph II samples. 1: Example G; 2: Example E; 3: Example F.

Figure 16 shows the comparison of X-ray powder diffraction patterns of crystalline 2'-*O*-fucosyllactose polymorphs I and II. 1: Example E; 2: Example F; 3: Example A, item 1.

Figure 17 shows the IR spectrum of 2'-*O*-fucosyllactose polymorph II.

Figure 18 shows the DSC thermogram of 2'-*O*-fucosyllactose polymorph II according to example E.

Figure 19 shows the DSC thermogram of 2'-*O*-fucosyllactose polymorph II according to example F.

Figure 20 shows the DSC thermogram of 2'-*O*-fucosyllactose polymorph II according to example G.

DETAILED DISCLOSURE OF THE INVENTION

The present inventors have found that 2'-*O*-fucosyllactose can be obtained in different crystalline forms.

Crystalline 2'-*O*-fucosyllactose polymorph I, either as polycrystalline material or as single crystal, comprises X-ray powder diffraction reflections, based on a measurement using CuKα radiation, at 21.34±0.20, 20.92±0.20 and 18.37±0.20 2Θ angles, more preferably at 21.34±0.20, 20.92±0.20, 18.37±0.20 and 16.70±0.20 2Θ angles, even more preferably at 21.34±0.20, 20.92±0.20, 18.37±0.20, 16.70±0.20 and 9.91±0.20 2Θ angles, most preferably at 21.34±0.20, 20.92±0.20, 18.37±0.20, 16.70±0.20, 9.91±0.20 and 13.13±0.20 2Θ angles, in particular at 21.34±0.20, 20.92±0.20, 18.37±0.20, 16.70±0.20,

9.91±0.20, 13.13±0.20, 7.87±0.20 and 8.90±0.20 2Θ angles. List of peaks of the XRPD pattern of crystalline 2'-*O*-fucosyllactose polymorph I is reported in Table 1.

2Θ	rel.	2Θ	rel.
7.87	23	27.98	8
8.90	22	28.58	6
9.91	31	29.24	3
12.46	13	30.20	2
13.13	23	30.57	3
13.61	13	31.58	4
13.84	5	31.74	4
15.80	3	33.49	6
16.70	31	33.88	2
17.19	13	34.30	3
18.37	38	35.68	9
18.54	16	36.12	8
19.34	6	36.31	9
19.76	7	36.75	4
20.40	6	37.36	3
20.92	46	37.64	3
21.34	100	38.28	2
21.79	11	39.73	4
22.22	3	40.22	5
22.68	4	40.43	5
23.75	3	40.93	4
25.10	16	41.76	2
26.01	14	42.54	3
26.48	4	43.64	2
26.83	7		

Table 1. List of peaks of the XRPD pattern of crystalline 2'-*O*-fucosyllactose polymorph I

The XRPD patterns of different samples of crystalline 2'-*O*-fucosyllactose polymorph I are shown in Figs. 1-4.

Crystalline 2'-*O*-fucosyllactose polymorph I has a characteristic IR peak at 3428±4 cm^{-1}, preferably has characteristic IR peaks at 3428±4 and 1021±4 cm^{-1}, more preferably at 3428±4, 1021±4 and 1039±4 cm^{-1}, even more preferably at 3428±4, 1021±4, 1039±4 and 1066±4 cm^{-1}, in particular at 3428±4, 1021±4, 1039±4, 1066±4, 1088±4, 1113±4, 1133±4, 1165±4, 1346±4, 1389±4, 1451±4, 2916±4, 2956±4 and 2975±4 cm^{-1}.

The IR spectrum of crystalline 2'-*O*-fucosyllactose polymorph I is shown in Fig. 7.

The novel crystalline polymorph I of 2-FL can be considered as an anomeric mixture of α- and β-anomers or even pure form of one of the anomers. If 2-FL polymorph I is isolated as a polycrystalline material, it forms a mixture of α- and β-anomers, wherein the α-anomer is predominant over the β-anomer and at most 30 % of β-anomer, preferably 7-25 % of β-anomer is present according to solid-state ^{13}C-NMR measurements. If 2'-*O*-fucosyllactose polymorph I is obtained as single crystal, it exists in the monoclinic system, space group $P2_1$, and has the following crystal cell parameters: a= 10.1781(11) Å, b= 9.1990(9) Å, c= 11.7332(13) Å, α= 90.00°, β= 107.871(3)°, γ= 90.00°. No constitutional water and/or solvent are incorporated in the crystal structure. The anomeric OH-group occupies axial position that is it concerns *O*-(α-L-fucopyranosyl)-(1→2)-*O*-(β-D-galactopyranosyl)-(1→4)-α-D-glucose (see Fig. 6). The details of crystal data and structure refinement for crystalline 2'-*O*-fucosyllactose polymorph I are given in Table 2.

DATA	crystalline 2'-*O*-fucosyllactose polymorph I
Empirical formula	$C_{18}H_{31}O_{15}$
Formula weight	487.43
Temperature	93(2) K
Radiation and wavelength	Mo-K_α, λ =0.71075 Å
Crystal system	monoclinic
Space group	$P\,2_1$
Unit cell dimensions	a = 10.1781(11) Å
	b = 9.1990(9) Å
	c = 11.7332(13) Å
	α = 90.00°
	β = 107.871(3)°
	γ = 90.00°
Volume	1045.55(19) Å^3
Z	2
Density (calculated)	1.548 g/cm^3
Absorption coefficient, μ	0.137 mm^{-1}
F(000)	518
Crystal colour	colourless
Crystal description	prism
Crystal size	0.13 x 0.05 x 0.05 mm
Absorption correction	numerical
Max. and min. transmission	0.990 and 0.977
Θ-range for data collection	3.05 ≤ θ ≤ 21.49°
Index ranges	-10 ≤ h ≤ 10;-9 ≤ k ≤ 9;-12 ≤ l ≤ 12
Reflections collected	8050

Completeness to 2θ	0.997
Independent reflections	2399 [*R*(int) =0.1322]
Reflections $I>2\sigma(I)$	1424
Refinement method	full-matrix least-squares on *F*2
Data / restraints / parameters	2399 /19 /312
Goodness-of-fit on *F*2	0.918
Extinction coefficient	0.018(3)
Final *R* indices [$I>2\sigma(I)$]	*R*1 = 0.0604, *wR*2 = 0.1124
R indices (all data)	*R*1 = 0.1093, *wR*2 = 0.1319
Max. and mean shift/esd	0.544; 0.005
Largest diff. peak and hole	0.26 and -0.29 e.Å^{-3}

Table 2. Single crystal parameters for crystalline 2'-*O*-fucosyllactose polymorph I

The tests and procedures used to obtain the data included in Table 2 are standard in the art and a person skilled in the art would know how to carry out these tests based on this specification and his/her knowledge of the art.

The XRPD patterns of crystalline 2'-*O*-fucosyllactose polymorph I having different α/β ratios and the simulated powder pattern of the single crystal are identical to each other showing that the different samples belong to the one and same crystalline polymorph (see Fig. 5).

Crystalline 2'-*O*-fucosyllactose polymorph I containing 20±3 % of β-anomer displays, in DSC investigations, an endothermic reaction with a peak maximum at 260±5 °C, more preferably at 260±4 °C, even more preferably at 260±3 °C, most preferably at 260±2 °C, in particular at 260±1 °C (see Fig. 8). Crystalline 2-FL sample having 12±3 % of β-anomer shows an endothermic peak maximum at 246±5 °C, more preferably at 246±4 °C, even more preferably at 246±3 °C, most preferably at 246±2 °C, in particular at 246±1 °C (see Fig. 9).

Preferably the crystalline 2-FL polymorph I is substantially free from organic solvent and/or water. The expression "substantially free from organic solvent and/or water" intends to mean that the content of organic solvent(s) and/or water is at most 1000 ppm, preferably at most 800 ppm, more preferably at most 600 ppm, most preferably at most 400 ppm and in particular at most 200 ppm.

According to another preferred embodiment the crystalline 2-FL polymorph I is substantially pure. The expression "substantially pure" intends to mean that the crystalline 2-FL polymorph I contains less than 10 w/w% of impurity, preferably less than 5 w/w% of impurity, more preferably less than 1 w/w% of impurity, most preferably less than 0.5 w/w% of impurity, in particular less than 0.1 w/w% of impurity, wherein "impurity" refers to any physical entity

different to the crystalline 2-FL polymorph I, such as amorphous 2-FL, different 2-FL polymorph(s), unreacted intermediate(s) remained from the synthesis of 2-FL, by-product(s), degradation product(s), inorganic salt(s) and/or other contaminations different to organic solvent(s) and/or water.

In order to perform comparative studies huge effort was allocated and many attempts were carried out to obtain crystalline 2-FL according to the literature method, but the procedures have not worked and the present inventors have never been able to reproduce the methods described by Kuhn. In addition, methods comprising steps such as "prolonged" storage or storage for "several" weeks do not hold out much hopes of reproduction. However, the inventors of the present application were able to produce crystalline 2-FL polymorphs.

Thus the present invention provides a process for preparing crystalline 2-FL polymorph I by crystallization from a solvent system containing one or more C_1-C_3 alcohols and optionally water in the absence of seed crystals. Term "C_1-C_3 alcohol" refers to mono- or dihydroxy alkanes having 1 to 3 carbon atoms, that is methanol, ethanol, *n*-propanol, *i*-propanol, ethylene glycol, 1,2-propanediol and 1,3-propanediol, preferably monohydroxy alkanes having 1 to 3 carbon atoms, more preferably methanol or ethanol. According to another preferred embodiment the solvent system may further contain water. The water content in the overall volume of the solvent system may preferably range up to 30 v/v%, more preferably up to 15 v/v%, most preferably up to 5 v/v%.

In a preferred realization 2-FL to be crystallized is dissolved in hot (5-10 °C less than boiling temperature) or boiling aqueous alcohol(s), then to this mixture hot or boiling same or different alcohol(s) is/are added gradually. The solution is allowed to cool to room temperature (rt) and the stirring is continued for 12-24 h. The precipitated crystals are collected by filtration and washed with cold solvent(s). Especially favoured alcohols are methanol and methanol/ethanol mixture.

According to another preferred embodiment, 2-FL to be crystallized is dissolved in hot or boiling alcohol(s), then to this mixture hot or boiling same or different alcohol(s) containing water is/are added gradually. The solution is allowed to cool to rt and the stirring is continued for 12-24 h. The precipitated crystals are collected by filtration and washed with cold solvent(s). Especially favoured alcohols are methanol and methanol/ethanol mixture.

In a further preferred process 2-FL to be crystallized is dissolved in hot or boiling aqueous alcohol(s), then to this mixture hot or boiling same or different alcohol(s) containing water is/are added gradually. The solution is allowed to cool to rt and the stirring is continued for

12-24 h. The precipitated crystals are collected by filtration and washed with cold solvent(s). Especially favoured alcohols are methanol and methanol/ethanol mixture.

According to a further preferred embodiment 2-FL in aqueous methanol, obtained in catalytic hydrogenolysis of benzylated 2-FL described in the international applications WO 2010/115934 or WO 2010/115935, is diluted with ethanol or isopropanol and the solution is allowed to stand and crystallize.

More preferably, *O*-(2-*O*-benzyl-α-L-fucopyranosyl)-(1→2)-*O*-(β-D-galactopyranosyl)-(1→4)-D-glucose (see international application WO 2010/115935) is subjected to catalytic hydrogenolysis in methanol in the presence of an acid such as cc. HCl. Before filtration of the catalyst the acid may be neutralized by a base, optionally in the form of an aqueous solution of the base, the solvents are evaporated partially and water is optionally added to the methanolic concentrate, then the solution is stirred or allowed to stand and crystallize.

The present invention provides another process for producing the crystalline 2-FL polymorph I, characterized in that the crystallization is carried out from a solvent system containing one or more C_1-C_6 alcohols and optionally water in the presence of seed crystals of polymorph I. Term "C_1-C_6 alcohol" refers to mono- or dihydroxy alkanes having 1 to 6 carbon atoms, such as methanol, ethanol, *n*-propanol, *i*-propanol, *n*-butanol, *i*-butanol, *s*-butanol, *t*-butanol, amylalcohol, *n*-hexanol ethylene glycol, propylene glycol, etc. Preferred C_1-C_6 alcohols are C_1-C_6 monohydroxy-alkanes, more preferably C_1-C_4 monohydroxy-alkanes such as methanol, ethanol, *n*-propanol, *i*-propanol, *n*-butanol, *i*-butanol, *s*-butanol and *t*-butanol. An even more preferred solvent system contains methanol, ethanol, *n*-propanol, *i*-propanol or mixtures thereof, in particular methanol or methanol/isopropanol.

In a preferred embodiment 2-FL to be crystallized is dissolved in hot (5-10 °C less than boiling temperature) or boiling alcohol under agitation until a clear solution is obtained. This solution is allowed to cool to rt, seed crystals of polymorph I are added and the stirring is continued for 12-24 h. The precipitated crystals are collected by filtration and washed with the cold solvent.

In another preferred realization 2-FL to be crystallized is dissolved in hot or boiling alcohol under agitation, then to this mixture hot or boiling another alcohol(s) is/are added gradually until a clear solution is obtained. This solution is allowed to cool to rt, seed crystals of polymorph I are added and the stirring is continued for 12-24 h. The precipitated crystals are collected by filtration and washed with the cold solvent.

According to another preferred example the solvent system further contains water. The water content in the overall volume of the solvent system may range up to 30 v/v%, preferably up to 20 v/v%, more preferably up to 10 v/v%.

In an especially preferred realization 2-FL to be crystallized is dissolved in hot (5-10 °C less than boiling temperature) or boiling aqueous alcohol(s), then to this mixture hot or boiling same or different alcohol(s) is/are added gradually. The solution is allowed to cool to rt, seed crystals of polymorph I are added and the stirring is continued for 12-24 h. The precipitated crystals are collected by filtration and washed with cold solvent(s). Especially favoured alcohols are methanol, ethanol and methanol/isopropanol mixture.

According to another preferred embodiment, 2-FL to be crystallized is dissolved in hot or boiling alcohol(s), then to this mixture hot or boiling same or different alcohol(s) containing water is/are added gradually. The solution is allowed to cool to rt, seed crystals of polymorph I are added and the stirring is continued for 12-24 h. The precipitated crystals are collected by filtration and washed with cold solvent(s). Especially favoured alcohols are methanol, ethanol and methanol/isopropanol mixture.

In a further preferred process 2-FL to be crystallized is dissolved in hot or boiling aqueous alcohol(s), then to this mixture hot or boiling same or different alcohol(s) containing water is/are added gradually. The solution is allowed to cool to rt, seed crystals of polymorph I are added and the stirring is continued for 12-24 h. The precipitated crystals are collected by filtration and washed with cold solvent(s). Especially favoured alcohols are methanol, ethanol and methanol/isopropanol mixture.

2-FL in amorphous solid form might be prepared by procedures described in the international applications WO 2010/115934 or WO 2010/115935.

In another aspect of the present invention crystalline 2'-*O*-fucosyllactose polymorph II comprises X-ray powder diffraction reflections, based on a measurement using CuKα radiation, at 16.98±0.20, 13.65±0.20 and 18.32±0.20 2Θ angles, more preferably at 16.98±0.20, 13.65±0.20, 18.32±0.20 and 21.70±0.20 2Θ angles, even more preferably at 16.98±0.20, 13.65±0.20, 18.32±0.20, 21.70±0.20 and 15.22±0.20 2Θ angles, most preferably at 16.98±0.20, 13.65±0.20, 18.32±0.20, 21.70±0.20, 15.22±0.20 and 20.63±0.20 2Θ angles, in particular at 16.98±0.20, 13.65±0.20, 18.32±0.20, 21.70±0.20, 15.22±0.20, 20.63±0.20 and 11.94±0.20 2Θ angles. List of peaks of the XRPD pattern of crystalline 2'-*O*-fucosyllactose polymorph II is reported in Table 3.

2Θ	rel.	2Θ	rel.
7.89	7	24.87	11
9.14	12	25.33	33
9.81	8	25.80	32
10.0	6	26.12	10
10.38	3	26.79	11
10.68	1	27.46	5
11.73	18	27.62	5
11.94	34	28.00	6
12.17	20	28.61	10
12.48	2	28.94	4
13.23	6	29.25	5
13.65	89	29.64	7
14.12	26	30.43	9
15.22	68	30.68	7
15.86	18	31.67	16
16.29	4	32.24	5
16.98	100	32.74	8
17.32	35	32.94	5
18.12	35	33.32	7
18.32	85	33.70	9
18.96	33	33.92	6
19.29	26	34.32	10
19.70	19	34.55	8
19.80	19	35.07	6
20.11	13	35.65	7
20.63	40	35.78	5
21.44	25	36.05	4
21.70	67	36.41	14
21.93	20	36.50	14
22.29	10	36.60	12
22.58	13	37.18	10
23.16	7	37.61	8
23.55	16	38.25	9
23.83	16	38.48	7
24.04	16	39.68	8
24.60	11		

Table 3. List of peaks of the XRPD pattern of crystalline 2'-*O*-fucosyllactose polymorph II

The XRPD patterns of different samples of crystalline 2'-*O*-fucosyllactose polymorph II are shown in Figs. 12-14.

Crystalline 2'-*O*-fucosyllactose polymorph II according to the present invention has a characteristic IR peak at 3571±4 cm^{-1}, preferably has characteristic IR peaks at 3571±4 and 1042±4 cm^{-1}, more preferably at 3571±4, 1042±4 and 1412±4 cm^{-1}, even more preferably

at 3571±4, 1042±4, 1412±4 and 1255±4 cm^{-1}, in particular at 3571±4, 964±4, 1042±4, 1072±4, 1124±4, 1154±4, 1255±4, 1295±4, 1342±4, 1412±4, 2877±4, 2906±4, 2956±4, 3333±4 and 3442±4 cm^{-1}.

The IR spectrum of 2-FL polymorph II is shown in Fig. 17.

The crystalline 2-FL polymorph II can be considered as an anomeric mixture of α- and β-anomers or even pure form of one of the anomers. No constitutional water and/or solvent are incorporated in the crystal structure.

The XRPD patterns of crystalline 2'-*O*-fucosyllactose polymorph II obtained under different conditions are identical to each other showing that the different samples belong to the one and same crystalline polymorph (see Fig. 15).

Crystalline 2'-*O*-fucosyllactose polymorph II displays, in DSC investigations, an endothermic reaction with a peak maximum at 259.5±5 °C, more preferably at 259.5±4 °C, even more preferably at 259.5±3 °C, most preferably at 259.5±2 °C (see Figs. 18-20).

Preferably, the crystalline 2-FL polymorph II is substantially free from organic solvent and/or water. The expression "substantially free from organic solvent and/or water" intends to mean that the content of organic solvent(s) and/or water is at most 1000 ppm, preferably at most 800 ppm, more preferably at most 600 ppm, most preferably at most 400 ppm and in particular at most 200 ppm.

According to another preferred embodiment the crystalline 2-FL polymorph II is substantially pure. The expression "substantially pure" intends to mean that the crystalline 2-FL polymorph II contains less than 10 w/w% of impurity, preferably less than 5 w/w% of impurity, more preferably less than 1 w/w% of impurity, most preferably less than 0.5 w/w% of impurity, in particular less than 0.1 w/w% of impurity, wherein "impurity" refers to any physical entity different to crystalline 2-FL polymorph II, such as amorphous 2-FL, different 2-FL polymorph(s), unreacted intermediate(s) remained from the synthesis of 2-FL, by-product(s), degradation product(s), inorganic salt(s) and/or other contaminations different to organic solvent(s) and/or water.

The present invention provides method for producing the crystalline 2-FL polymorph II, characterized in that the crystallization is carried out from a solvent system comprising one or more C_1-C_6 alcohols in the presence of seed crystals of polymorph II. Term "C_1-C_6 alcohol" refers to mono- or dihydroxy alkanes having 1 to 6 carbon atoms, such as methanol, ethanol, *n*-propanol, *i*-propanol, *n*-butanol, *i*-butanol, *s*-butanol, *t*-butanol, amylalcohol, *n*-hexanol

ethylene glycol, propylene glycol, etc. Preferred C_1-C_6 alcohols are C_1-C_6 monohydroxy-alkanes, more preferably C_1-C_4 monohydroxy-alkanes such as methanol, ethanol, *n*-propanol, *i*-propanol, *n*-butanol, *i*-butanol, *s*-butanol and *t*-butanol. An even more preferred solvent system comprises methanol, ethanol, *n*-propanol, *i*-propanol or mixtures thereof, in particular methanol.

In a preferred embodiment 2-FL to be crystallized is dissolved in hot (5-10 °C less than boiling temperature) or boiling alcohol under agitation until a clear solution is obtained. This solution is allowed to cool to rt, seed crystals of polymorph II are added and the stirring is continued for 12-24 h. The precipitated crystals are collected by filtration and washed with the cold solvent.

According to another preferred example the solvent system further contains water. The water content in the overall volume of the solvent system may range up to 60 v/v%, preferably up to 55 v/v%, more preferably between 40-55 v/v%.

In an especially preferred realization 2-FL to be crystallized is dissolved in hot (40-80 °C) aqueous alcohol. The solution is allowed to cool to rt, seed crystals of polymorph II are added and the stirring is continued for 12-24 h. The precipitated crystals are collected by filtration and washed with cold solvent(s). Especially favoured alcohol is methanol.

According to another method for producing the crystalline 2-FL polymorph II syrupy 2-FL, solid 2-FL comprising amorphous 2-FL or any 2-FL polymorph(s) different to polymorph II or mixture of amorphous 2-FL and any 2-FL polymorph(s) different to polymorph II is suspended in one or more less polar aprotic organic solvent and stirred for 6-72 hours. Optionally, the solid 2-FL to be (re)crystallized may also contain 2-FL polymorph II.

Less polar aprotic organic solvent means aprotic organic solvents having dielectric constant less than approx. 21. For example such typical solvents are esters, ketones, ethers, hydrocarbons and halogenated hydrocarbons.

Esters preferably mean esters of C_1-C_6 carboxylic acids with C_1-C_6 alcohols, more preferably esters of acetic acid with C_1-C_6 alcohols such as methyl acetate, ethyl acetate, n-propyl acetate, i-propyl acetate, n-butyl acetate, i-butyl acetate amyl acetate, hexyl acetate and the like.

Ketones preferably mean open chain or cyclic ketones with 3-6 carbon atoms, more preferably acetone or methyl ethyl ketone.

Ethers preferably mean open chain or cyclic ethers with 2-6 carbon atoms, more preferably diethyl ether, methyl t-butyl ether, THF or dioxane.

Hydrocarbons preferably means alkanes (linear or branched) or cycloalkanes having 5-7 carbon atoms, more preferably n-pentane, n-hexane or cyclohexane. Moreover hydrocarbons relate to aromatic hydrocarbons such as benzene, toluene and xylenes, as well.

Halogenated hydrocarbons mean hydrocarbons defined above substituted with one or more halogen atom selected from fluoro, chloro and bromo, more preferably dichloromethane, chloroform, tetrachloromethane, 1,2-dichloroethane or chlorobenzene.

In a preferred embodiment an ester type solvent is used as less polar aprotic solvent. In a more preferred embodiment the ester type solvent may further contain water. The water content in the overall volume of the solvent system may range up to 5 v/v%, preferably up to 2 v/v%, more preferably between 1-2 v/v%.

According to another preferred embodiment the suspension is stirred at a temperature within the range of 0 °C to reflux, preferably 20 °C to 80 °C.

In an especially preferred embodiment ethyl acetate is the solvent of choice which may contain 1-2 v/v% of water. The suspension can be made using pure ethyl acetate or aqueous ethyl acetate. Alternatively the water may be added to the suspension continuously or sequentially. The suspension is then heated up slowly under stirring to 60-75 °C, preferably 65-70 °C and kept at this temperature for 6-24 hours, preferably 10-14 hours.

Crystalline 2-FL polymorph I and polymorph II, based on the evidence of their powder diffraction patterns, represent different crystalline modifications (see Fig. 16). Moreover, both crystalline modifications can be produced readily and a reproducible manner.

In a further embodiment crystalline 2-FL polymorph I and/or crystalline 2-FL polymorph II is suitable for pharmaceutical use. 2-FL acts as prophylactic and therapeutic agent that inhibits diseases caused by mucosal pathogens like *Campylobacter*, caliciviruses and rotavirus, which are responsible for diarrhoea especially in infants, or diseases caused by respiratory pathogens provoking pneumonia. Through its immunomodulatory effect 2-FL benefits the abnormal immune response found in some monocyte-mediated diseases. In humans and animals, by dosing with 2-FL it is possible to promote insulin secretion, suppress the elevation of a blood glucose level, ameliorate diabetes mellitus, promote growth and increase an insulin level in breast milk. Furthermore the combination of 2-FL with one or more *Bifidobacterium* species as probiotic(s) such as *Bifidobacterium lactis*, *Bifidobacterium*

infantis, *Bifidobacterium breve* or *Bifidobacterium longum*, is suitable for use in the prevention of opportunistic infections in immune-compromised individuals.

In another aspect, the present invention provides pharmaceutical composition comprising crystalline 2'-*O*-fucosyllactose polymorph I and/or crystalline 2-FL polymorph II as active ingredient and one or more pharmaceutically acceptable carriers including but not limited to additives, adjuvants, excipients and diluents (water, gelatine, talc, sugars, starch, gum arabic, vegetable gums, vegetable oils, polyalkylene glycols, flavouring agents, preservatives, stabilizers, emulsifying agents, lubricants, colorants, fillers, wetting agents, etc.). Suitable carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field. The dosage form for administration includes, for example, tablets, powders, granules, pills, suspensions, emulsions, infusions, capsules, syrups, injections, liquids, elixirs, extracts and tincture. Pharmaceutical compositions comprising crystalline 2-FL polymorph I and/or crystalline 2-FL polymorph II inhibits diseases caused by mucosal pathogens like *Campylobacter*, caliciviruses and rotavirus, which are responsible for diarrhoea especially in infants, or diseases caused by respiratory pathogens provoking pneumonia, influences the abnormal immune response found in some monocyte-mediated diseases, promotes insulin secretion, suppresses the elevation of a blood glucose level, ameliorates diabetes mellitus, promotes growth and increases an insulin level in breast milk The pharmaceutical composition comprising crystalline 2-FL polymorph I and/or crystalline 2-FL polymorph II and one or more *Bifidobacterium* species as probiotic(s) such as *Bifidobacterium lactis*, *Bifidobacterium infantis*, *Bifidobacterium breve* or *Bifidobacterium longum*, is suitable for use in the prevention of opportunistic infections in immune-compromised individuals.

In a further embodiment crystalline 2-FL polymorph I and/or crystalline 2-FL polymorph II is used for the preparation of pharmaceutical compositions. Pharmaceutical compositions can be manufacture by means of any usual manner known in the art, e.g. described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field.

In a further embodiment it is provided nutritional formulations comprising crystalline 2-FL polymorph I and/or crystalline 2-FL polymorph II such as foods, drinks or feeds. The nutritional formulation may contain edible micronutrients, vitamins and minerals as well. The amounts of such ingredient may vary depending on whether the formulation is intended for use with normal, healthy infants, children, adults or subjects having specialized needs (e.g. suffering from metabolic disorders). Micronutrients include for example edible oils, fats or fatty acids (such as coconut oil, soy-bean oil, monoglycerides, diglycerides, palm olein, sunflower oil, fish oil, linoleic acid, linolenic acid etc.), carbohydrates (such as glucose, fructose, sucrose, maltodextrin, starch, hydrolized cornstarch, etc.) and proteins from casein,

soy-bean, whey or skim milk, or hydrolysates of these proteins, but protein from other source (either intact or hydrolysed) may be used as well. Vitamins may be chosen from the group consisting of vitamin A, B1, B2, B5, B6, B12, C, D, E, H, K, folic acid, inositol and nicotinic acid. The nutritional formula may contain the following minerals and trace elements: Ca, P, K, Na, Cl, Mg, Mn, Fe, Cu, Zn, Se, Cr or I.

In a preferred embodiment the nutritional formulation is an infant formula. Infant formula means a foodstuff intended for particular nutritional use by infants during the first 4-6 months of life and satisfying by itself the nutritional requirements of infants. It may contain one or more probiotic *Bifidobacterium* species, prebiotics such as fructooligosaccharides and galactooligosaccharides, proteins from casein, soy-bean, whey or skim milk, carbohydrates such as lactose, saccharose, maltodextrin, starch or mixtures thereof, lipids (e.g. palm olein, sunflower oil, safflower oil) and vitamins and minerals essential in a daily diet. The infant formula contains crystalline 2-FL polymorph I and/or crystalline 2-FL polymorph II in a total amount of 0.1-3.0 g/100 g formula.

In another preferred embodiment the nutritional formulation may be a food supplement including crystalline 2-FL polymorph I and/or crystalline 2-FL polymorph II. The food supplement may comprise one or more probiotics in an amount sufficient to achieve the desired effect in an individual, preferably in children and adults. The food supplement may also contain vitamins, minerals, trace elements and other micronutritients as well. The food supplement may be for example in the form of tablets, capsules, pastilles or a liquid. The supplement may contain conventional additives selected from but not limited to binders, coatings, emulsifiers, solubilising agents, encapsulating agents, film forming agents, adsorbents, carriers, fillers, dispersing agents, wetting agents, jellifying agents, gel forming agents, etc. The daily dose of 2-FL ranges from 0.1 to 3.0 g.

According to a more preferred embodiment the food supplement is digestive health functional food as the administration of 2-FL provides a beneficial effect on digestive health. Digestive health functional food is a processed food used with intention enhance and preserve digestive health by crystalline 2-FL polymorph I and/or crystalline 2-FL polymorph II as physiologically functional ingredient or component in forms of tablet, capsule, powder, etc. Different terms such as dietary supplement, nutraceutical, designed food, health product may also be used to refer to functional food.

In a further embodiment crystalline 2-FL polymorph I and/or crystalline 2-FL polymorph II is used for the preparation of nutritional formulation including foods, drinks and feeds, preferably infant formulas, food supplements and digestive health functional food. The nutritional formulation may be prepared in any usual manner. For example, it may be

prepared by admixing micronutrient components in appropriate proportions. Then the vitamins and minerals are added, but to avoid thermal degradation or decomposition heat sensitive vitamins can be added after homogenization. Lipophilic vitamins may be dissolved in the fat source before mixing. A liquid mixture is formed using water, whose temperature is preferably about between 50-80 °C to help dissolution or dispersal of the ingredients. Crystalline 2-FL polymorph I and/or crystalline 2-FL polymorph II can be added at this stage. The resulting mixture is then homogenized by flash heating to about 80-150 °C by means of steam injection, heat exchanger or autoclave. This thermal treatment reduces significantly the bacterial loads as well. The hot mixture is then cooled rapidly to about 60-80 °C. If needed, further homogenization may be carried out at this temperature under high pressure of about 2-30 MPa. After cooling heat sensitive constituents may be added at this stage, and the pH and the content of the solids are conveniently adjusted. The resulting mixture is then dried by conventional method such as spray drying or freeze drying to powder. Probiotics may be added at this point by dry-mixing.

Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not to be limiting thereof.

EXAMPLES

Crystallization procedures

Polymorph I

A) Amorphous 2-FL was dissolved in a first hot or boiling solvent and optionally a second hot or boiling solvent was added gradually under stirring. The solution was allowed to cool to rt, optionally seeded with polymorph I and the stirring was continued for 12-24 h. The precipitated crystals were collected by filtration, washed with cold solvent(s) and dried. The solvent used are listed in the table below. The yields range 63-90 %.

item	first solvent	second solvent	seeding
1.	hot 80 % aqueous methanol (1 volume)	boiling methanol (2 volumes)	no
2.	hot 80 % aqueous methanol (1 volume)	boiling methanol (2 volumes)	yes
3.	boiling methanol (2 volumes)	-	yes

4.	boiling methanol (5 volumes)	hot isopropanol (2.5 volumes)	yes

The sample according to item 1 contains 20±3 % of β-anomer according to solid-state ^{13}C-NMR measurement (see Fig. 10).

B) 10.0 g of *O*-(2-*O*-benzyl-α-L-fucopyranosyl)-(1→2)-*O*-(β-D-galactopyranosyl)-(1→4)-D-glucose in methanol (40 ml) and water (6.5 ml) were subjected to catalytic hydrogenation in the presence of 10 % palladium on charcoal (850 mg) according to the international application WO 2010/115935. After removing the catalyst by filtration the filtrate was diluted with 2.5-fold volume of ethanol compared to the filtrate. The solution was allowed to stand at rt for 2 days and the crystals precipitated were collected.

C) *O*-(2-*O*-Benzyl-α-L-fucopyranosyl)-(1→2)-*O*-(β-D-galactopyranosyl)-(1→4)-D-glucose (200 g) was dissolved in methanol (1200 ml) and cc. HCl solution (4 ml) in methanol (200 ml) was added. After addition of a slurry of 10 % Pd/C (10 g) in methanol (100 ml), the mixture was stirred under hydrogen atmosphere at rt and 3-3,5 bar for 1 hour. The catalyst was filtered off and washed with methanol, the filtrate was concentrated to a solution that weights approx. 600 g, then 10 ml of water was added. Crystals precipitate under stirring which were collected by filtration, washed with methanol and dried to yield 113 g of product (67 %). The sample contains 12±3 % of β-anomer according to solid-state ^{13}C-NMR measurement (see Fig. 11).

D) *O*-(2-*O*-Benzyl-α-L-fucopyranosyl)-(1→2)-*O*-(β-D-galactopyranosyl)-(1→4)-D-glucose (200 g) was dissolved in methanol (1400 ml) and cc. HCl solution (4 ml) was added. After addition of a slurry of 10 % Pd/C (10 g) in methanol (100 ml), the mixture was stirred under hydrogen atmosphere at rt and 3,5-4 bar for 1 hour. The reaction mixture was neutralized with sodium carbonate (2.0 g in 30 ml of methanol), then the catalyst was filtered off and washed with methanol, the filtrate was concentrated to a solution that weights approx. 600 g, then 10 ml of water was added. Crystals precipitate under stirring which were collected by filtration, washed with methanol and dried to yield 102 g of product (61 %).

Polymorph II

E) Amorphous 2-FL (50 g) was dissolved in mixture of methanol (25 ml) and water (30 ml) and heated to 76 °C. The solution was allowed to cool to rt under stirring while it was seeded with polymorph II to initiate crystallization. The stirring was continued for 12-24 h, the precipitated crystals were collected by filtration, washed with cold solvent(s) and dried to give 40 g of white crystals. HPLC assay: 99.9%.

F) 2-FL polymorph I (50 g) was dissolved in mixture of methanol (35 ml) and water (26 ml) and heated to 40 °C. The solution was allowed to cool to rt under stirring while it was seeded with polymorph II to initiate crystallization. The stirring was continued for 12-24 h, the precipitated crystals were collected by filtration, washed with cold solvent(s) and dried to give 26 g of white crystals. HPLC assay: 98.2%.

G) Polymorph I (16.1 g) was suspended in ethyl acetate (80 ml) and water (8 ml), and stirred at 65-70 °C for 12 h. The solid was filtered and dried under vacuum to give 15.9 g of white crystals. HPLC assay: 98.6%.

X-Ray Powder Diffraction

XRPD investigations were conducted with a Philips PW 1830/PW1050 instrument in transmission geometry, using CuKα radiation made monochromatic by means of a graphite monochromator. D-spacings were calculated from the 2Θ values, based on a wavelength of 1.54186 Å. As a general rule the 2Θ values have an error rate of ± 0.2 Å.

DSC Analysis

The measurements were carried out on a SETARAM Labsys Evo TG-DSC thermoanalyzer, in flowing high purity (6.0) helium atmosphere (flow rate 20 ml/min) in the temperature range of 30-300 °C with a constant heating rate of 10 K/min, using standard 100 µl platinum crucible. Sample amounts varied between 5-10 mg.

An example of infant formula

Nutrient	per 100 kcal	per litre
Energy (kcal)	100	670
Protein (g)	1.83	12.3
Fat (g)	5.3	35.7
Linoleic acid (g)	0.79	5.3
α-Linolenic acid (mg)	101	675
Lactose (g)	11.2	74.7
Prebiotic (70 % FOS, 30 % inulin) (g)	0.64	4.3
Minerals (g)	0.37	2.5
Na (mg)	23	150
K (mg)	89	590
Cl (mg)	64	430
Ca (mg)	62	410
P (mg)	31	210
Mg (mg)	7	50

Mn (µg)	8	50
Se (µg)	2	13
Vitamin A (µg RE)	105	700
Vitamin D (µg)	1.5	10
Vitamin E (mg TE)	0.8	5.4
Vitamin K1 (µg)	8	54
Vitamin C (mg)	10	67
Vitamin B1 (mg)	0.07	0.47
Vitamin B2 (mg)	0.15	1.0
Niacin (mg)	1	6.7
Vitamin B6 (mg)	0.075	0.50
Folic acid (µg)	9	60
Pantothenic acid (mg)	0.45	3
Vitamin B12 (µg)	0.3	2
Biotin (µg)	2.2	15
Choline (mg)	10	67
Fe (mg)	1.2	8
I (µg)	15	100
Cu (mg)	0.06	0.4
Zn (mg)	0.7	5
2-FL according to the present invention (mg)	0.3	2.0
B. lactis CNCM 1-3446	$2 \cdot 10^7$ cfu/g of powder, live bacteria	

An example of cake

cake flour	100 g
starch	74 g
water	14 ml
2-FL according to the present invention	30 g
baking powder	2 teaspoons
salt	2 teaspoons
egg	1
butter	80 g
milk	2 tablespoons

Approx. 30 cookies can be produced from the ingredients above.

An example of powder milk

2-FL according to the present invention	20 g
skim milk	5 kg
whey protein concentrate	158 g
lactose	924 g
le vitamin mixture	75 g
minerals	75 g

lipophilic vitamin	578 g

The ingredients are mixed, homogenized, sterilized and dried by means of routine methodologies to produce powder milk.

CLAIMS

1. Crystalline 2'-*O*-fucosyllactose polymorph II, characterized in that it displays X-ray powder diffraction reflections, based on a measurement using CuKα radiation, at 16.98±0.20, 13.65±0.20 and 18.32±0.20 2Θ angles, more preferably at 16.98±0.20, 13.65±0.20, 18.32±0.20 and 21.70±0.20 2Θ angles, even more preferably at 16.98±0.20, 13.65±0.20, 18.32±0.20, 21.70±0.20 and 15.22±0.20 2Θ angles, most preferably at 16.98±0.20, 13.65±0.20, 18.32±0.20, 21.70±0.20, 15.22±0.20 and 20.63±0.20 2Θ angles, in particular at 16.98±0.20, 13.65±0.20, 18.32±0.20, 21.70±0.20, 15.22±0.20, 20.63±0.20 and 11.94±0.20 2Θ angles.

2. Crystalline 2'-*O*-fucosyllactose polymorph II according to claim 1 which is substantially pure.

3. Crystalline 2'-*O*-fucosyllactose polymorph II according to any of the claims 1 or 2 which is substantially free from organic solvent and/or water.

4. A method for producing crystalline 2'-*O*-fucosyllactose polymorph II according to any of the claims 1 to 3, characterized in that the crystallization is carried out from a solvent system comprising one or more C_1-C_6 alcohols in the presence of seed crystals of crystalline 2'-*O*-fucosyllactose polymorph II according to any of the claims 1 to 3.

5. The method according to claim 4, wherein the C_1-C_6 alcohol is methanol and/or ethanol.

6. The method according to any of the claims 4 or 5, wherein the solvent system further contains water.

7. A method for producing crystalline 2'-*O*-fucosyllactose polymorph II according to any of the claims 1 to 3, characterized in that syrupy 2-FL, solid 2-FL comprising amorphous 2-FL or any 2-FL polymorph(s) different to polymorph II, or a mixture of amorphous 2-FL and any 2-FL polymorph(s) different to polymorph II is suspended in one or more less polar aprotic organic solvent.

8. The method according to claim 7 wherein the less polar aprotic organic solvent is an ester type solvent, preferably an ester of acetic acid with a C_1-C_6 alcohol, more preferably ethyl acetate.

9. 2'-*O*-Fucosyllactose polymorph I in polycrystalline or single crystal form, characterized in that it displays X-ray powder diffraction reflections, based on a measurement using CuKα radiation, at 21.34±0.20, 20.92±0.20 and 18.37±0.20 2Θ angles, more preferably at 21.34±0.20, 20.92±0.20, 18.37±0.20 and 16.70±0.20 2Θ angles, even more preferably at 21.34±0.20, 20.92±0.20, 18.37±0.20, 16.70±0.20 and 9.91±0.20 2Θ angles, most preferably at 21.34±0.20, 20.92±0.20, 18.37±0.20, 16.70±0.20, 9.91±0.20 and 13.13±0.20 2Θ angles, in particular at 21.34±0.20, 20.92±0.20, 18.37±0.20, 16.70±0.20, 9.91±0.20, 13.13±0.20, 7.87±0.20 and 8.90±0.20 2Θ angles.

10. Crystalline 2'-*O*-fucosyllactose polymorph I according to claim 9 in single crystal form, characterized in that it has monoclinic crystals, space group $P\,2_1$, preferably with the following cell parameters: a = 10.1781(11) Å, b = 9.1990(9) Å, c = 11.7332(13) Å, α= 90.00°, β = 107.871(3)°, γ = 90.00°.

11. Crystalline 2'-*O*-fucosyllactose polymorph I according to claim 9, characterized in that it contains at most 30 % of β-anomer, preferably 7-25 % of β-anomer.

12. Crystalline 2'-*O*-fucosyllactose polymorph I according to any of the claims 9 to 11 which is substantially pure.

13. Crystalline 2'-*O*-fucosyllactose polymorph I according to any of the claims 9 to 12 which is substantially free from organic solvent and/or water.

14. A method for producing crystalline 2'-*O*-fucosyllactose polymorph I according to any of the claims 9 to 13, characterized in that the crystallization is carried out from a solvent system containing one or more C_1-C_3 alcohols and optionally water, in the absence of seed crystals.

15. The method according to claim 14, wherein the C_1-C_3 alcohol is methanol and/or ethanol.

16. The method according to any of the claims 14 or 15, wherein the solvent system further contains water.

17. Crystalline 2'-*O*-fucosyllactose polymorph II according to any of the claims 1 to 3 and/or polymorph I according to any of the claims 9 to 13 as a pharmaceutical agent.

18. Use of crystalline 2'-*O*-fucosyllactose polymorph II according to any of the claims 1 to 3 and/or polymorph I according to any of the claims 9 to 13 for the preparation of pharmaceutical compositions.

19. Pharmaceutical compositions comprising crystalline 2'-*O*-fucosyllactose polymorph II according to any of the claims 1 to 3 and/or polymorph I according to any of the claims 9 to 13, and one or more pharmaceutically acceptable carriers.

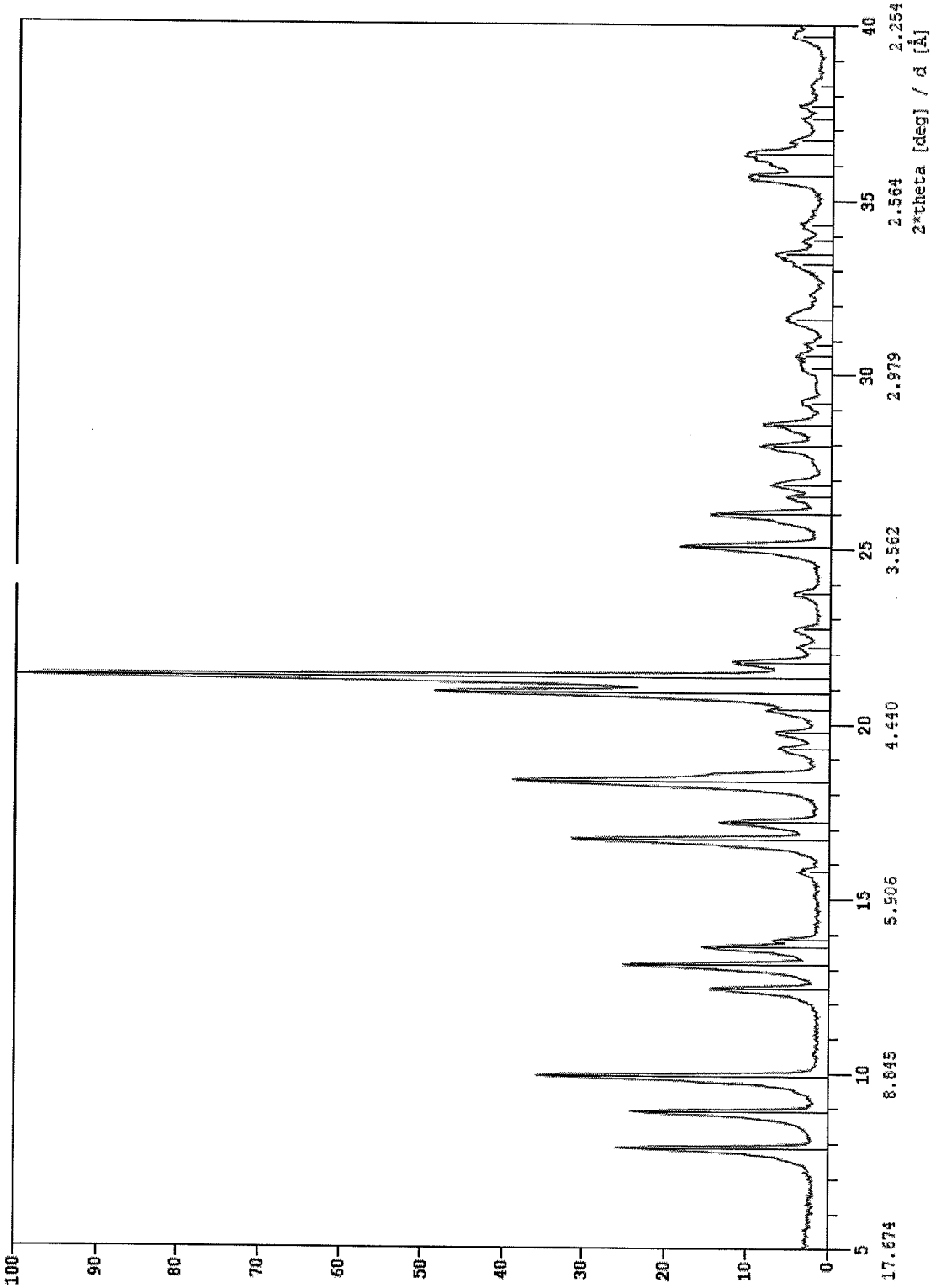
20. Nutritional formulations comprising crystalline 2'-*O*-fucosyllactose polymorph II according to any of the claims 1 to 3 and/or polymorph I according to any of the claims 9 to 13.

21. A nutritional formulation according to claim 20, which is an infant formula.

22. A nutritional formulation according to claim 20, which is a food supplement.

23. A food supplement according to claim 22, which is a digestive health functional food.

24. Use of crystalline 2'-*O*-fucosyllactose polymorph II according to any of the claims 1 to 3 and/or polymorph I according to any of the claims 9 to 13 for the preparation of nutritional formulations.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90
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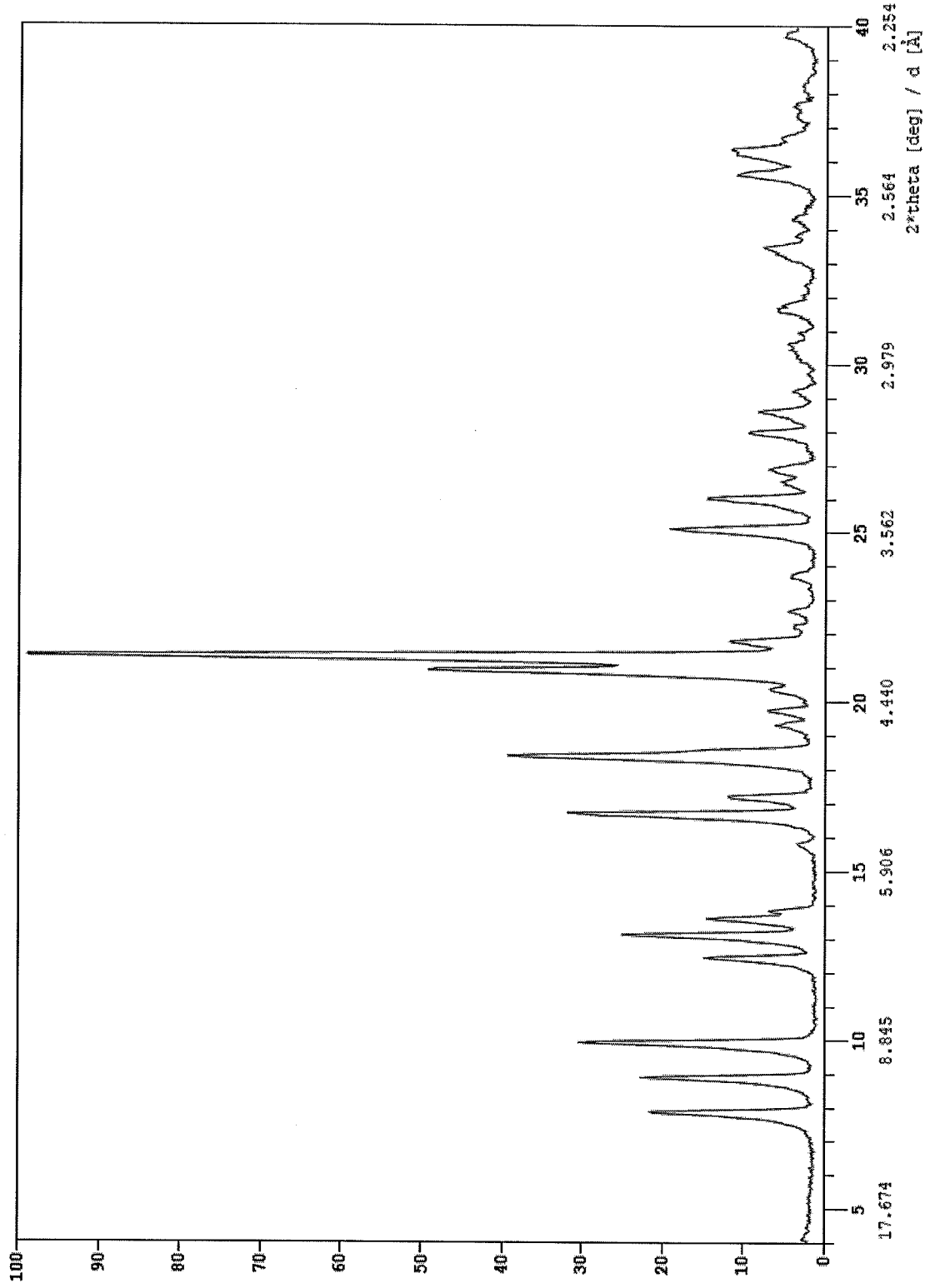
40
30
20
10
0
5
10
15
20
25
30
35
40
17.674
8.845
5.906
4.440
3.562
2.979
2.564
2.254
2*theta [deg] / d [Å]

Figure 1.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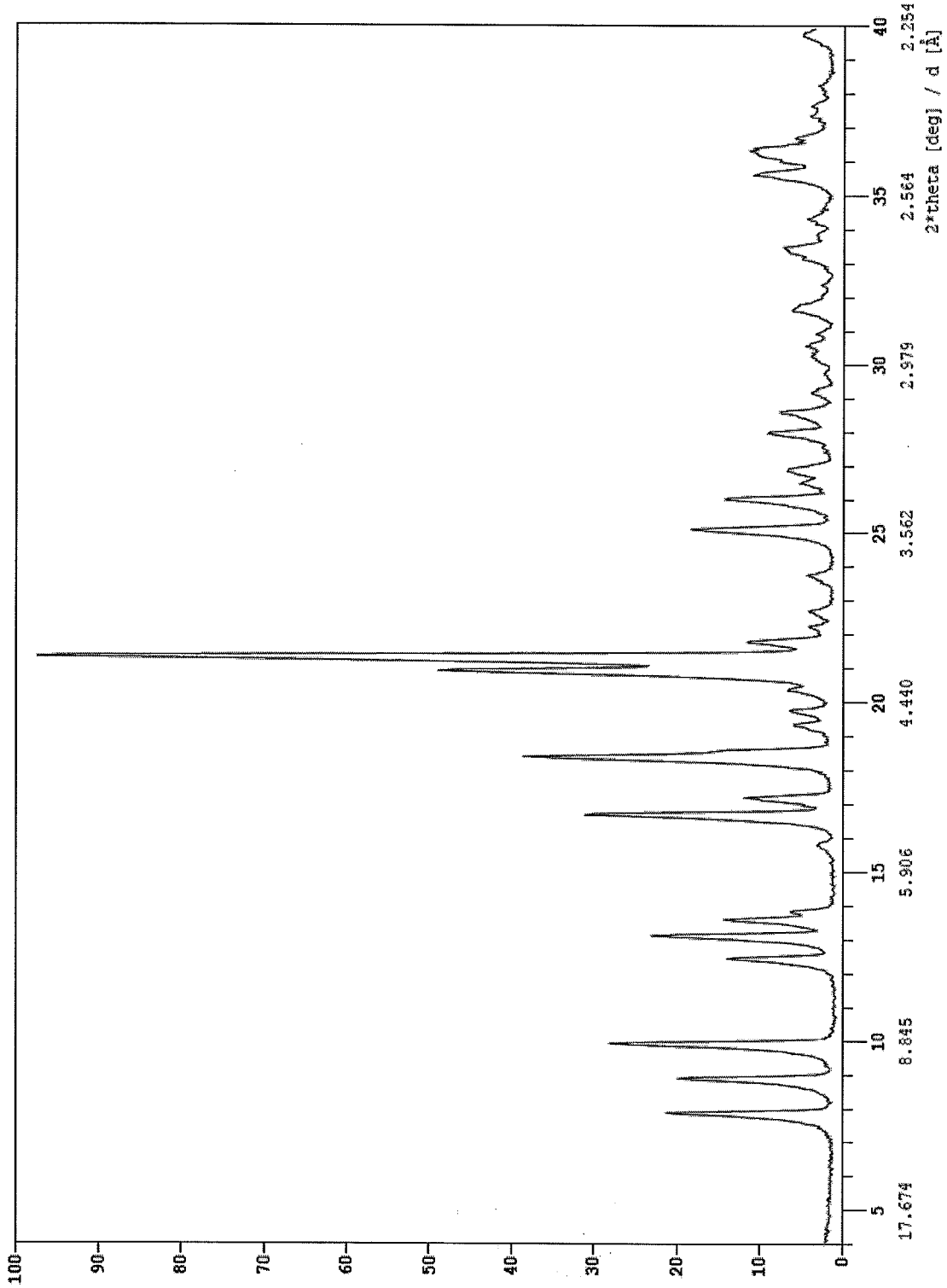
8.845
5.906
4.440
3.562
2.979
2.564
2.254
2*theta [deg] / d [Å]

Figure 2.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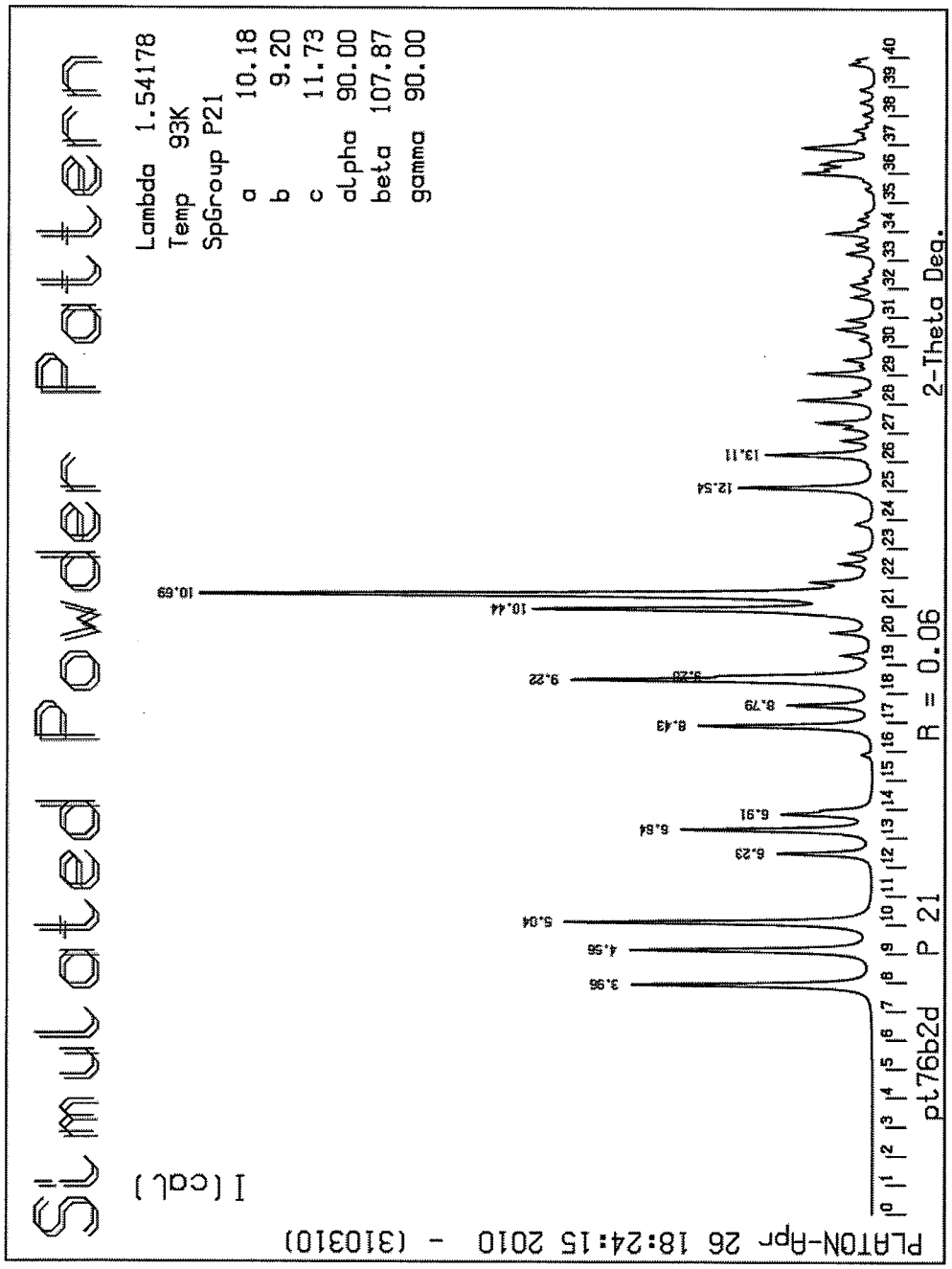
8.845
5.906
4.440
3.562
2.979
2.564
2.254
2*theta [deg] / d [Å]

Figure 3.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I (calc)
PLATON-Apr 26 18:24:15 2010 - (31031O)
pt76b2d P 21
R = 0.06
2-Theta Deg.
10.69
10.44
9.22
8.79
8.43
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6.84
6.23
5.04
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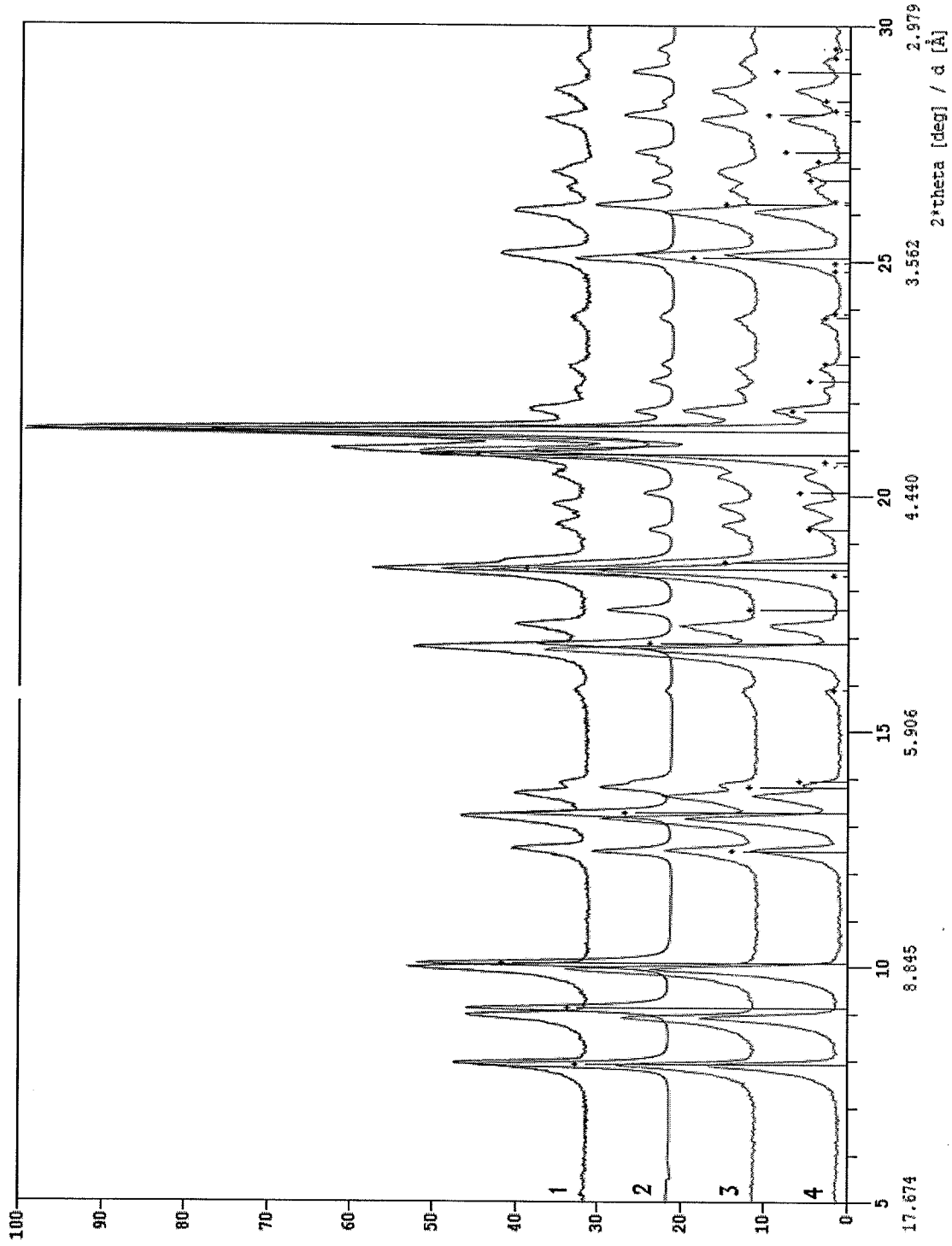
12.54
13.11

Figure 4.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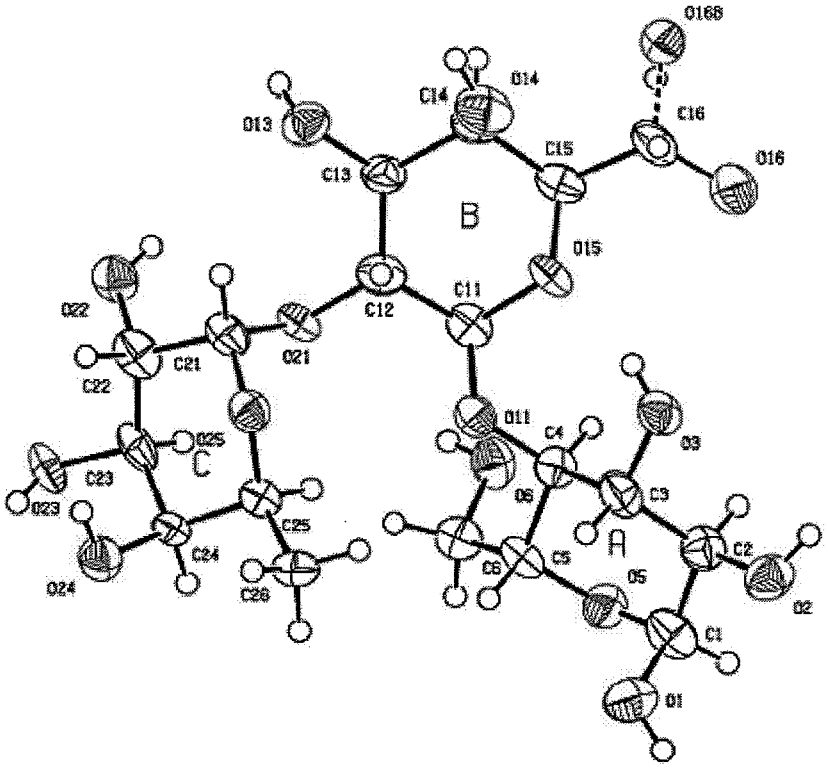
8.845
5.906
4.440
3.562
2.979
2*theta [deg] / d [Å]

Figure 5.


O16B
C14
O14
O13
C16
C15
O16
C13
B
O15
C11
C12
O22
O21
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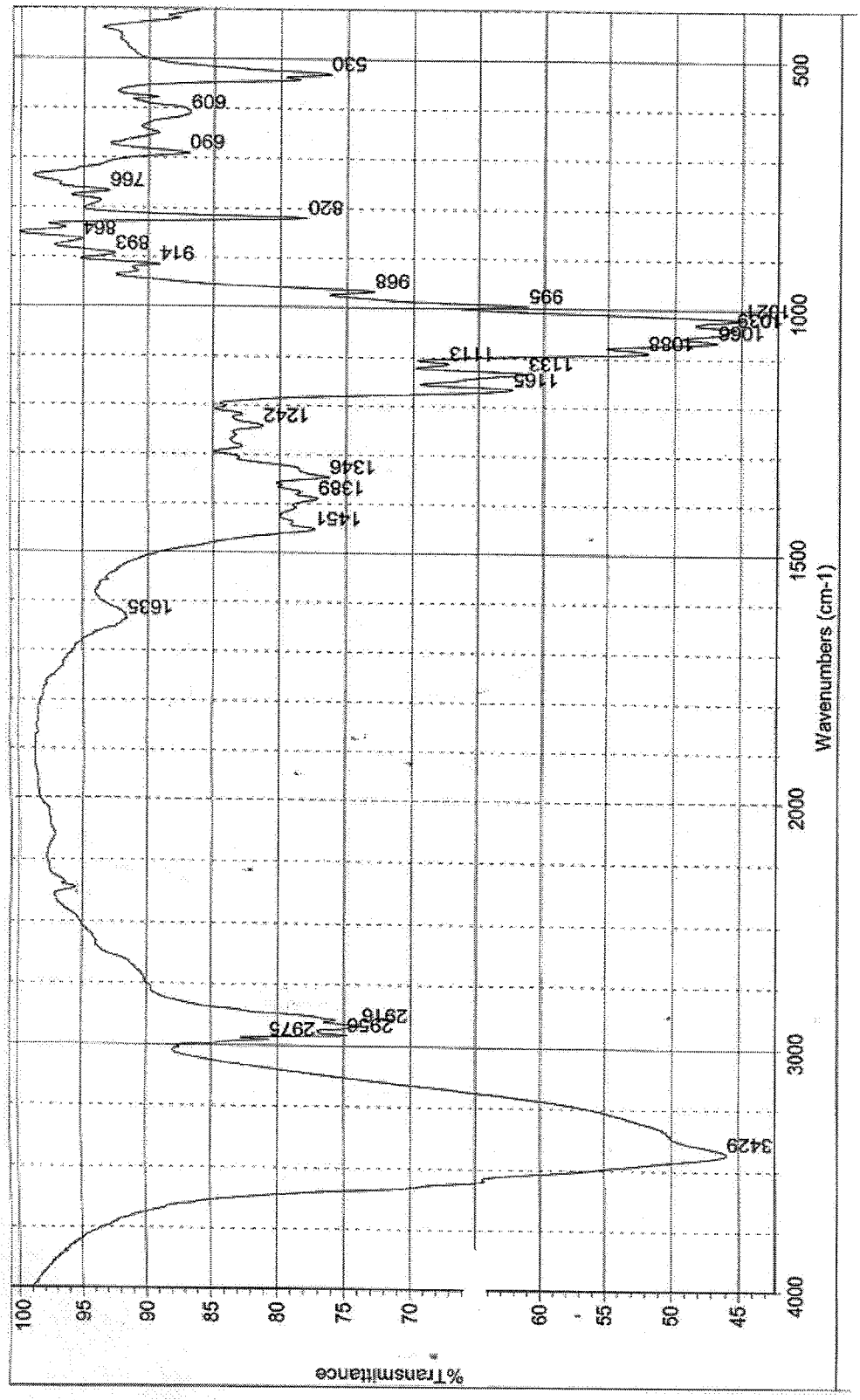
O11
C4
O3
O25
C23
C
O6
C3
O23
C25
A
C2
C24
C5
O5
O24
C26
C1
O2
O1

Figure 6.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1113
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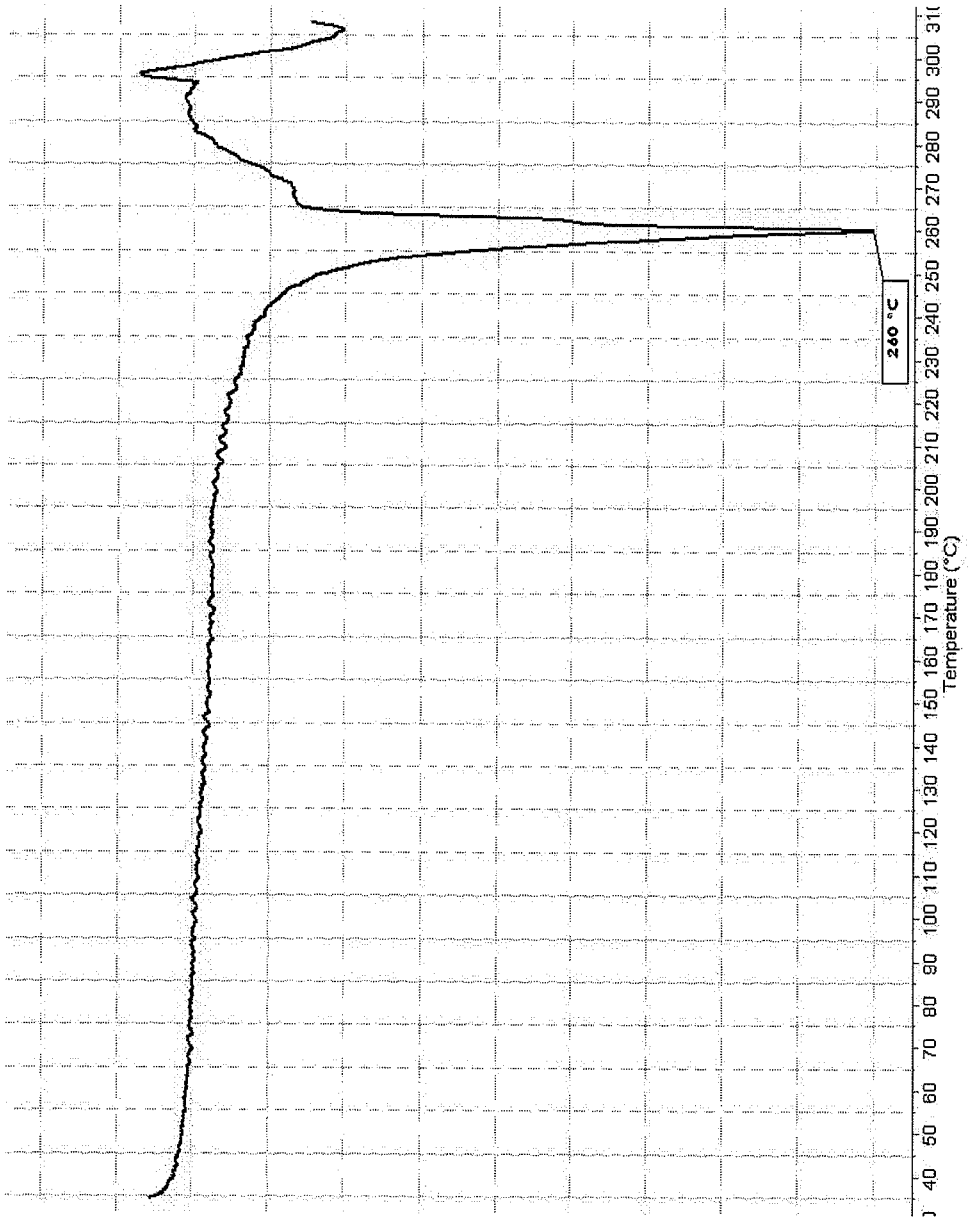
1389
1451
1635
2916
2956
2975
3429
Wavenumbers (cm-1)
%Transmittance

Figure 7.


260 °C
Temperature (°C)
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100
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290
300

Figure 8.

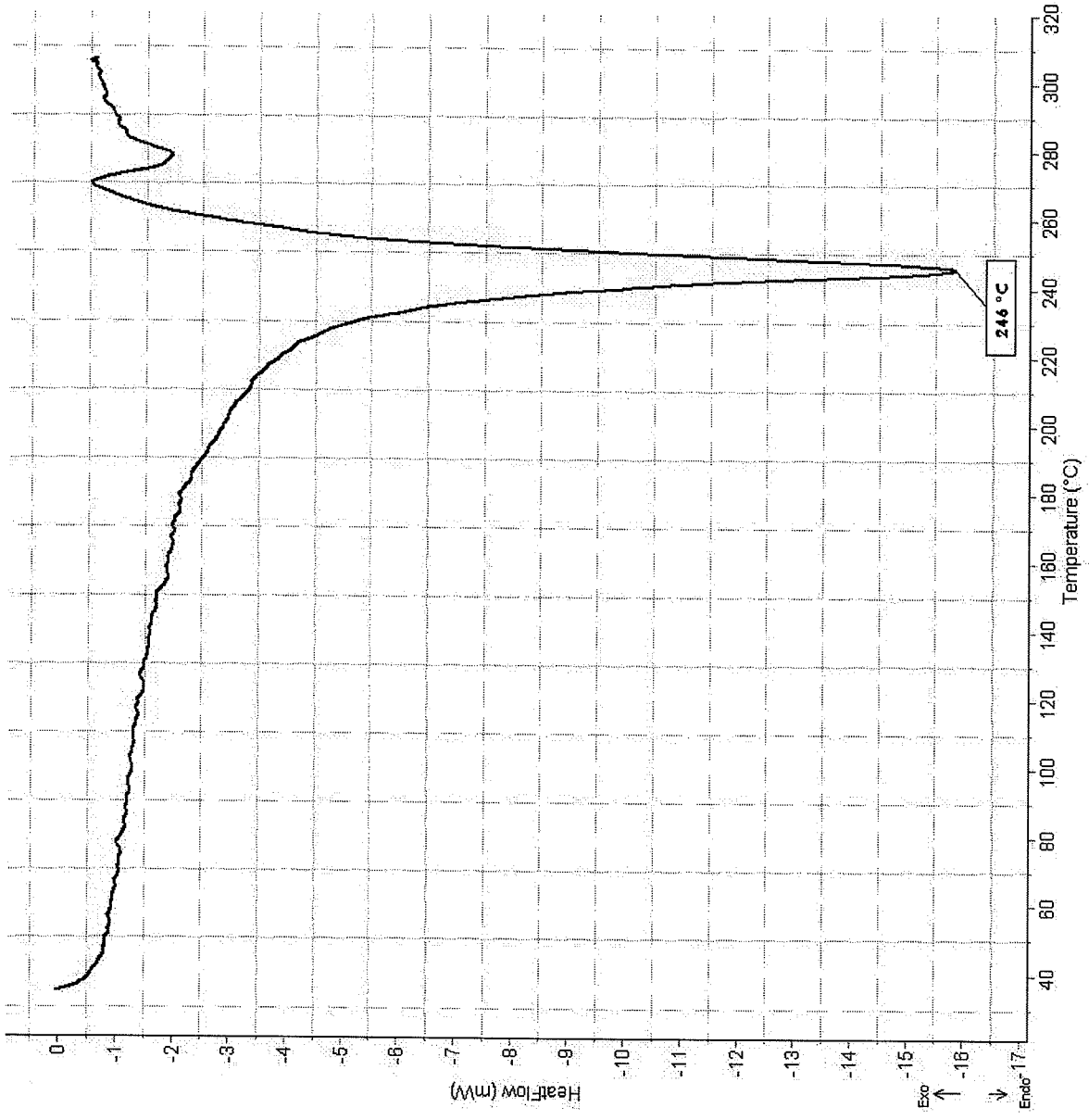


Figure 9.

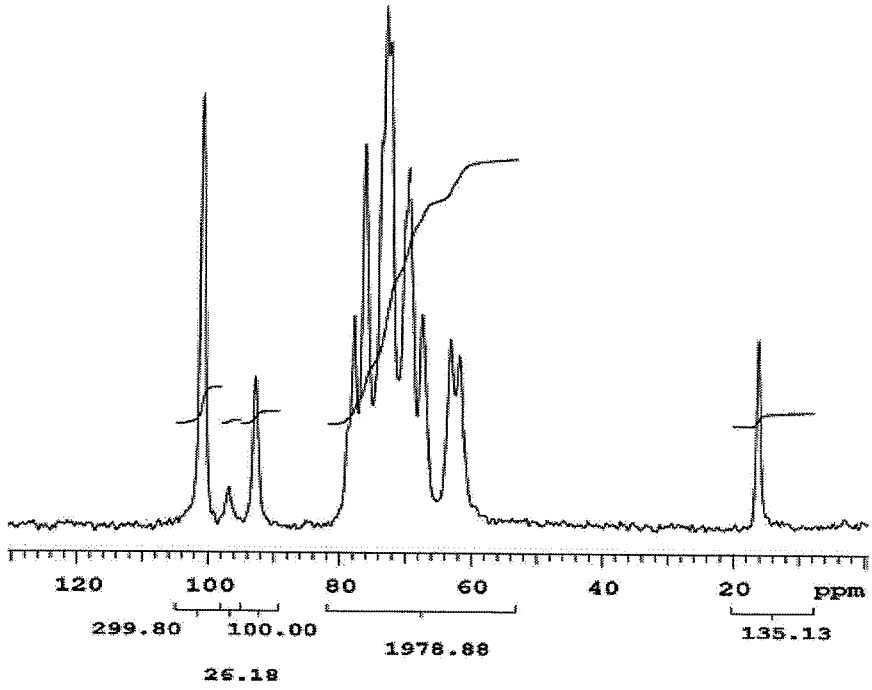
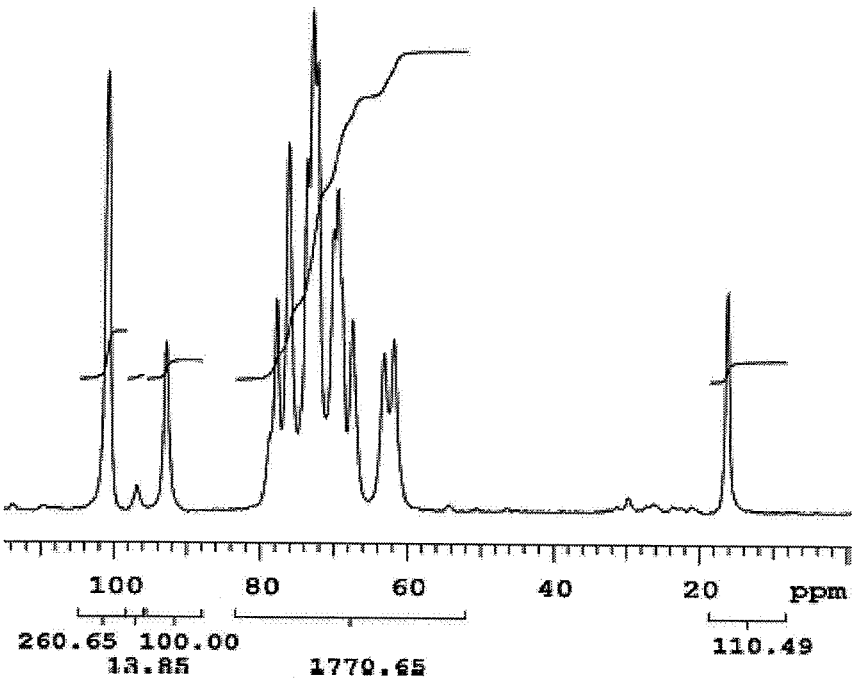


Figure 10.



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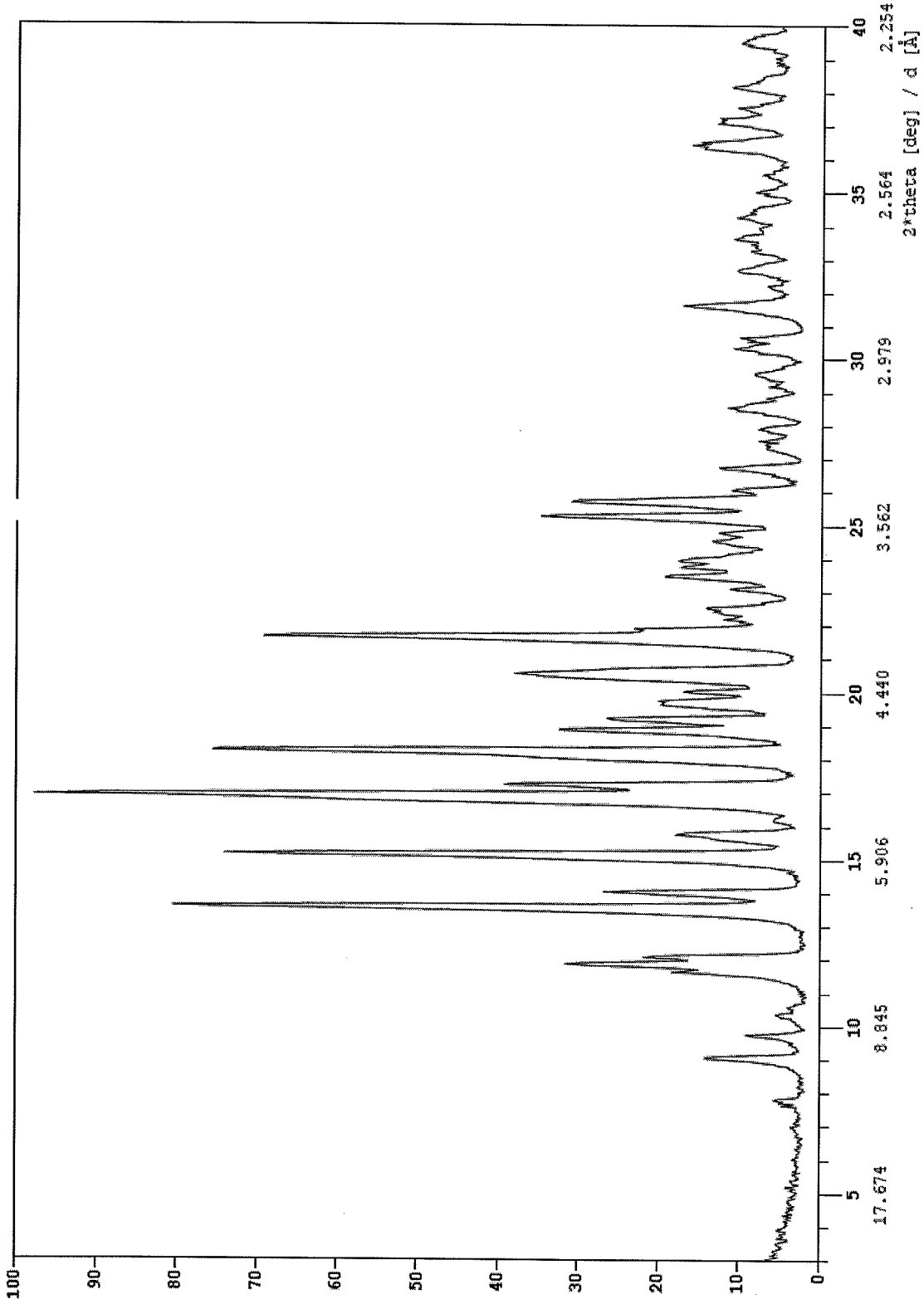
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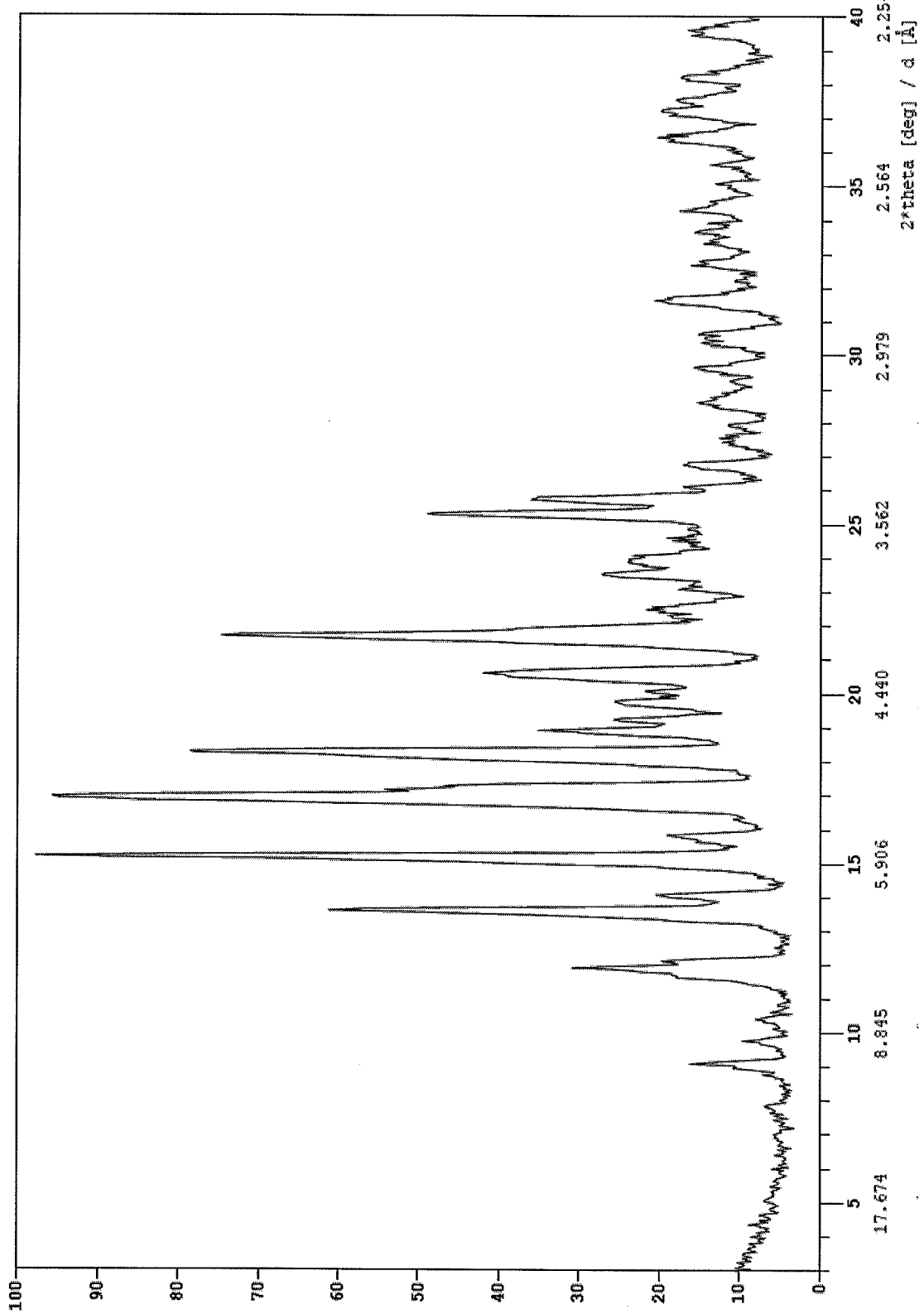
5.906
4.440
3.562
2.979
2.564
2.254
2*theta [deg] / d [Å]

Figure 12.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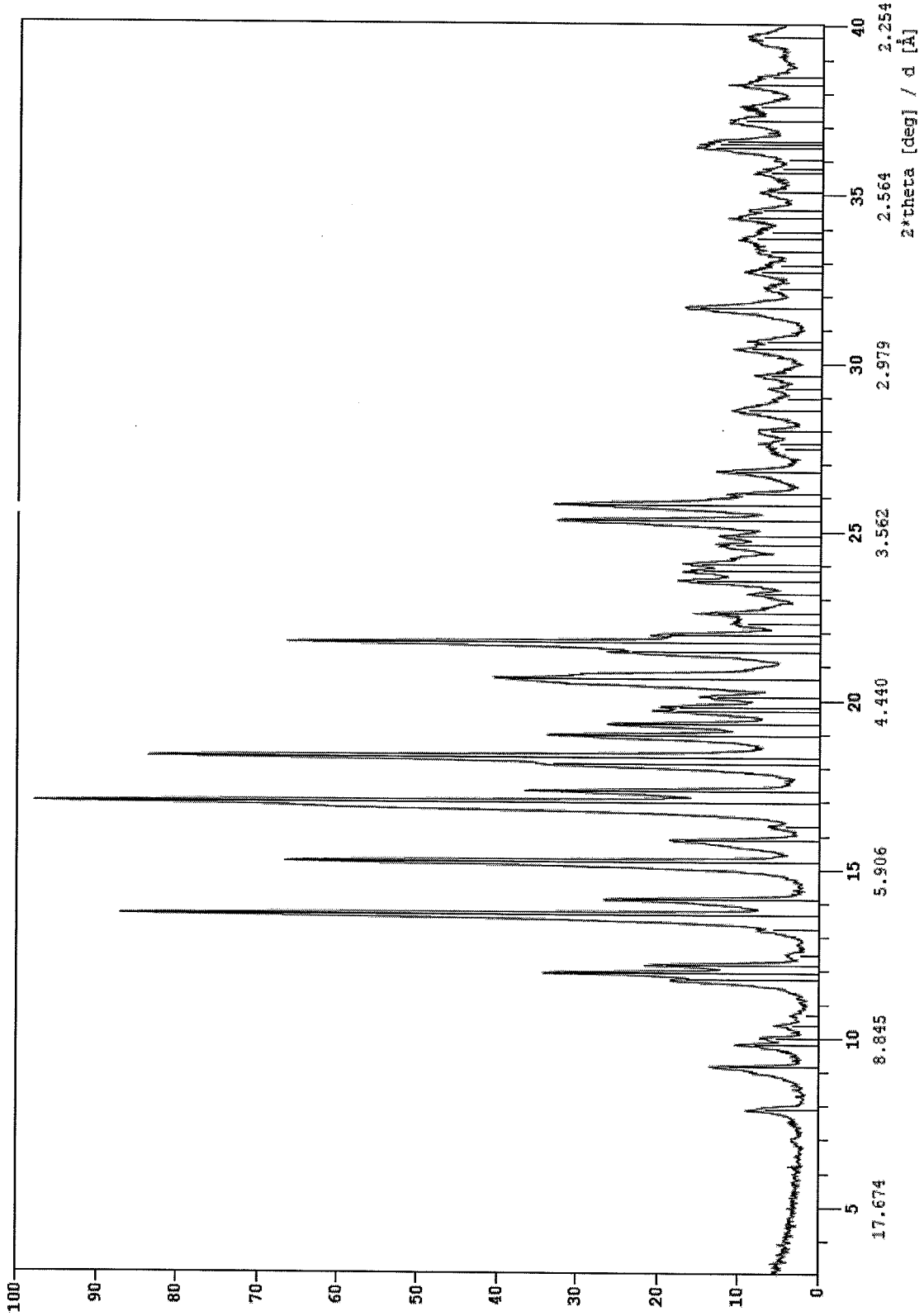
2*theta [deg] / d [Å]

Figure 13.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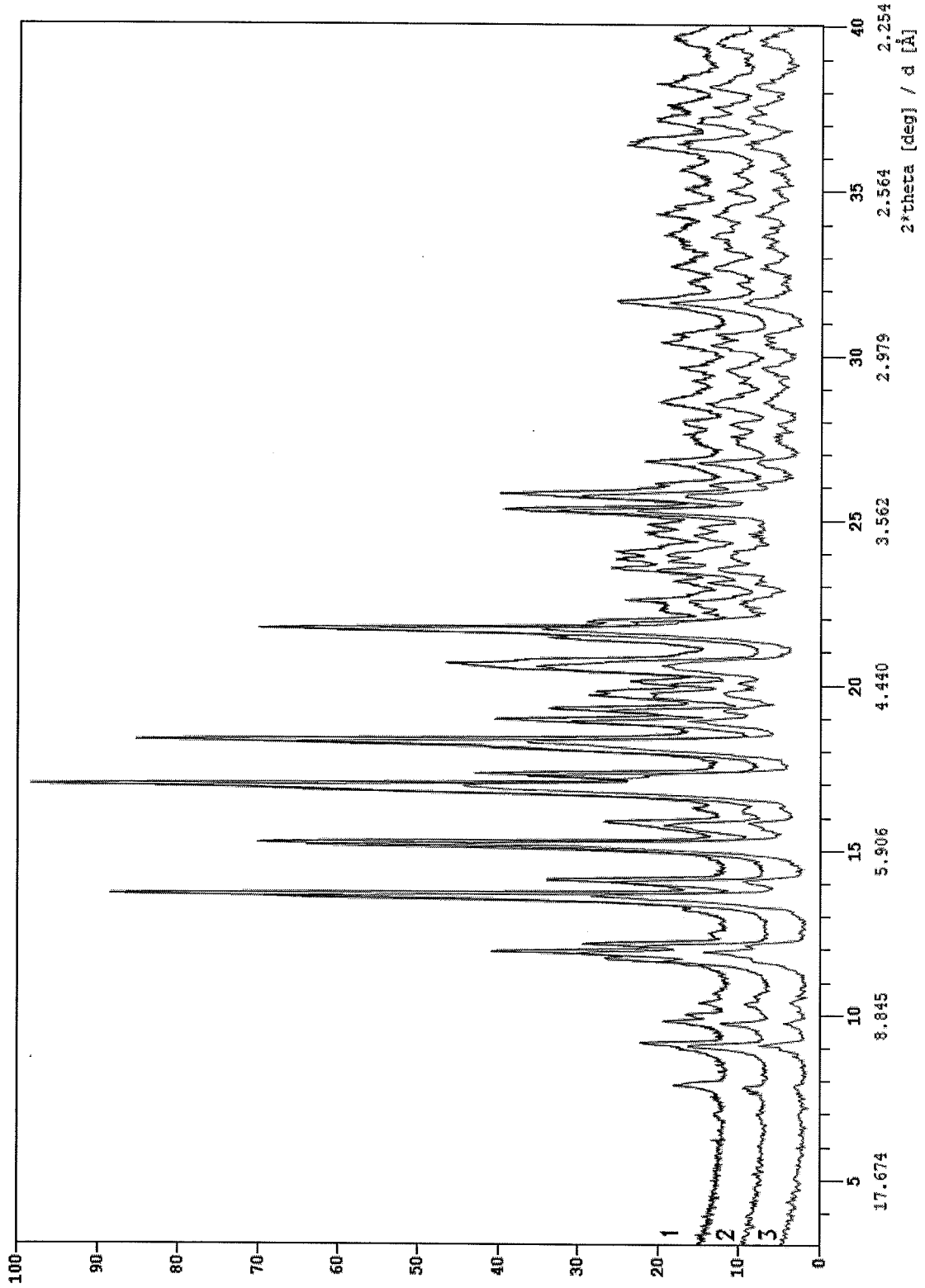
2*theta [deg] / d [Å]

Figure 14.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4.440
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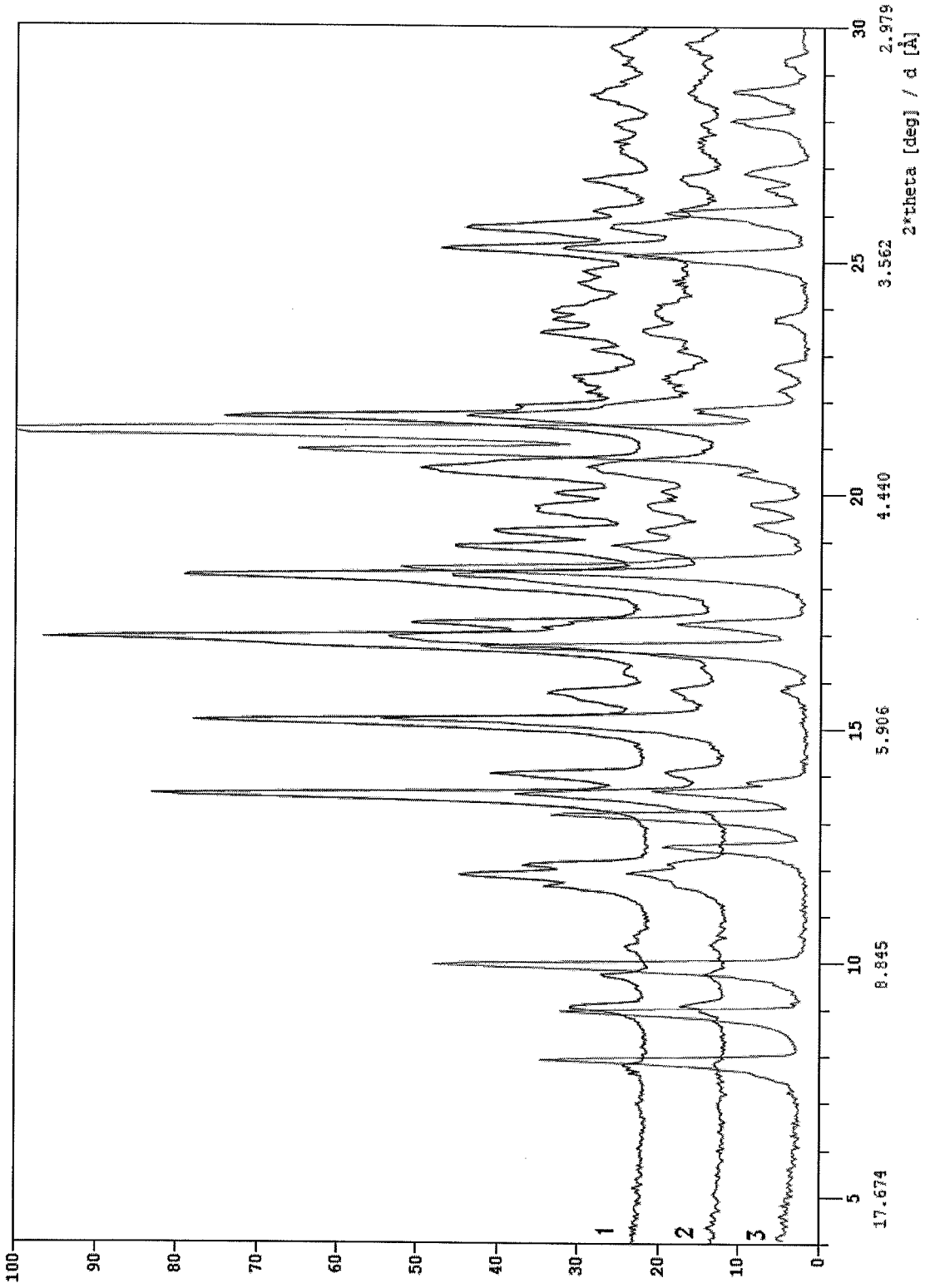
2.564
2.254
2*theta [deg] / d [Å]

Figure 15.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17.674
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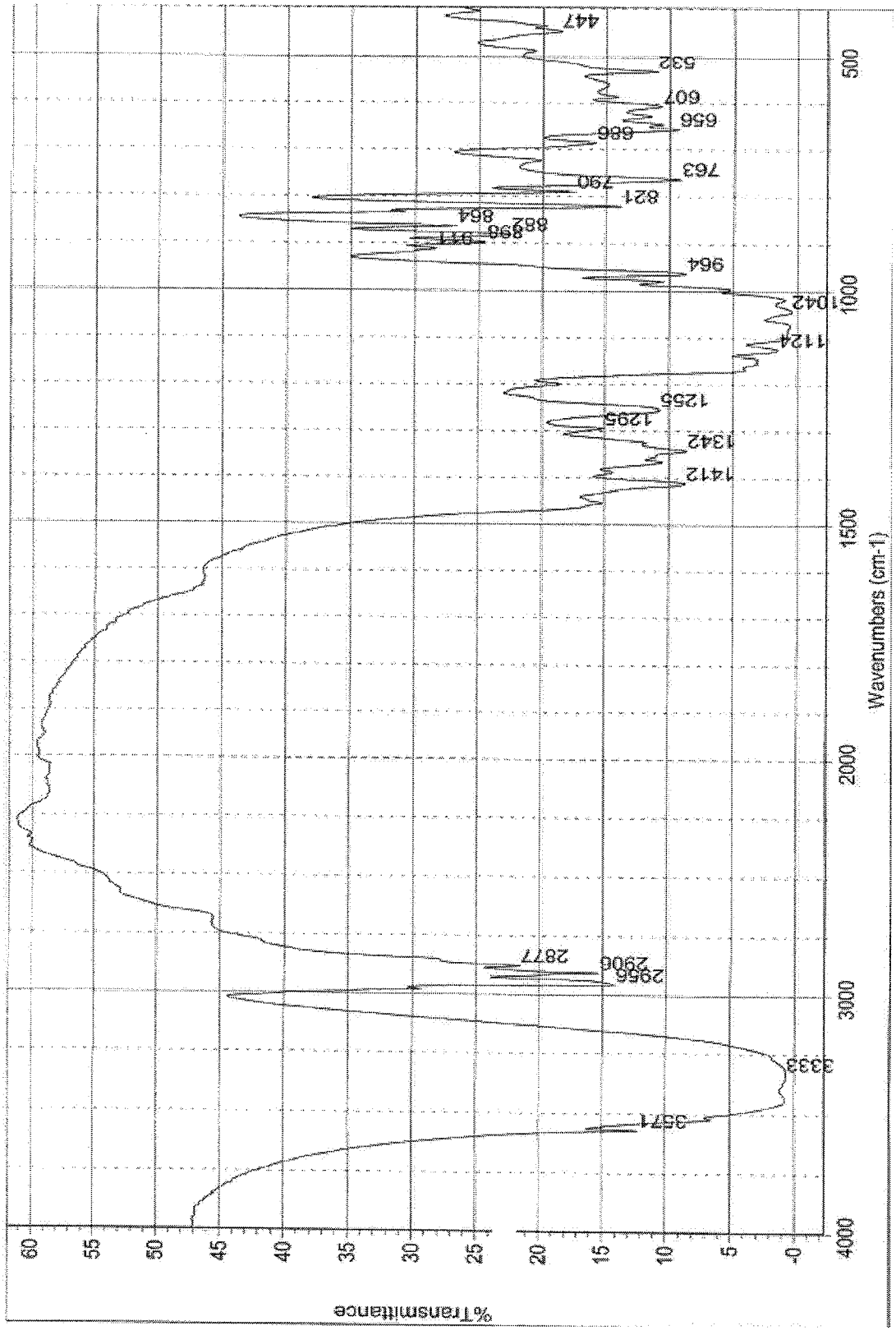
5.906
4.440
3.562
2.979
2*theta [deg] / d [Å]

Figure 16.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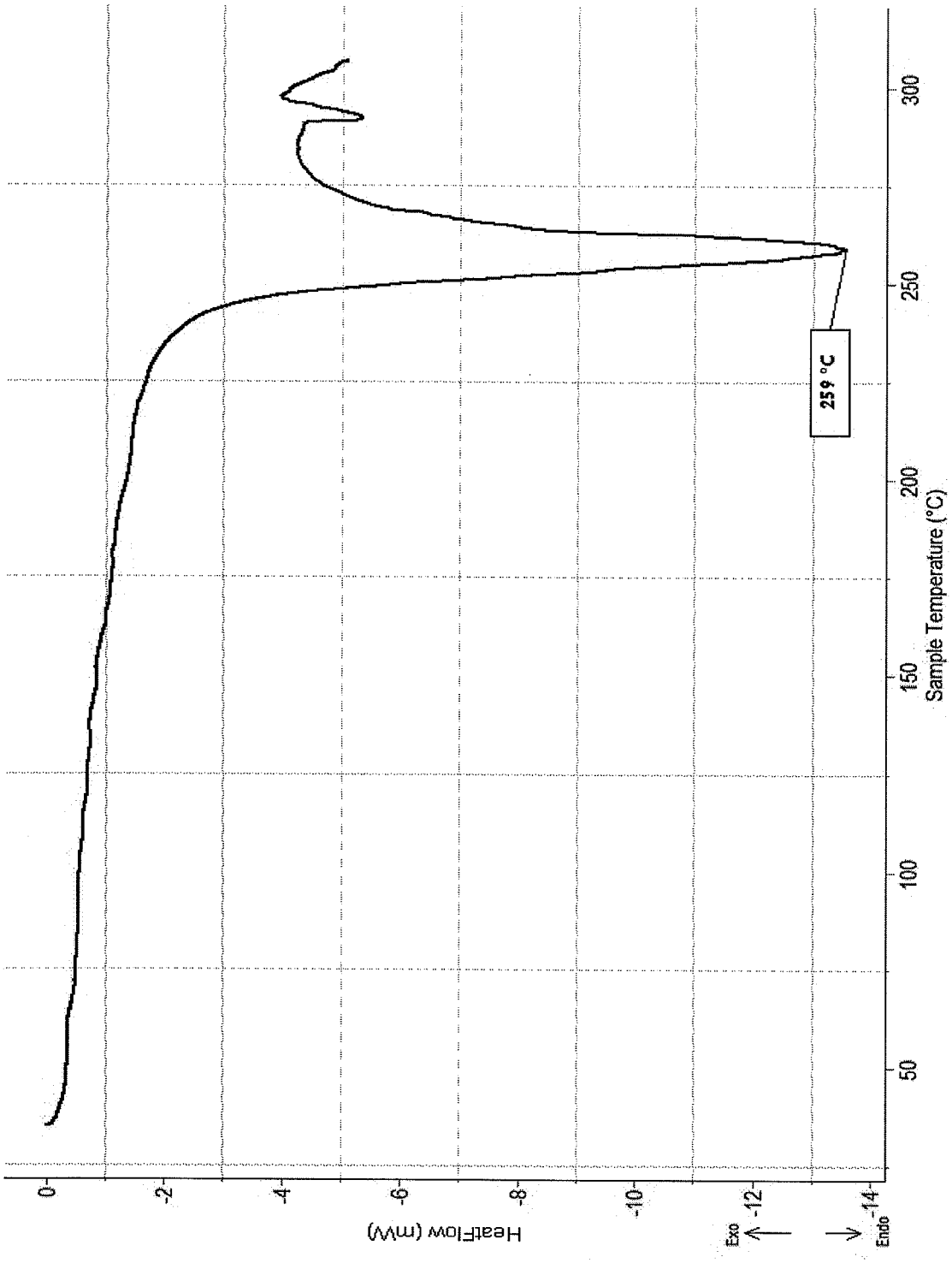
2956
3333
3571
%Transmittance
Wavenumbers (cm-1)

Figure 17.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HeatFlow (mW)
Exo
Endo
Sample Temperature (°C)
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-6
-8
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-14
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100
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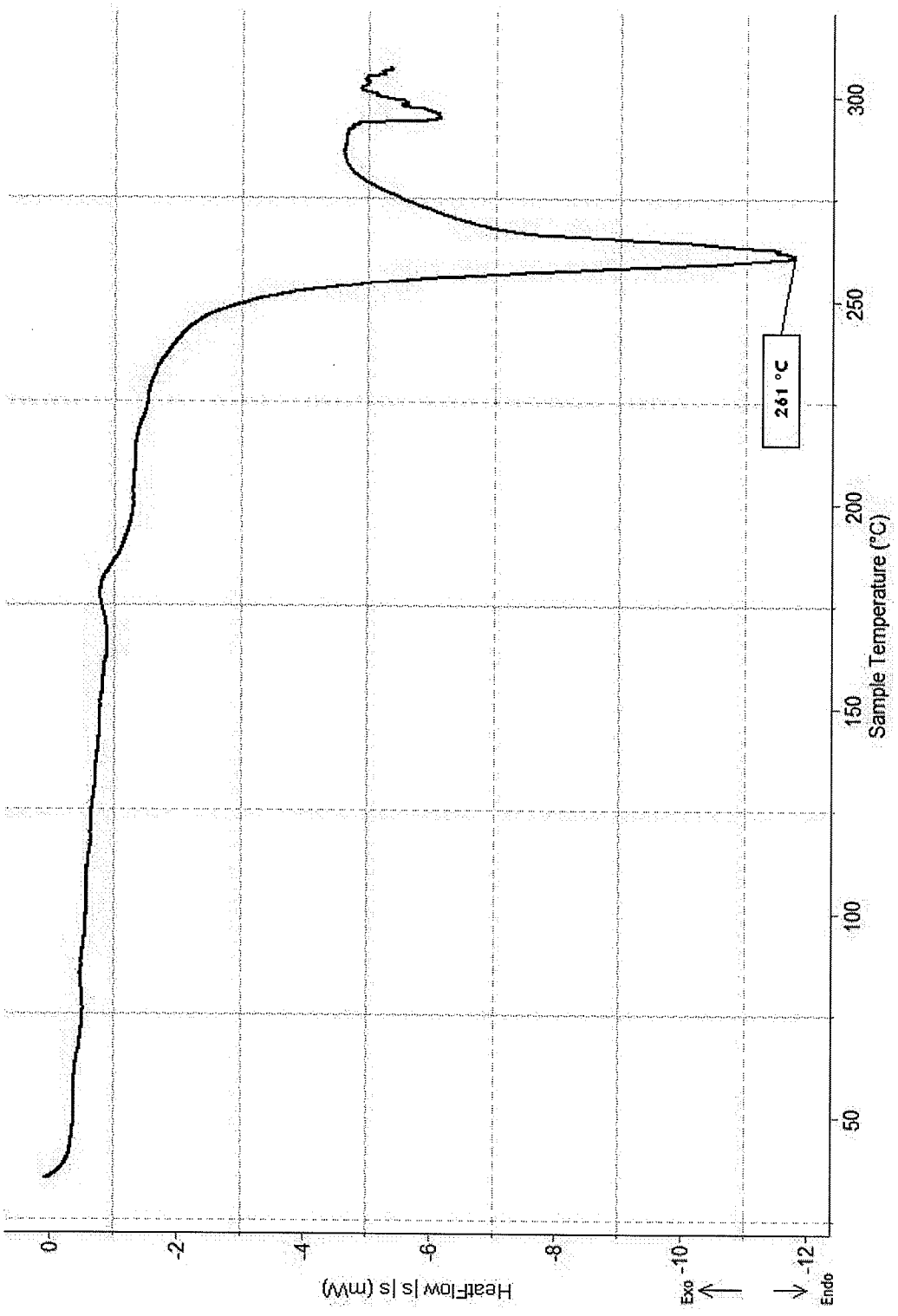
200
250
300

Figure 18.


261 °C
Sample Temperature (°C)
HeatFlow | s | s (mW)
Exo
Endo
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-2
-4
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-8
-10
-12
50
100
150
200
250
300

Figure 19.

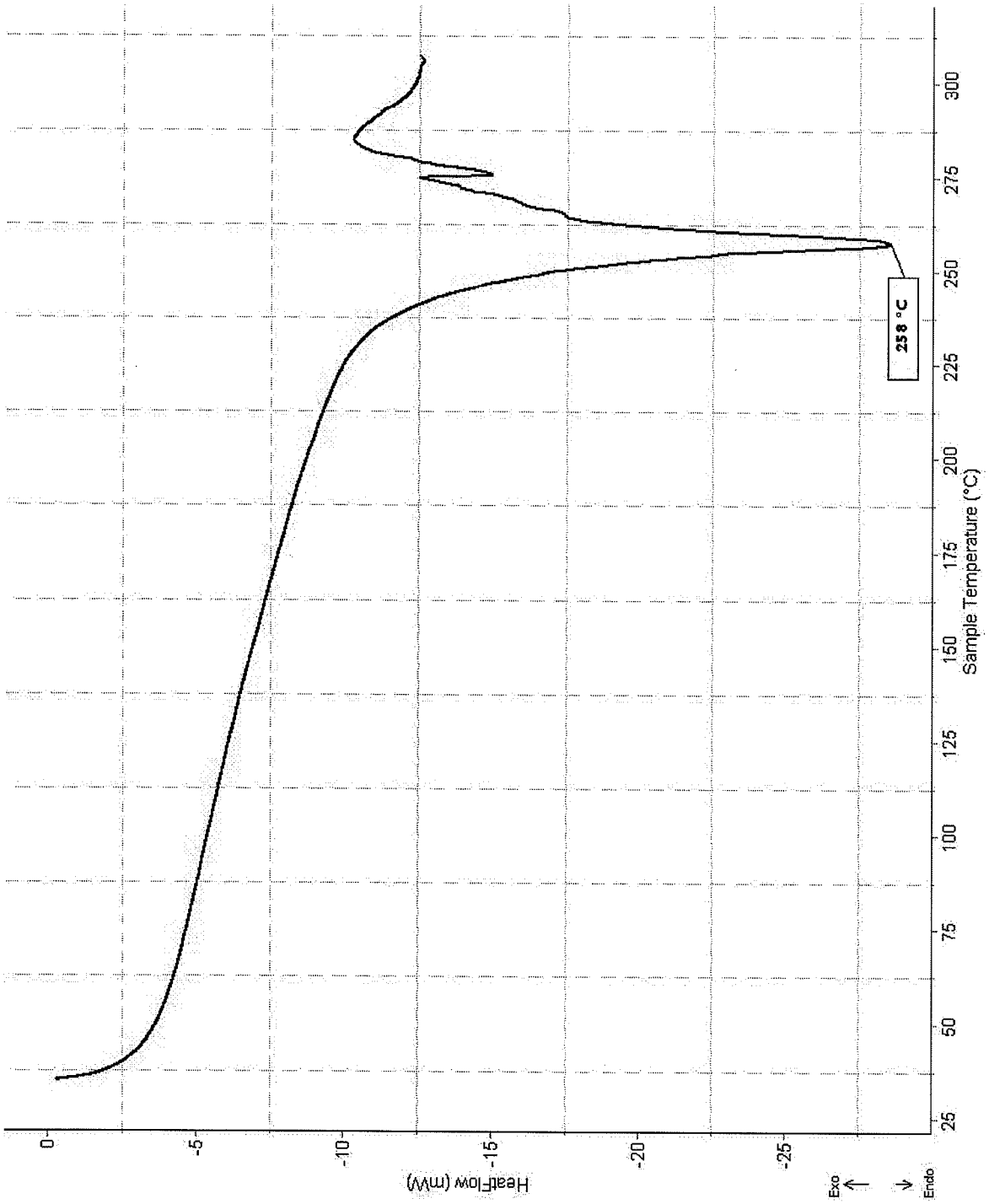


Figure 20.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK2011/050192

A. CLASSIFICATION OF SUBJECT MATTER
IPC (2006.01): C07H 3/06, C07H 1/06, A61K 31/702, A23L 1/09, A23L 1/29

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C07H, A61K, A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
DK, NO, SE, FI: Classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

REGISTRY, HCAPLUS, REAXYS, WPI, EPODOC, TXTE, TXTG, TXTF

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 2010/070616 A2 (INALCO S.P.A.) 2010.06.24 See Examples 20-21	1-24
X	KUHN, R. et al.: "Kristalliserte Fucosido-lactose", Chem. Ber., 1956, Vol. 89, No. 11, page 2513	1-24
A	KUHN, R. et al.: "Fucosido-lactose, das Trisaccharid der Frauenmilch", Chem. Ber., 1955, Vol. 88, No. 8, pages 1135-1146	
A	ABBAS, S. A. et al.: " Synthesis of O-a-L-Fucopyranosyl-(1-2) -O-b-D-Galactopyranosyl-(1-4)-D-Glucopyranose (2'-O-a-L-Fucopyranosyl-lactose)", Carbohydr. Res., 1981, Vol. 88, No. 1, pages 51-60	
A	FERNANDEZ-MAYORALES, A. et al.: "Synthesis of 3- and 2'-fucosyl-lactose and 3,2'-difucosyl-lactose from partially benzylated lactose derivatives", Carbohydr. Res. 1986, Vol. 154, No. 1, pages 93-101	
A	WO 2010/115934 A1 (GLYCOM A/S) 2010.10.14	

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21/06/2011	04/07/2011
Name and mailing address of the ISA/ Nordic Patent Institute, Helgeshøj Allé 81, DK-2630 Taastrup, Denmark Facsimile No. +45 4350 8008	Authorized officer Anne Bülow Find Telephone No. +45 4350 8125

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK2011/050192

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Continuation of box No. III:

The international Searching Authority found that the application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept. The result is a lack of unity between:

Invention 1 (claims 1-8 and claims 17-24 (in part)):
Crystalline 2'-O-fucosyllactose polymorph II, methods for producing said polymorph, pharmaceutical or nutritional compositions comprising polymorph II and the use of polymorph II in pharmaceutical or nutritional compositions.

Invention 2 (claims 9-16 and claims 17-24 (in part)):
Crystalline 2'-O-fucosyllactose polymorph I, methods for producing said polymorph, pharmaceutical or nutritional compositions comprising polymorph I and the use of polymorph I in pharmaceutical or nutritional compositions.

The special technical feature linking inventions 1 and 2 is regarded to be a crystalline polymorph of 2'-O-fucosyllactose. However, Kuhn, R. et al. (1956) has already described a crystalline form of 2'-O-fucosyllactose. Accordingly, a lack of unity arise between above inventions 1 and 2 as the technical feature linking the inventions, is known.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/DK2011/050192

Patent documents cited in search report	Publication date	Patent family member(s)	Publication date
WO2010070616 A2	2010.06.24	ITFI20080244 A1	2010.06.19
WO2010115934 A1	2010.10.14	WO2010115935 A1	2010.10.14

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 August 2011 (25.08.2011)

PCT

(10) International Publication Number
WO 2011/100980 A1

(51) International Patent Classification:
C07H 1/00 (2006.01) *C07H 15/18* (2006.01)
C07H 5/04 (2006.01) *A61K 31/702* (2006.01)
C07H 15/203 (2006.01) *A23L 1/29* (2006.01)

(21) International Application Number:
PCT/DK2011/050053

(22) International Filing Date:
21 February 2011 (21.02.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 2010 70060 19 February 2010 (19.02.2010) DK

(71) Applicant *(for all designated States except US)*: **GLYCOM A/S** [DK/DK]; Danmarks Tekniske Universitet, Bygning 201, DK-2800 Lyngby (DK).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **BAJZA, István** [SK/HU]; K. Tóth Kálmán u. 18/B, H-4031 Debrecen (HU). **DEKANY, Gyula** [HU/AU]; 46 Furness Crescent, Sinnamon Park, Queensland 4073 (AU). **ÁGOSTON, Károly** [HU/HU]; Berkes ltp. 58 , H-8000 Székesfehérvár (HU). **PÉREZ, Ignacio Figueroa** [CU/DK]; Bymidten 73D,2 , DK-3500 Værløse (DK). **BOUTET, Julien** [FR/FR]; Les Pesquies, Rue de la Croix Cholet , F-44770 La Plaine sur Mer (FR). **HEDEROS, Markus** [SE/SE]; Byggmästaregatan 103 , S-23343 Svedala (SE). **HORVÁTH, Ferenc** [HU/HU]; Forrás út 44 , H-2098 Pilisszentkereszt (HU). **KOVÁCS-PÉNZES, Piroska** [HU/HU]; Kéve u. 4 , H-5100 Jászberény (HU). **KRÖGER, Lars** [DE/DE]; Herzog-Alf-Weg 36 , 22457 Hamburg (DE). **RÖHRIG, Christoph** [DE/DE]; Drei-Lerchen 4 , 78357 Mühlingen (DE). **SCHROVEN, Andreas** [DE/DE]; Passatweg 5 , 26676 Barssel (DE). **VRASIDAS, Ioannis** [GR/GR]; Papapetrou 17 , GR-55131 Thessaloniki (GR). **TRINKA, Péter** [HU/HU]; Karinthy Frigyes u. 4-6 , H-1111 Budapest (HU). **KALMÁR, László** [HU/HU]; Dózsa Gy. u. 4 , H-4119 Váncsod (HU). **KOVÁCS, Imre** [HU/HU]; Ecsedi I. u. 3 , H-4034 Debrecen (HU). **DEMKÓ, Sándor** [HU/HU]; Békessy B. u. 7. 1/6 , H-4032 Debrecen (HU). **ÁGOSTON, Ágnes** [HU/HU]; Berkes ltp. 58 , H-8000 Székesfehérvár (HU). **RISINGER, Christian** [SE/DE]; Brugger Str. 98, 78628 Rottweil (DE).

(74) Agents: **THORSEN, Jesper** et al.; Inspicos A/S, P.O. Box 45, Kogle Allé 2, DK-2970 Hørsholm (DK).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

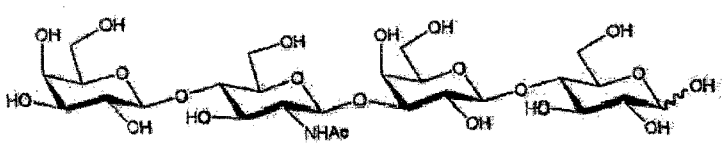
— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

(54) Title: A METHOD FOR PREPARATION OF THE TETRASACCHARIDE LACTO-N-NEOTETRAOSE (LNNT) CONTAINING N-ACETYLLACTOSAMINE

(I)



(57) Abstract: The present invention relates to a method for preparation of the tetrasaccharide lacto-N-neotetraose (LNnt, formula (I)) especially in large scale, as well as intermediates in the synthesis, a new crystal form (polymorph) of LNnt, and the use thereof in pharmaceutical or nutritional compositions.

WO 2011/100980 A1

A method for preparation of the tetrasaccharide lacto-N-neotetraose (LNnt) containing N-acetyllactosamine

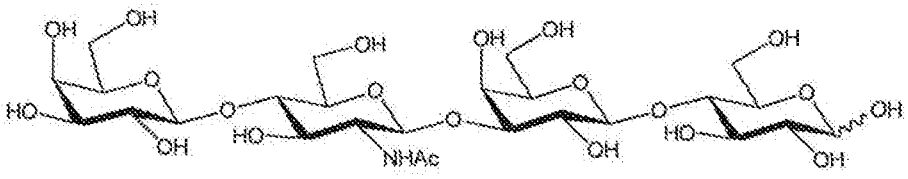
FIELD OF THE INVENTION

The present invention relates to a method for the preparation of Galpβ1-4GlcNAcpβ1-3Galpβ1-4Glc (LNnT), especially in large scale, as well as to intermediates and a new crystal form (polymorph) of LNnT.

BACKGROUND OF THE INVENTION

During the past decades the interest for preparation and commercialisation of human milk oligosaccharides (HMOs) has been increasing steadily. The importance of HMOs is directly linked to their unique biological activities such as antibacterial, antiviral, immune system and cognitive development enhancing activities.

The tetrasaccharide Galpβ1-4GlcNAcpβ1-3Galpβ1-4Glc (lacto-*N*-neotetraose, LNnT, Scheme 1) is one of the oligosaccharides occurring in human milk [Kuhn et al. *Chem. Ber.* **1962**, *95*, 513 and 518, Kobata *Methods Enzymol.* **1972**, *28*, 262]. LNnT act as bacterial receptor for pneumococci and it was found to be useful in the recognition of the acceptor specificity of glycosyltransferases, the substrate specificity of glycosidases and the structure of antigenic determinants. Furthermore LNnT represents a core structural element of more complex oligosaccharides, in glycolipids and in glycoproteins (paragloboside, 6-sialyl-LNnT, etc.) with various physiological activities.



Scheme 1. Lacto-*N*-neotetraose, LNnT

To date, access to large volumes of LNnT has not been possible by using isolation, biotechnology and synthetic methodologies. The isolation of LNnT from human milk is

rather difficult even in milligram quantities due to the presence of a large number of similar oligosaccharides.

Enzymatic synthesis of LNnT consists of incubation of lacto-*N*-triose II with UDP-galactose in the presence of a galactosyltransferase [Sabesan et al. *J. Am. Chem. Soc.* **1986**, *108*, 2068; EP-A-870841] or with lactose in the presence of β-D-galactosidase [Murata et al. *Glycoconj. J.* **1999**, *16*, 189; JP 10-234394 A]. Enzymatic galactosylation of the proper trisaccharide bound to solid support was also elaborated [Blixt et al. *Carbohydr. Res.* **1999**, *319*, 80; Renaudie et al. *ibid.* **2004**, *339*, 693]. These complex enzymatic systems represent very expensive methodologies and difficult purification protocols for scale-up productions of LNnT.

Total synthetic procedures towards LNnT published [Zurabyan et al. *Soviet J. Bioorg. Chem.* **1978**, *4*, 679; Paulsen et al. *Carbohydr. Chem.* **1987**, *169*, 105; Aly et al. *ibid.* **1999**, *316*, 121] comprise plenty of reaction steps, protecting group manipulations and chromatographic purification, poor yields, and provide only milligram quantity of LNnT, thus they do not offer attractive techniques for large scale preparation. Synthesis of 1-*O*-benzyl-LNnT as benzyl analogue of paragloboside has also been published [Ponpipom et al. *Tetrahedron Lett.* **1978**, *20*, 1717].

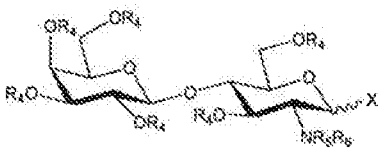
When isolated from natural source [Kuhn et al. *Chem. Ber.* **1962**, *95*, 513] or made by enzymatic way [EP-A-1405856] LNnT was characterized as a crystalline material.

With respect of LNnT and intermediates towards it there is still a need for crystalline products which may simplify isolation, purification and formulation problems so far envisaged.

SUMMARY OF THE INVENTION

The first aspect of the present invention relates a method for the preparation of Galpβ1-4GlcNAcpβ1-3Galpβ1-4Glc (LNnT) comprising the steps of:

a) reaction of a donor characterized by general formula **5**



general formula **5**

wherein R_4 is optionally substituted acyl,

$-NR_5R_6$ is selected from $-NAc_2$, -NH-haloacyl, phthalimide, tetrachlorophthalimide, 2,3-diphenylmaleimide and 2,3-dimethylmaleimide,

X is selected from halogen, $-OC(=NH)CCl_3$, -OAc, -OBz and $-SR_7$, wherein R_7 is selected from alkyl and optionally substituted phenyl,

with an acceptor of general formula **6**

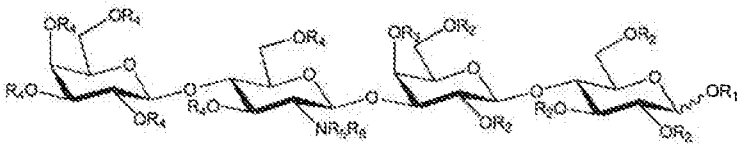
general formula **6**

wherein R_1 is a group removable by catalytic hydrogenolysis,

R_2 is optionally substituted acyl and

R_3 is selected from optionally substituted acyl or H,

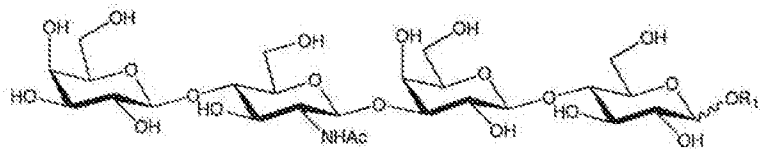
to yield a compound of general formula **4**



general formula **4**

wherein R_1, R_2, R_3, R_4 and $-NR_5R_6$ are as defined above,

b) converting the compound of general formula **4** into a compound of general formula **1**



general formula **1**

wherein R_1 is as defined above,

c) crystallizing the compound of general formula **1**, and

d) subsequently subjecting the compound of general formula **1** to catalytic reduction.

The second aspect of the present invention provides a polymorph of Galpβ1-4GlcNAcpβ1-3Galpβ1-4Glc (LNnT).

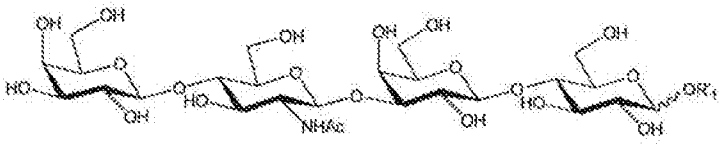
The third aspect of the present invention relates to the polymorph of Galpβ1-4GlcNAcpβ1-3Galpβ1-4Glc (LNnT) for use as pharmaceutical agent.

The fourth aspect of the present invention provides the polymorph of Galpβ1-4GlcNAcpβ1-3Galpβ1-4Glc (LNnT) for use as nutritional additive.

The fifth aspect of the present invention relates to a pharmaceutical composition comprising the polymorph of Galpβ1-4GlcNAcpβ1-3Galpβ1-4Glc.

The sixth aspect of the present invention provides a nutritional composition comprising the polymorph of Galpβ1-4GlcNAcpβ1-3Galpβ1-4Glc.

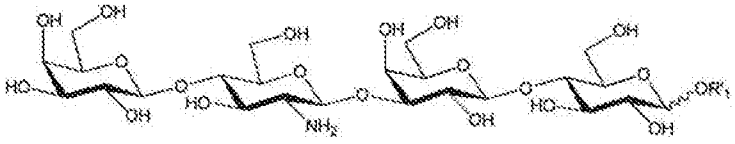
The seventh aspect of the present invention relates to a compound of general formula **1'**



general formula **1'**

wherein R'_1 is selected from substituted benzyl, optionally substituted benzhydryl, optionally substituted trityl and optionally substituted naphthylmethyl.

The eighth aspect of the present invention provides a compound of general formula **2'**



general formula **2'**

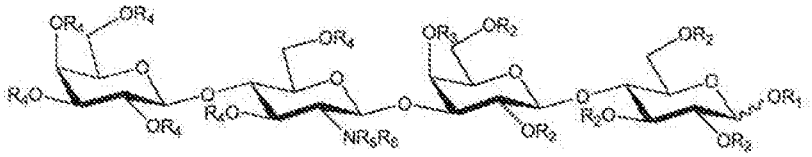
wherein R'_1 is selected from substituted benzyl, optionally substituted benzhydryl, optionally substituted trityl and optionally substituted naphthylmethyl.

The ninth aspect of the present invention relates to a compound of general formula **3**

general formula **3**

wherein R_1 is selected from optionally substituted benzyl, optionally substituted benzhydryl, optionally substituted trityl and optionally substituted naphthylmethyl, and $-NR_5R_6$ is selected from -NH-haloacyl, phthalimide, tetrachlorophthalimide, 2,3-diphenylmaleimide and 2,3-dimethylmaleimide.

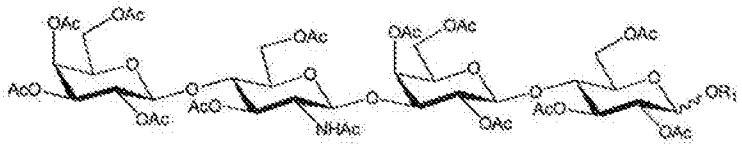
The tenth aspect of the present invention provides a compound of general formula **4'**



general formula **4'**

wherein R_1 is selected from optionally substituted benzyl, optionally substituted benzhydryl, optionally substituted trityl and optionally substituted naphthylmethyl, and each of R_2 and R_4 are independently optionally substituted acyl, R_3 is selected from optionally substituted acyl and H, $-NR_5R_6$ is selected from $-NAc_2$, -NH-haloacyl, tetrachlorophthalimide, 2,3-diphenylmaleimide and 2,3-dimethylmaleimide.

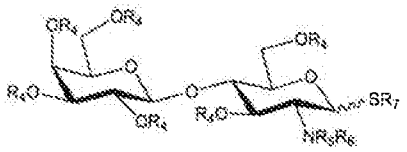
The eleventh aspect of the present invention relates to a compound of general formula **4a**



general formula **4a**

wherein R_1 is selected from optionally substituted benzyl, optionally substituted benzhydryl, optionally substituted trityl and optionally substituted naphthylmethyl.

The twelfth aspect aspect of the present invention provides a compound of general formula **5'**



general formula **5'**

wherein R_4 is optionally substituted acyl, preferably acetyl, $-NR_5R_6$ is –NH-haloacyl, preferably –NH-trichloroacetyl or –NH-trifluoroacetyl, and R_7 is optionally substituted phenyl, preferably phenyl.

The thirteenth aspect of the present invention relates to a compound of general formula **6'**

general formula **6'**

wherein R_1 is selected from optionally substituted benzyl, optionally substituted benzhydryl, optionally substituted trityl and optionally substituted naphthylmethyl, R_2 is optionally substituted benzoyl, and R_3 is selected from optionally substituted acyl and H.

BRIEF DESCRIPTION OF THE FIGURES

The invention will be described in further detail hereinafter with reference to the accompanying figures, in which:

Figure 1 shows the X-ray powder diffraction pattern of crystalline LNnT prepared according to example 34.

Figure 2 shows the comparison of X-ray powder diffraction pattern of crystalline LNnT prepared according to 34 and EP-A-1405856 (continuous curve: diffraction pattern of crystalline LNnT prepared according to example 34; vertical lines: data taken from EP-A-1405856).

Figure 3 shows the solid-state ^{13}C-NMR spectrum of crystalline LNnT prepared according to example 34.

DETAILED DISCLOSURE OF THE INVENTION

Throughout the present description, the term "alkyl", either alone or when attached to another atom or group, means a linear or branched hydrocarbon group with 1-6 carbon atoms, like methyl, ethyl, *n*-propyl, i-propyl, *n*-butyl, i-butyl, *s*-butyl, *t*-butyl, etc.

In the present application the term "aryl" refers to homoaromatic groups like phenyl or naphthyl. Preferably, aryl means phenyl.

In the present description, the term "acyl" represent an R-C(=O)-, wherein R may be H, alkyl or aryl, like formyl, acetyl, propionyl, butyryl, pivaloyl, benzoyl, etc. The alkyl and aryl residues both may be substituted.

For the purpose of this specification with claims, the term "optionally substituted" means that the group in question may either carry a substituent or may be unsubstituted.

For the purpose of this specification with claims, the term "substituted" means that the group in question is substituted with a group which typically modifies the general chemical characteristics of the chain or ring. The substituents can be used to modify characteristics of the molecule as a whole, such as stability, solubility, and ability to form crystals. The person skilled in the art will be aware of other suitable substituents of a similar size and charge characteristics, which could be used as alternatives in a given situation.

More generally in connection with the terms "alkyl", "aryl" and "acyl" the term "optionally substituted" is intended to mean that the group in question may be substituted one or several times, preferably 1-5 times, more preferably 1-3 times with group(s) selected from alkyl (only for aryl and aromatic acyl), hydroxy, alkoxy (i.e. alkyl-oxy), carboxy, oxo (forming a keto or aldehyde functionality), alkoxycarbonyl, alkylcarbonyl, formyl, aryl, aryloxy-carbonyl, aryloxy, arylamino, arylcarbonyl, amino, mono- and dialkylamino, carbamoyl, mono- and dialkyl-aminocarbonyl, alkylcarbonylamino, cyano, alkanoyloxy, nitro, alkylthio and halogen (F, Cl, Br, I).

The expression "group removable by catalytic hydrogenation" refers to groups, whose C-O bond is cleaved by addition of hydrogen in the presence of catalytic amounts of palladium, Raney nickel or another appropriate metal catalyst known for use in hydrogenolysis, resulting in the regeneration of the OH group. Groups of this type are well known to the skilled man and thoroughly discussed [e.g. P.G.M. Wuts and T.W. Greene: *Protective Groups in Organic Synthesis*, John Wiley & Sons (2007)]. Suitable groups include benzyl, diphenylmethyl (benzhydryl), 1-naphthylmethyl, 2-naphthylmethyl or triphenylmethyl (trityl) groups, each of which may be optionally substituted by one or more groups selected from: alkyl, alkoxy, phenyl, amino, acylamino, alkylamino, dialkylamino, nitro, carboxyl, alkoxycarbonyl, carbamoyl, *N*-alkylcarbamoyl, *N*,*N*-dialkylcarbamoyl, azido, halogenalkyl or halogen. Preferably, such substitution, if present, is on the aromatic ring(s). Particularly preferred protecting group is benzyl optionally substituted with one or more groups selected from alkyl or halogen. More preferably, the protecting group is selected from unsubstituted benzyl, 4-chlorobenzyl and 4-methylbenzyl. These particularly preferred and more preferable protecting groups have the advantage that the by-products of the hydrogenolysis are exclusively toluene or substituted toluene. Such by-products can easily be removed even in multi ton scales from water soluble oligosaccharide products via evaporation and/or extraction processes.

In connection with the -NH-haloacyl in group $-NR_5R_6$ the term "haloacyl" refers to halogenated acyl groups of formula $C_nH_xX_y$-C(=O)-, wherein integer n is 1, 2 or 3, $x+y=2n+1$ with the proviso that $x<y$, and X is F, Cl and Br, such as dichloroacetyl, trichloroacetyl, trifluoroacetyl, heptafluorobutyryl and the like.

The present invention represents a commercial approach suitable for large scale manufacture of LNnT, i.e. typically for the manufacture of batches of at least 1 kg of LNnT, such as at least 5 kg, or at least 50 kg, or even at least 200 kg, e.g. at least 1 ton, of LNnT. The successful strategy is based upon the introduction of relevant crystalline intermediates permitting simple and robust purification methodologies. Crystallization or recrystallization is one of the simplest and cheapest methods to separate a product from contaminations and obtain pure substance. In addition, providing one or more crystalline modifications (polymorphs) of a solid is an important factor in product development, because the different crystalline forms affect the compound's properties - for example thermodynamic stability, solubility, density, hygroscopicity, electrical properties (such as dielectric constant, conductivity), mechanical properties (such as friability, hardness, breaking strength, elasticity), optical properties (such as colour, transparency, refraction), etc. - diversely. It enlarges the repertoire of materials that a scientist has available for improving the product's characteristics.

The present invention provides in a first aspect a method for the preparation of Galpβ1-4GlcNAcpβ1-3Galpβ1-4Glc comprising the steps of:

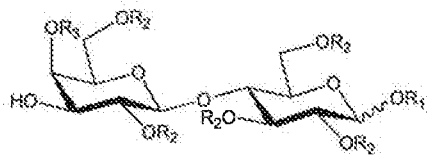
a) reaction of a donor characterized by general formula **5**

general formula **5**

$-NR_5R_6$ is selected from $-NAc_2$, -NH-haloacyl, phthalimide, tetrachlorophthalimide, 2,3-diphenylmaleimide and 2,3-dimethylmaleimide,

X is selected from halogen, $-OC(=NH)CCl_3$, -OAc, -OBz and $-SR_7$, wherein R_7 is selected from alkyl and optionally substituted phenyl,

with an acceptor of general formula **6**



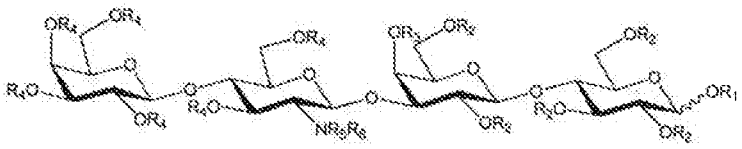
general formula 6

wherein R_1 is a group removable by catalytic hydrogenolysis,

R_2 is optionally substituted acyl and

R_3 is selected from optionally substituted acyl or H,

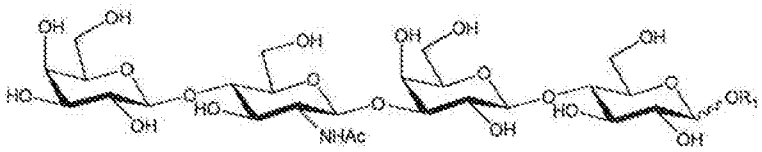
to yield a compound of general formula **4**



general formula 4

wherein R_1, R_2, R_3, R_4 and $-NR_5R_6$ are as defined above,

b) converting the compound of general formula **4** into a compound of general formula **1**



general formula 1

wherein R_1 is as defined above,

c) crystallizing the compound of general formula **1**, and

d) subsequently subjecting the compound of general formula **1** to catalytic reduction.

With regard to step a) the fully protected LNnT derivatives according to general formula **4** are synthesized in the reaction of a compound of general formula **5** (donor) with a compound of general formula **6** (acceptor) under glycosylation condition.

The coupling of the lactose acceptor of general formula **6** with the lactosaminyl donor of general formula **5** can be carried out an aprotic solvent or in a mixture of aprotic solvents in the presence of an activator (promoter or catalyst) so as to lead to the desired glycosylated product. The new interglycosidic linkage is formed by the nucleophilic displacement of the leaving group X of donor according to general formula **5** with the 3'-OH group of the acceptor according to general formula **6**. Other functional groups in both participating reactants have to be masked with protecting groups. In addition the present inventors realized that regioselective glycosylation can be achieved on acceptor of general formula **6**, wherein R_3 is H. In such dihydroxy acceptors the reactivity of the equatorial 3'-OH and the axial 4'-OH is different: the equatorial OH-group may act as stronger nucleophile under glycosylation conditions. Thus with careful selection of the conditions such as donor reactivity, solvent, temperature, nature of promoter, means of addition of reactants/promoters and like the reaction can be driven to the formation of the desired 1-3 interglycosidic linkage instead of 1-4 coupling. Particular care has to be taken with regard to the stereoselectivity. The stereochemical outcome may be affected by different factors like the presence or absence of a participating group at C-2 of the donor, the nature of the leaving group X, solvent effect, nature of the protective groups on both the donor and acceptor, nature of the promoters or catalysts, temperature, pressure, steric interactions between the donor and acceptor, and like. In case of glucosaminyl or lactosaminyl derivatives an array of anomeric activation for glycosylation is developed and available to a skilled person engaged in carbohydrate chemistry. These methodologies are expansively discussed by reviews and handbooks, for instance by Demchenko (Ed.): Handbook of Chemical Glycosylation, Wiley (2008). For the sake of examples some general considerations are briefly mentioned below depending on the X-group.

The glycosyl halides (X means F, Cl, Br, I) are frequently used in glycosylation reaction because of their easy accessibility and satisfactory reactivity. Typically, anomeric halides follow the reactivity order F<Cl<Br<I for nucleophilic displacement. The glycosylation reactions are generally promoted by heavy metal ion, mainly mercury or silver, and Lewis acids.

Glycosyl trichloroacetimidates (X= $-OC(=NH)CCl_3$) can be easily prepared by the addition of the free anomeric OH to trichloroacetonitrile under inorganic or organic base catalysis. In

a typical glycosidation reaction catalytic amount of Lewis acid, such as trimethylsilyl triflate or BF_3-etherate, promotes the coupling.

Glycosyl acetates or benzoates (X represents –OAc or –OBz) in glycosylation reaction are first subjected to electrophilic activation providing a reactive intermediate, then treated with the nucleophilic OH-acceptor. Typical activators of choice are Bronsted acids (such as TsOH, $HClO_4$, sulfamic acid), Lewis acids (such as $ZnCl_2$, $SnCl_4$, triflate salts, BF_3-etherate, trityl perchlorate, $AlCl_3$, triflic anhydride) and their mixtures.

Thioglycosides (X denotes alkylthio- or phenylthio-group) can be activated by thiofilic promoters such as mercury(II) salts, Br_2, I_2, NBS, NIS, triflic acid, triflate salts, BF_3-etherate, trimethylsilyl triflate, dimethyl-methlythio sulphonium triflate, phenylselenyl triflate, iodonium dicollidine perchlorate, tetrabutylammonium iodide or mixtures thereof, in condensation reactions, preferably by Br_2, NBS, NIS and triflate salts.

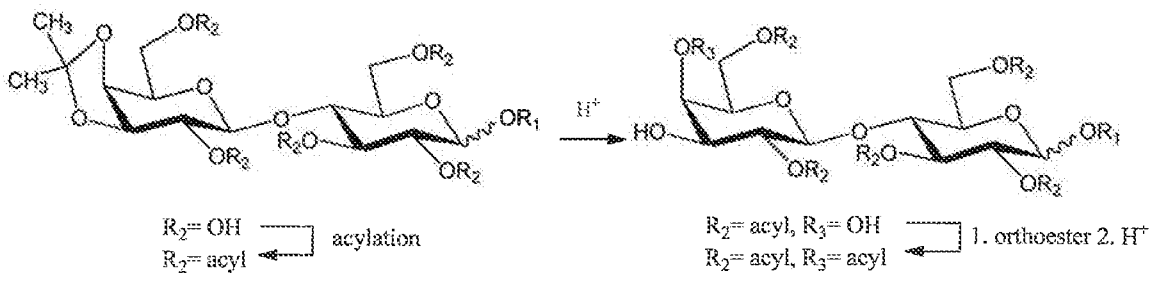
In a preferred embodiment the glycosyl donor is a compound of general formula **5**, wherein R_4 is optionally substituted acyl, $-NR_5R_6$ is –NH-haloacyl and X is $–SR_7$, wherein R_7 is selected from optionally substituted alkyl or optionally substituted phenyl; more preferably R_7 is optionally substituted phenyl; even more preferably R_4 is acetyl, $-NR_5R_6$ is selected from –NH-trichloroacetyl and –NH-trifluoroacetyl, R_7 is phenyl and $–SR_7$ is in β. The glycosylation is carried out in aprotic solvent(s) like chloroform, dichloromethane, toluene, dioxane, THF, acetonitrile or mixture thereof, preferably chloroform or dichloromethane, under the activation of NIS, NBS, Br_2, triflic acid, silver triflate, BF_3-etherate or mixture thereof.

In a further preferred embodiment the glycosyl acceptor is a compound of general formula **6**, in which R_1 is optionally substituted benzyl and R_3 is selected from H and optionally substituted benzoyl; more preferably R_1 is benzyl, R_2 is benzoyl optionally substituted with chloro and R_3 is selected from H and benzoyl optionally substituted with chloro, and OR_1 is in β.

The donors characterized by general formula **5** can be produced by conventional methodologies known in the art. An approach can imply the galactosylation of the protected glucosamine thioglycoside [e.g. Sherman et al. *Carbohydr. Res.* **2001**, *336*, 13], or of a

methyl glucosaminide derivative [e.g. Kochetkov et al. *Tetrahedron* **1987**, *43*, 3109]. Another access is based on the derivatization of the double bond of D-lactal hexaacetate, either by azidonitration followed by reduction of the 2-azido group with subsequent protection of the amine formed and nitrate-halogenide exchange on the anomeric carbon [e.g. Lemieux et al. *Can. J. Chem.* **1982**, *60*, 63], or by nitroso-chlorination followed by acetylation of the formed oxime and diborane reduction [e.g. Ponpipom et al. *Tetrahedron Lett.* **1978**, *20*, 1717], both methods leading to protected lactosaminyl halogenides. Functionalization of lactosamine is also conceivable (*O*-acetylation followed by amine protection and anomeric acetate-halogen interconversion). Further anomeric activations can be envisaged by the formation of thioglycosides from halides [e.g. Sherman et al. *Carbohydr. Res.* **2001**, *336*, 13] or from acetates [e.g. Sato et al. *Tetrahedron Lett.* **1988**, *29*, 4759]. Another frequently used glycosyl donors are trichloroacetimidates [e.g. Sato et al. *Tetrahedron Lett.* **1988**, *29*, 4759], and the application of lactosaminyl fluorides – synthesized from the corresponding glycosyl azides - as donors is also published [e.g. Bröder et al. *Carbohydr. Res.* **1993**, *249*, 221]. The literature examples mentioned above just illustrate some possible pathways without limitation, and the skilled person is capable of combining them to achieve the desired embodiments characterized by general formula **5**.

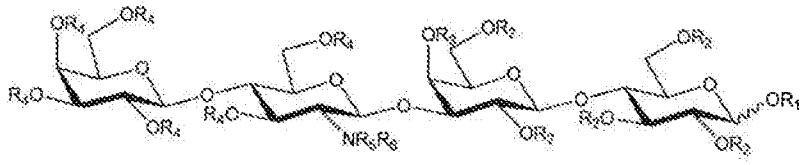
Compounds of general formula **6** are available by the following manipulations. Starting from the common octa-*O*-acetyl lactose or hepta-*O*-acetyl lactosyl bromide the corresponding lactoside can be formed with R_1OH under Lewis-acid (e.g. mercury salt, BF_3-etherate) activation. By de-*O*-acetylation (e.g. Zemplén-deprotection, aminolysis or basic hydrolysis) followed by regioselective acetonidation with dimethoxypropane in the presence of acid catalyst the 3',4'-protected lactoside may be obtained, which is then acylated with R_2-halogenide or $(R_2)_2O$ (anhydride) under usual conditions. The resulting derivative may be hydrolized with acid to remove isopropylidene giving a diol (compounds of general formula **6**, wherein R_3 is OH) which is treated with an orthoester derived from R_3OH. A cyclic orthoester thus obtained is subsequently rearranged with acid catalyst to another compound of general formula **6**, wherein R_3 is acyl [see e.g. Paulsen et al. *Carbohydr. Res.* **1985**, *137*, 39; Lubineau et al. *ibid.* **1997**, *305*, 501; and references cited therein] (Scheme 2.).



general formula **6**

Scheme 2.

In step b) of the first aspect of the present invention compounds of general formula **4**



general formula **4**

wherein R_1 is a group removable by catalytic hydrogenolysis, each of R_2 and R_4 are independently optionally substituted acyl groups, R_3 is selected from optionally substituted acyl or H, $-NR_5R_6$ is selected from $-NAc_2$, -NH-haloacyl, phthalimide, tetrachlorophthalimide, 2,3-diphenylmaleimide and 2,3-dimethylmaleimide, are converted into compounds of general formula **1** comprising the steps:

ba) base catalyzed transesterification deprotection or basic hydrolysis of the compound of general formula **4**, wherein $-NR_5R_6$ is $-NAc_2$, to give a compound of general formula **1** or

bb) base catalyzed transesterification deprotection of a compound of general formula **4**, wherein $-NR_5R_6$ is selected from -NH-haloacyl, phthalimide, tetrachlorophthalimide, 2,3-diphenylmaleimide and 2,3-dimethylmaleimide, to give a compound of general formula **3**

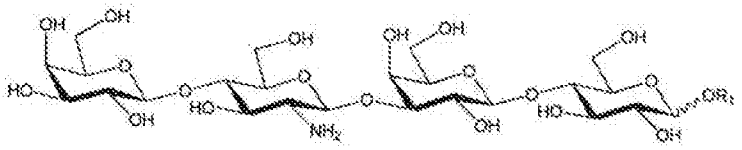
general formula 3

wherein R_1 and $-NR_5R_6$ are defined as above, which compound of general formula **3** is subjected to basic hydrolysis or aminolysis to give rise to a compound of general formula **2**

general formula 2

wherein R_1 is as defined above, which compound of general formula **2** is converted into the compound of general formula **1**, or

bc) basic hydrolysis of the compound of general formula **4**, wherein $-NR_5R_6$ is selected from -NH-haloacyl, 2,3-diphenylmaleimide and 2,3-dimethylmaleimide, to give a compound of general formula **2**



general formula 2

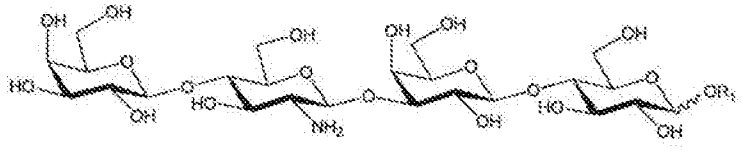
wherein R_1 is as defined above, which compound of general formula **2** is converted into the compound of general formula **1**, or

bd) basic hydrolysis of the compound of general formula **4**, wherein $-NR_5R_6$ is selected from phthalimide and tetrachlorophthalimide, followed by aminolysis to give a compound of general formula **2**

general formula **2**

wherein R_1 is defined as above, which compound of general formula **2** is converted into the compound of general formula **1**, or

be) aminolysis of the compound of general formula **4**, wherein $-NR_5R_6$ is selected from $-NAc_2$, -NH-haloacyl, phthalimide, tetrachlorophthalimide, 2,3-diphenylmaleimide and 2,3-dimethylmaleimide, to give a compound of general formula **2**



general formula **2**

wherein R_1 is defined as above, which compound of general formula **2** is converted into the compound of general formula **1**.

The term "base catalyzed transesterification deprotection" means a reaction, where the acyl protective groups from hydroxyls are removed in an alcohol solvent such as methanol, ethanol, propanol, *t*-butanol, etc. in the presence of an alcoholate like NaOMe, NaOEt, KO^tBu, etc. at 20-100 °C temperatures. The alcohol and the alcoholate should be matched. The use of co-solvent as toluene or xylene might be beneficial in order to control particle size

of the product and to avoid gel formations. Under this condition only *O*-acyls can be deprotected or when both R_5 and R_6 are acetyls, one of the acyl groups is also removed to give a compound having a –NHAc substituent. The -NH-haloacyl and cyclic imide protective groups remain intact under the condition of base catalyzed transesterification deprotection. In a preferred embodiment catalytic amount of NaOMe is used in methanol (Zemplén de-*O*-acylation).

The term "basic hydrolysis" generally means base catalyzed hydrolysis in water, alcohol or water-organic solvent mixtures, in homogeneous or heterogeneous reaction conditions at temperatures varying from 0–100 °C. The base of choice is generally a strong base, e.g. LiOH, NaOH, KOH, $Ba(OH)_2$, K_2CO_3, basic ion exchange resins, tetraalkylammonium hydroxides, etc. The bases can be used in the form of an aqueous solution as well. This condition affects *O*-acyls, *N*-haloacyls, 2,3-diphenylmaleimide and 2,3-dimethylmaleimide. If R_5 and R_6 are both acetyl, one of the acyl groups is also removed. In a preferred embodiment the base is NaOH and the solvent is methanol.

The term "aminolysis" or *N*-acyl transfer based deprotection means a treatment with ammonia, hydrazine, substituted hydrazine, ethylene diamine or primary amines in water, alcohol or water-organic solvent mixtures at 20-120 °C temperatures. Under this condition all of the *O*- and *N*-protecting acyl groups, including cyclic imides, can be readily removed.

According to another embodiment a compound of general formula **2** obtained is *N*-acetylated. Selective *N*-acetylation in the presence of one or more hydroxyls is a well-known reaction and performing such reaction takes part of the skilled person's general knowledge. It involves reaction of the amine with slight excess of acetic anhydride or acetyl chloride (≈1.5-3 equiv.) at about 0-35 °C with or without added base. The eventually formed overacetylated by-product(s) can be readily transformed into the desired compounds of general formula **1** with e.g. NaOH/MeOH or NaOMe/MeOH treatment. In another method, derivatives according to general formula **2** are peracetylated, that is the free amino group and all the free hydroxyl groups are acetylated. It belongs to the skilled person's competence to perform the reaction until all of the groups to be protected are acetylated. The compound is treated with acetic anhydride or acetyl chloride, preferably acetic anhydride, in the presence of a base, preferably pyridine, triethylamine or Hünig's base, to give a group of fully protected tetrasaccharides of general formula **4**, which is characterized by general formula **4a**

general formula **4a**

wherein R_1 is defined as above. The peracetylated derivative **4a** is then subjected to base catalyzed transesterification deprotection or basic hydrolysis (vide supra), preferably to Zemplén de-*O*-acetylation, to give rise to a compound of general formula **1**.

Once compounds of general formula **1** are prepared by taking whatever route specified above, they are isolated in crystalline form. The present inventors are realized that compounds of general formula **1** are crystalline materials. As compounds of general formula **1** are the final intermediates en route to LNnT and the last deprotective step runs practically without by-product formation, their purity is proportional to that of the target product LNnT.

The crystallization is carried out from a solvent system comprising one or more C_1-C_6 alcohols in the absence of seed crystals. Term "C_1-C_6 alcohol" refers to alcohols having 1 to 6 carbon atoms, that is methanol, ethanol, *n*-propanol, *i*-propanol, *t*-butanol, *i*-amylalcohol, etc. Preferably methanol or ethanol is chosen. More preferably the solvent system may further contain water. The water content in the overall volume of the solvent system may preferably range up to 30 v/v%, more preferably 15-25 v/v%.

In a preferred realization 1-*O*-benzyl LNnT is crystallized form aqueous methanol or ethanol.

In step d) of the first aspect of the present invention a compound of general formula **1** is subjected to catalytic reduction leading to LNnT.

Removal of the R_1-group typically takes place in a protic solvent or in a mixture of protic solvents. A protic solvent may be selected from a group consisting of water, acetic acid or C_1-C_6 alcohol. Mixture of one or more protic solvents with one or more proper aprotic organic solvents miscible partially or fully with the protic solvent(s) (such as THF, dioxane, ethyl acetate, acetone, etc.) may also be applied. Water, one or more C_1-C_6 alcohols or a mixture of water and one or more C_1-C_6 alcohols are preferably used as solvent system. Solutions containing the carbohydrate derivatives in any concentration or suspensions of the carbohydrate derivatives with the solvent(s) used are also applicable. The reaction mixture is

stirred at 10-100 °C temperature range, preferably between 20-70 °C in hydrogen atmosphere of 1-50 bar in the presence of a catalyst such as palladium, Raney nickel or any other appropriate metal catalyst, preferably palladium on charcoal or palladium black, until reaching the completion of the reaction. Catalyst metal concentrations generally range from 0.1 % to 10 % based on the weight of carbohydrate. Preferably, the catalyst concentrations range from 0.15 % to 5 %, more preferably 0.25 % to 2.25 %. Transfer hydrogenation may also be performed, when the hydrogen is generated *in situ* from cyclohexene, cyclohexadiene, formic acid or ammonium formate. Addition of organic or inorganic bases/acids and/or basic and/or acidic ion exchange resins can also be used to improve the kinetics of the hydrogenolysis. The use of basic substances is especially preferred when halogen substituents are present on the substituted benzyl moieties of the precursors. Preferred organic bases are including but not limited to triethylamine, diisopropyl ethylamine, ammonia, ammonium carbamate, diethylamine, etc. Preferred organic/inorganic acids are including but not limited to formic acid, acetic acid, propionic acid, chloroacetic acid, dichloroacetic acid, triflouroacetic acid, HCl, HBr, etc. The conditions proposed above allow simple, convenient and delicate removal of the solvent(s) giving rise to pure LNnT. LNnT can be isolated from the reaction mixture using conventional work-up procedures in crystalline, amorphous solid, syrupy form or concentrated aqueous solution.

In a preferred embodiment 1-*O*-benzyl LNnT is subjected to catalytic hydrogenolysis to give LNnT. The hydrogenation can be performed in water or in aqueous alcohol, preferably in water/methanol or water/ethanol mixture (alcohol content: 10-50 v/v %) at 15-65 °C, preferably between 60-65 °C. The concentration of the starting material can vary between 140-230 g/l and the catalyst concentration may range from 0.4 % to 1.2 % (weight of the metal content based on the weight of the carbohydrate).

Both solid forms of LNnT such as amorphous/freeze dried/spray dried and liquid forms of LNnT such as aqueous solutions/syrups provided by the present invention have high LNnT purity suitable for infant nutritional use including but not limited to infant formulas, infant cereals, clinical infant nutritional products etc. In general, both solid and liquid forms of LNnT produced by the methodologies of the present invention are suitable for general nutritional use for infants, toddlers, children, adults and elderly. Both solid and liquid forms of LNnT provided by the present invention can also be used as food additives, dietary

supplements, a component of alcoholic and non alcoholic beverages such as soft drinks, fruit juices, bottled water, wine, beer etc. Both solid and liquid forms of LNnT provided by the present invention can also be used as a therapeutic agent in broad therapeutic application areas including but not limited to prevent bacterial and viral infections, to avoid diarrhoea, to enhance immune system and brain development, etc. Both solid and liquid forms of LNnT provided by the present invention can also be used in veterinary applications including but not limited to fight against infectious diseases of domesticated animals. LNnT provided by the present invention can also be used as a crucial monomer for the preparation of polymeric/polymer mounted products providing multivalent binding for bacteria and viruses. LNnT provided by the present invention can also be used for the preparation of other human milk oligosaccharides by applying chemical and/or enzymatic methodologies including but not limited to simple structural modifications of further fucosylation, further sialylation, further extension of the core structure via *N*-acetyl lactosaminylation/*N*-acetylisolactosamylation, etc.

Another aspect of the present invention relates to a novel crystalline polymorph of LNnT. The novel crystalline LNnT comprises X-ray powder diffraction reflections, based on a measurement using CuKα radiation, at 20.32±0.20 2Θ angle, preferably at 20.32±0.20 and 19.10±0.20 2Θ angles, more preferably at 20.32±0.20, 19.10±0.20 and 7.98±0.20 2Θ angles, even more preferably at 20.32±0.20, 19.10±0.20, 7.98±0.20 and 21.03±0.20 2Θ angles, most preferably 20.32±0.20, 19.10±0.20, 7.98±0.20, 21.03±0.20 and 20.95±0.20 2Θ angles, in particular 20.32±0.20, 19.10±0.20, 7.98±0.20, 21.03±0.20, 20.95±0.20 and 5.66±0.20 2Θ angles. List of peaks of the XRPD pattern of crystalline LNnT prepared according to example 34 is reported in Table 1.

2Θ	rel. intensity	2Θ	rel. intensity
5.66	20	21.03	29
6.78	7	21.88	14
7.98	67	22.08	17
9.10	2	22.32	16
10.16	3	23.62	12
11.58	9	25.22	14
11.76	9	25.57	17
14.00	5	25.64	17
16.07	5	26.50	11
17.20	11	27.25	8
17.98	9	27.94	6
19.10	77	29.99	5
20.32	100	31.66	5
20.95	27	33.94	7

Table 1.

The novel crystalline form of LNnT can be considered as an anomeric mixture of α- and β-anomers or even pure form of one of the anomers. If LNnT is isolated as a polycrystalline material, it forms a mixture of α- and β-anomers, wherein the α-anomer is predominant over the β-anomer (ratio: approx. 5:2) according to solid-state ^{13}C-NMR measurements (see Figure 3).

Kuhn et al. [*Chem. Ber.* **1962**, *95*, 513] reported a melting point of 214-218 °C (dec.) for crystalline LNnT. The melting point of LNnT polymorph according to the present invention is 226-230 °C (dec). The remarkable difference between the melting points implies that it concerns different polymorphs.

In European application EP-A-1405856 crystals of LNnT were obtained from aqueous acetone whose powder X-ray diffraction data measured differ significantly from those of the crystals of the present application. Comparison of the data is shown in Figure 2.

Preferably the crystalline LNnT according to the present invention is substantially free from organic solvent. The expression "substantially free from organic solvent" intends to mean that the content of organic solvent(s) is at most 1000 ppm, preferably at most 800 ppm, more preferably at most 600 ppm, most preferably at most 400 ppm and in particular at most 200 ppm.

According to another preferred embodiment the crystalline LNnT claimed in the present application is substantially pure. The expression "substantially pure" intends to mean that the crystalline LNnT (the novel polymorph) contains less than 10 w/w% of impurity, preferably less than 5 w/w% of impurity, more preferably less than 1 w/w% of impurity, most preferably less than 0.5 w/w% of impurity, in particular less than 0.1 w/w% of impurity, wherein "impurity" refers to any physical entity different to the crystalline LNnT described in the present application, such as amorphous LNnT, different LNnT polymorph(s), unreacted intermediate(s) remained from the synthesis of LNnT, by-product(s), degradation product(s), inorganic salt(s) and/or other contaminations different to organic solvent(s).

The present invention also provides a process for preparing crystalline LNnT by crystallization from a solvent system comprising one or more C_1-C_6 alcohols. More preferably the solvent system may further contain water. The water content in the overall volume of the solvent system may preferably range up to 40 v/v %. The crystallization preferably can be carried out using 3-15 volumes of the solvent mixture.

In a typical crystallization a solution of LNnT in water/methanol or in water/ethanol, preferably in water/methanol (≈ 1:1) mixture, taken either from the reaction mixture of the hydrogenation after removing the catalyst or prepared freshly (concentration: 140-180 g/l), is warmed to 50-60 °C to which hot methanol (up to 115-250 % of the starting volume) is added in 2-4 portions under stirring and gradual chilling to 35-45 °C. The crystallization may be initiated by adding seeding crystals. The resulting warm suspension is then carefully cooled to 0-8 °C and the stirring is optionally continued for 2-5 hours. The crystalline material formed is separated by filtration.

In a further embodiment crystalline LNnT according to the present invention is suitable for pharmaceutical and nutritional use. LNnT alone or in combination with other *N*-acetyllactosamine and/or fucose and/or sialic acid containing human milk oligosaccharides is particularly effective in the education and/or maturation of the immune system of neonatal infants, and have preventive effect against secondary infections following viral infections such as influenza. The use of LNnT as prebiotic enhances the beneficial effects and efficiency of probiotics, such as *Lactobacillus* and *Bifidobacterium* species, in promoting the development of an early bifidogenic intestinal microbiota in infants, in reducing the risk of

development or allergy and/or asthma in infants, in preventing and treating pathogenic infections in such as diarrhoea in infants.

In another aspect, the present invention provides pharmaceutical composition comprising crystalline LNnT claimed as active ingredient and one or more pharmaceutically acceptable carriers including but not limited to additives, adjuvants, excipients and diluents (water, gelatine, talc, sugars, starch, gum arabic, vegetable gums, vegetable oils, polyalkylene glycols, flavouring agents, preservatives, stabilizers, emulsifying agents, lubricants, colorants, fillers, wetting agents, etc.). Suitable carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field. The dosage form for administration includes, for example, tablets, powders, granules, pills, suspensions, emulsions, infusions, capsules, syrups, injections, liquids, elixirs, extracts and tincture.

In a further embodiment crystalline LNnT according to the present invention is used for the preparation of pharmaceutical compositions. Pharmaceutical compositions can be manufacture by means of any usual manner known in the art, e.g. described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field.

In a further embodiment it is provided nutritional formulations comprising crystalline LNnT according to the present invention such as foods, drinks or feeds. The nutritional formulation may contain edible micronutrients, vitamins and minerals as well. The amounts of such ingredient may vary depending on whether the formulation is intended for use with normal, healthy infants, children, adults or subjects having specialized needs (e.g. suffering from metabolic disorders). Micronutrients include for example edible oils, fats or fatty acids (such as coconut oil, soy-bean oil, monoglycerides, diglycerides, palm olein, sunflower oil, fish oil, linoleic acid, linolenic acid etc.), carbohydrates (such as glucose, fructose, sucrose, maltodextrin, starch, hydrolized cornstarch, etc.) and proteins from casein, soy-bean, whey or skim milk, or hydrolysates of these proteins, but protein from other source (either intact or hydrolysed) may be used as well. Vitamins may be chosen from the group consisting of vitamin A, B1, B2, B5, B6, B12, C, D, E, H, K, folic acid, inositol and nicotinic acid. The nutritional formula may contain the following minerals and trace elements: Ca, P, K, Na, Cl, Mg, Mn, Fe, Cu, Zn, Se, Cr or I.

In a preferred embodiment the nutritional formulation is an infant formula. Infant formula means a foodstuff intended for particular nutritional use by infants during the first 4-6 months of life and satisfying by itself the nutritional requirements of infants. It may contain one or more probiotic *Bifidobacterium* species, prebiotics such as fructooligosaccharides and galactooligosaccharides, proteins from casein, soy-bean, whey or skim milk, carbohydrates such as lactose, saccharose, maltodextrin, starch or mixtures thereof, lipids (e.g. palm olein, sunflower oil, safflower oil) and vitamins and minerals essential in a daily diet. The infant formula contains crystalline LNnT according to the present invention in a total amount of 0.1-3.0 g/100 g formula.

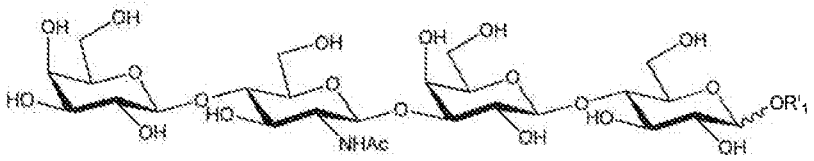
In another preferred embodiment the nutritional formulation may be a food supplement including crystalline LNnT according to the present invention. The food supplement may comprise one or more probiotics in an amount sufficient to achieve the desired effect in an individual, preferably in children and adults. The food supplement may also contain vitamins, minerals, trace elements and other micronutritients as well. The food supplement may be for example in the form of tablets, capsules, pastilles or a liquid. The supplement may contain conventional additives selected from but not limited to binders, coatings, emulsifiers, solubilising agents, encapsulating agents, film forming agents, adsorbents, carriers, fillers, dispersing agents, wetting agents, jellifying agents, gel forming agents, etc. The daily dose of LNnT ranges from 0.1 to 3.0 g.

According to a more preferred embodiment the food supplement is digestive health functional food as the administration of LNnT provides a beneficial effect on digestive health. Digestive health functional food is a processed food used with intention enhance and preserve digestive health by crystalline LNnT according to the present invention as physiologically functional ingredient or component in forms of tablet, capsule, powder, etc. Different terms such as dietary supplement, nutraceutical, designed food, health product may also be used to refer to functional food.

In a further embodiment crystalline LNnT according to the present invention is used for the preparation of nutritional formulation including foods, drinks and feeds, preferably infant formulas, food supplements and digestive health functional food. The nutritional formulation may be prepared in any usual manner.

Compounds of general formulae **1**, **2**, **3**, **4**, **5**, and **6** are believed to be valuable synthetic intermediates towards LNnT. The present inventors surprisingly recognized some of the compounds of general formulae **1**, **2**, **3**, **4**, **5**, and **6** can be obtained in crystalline form. Crystallization or recrystallization is one of the simplest and cheapest methods to isolate a product from a reaction mixture, separate it from contaminations and obtain pure substance. Isolation or purification that uses crystallization makes the whole technological process robust and cost-effective, thus it is advantageous and attractive compared to other procedures. The present invention has a great commercial value in large scale production of LNnT providing high purity of intermediates, which cannot be achieved by any other known purification methods. Although some other intermediates have not shown the ability to crystallize, they can be prepared in clean, high-yielding and less by-product forming reactions where usual work-up (extraction, evaporation, precipitation, etc.) procedures have been sufficient to obtain high purity products which have been used without further purification in the next step.

Thus it is provided valuable LNnT intermediates of general formula **1'**



general formula **1'**

wherein R'_1 is selected from substituted benzyl, optionally substituted benzhydryl, optionally substituted trityl and optionally substituted naphthylmethyl.

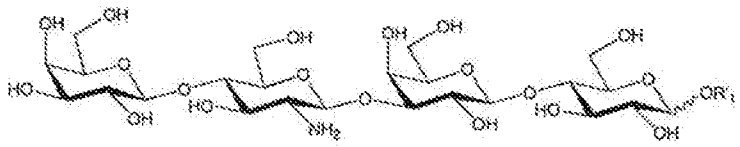
It is strongly emphasised that novel derivatives characterized by general formula **1'** can be considered as sole chemical entities such as either α or β anomers or even an anomeric mixture of α and β isomers, preferably as β-anomer. Novel LNnT intermediates of general formula **1'** can be characterized as crystalline solids, oils, syrups, precipitated amorphous material or spray dried products. If crystalline, compounds of general formula **1'** might exist either in anhydrous or in hydrated crystalline forms by incorporating one or several molecules of water into their crystal structures. Similarly, novel compounds characterized by

general formula **1'** might exist as crystalline substances incorporating ligands such as organic molecules and/or ions into their crystal structures.

In a preferred embodiment R_1 is substituted benzyl, preferably 4-chlorobenzyl or 4-methylbenzyl.

Novel compounds of general formula **1'** provided by the present invention can be used for the preparation of LNnT itself and other LNnT derivatives by using chemical/enzymatic methodologies known in the Art. Novel compounds of general formulas **1'** can also be used as advanced precursors/intermediates for the production/preparation of numerous human milk oligosaccharides. Novel compounds of general formulas **1'** can also be considered as valuable intermediates for the synthesis of complex oligosaccharides/glycoconjugates suitable for therapeutic/nutritional use.

It is provided valuable LNnT intermediates of general formula **2'**



general formula 2'

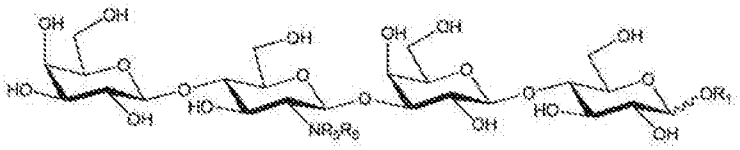
wherein R'_1 is selected from substituted benzyl, optionally substituted benzhydryl, optionally substituted trityl and optionally substituted naphthylmethyl.

It is strongly emphasised that novel derivatives characterized by general formula **2'** can be considered as sole chemical entities such as either α or β anomers or even an anomeric mixture of α and β isomers, preferably as β-anomer. Novel LNnT intermediates of general formula **2'** can be characterized as crystalline solids, oils, syrups, precipitated amorphous material or spray dried products. If crystalline, compounds of general formula **2'** might exist either in anhydrous or in hydrated crystalline forms by incorporating one or several molecules of water into their crystal structures. Similarly, novel compounds characterized by general formula **2'** might exist as crystalline substances incorporating ligands such as organic molecules and/or ions into their crystal structures.

In a preferred embodiment R_1 is substituted benzyl, preferably 4-chlorobenzyl or 4-methylbenzyl.

Novel compounds of general formula **2'** provided by the present invention can be used for the preparation of LNnT itself and other LNnT derivatives by using chemical/enzymatic methodologies known in the Art. Novel compounds of general formulas **2'** can also be used as advanced precursors/intermediates for the production/preparation of numerous human milk oligosaccharides. Novel compounds of general formulas **2'** can also be considered as valuable intermediates for the synthesis of complex oligosaccharides/glycoconjugates suitable for therapeutic/nutritional use.

Furthermore it is provided compounds of general formula **3**



general formula **3**

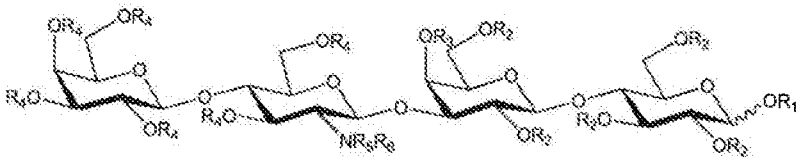
wherein R_1 is selected from optionally substituted benzyl, optionally substituted benzhydryl, optionally substituted trityl and optionally substituted naphthylmethyl, and $-NR_5R_6$ is selected from -NH-haloacyl, phthalimide, tetrachlorophthalimide, 2,3-diphenylmaleimide and 2,3-dimethylmaleimide.

It is strongly emphasised that novel derivatives characterized by general formula **3** can be considered as sole chemical entities such as either α or β anomers or even an anomeric mixture of α and β isomers, preferably as β-anomer. Novel LNnT intermediates of general formula **3** can be characterized as crystalline solids, oils, syrups, precipitated amorphous material or spray dried products. If crystalline, compounds of general formula **3** might exist either in anhydrous or in hydrated crystalline forms by incorporating one or several molecules of water into their crystal structures. Similarly, novel compounds characterized by general formula **3** might exist as crystalline substances incorporating ligands such as organic molecules and/or ions into their crystal structures.

In a preferred embodiment R_1 is selected from benzyl, 4-methylbenzyl and 4-chlorobenzyl, preferably benzyl and $-NR_5R_6$ is -NH-haloacyl, preferably –NH-trichloroacetyl.

Novel compounds of general formula **3** provided by the present invention can be used for the preparation of LNnT itself and other LNnT derivatives by using chemical/enzymatic methodologies known in the Art. Novel compounds of general formulas **3** can also be used as advanced precursors/intermediates for the production/preparation of numerous human milk oligosaccharides. Novel compounds of general formulas **3** can also be considered as valuable intermediates for the synthesis of complex oligosaccharides/glycoconjugates suitable for therapeutic/nutritional use.

Moreover it is provided compounds of general formula **4'**



general formula **4'**

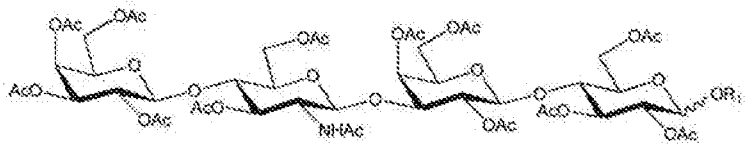
wherein R_1 is selected from optionally substituted benzyl, optionally substituted benzhydryl, optionally substituted trityl and optionally substituted naphthylmethyl, and each of R_2 and R_4 are independently optionally substituted acyl, R_3 is selected from optionally substituted acyl and H, $-NR_5R_6$ is selected from $-NAc_2$, -NH-haloacyl, tetrachlorophthalimide, 2,3-diphenylmaleimide and 2,3-dimethylmaleimide.

It is strongly emphasised that novel derivatives characterized by general formula **4'** can be considered as sole chemical entities such as either α or β anomers or even an anomeric mixture of α and β isomers, preferably as β-anomer. Novel LNnT intermediates of general formula **4'** can be characterized as crystalline solids, oils, syrups, precipitated amorphous material or spray dried products. If crystalline, compounds of general formula **4'** might exist either in anhydrous or in hydrated crystalline forms by incorporating one or several molecules of water into their crystal structures. Similarly, novel compounds characterized by general formula **4'** might exist as crystalline substances incorporating ligands such as organic molecules and/or ions into their crystal structures.

In a preferred embodiment R_1 is selected from benzyl, 4-methylbenzyl and 4-chlorobenzyl, preferably benzyl, R_2 is benzoyl optionally substituted by chloro, R_3 is optionally substituted benzoyl, preferably benzoyl, R_4 is acetyl and $-NR_5R_6$ is –NH-haloacyl, preferably –NH-trichloroacetyl.

Novel compounds of general formula **4'** provided by the present invention can be used for the preparation of LNnT itself and other LNnT derivatives by using chemical/enzymatic methodologies known in the Art. Novel compounds of general formulas **4'** can also be used as advanced precursors/intermediates for the production/preparation of numerous human milk oligosaccharides. Novel compounds of general formulas **4'** can also be considered as valuable intermediates for the synthesis of complex oligosaccharides/glycoconjugates suitable for therapeutic/nutritional use.

Moreover it is provided compounds of general formula **4a**



general formula **4a**

wherein R_1 is selected from optionally substituted benzyl, optionally substituted benzhydryl, optionally substituted trityl and optionally substituted naphthylmethyl.

It is strongly emphasised that novel derivatives characterized by general formula **4a** can be considered as sole chemical entities such as either α or β anomers or even an anomeric mixture of α and β isomers, preferably as β-anomer. Novel LNnT intermediates of general formula **4a** can be characterized as crystalline solids, oils, syrups, precipitated amorphous material or spray dried products. If crystalline, compounds of general formula **4a** might exist either in anhydrous or in hydrated crystalline forms by incorporating one or several molecules of water into their crystal structures. Similarly, novel compounds characterized by general formula **4a** might exist as crystalline substances incorporating ligands such as organic molecules and/or ions into their crystal structures.

In a preferred embodiment R_1 is selected from benzyl, 4-methylbenzyl and 4-chlorobenzyl, preferably benzyl.

Novel compounds of general formula **4a** provided by the present invention can be used for the preparation of LNnT itself, especially when selective *N*-acetylation of the compounds of general formula **2** is not efficient, and other LNnT derivatives by using chemical/enzymatic methodologies known in the Art. Novel compounds of general formulas **4a** can also be used as advanced precursors/intermediates for the production/preparation of numerous human milk oligosaccharides. Novel compounds of general formulas **4a** can also be considered as valuable intermediates for the synthesis of complex oligosaccharides/glycoconjugates suitable for therapeutic/nutritional use.

Another aspect of the invention relates to the novel crystalline lactosaminyl donor characterized by the general formula **5'**

general formula **5'**

wherein R_4 is optionally substituted acyl, $-NR_5R_6$ is –NH-haloacyl, and R_7 is optionally substituted phenyl.

It is strongly emphasised that novel derivatives characterized by general formula **5'** can be considered as sole chemical entities such as either α or β anomers or even an anomeric mixture of α and β isomers, preferably as β-anomer. Novel LNnT intermediates of general formula **5'** can be characterized as crystalline solids, oils, syrups, precipitated amorphous material or spray dried products. If crystalline, compounds of general formula **5'** might exist either in anhydrous or in hydrated crystalline forms by incorporating one or several molecules of water into their crystal structures. Similarly, novel compounds characterized by general formula **5'** might exist as crystalline substances incorporating ligands such as organic molecules and/or ions into their crystal structures.

In preferred embodiments R_4 is acetyl, $-NR_5R_6$ is –NH-trichloroacetyl or –NH-trifluoroacetyl, R_7 is phenyl and $–SR_7$ in β.

Compounds of general formula **5'** are stable, can be stored for longer period of time without significant decomposition, can be easily activated in glycosylation reactions and shows excellent β-selectivity. As other β-selective lactosaminyl donors are known not to be solid and/or stable, compounds of general formula **5'** according to the present application has obviously advantageous applicability in lactosaminylation reactions and thus represent a valuable donor tool in syntheses where lactosamine containing oligosaccharides are targeted, especially in large or industrial scale.

Compounds of general formula **5'** can be prepared as follows: lactosamine hydrochloride is acetylated in Ac_2O/HBr/AcOH to give 1,3,6,2',3',4'6'-hepta-*O*-acetyl-lactosamine hydrochloride which is *N*-acylated with haloacyl halogenide or anhydride to the –NH-haloacyl derivative. Bromosugar formation with HBr/AcOH followed by thiolysis with R_7SH thiophenol derivative readily gives compounds of general formula **5'**. The acetyl groups can be changed to other appropriate acyl groups by base catalyzed transesterification reaction followed by subsequent acylation.

The present invention thus provides the valuable compounds of general formula **6'**

general formula 6'

wherein R_1 is selected from optionally substituted benzyl, optionally substituted benzhydryl, optionally substituted trityl and optionally substituted naphthylmethyl, R_2 is optionally substituted benzoyl, and R_3 is selected from optionally substituted benzoyl and H.

The preferred embodiments encompass compounds of general formula **6'**, wherein R_1 is optionally substituted benzyl, preferably benzyl, 4-methylbenzyl or 4-chlorobenzyl, R_2 is optionally substituted benzoyl, preferably benzoyl or 4-chlorobenzoyl, and R_3 means benzoyl or H.

The present inventors realized that acetyl group as R_3 are inconvenient protective group when compounds of general formula **6** act as glycosyl acceptor in glycosidation reactions. Under the conditions of coupling acetyl migration always occurred to give complex mixture containing substances with similar physical characteristics which compounds can be separated only by lengthy and/or sophisticated and/or laborious techniques, e.g. chromatography. Choosing bulkier acyl protective group that don't tend or tend less to migrate results in an acceptor whose coupling product is formed almost exclusively in glycosidation, making the work-up procedure and isolation process of the desired compounds simpler, quick, powerful and cost-effective, e.g. by crystallization, which is one of the paramount concerns in large scale preparation or industrial process.

Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not to be limiting thereof.

EXAMPLES

Example 1

Suspension of 10 g of benzyl β-D-lactoside in acetone (50 ml), dimethoxypropane (3.5 ml) and TMSCl (7 ml) was stirred at rt for 5 h. The mixture was diluted with ethyl acetate (50 ml), filtered, and the cake was washed with ethyl acetate (2 x 30 ml). The wet cake was dissolved in pyridine (36 ml) and dry DCM (50 ml) and 4-chlorobenzoyl chloride (22 ml) was added slowly to maintain the temperature between 40-45 °C. After overnight stirring methanol (10 ml) and DCM (10 ml) were added and extractive work-up was made (2 x 1M HCl, 1 x water, 1 x sat. $NaHCO_3$). The combined organic phase was concentrated and as thick syrup it was poured to 50 ml of isopropanol under intensive stirring. The solid was filtered, washed with isopropanol and dried to result in 20.0 g of benzyl 2,3,6,2',6'-penta-*O*-(4-chlorobenzoyl)-3',4'-di-*O*-isopropylidene-β-D-lactoside (72 %). $[\alpha]_D$ = +58.4° (c = 1 DCM), Mp: 184 °C.

Example 2

10 g of benzyl 2,3,6,2',6'-penta-*O*-(4-chlorobenzoyl)-3',4'-di-*O*-isopropylidene-β-D-lactoside was dissolved in DCM (20 ml), acetonitrile (2 ml) and 50 % $HClO_4$ (1 ml) and the mixture was stirred at rt for 30 min. The solution was extracted with sat. $NaHCO_3$ (2 x 10 ml), dried, filtered and concentrated. The obtained material was redissolved in ethyl acetate (10 ml) and diluted with hexane (50 ml). The suspension was stirred at rt for 30 min, the filtered to yield 5.4 g of benzyl 2,3,6,2',6'-penta-*O*-(4-chlorobenzoyl)-β-D-lactoside as white crystals. $[\alpha]_D$ = +58.65° (c = 1 DCM), Mp: 200-201 °C.

Example 3

Analogously prepared with propionyl chloride according to examples 1 and 2: benzyl 2,3,6,2',6'-penta-*O*-propionyl-β-D-lactoside.

^{1}H NMR ($CDCl_3$, 300 MHz) δ: 7.346-7.227 (5H, Ph), 5.140 (1H, dd, J = 9.1, 9.7, H-3), 4.980 (1H, dd, J = 7.9, 9.7, H-2), 4.881 (1H, dd, J = 7.9 9.7, H-2'), 4.838 (1H, d, J = 12.3, ½C$\underline{H}_2$Ph), 4.570 (1H, d, J = 12.3, ½C$\underline{H}_2$Ph), 4.495 (1H, dd, J = 1.8, 11.7, H-6a or H-6a'), 4.343-4.282 (2H, unresolved, H-6a or H-6a', H-1), 4.217-4.155 (2H, unresolved, H-6b, H-6b'), 3.821 (1H, d, J = 3.4, H-4'), 3.743 (1H, dd, J = 9.8, 9.8, H-4), 3.606-3.537 (3H, unresolved, H-3', H-5, H-5'), 2.438-2.194 (10H, m, 5×C$\underline{H}_2$CH$_3$), 1.263-1.036 (15H, m, 5×CH$_2$C$\underline{H}_3$).

^{13}C NMR ($CDCl_3$, 75.4 MHz) δ: 174.7, 174.3, 173.9, 173.8, 172.9, 171.2 (6×CO), 136.6-127.9 (Ph), 100.6 (C-1'), 99.1 (C-1), 75.9 (C-4), 73.3, 72.9, 72.7, 72.3, 72.2 (C-2', C-3, C-3', C-5, C-5'), 71.2 (C-2), 70.6 (C$\underline{H}_2$Ph), 68.4 (C-4'), 62.2, 62.0 (C-6, C-6').

Example 4

Analogously prepared with benzoyl chloride according to examples 1 and 2: benzyl 2,3,6,2',6'-penta-*O*-benzoyl-β-D-lactoside.

^{1}H NMR. ($CDCl_3$) δ: 8.20-7.10 (m, 30 H, aromatic), 5.55 (dd, 1 H, $J_{2,3}$ 9.82 Hz, $J_{3,4}$ 9.82 Hz, H-3), 5.49 (dd, 1 H, $J_{1,2}$ 9.78 Hz, H-2), 5.35 (dd, 1 H, $J_{1',2'}$ 7.85 Hz, $J_{2',3'}$ 9.64 Hz, H-2'), 4.81

and 4.57 (ABq, 2 H, J_{gem} 12.59 Hz, -C<u>H</u>$_2$Ph), 4.62 (d, 1 H, H-1), 4.57 (d, 1 H, H-1′), 4.52 (m, 2 H, H-6), 4.12 (m, 1 H, H-4), 3.80 (m, 1 H, H-4′), 3.75 (m, 1 H, H-5), 3.70 (m, 1 H, H-3′).

^{13}C NMR ($CDCl_3$) δ: 166.51, 166.31, 166.18, 166.16 and 165.45 (5 × CO), 101.23 (C-1′), 99.09 (C-1), 76.49 (C-4), 73.81 (C-2′), 73.26, 73.22, 73.19 and 72.87 (C-3, C-5, C-3′ and C-5′), 72.80 (C-2), 71.77 (-<u>C</u>H$_2$Ph), 68.81 (C-4′), 62.85 and 61.99 (C-6 and C-6′).

Example 5

Analogously prepared from 4-methylbenzyl β-D-lactoside according to examples 1 and 2: 4-methylbenzyl 2,3,6,2',6'-penta-*O*-(4-chlorobenzoyl)-β-D-lactoside.

^{1}H NMR ($CDCl_3$): 7.8, 7.4, 7.2, 7.0 (m), 5.4 (m, 3H), 4.76 (d, 1H), 4.5 (m, 5H), 4.1 (dd, 1H), 4.0 (m, 2H), 3.84 (m, 1H), 3.72 (m, 2H), 3.6 (m, 2H), 3.48 (m, 1H), 3.3 (d, 1H).

^{13}C NMR ($CDCl_3$): 165.3, 165.2, 165.1, 164.3, 139.9, 139.8, 137.9, 133.2, 131.3, 131.1, 130.9, 130.9, 129.1, 129.0, 128.9, 128.8, 128.7, 128.1, 127.9, 127.8, 127.7, 127.5, 127.4, 101.1, 98.3, 76.4, 73.6, 73.4, 72.9, 72.6, 72.3, 71.7, 70.4, 68.6, 62.9, 62.0, 60.4, 21.1, 14.2.

Example 6

To a mixture of 116.6 g of compound according to example 2 in toluene (600 ml) trimethyl ortobenzoate (120 ml) and camphenesulphonic acid (4 g) were added. The mixture was stirred vigorously at rt for 3 h, then 80 % acetic acid (160 ml) was added. After further 1 h of stirring the biphasic mixture obtained was separated, the organic phase was diluted with toluene (600 ml), washed with water (800 ml) and sat. $NaHCO_3$ (2 x 600 ml), dried, filtered and evaporated. The resulting oil was dropped into 600 ml of heptane and seeded. The white crystalline compound was filtered, washed and dried to yield 110.3 g of acceptor (a compound of general formula **6**, wherein R_1= Bn, R_2= 4-chlorobenzoyl, R_3= benzoyl, OR_1 in β). $[\alpha]_D$ = +17.13° (c = 1 DCM), Mp 156-157 °C.

Example 7

Analogously prepared from compound of example 4: a compound of general formula **6**, wherein R_1= Bn, R_2= R_3= benzoyl, OR_1 in β.

^{1}H NMR. ($CDCl_3$) δ: 8.20-7.00 (m, 35 H, aromatic), 5.67 (dd, 1 H, $J_{2,3}$ 9.77 Hz, $J_{3,4}$ 9.11 Hz, H-3), 5.55 (dd, 1 H, $J_{1,2}$ 7.81 Hz, H-2), 5.47 (dd, 1 H, $J_{3',4'}$ 3.45 Hz, $J_{4',5'}$ <1 Hz, H-4′), 5.30 (dd, 1 H, $J_{1',2'}$ 7.82 Hz, $J_{2',3'}$ 9.97 Hz, H-2′), 4.85 and 4.60 (ABq, 2 H, J_{gem} 12.59 Hz, -C<u>H</u>$_2$Ph), 4.72 (d, 1 H, H-1), 4.70 (d, 1 H, H-1′), 4.60 (m, 2 H, H-6), 4.21 (m, 1 H, H-4), 3.93 (m, 1 H, H-3′), 3.82 (m, 1 H, H-5), 3.75-3.45 (m, 3 H, H-5′ and H-6′) 2.70 (d, 1 H, $J_{3',OH}$ 6.68 Hz, 3′-OH).

^{13}C NMR ($CDCl_3$) δ: 166.67, 166.26, 166.03, 165.90, 165.68 and 165.41 (6 × CO), 100.79 (C-1′), 99.35 (C-1), 76.16 (C-4), 73.89 (C-2′), 73.26 (C-5), 72.96 (C-3), 72.11 (C-3′), 71.79 (C-2), 71.76 (C-5′), 70.73 (-<u>C</u>H$_2$Ph), 70.22 (C-4′), 62.87 and 61.68 (C-6 and C-6′).

Example 8

Analogously prepared from compound of example 5: a compound of general formula **6**, wherein R_1= 4-methylbenzyl, R_2= 4-chlorobenzoyl, R_3= benzoyl, OR_1 in β.

^{1}H NMR ($CDCl_3$): 7.92-7.0 (m, aromatic H), 5.6 (dd, 1H), 5.5 (d, 1H), 5.41 (dd, 1H), 5.3 (dd, 1H), 4.8 (d, 1H), 4.68 (m, 2H), 4.52 (m, 2H), 4.08 (m), 4.0 (m, 2H), 3.78 (m, 3H), 3.6 (m, 1H).

^{13}C NMR ($CDCl_3$): 165.7, 165.2, 165.1, 164.7, 164.5, 164.3 (Bz and ClBz carbonyls), 140.1, 140.02, 139.9, 139.7, 139.6, 137.8, 133.7, 133.1, 131.2-127.2 aromatic carbons, 100.6, 98.6 (anomeric carbons), 75.9, 73.6, 73.1, 72.9, 71.7, 71.4, 71.3, 70.5, 69.9, 62.9, 61.7, 45.9, 42.7, 42.4, 14.1.

Example 9

Analogously prepared from 4-chlorobenzyl β-D-lactoside according to examples 1, 2 and 6: a compound of general formula **6**, wherein R_1= 4-chlorobenzyl, R_2= 4-chlorobenzoyl, R_3= benzoyl OR_1 in β.

^{1}H NMR ($CDCl_3$): 7.89-7.0 (m, aromatic H), 5.63 (dd, 1H), 5.5 (d, 1H), 5.42 (dd, 1H), 5.28 (dd, 1H), 4.78 (d, 1H), 4.68 (dd, 1H), 4.54 (m, 3H), 4.1 (m, 2H), 3.98 (m, 1H), 3.8 (m, 2 H), 3.6 (m, 2H).

^{13}C NMR ($CDCl_3$): 165.8, 165.2, 164.8, 164.5, 164. 3, 140.2, 140.0, 139.8, 134.9, 133.9, 133.8. 131.2, 131.1, 130.9, 130.9, 130.8, 129.9, 129.0, 128.9, 128.8, 128.7, 128.6, 128.5, 127.7, 127.4, 127.3, 100.6, 99.2, 75.8, 73.6, 73.0, 71.8, 71.5, 71.4, 70.0, 69.9, 62.8, 61.6, 57.9, 56.1, 48.1, 46.0, 42.7, 42.5.

Example 10

10 g of lactosamine hydrochloride was dissolved Ac_2O (50 ml) and 10 ml of 30 % HBr/AcOH was added at 0 °C. The reaction mixture was allowed to warm to rt, stirred for 10 h and poured into 200 ml of tert-butyl methyl ether (MTBE). The precipitation was filtered, washed with MTBE and dried to give 12.4 g of 1,3,6,2',3',4'6'-hepta-*O*-acetyl-lactosamine hydrochloride as light brown powder. ^{1}H NMR ($CDCl_3$) δ: 5.57 and 5.22 (2 m, 1 H, H-1 α and β), 5.57, 5.29, 4.98 and 4.89 (4 m, each 1 H, H-3, H-2′, H-3′ and H-4′), 4.41 (m, 1 H, H-1′), 4.37 and 4.06 (2 m, 1 H and 3 H, H-6 and H-6′), 3.83, 3.82 and 3.73 (3 m, each 1 H, H-4, H-5 and H-5′), 3.40 (m, 1 H, H-2), 2.10, 2.10, 2.06, 2.04, 1.98, 1.90 and 1.89 (7 s, each 3 H, 7 × -OAc). ^{13}C NMR ($CDCl_3$) δ: 100.87 (C-1′), 92.91 and 88.93 (C-1 α and β), 75.64, 73.03 and 71.01 (C-4, C-5 and C-5′), 61.72 and 60.72 (C-6 and C-6′), 54.08 (C-2).

Example 11

16.4 g of 1,3,6,2',3',4'6'-hepta-*O*-acetyl-lactosamine hydrochloride was dissolved in DCM (30 ml) and cooled to 0 °C. First 2.95 ml of trichloroacetyl chloride then slowly 7.6 ml of triethyl amine were added and the stirring was continued for 30 min at 0 °C. The reaction mixture was diluted with DCM (10 ml), washed with water (2 x 20 ml) and brine (20 ml), dried on Na_2SO_4 and filtered. A mixture of this solution with Ac_2O (100 µl) was dropped to a

solution of DCM (15 ml), HBr/AcOH (15 ml) and Ac_2O (1 ml) at 0 °C and was kept in the same temperature for 30 min. The reaction mixture was diluted with DCM (10 ml), washed with cold water (3 x 20 ml) and cold sat. $NaHCO_3$ (20 ml). To the organic phase 100 ml of half sat. Na_2CO_3 solution, 4.3 ml of thiophenol and 100 mg of tetrabutyl ammonium hydrogensulphate were added and the biphasic mixture was stirred at rt for 30 min. After separation the organic phase was washed with brine (2 x 20 ml), and after addition of ethyl acetate (50 ml) the crystal formed was filtered (6.3 g). From the concentrated mother liquor further 3.2 g of crystal was precipitated by adding MTBE (50 ml). Combined yield: 9.5 g of product (a compound of general formula **5**, wherein R_4= acetyl, -NR_5R_6= -NH-trichloroacetyl, X= β-SPh). ^{1}H NMR. ($CDCl_3$) δ: 7.50 and 7.30 (2 m, 5 H, aromatic), 7.08 (d, 1 H, $J_{NH,2}$ 9.37 Hz, NH), 5.33 (dd, 1 H, $J_{3',4'}$ 3.19 Hz, $J_{4',5'}$ 0.65 Hz, H-4′), 5.21 (dd, 1 H, $J_{2,3}$ 8.34 Hz, $J_{3,4}$ 10.12 Hz, H-3), 5.08 (dd, 1 H, $J_{1',2'}$ 7.83 Hz, $J_{2',3'}$ 10.47 Hz, H-2′), 4.94 (dd, 1 H, H-3′), 4.75 (d, 1 H, $J_{1,2}$ 10.34 Hz, H-1), 4.55 and 4.09 (2 m, 4 H, H-6 and H-6′), 4.47 (d, 1 H, H-1′), 4.00 (m, 1 H, H-2), 3.87, 3.78 and 3.67 (3 m, each 1 H, H-4, H-5 and H-5′), 2.16, 2.11, 2.10, 2.04, 2.03 and 1.96 (6 s, each 3 H, 6 × -OAc). ^{13}C NMR ($CDCl_3$) δ: 100.47 (C-1′), 85.1 (C-1), 75.99, 75.41 and 69.97 (C-4, C-5 and C-5′), 72.50 (C-3), 70.17 (C-3′), 68.42 (C-2′), 65.87 (C-4′), 61.43 and 60.26 (C-6 and C-6′), 53.66 (C-2), 21.35, 21.11, 21.11, 20.98, 20.87 and 20.75 (6 × OAc). Mp.: 247-249 °C, $[\alpha]_D$ = -13,9° (c = 0,58 $CHCl_3$).

Example 12

Similarly prepared according to example 11: compound of general formula **5**, wherein R_4= acetyl, -NR_5R_6= -NH-dichloroacetyl, X= β-SPh.

^{1}H NMR ($CDCl_3$) δ: 7.50 and 7.30 (2 m, 5 H, aromatic), 7.08 (d, 1 H, $J_{NH,2}$ 9.37 Hz, NH), 5.33 (dd, 1 H, $J_{3',4'}$ 3.19 Hz, $J_{4',5'}$ 0.65 Hz, H-4′), 5.21 (dd, 1 H, $J_{2,3}$ 8.34 Hz, $J_{3,4}$ 10.12 Hz, H-3), 5.08 (dd, 1 H, $J_{1',2'}$ 7.83 Hz, $J_{2',3'}$ 10.47 Hz, H-2′), 4.94 (dd, 1 H, H-3′), 4.75 (d, 1 H, $J_{1,2}$ 10.34 Hz, H-1), 4.55 and 4.09 (2 m, 4 H, H-6 and H-6′), 4.47 (d, 1 H, H-1′), 4.00 (m, 1 H, H-2), 3.87, 3.78 and 3.67 (3 m, each 1 H, H-4, H-5 and H-5′), 2.16, 2.11, 2.10, 2.04, 2.03 and 1.96 (6 s, each 3 H, 6 × -OAc).

^{13}C NMR ($CDCl_3$) δ: 100.47 (C-1′), 85.1 (C-1), 75.99, 75.41 and 69.97 (C-4, C-5 and C-5′), 72.50 (C-3), 70.17 (C-3′), 68.42 (C-2′), 65.87 (C-4′), 61.43 and 60.26 (C-6 and C-6′), 53.66 (C-2), 21.35, 21.11, 21.11, 20.98, 20.87 and 20.75 (6 × OAc).

Example 13

Similarly prepared according to example 11: compound of general formula **5**, wherein R_4= acetyl, -NR_5R_6= -NH-trifluoroacetyl, X= β-SPh.

^{1}H NMR ($CDCl_3$) δ: 7.79 (d, 1 H, $J_{NH,2}$ 9.74 Hz, NH), 7.45 and 7.29 (2 m, 5 H, aromatic), 5.35 (dd, 1 H, $J_{3',4'}$ 3.10 Hz, $J_{4',5'}$ <1 Hz, H-4′), 5.24 (m, 1 H, H-3), 5.05 (dd, 1 H, $J_{1',2'}$ 7.59 Hz, $J_{2',3'}$ 10.44 Hz, H-2′), 4.96 (dd, 1 H, H-3′), 4.82 (d, 1 H, $J_{1,2}$ 10.35 Hz, H-1), 4.46 (d, 1 H, H-1′), 4.65 and 4.10-3.88 (m, 7 H, H-2, H-5, H-6, H-5′ and H-6′), 3.76 (m, 1 H, H-4), 2.11, 2.06, 2.05, 2.04, 2.03 and 1.99 (6 s, each 3 H, 6 × -OAc).

^{13}C NMR ($CDCl_3$) δ: 171.67, 170.63, 170.56, 170.41, 170.41, and 169.48 (6 × OAc), 101.64 (C-1′), 84.44 (C-1), 76.78 (C-4), 76.25 (C-5), 71.41 (C-3), 71.09 and 70.84 (C-3′ and C-5′), 69.22 (C-2′), 66.70 (C-4′), 62.06 and 60.87 (C-6 and C-6′), 52.72 (C-2).

Example 14

Similarly prepared according to example 11: compound of general formula **5**, wherein R_4= 4-chlorobenzoyl, -NR_5R_6= -NH-dichloroacetyl, X= β-SPh.

^{1}H NMR ($CDCl_3$) δ: 7.92-7.05 (m, 29 H, aromatic), 6.65 (d, 1 H, $J_{NH,2}$ 9.45 Hz, NH), 5.74 (s, 1 H, -$CHCl_2$), 5.65 (dd, 1 H, $J_{3',4'}$ 3.39 Hz, $J_{4',5'}$ <1 Hz, H-4′), 5.55 (dd, 1 H, $J_{1',2'}$ 7.89 Hz, $J_{2',3'}$ 10.34 Hz, H-2′), 5.53 (dd, 1 H, $J_{2,3}$ 9.01 Hz, $J_{3,4}$ 10.24 Hz, H-3), 5.32 (dd, 1 H, H-3′), 4.80 (d, 1 H, $J_{1,2}$ 10.31 Hz, H-1), 4.78 (d, 1 H, H-1′), 4.52 and 4.35 (m, 2 H, H-6), 4.01 (m, 1 H, H-2), 3.91 (m, 1 H, H-4), 3.90 (m, 3 H, H-5′ and H-6′), 3.67 (m, 1 H, H-5).

^{13}C NMR ($CDCl_3$) δ: 165.42, 165.16, 164.89, 164.74, 164.37, 164.37 and 164.25 (7 × CO), 100.87 (C-1′), 86.27 (C-1), 76.98 (C-5), 75.99 (C-4), 73.99 (C-3), 71.67 (C-3′), 71.01 (C-5′), 70.12 (C-2′), 67.69 (C-4′), 66.36 (-$CHCl_2$), 62.71 and 61.05 (C-6 and C-6′), 53.42 (C-2).

Example 15

Similarly prepared according to example 11: compound of general formula **5**, wherein R_4= benzoyl, -NR_5R_6= -NH-dichloroacetyl, X= β-SPh.

^{1}H NMR ($CDCl_3$) δ: 8.18-7.08 (m, 35 H, aromatic), 6.84 (d, 1 H, $J_{NH,2}$ 9.50 Hz, NH), 5.82 (s, 1 H, -$CHCl_2$), 5.77 (dd, 1 H, $J_{3',4'}$ 3.40 Hz, $J_{4',5'}$ <1 Hz, H-4′), 5.69 (dd, 1 H, $J_{1',2'}$ 7.88 Hz, $J_{2',3'}$ 10.36 Hz, H-2′), 5.53 (dd, 1 H, $J_{2,3}$ 9.05 Hz, $J_{3,4}$ 10.27 Hz, H-3), 5.42 (dd, 1 H, H-3′), 4.91 (d, 1 H, $J_{1,2}$ 10.21 Hz, H-1), 4.91 (d, 1 H, H-1′), 4.65 and 4.44 (m, 2 H, H-6), 4.14 (m, 1 H, H-2), 4.05 (m, 1 H, H-4), 3.83 (m, 3 H, H-5′ and H-6′), 3.73 (m, 1 H, H-5).

^{13}C NMR ($CDCl_3$) δ: 166.51, 165.97, 165.84, 165.67, 165.45, 165.05 and 164.39 (7 × CO), 101.30 (C-1′), 86.63 (C-1), 76.86 (C-5), 75.85 (C-4), 73.91 (C-3), 71.90 (C-3′), 71.65 (C-5′), 70.11 (C-2′), 67.64 (C-4′), 66.25 (-$CHCl_2$), 62.79 and 61.28 (C-6 and C-6′), 53.72 (C-2).

Example 16

3.0 g of methyl thiolactosaminide is dissolved in 10 ml of DMF and to this solution 100 µl of triethyl amine and 6.0 ml of methyl trichloroacetate were added. The reaction mixture was stirred at rt for overnight, then concentrated to dryness, the concentrate was dissolved in methanol (10 ml), chilled and NaOMe was added until pH reached 9. This mixture was stirred for overnight, evaporated, then pyridine (20 ml) and Ac_2O (10 ml) were added. After 6 h the mixture was concentrated and chromatographed with hexane-acetone 6:4 to yield off-white foam (a compound of general formula **5**, wherein R_4= acetyl, -NR_5R_6= -NH-trichloroacetyl, X= SMe). For characterization of the anomers an analytical sample was chromatographed. α-anomer: ^{1}H NMR ($CDCl_3$) δ: 7.05 (d, 1 H, $J_{NH,2}$ 7.05 Hz, NH), 5.30 (m, 2 H, $J_{1,2}$ 5.38 Hz, H-1 and H-4′), 5.13 (dd, 1 H, $J_{2,3}$ 10.99 Hz, $J_{3,4}$ 8.81 Hz, H-3), 5.07 (dd, 1 H, $J_{1',2'}$ 7.83 Hz, $J_{2',3'}$ 10.42 Hz, H-2′), 4.92 (dd, 1 H, $J_{3',4'}$ 3.37 Hz, H-3′), 4.49 (d, 1 H, H-1′), 4.38-4.13 (m, 2 H, H-6′), 4.26 (m, 1 H, H-2), 4.21 (m, 1 H, H-5), 4.03 (m, 2 H, H-6), 3.85 (m, 1 H, H-5′), 3.76 (m, 1 H, H-4), 2.09, 2.09, 2.07, 2.02, 2.02, 2.01 and 1.99 (7 × s, each 3 H, 6 × OAc and SMe). ^{13}C NMR ($CDCl_3$) δ: 171.14, 170.28, 170.28, 170.01, 169.91, 169.03 and 161.75 (7 × CO), 100.96 (C-1′), 91.67 (TCA), 83.98 (C-1), 75.89 (C-4), 70.76 (C-3′), 70.75 (C-3), 70.43 (C-5′), 68.98 (C-5), 68.91 (C-2′) 66.46 (C-4′), 61.79 and 60.69

(C-6 and C-6′), 54.31 (C-2), 20.69, 20.59, 20.55, 20.47, 20.47 and 20.34 (6 × OAc), 13.51 (SMe). β-anomer: ^{1}H NMR. ($CDCl_3$) δ: 7.48 (d, 1 H, $J_{NH,2}$ 9.68 Hz, NH), 5.33 (m, 1 H, H-4′), 5.28 (dd, 1 H, $J_{2,3}$ 10.19 Hz, $J_{3,4}$ 9.12 Hz, H-3), 5.02 (dd, 1 H, $J_{1',2'}$ 7.64 Hz, $J_{2',3'}$ 10.40 Hz, H-2′), 4.92 (dd, 1 H, $J_{3',4'}$ 3.27 Hz, H-3′), 4.48 (m, 2 H, H-1 and H-1′), 4.15 (m, 1 H, H-2), 4.04 (m, 4 H, H-6 and H-6′), 3.86 (m, 1 H, H-5′), 3.80 (m, 1 H, H-4), 3.64 (m, 1 H, H-5), 2.15, 2.12, 2.10, 2.04, 2.03, 2.01 and 1.92 (7 × s, each 3 H, 6 × OAc and SMe). ^{13}C NMR ($CDCl_3$) δ: 170.97, 170.36, 170.25, 170.06, 169.94, 169.08 and 162.23 (7 × CO), 101.51 (C-1′), 92.23 (TCA), 83.03 (C-1), 76.66 and 76.66 (C-4 and C-5), 73.61 (C-3), 70.81 (C-3′), 70.55 (C-5′), 70.52 (C-2′), 66.41 (C-4′) 62.09 and 60.60 (C-6 and C-6′), 53.62 (C-2), 20.85, 20.80, 20.57, 20.56, 20.52 and 20.40 (6 × OAc), 11.25 (SMe).

Example 17

10 g (8.13 mmol) of the acceptor according to example 6 (a compound of general formula **6**, wherein R_1= Bn, R_2= 4-chlorobenzoyl, R_3= benzoyl, OR_1 in β) and 10 g (1.6 equiv.) of the donor according to example 16 (a compound of general formula **5**, wherein R_4= acetyl, -NR_5R_6= trichloroacetyl, X= SMe) were dissolved in 35 ml of dry $CHCl_3$ under argon. To this solution 3.7 g of NIS and 490 mg of AgOTf were added at rt, and the stirring was continued for approx. 20 min. Triethyl amine (5 ml) was added to the slurry, diluted with CH_2Cl_2 (500 ml) and then extracted 2x with sodium thiosulphate solution (10 %), the organic phase was separated, dried with $MgSO_4$, filtered, concentrated, and the syrup was chromatographed on a column of silica-gel, using gradient of CH_2Cl_2 : acetone 98:2 → 95:5. Yield: 12.7 g, 80 % (a compound of general formula **4**, wherein R_1= Bn, R_2= 4-chlorobenzoyl, R_3= benzoyl, R_4= acetyl, -NR_5R_6= -NH-trichloroacetyl, OR_1 in β). MS (ESP): 1972.1 $[M+Na]^+$, 1988.1 $[M+K]^+$, 1948.2 $[M-H]^-$, 1984.0 $[M+Cl]^-$. ^{13}C NMR ($CDCl_3$) δ: 101.2, 100.7, 100.0, 98.8 (anomeric carbons). Mp.: 139-142 °C.

Example 18

100 g (120.4 mmol) of the donor according to example 11 (a compound of general formula **5**, wherein R_4= acetyl, -NR_5R_6= -NH-trichloroacetyl, X= β-SPh) and 118 g (96.2 mmol) of the acceptor according to example 6 (a compound of general formula **6**, wherein R_1= Bn, R_2= 4-chlorobenzoyl, R_3= benzoyl, OR_1 in β) was dissolved in 250 ml of dry $CHCl_3$ under argon.

To this solution 38 g of NIS and 6 g of AgOTf were added at rt., and the stirring was continued for approx. 1 h. The slurry was diluted with CH_2Cl_2 (500 ml) and subjected to extractive work-up. The final volume of the organic phase was 1.5 L, 450 ml of 10 % $Na_2S_2O_3$-solution and 150 ml of saturated $NaHCO_3$-solution was used. After the concentration, 280 g of brown syrup was isolated, which was subjected to column chromatography yielding a compound of general formula **4**, wherein R_1= Bn, R_2= 4-chlorobenzoyl, R_3= benzoyl, R_4= acetyl, -NR_5R_6= -NH-trichloroacetyl, OR_1 in β (physical data are identical to those of compound of example 17).

Example 19

Analogously prepared according to example 18 from donor of example 11 and acceptor of example 8: compound of general formula **4**, wherein R_1= 4-methylbenzyl, R_2= 4-chlorobenzoyl, R_3= benzoyl, R_4= acetyl, -NR_5R_6= -NH-trichloroacetyl, OR_1 in β.

White crystals from MeOH

^{1}H data ($CDCl_3$): 8.0-7.25 (m, aromatic), 6.8-7.0 (m, aromatic), 6.4 (d, 1H, NH), 5.54 (m, 2H), 5.42 (dd, 1H), 5.38 (dd, 1H), 5.3 (d, 1H), 5.04 (m, 2H), 4.92 (dd, 1H), 4.74 (d, 1H), 4.6 (m, 4H), 4.4 (m, 4H), 4.0 (m, 6H), 3.7 (m, 5H), 3.4 (m, 2H).

^{13}C data ($CDCl_3$): 177.4 (NHTCA carbonyl), 170.3, 170.2, 170.1, 170.0, 169.9, 169.0 (OAc, carbonyl), 165.3, 165.1, 164.9, 164.4, 164.2, 163.5, 161.3 (OBz carbonyl), 140.2, 140.0, 139.9, 139.7, 139.4, 137.9, 133.4, 133.1, 131.2, 131.1, 130.9, 130.8, 130.7, 129.9, 129.1, 129.0, 128.9, 128.6, 128.5, 128.3, 127.9, 127.8, 127.7, 127.6, 127.5, 127.1 (aromatic carbons), 101.2, 100.7, 99.9, 98.6 (anomeric carbons), 91.7 (NHTCA CCl_3), 76.2, 75.4, 72.9, 72.8, 71.7, 71.6, 70.8, 70.7, 70.5, 69.5, 68.9, 66.5, 62.8, 62.1, 60.9, 60.7, 55.7, 29.5, 21.1, 20.6, 20.5, 20.4, 20.3.

Example 20

Analogously prepared according to example 18 from donor of example 11 and acceptor of example 9: compound of general formula **4**, wherein R_1= 4-chlorobenzyl, R_2= 4-chlorobenzoyl, R_3= benzoyl, R_4= acetyl, -NR_5R_6= -NH-trichloroacetyl, OR_1 in β.

^{1}H NMR ($CDCl_3$): 7.95-6.85 (m, aromatic H), 6.42 (d, 1H), 5.55 (m), 5.4 (m, 4H), 5.0 (m, 3H), 4.75 (d, 1H), 4.55 (m, 4H), 4.4 (m, 2H), 4.0 (m), 3.7 (m, 5H), 3.45 (m, 3 H).

^{13}C NMR ($CDCl_3$): 170.32, 170.0, 169.0, 165.1, 164.4, 163.6, 161.4, 140.3, 139.9, 139.5, 134.9, 133.9, 133.4, 131.0, 130.0, 128.9,128.6, 127.9, 127.6, 127.4, 127.1, 101.2, 100.7, 99.9, 99.2, 91.7, 76.2, 75.4, 73.0, 71.6, 70.8, 70.0, 69.6, 68.9, 68.7, 66.6, 65.4, 63.2, 62.7, 62.1, 60.8, 55.7, 53.5, 30.9.

Example 21

Glycosyl donor of example 11 (1.0 equiv.) and diol acceptor of example 2 (1.0 equiv.) was dissolved in 25 mL of CH_2Cl_2. The solution was cooled to 0 °C and NBS (1.2 equiv) was added. After 10 min. stirring 25 µL of trifluoromethanesulfonic acid was added slowly. After the reaction reached completion, it was worked up as usually. The crude product was purified by flash chromatography to give compound of general formula **4**, wherein R_1= benzyl, R_2= 4-chlorobenzoyl, R_3= H, R_4= acetyl, -NR_5R_6= -NH-trichloroacetyl, OR_1 in β (56 %).

^{13}C NMR (500 MHz): 170.3, 170.1, 170.0, 169.9, 169.0, 165.0, 164.9, 164.5, 164.3, 163.6, 162.0, 140.0, 139.9, 139.8, 139.7, 139.5, 137.7, 136.3, 131.2, 131.1, 131.0, 130.8, 130.7, 129.0, 128.9 (2 signals), 128.8, 128.6, 128.5, 128.3, 128.1, 128.0 (2 signals), 127.9, 127.6 (2 signals), 127.5, 127.2, 125.2, 101.1, 100.6, 99.4, 98.7, 91.6, 79.8, 75.9, 75.4, 73.2, 73.1, 72.8, 72.1, 71.9, 71.0, 70.7, 70.6, 70.5 (2 signals), 69.0, 67.4, 66.5, 62.8, 62.7, 61.7, 60.8, 55.8, 22.6, 21.3, 20.5 (2 signals), 20.4.

Example 22

10 g (5.1 mmol) of protected tetrasaccharide (example 17) was dissolved in MeOH (110 ml) and solution of NaOMe (1 M in MeOH) was added until pH 10. The solution was stirred at 40 °C for 5 h, then was neutralized by addition of Amberlite IR 120 H^+ resin, the resin was filtered off, and the filtrate was evaporated to dryness. The residue was dissolved in warm DMF (10 ml) and added dropwise to ^{i}Pr_2O (150 ml) and the suspension was stirred for additional 3 h. The precipitate was filtered off, washed with ^{i}Pr_2O (2 x 20 ml) and dried to yield 4.2 g of product (a compound of general formula **3**, wherein R_1= Bn, -NR_5R_6= -NH-

trichloroacetyl, OR_1 in β) as off-white powder (91%). MS (ESP): 900.1 $[M-H]^-$. ^{13}C NMR (D_2O) δ: 105.6, 105.5, 104.2, 103.7 (anomeric carbons). Mp.: 134,5-135 °C.

The same product could be prepared analogously from compound of example 21.

Example 23

Analogously prepared according to example 22 from compound of example 19: compound of general formula **3**, wherein R_1= 4-methylbenzyl, -NR_5R_6= -NH-trichloroacetyl, OR_1 in β.

^1H NMR (D_2O): 7.25 (dd, 4H, aromatic), 4.98 (d, 1H), 4.88 (d, 1H), 4.8 (s), 4.7 (d, 1H), 4.5 (dd, 1H), 4.44 (d, 1H), 4.18 d (1H), 3.9 (m), 3.78 (m), 3.6 (m), 3.36 (s).

^{13}C NMR (D_2O): 167.7, 141.5, 136.2, 132.0, 131.7, 105.7, 105.6, 104.2, 103.7, 94.3, 84.2, 81.1, 80.9, 78.1, 77.6, 77.5, 77.4, 77.1, 75.5, 75.3, 74.5, 74.1, 73.7, 72.9, 71.3, 71.0, 63.8, 63.7, 62.9, 62.6, 60.0, 51.6, 23.0.

Example 24

Analogously prepared according to example 22 from compound of example 20: compound of general formula **3**, wherein R_1= 4-chlorobenzyl, -NR_5R_6= -NH-trichloroacetyl, OR_1 in β.

^1H NMR (D_2O): 7.2 (m, 4H, aromatic), 4.96 (d, 1H), 4.88 (d, 1H), 4.72 (d, 1H), 4.62(d,1H), 4.52 (m, 2H), 4.42 (m, 1H), 4.16 (d, 1H), 3.9 (m), 3.7 (m), 3.65 (m), 3.58 (m).

^{13}C NMR (D_2O): 167.7 (NHTCA carbonyl), 137.9, 136.2, 132.9, 131.3, 105.7, 105.6, 104.3, 103.8 (4 x anomeric carbons), 94.3 (NHTCA CCl_3), 84.2, 81.4, 81.1, 80.9, 78.1, 77.6, 77.5, 77.4, 77.1, 75.5, 75.3, 74.6, 74.5, 73.7, 73.4, 72.9, 71.3, 71.0, 63.8, 63.7, 62.8, 62.6, 60.2, 60.0, 59.8.

Example 25

35 g of a compound of example 22 was dissolved in 110 ml of MeOH and 110 ml of aqueous KOH (7.5 g) solution and the mixture was stirred at rt. for 1 d. The mixture was then chilled with ice-bath, neutralized by HCl-gas and concentrated to dryness. The crude brown glass (a

compound of general formula **2**, wherein R_1= Bn, OR_1 in β, ^{13}C NMR (D_2O) δ: 107.1, 105.7, 105.4, 103.7 (anomeric carbons)) was then acetylated with pyridine (150 ml) and acetic anhydride (150 ml) at rt. for 1 d. The solution was concentrated, the syrup was dissolved in CH_2Cl_2, the organic phase was extracted with 1M HCl-solution and then with sat. $NaHCO_3$-solution, dried with $MgSO_4$, filtered and concentrated to yield 43 g of brown foam. This was subjected to column chromatography to give a compound of general formula **4a**, wherein R_1= Bn, OR_1 in β). ^{13}C NMR ($CDCl_3$) δ: 101.2, 100.8, 100.4, 99.2 (anomeric carbons).

Example 26

Analogously prepared according to the de-*N*-acylation step of example 25 from compound of example 23: compound of general formula **2** wherein R_1= 4-methylbenzyl, OR_1 in β.

^1H NMR (D_2O): 7.3 (dd, 4H, aromatic), 4.9 (d, 1H), 4.72 (dd, 1H), 4.5 (m), 4.18 (d, 1H), 3.95 (m), 3.8-3.55 (m), 3.34 (dd).

^{13}C NMR (D_2O): 184.1, 141.4, 136.6, 136.1, 131.9, 131.6, 131.4, 105.7, 105.3, 105.2, 100.0, 84.8, 81.0, 80.9, 78.0, 77.7, 77.5, 77.4, 77.1, 75.9, 75.5, 75.2, 74.4, 74.0, 73.6, 73.2, 72.7, 72.4, 71.2, 70.9, 63.7, 63.6, 62.5, 58.9, 25.9.

Example 27

Analogously prepared according to the de-*N*-acylation step of example 25 from compound of example 24: compound of general formula **2** wherein R_1= 4-chlorobenzyl, OR_1 in β.

^1H NMR (D_2O): 7.4 (s, 4H, aromatic), 4.9 (m, 2H), 4.75 (m), 4.52 (dd, 1H), 4.46 (dd, 1H), 4.18 (d, 1H), 3.96 (m), 3.86 (m), 3.7 (m), 3.62 (m).

^{13}C NMR (D_2O): 184.1, 137.9, 136.2, 132.9, 132.6, 131.3, 105.7, 105.2, 104.0, 103.8, 84.6, 80.9, 80.7, 78.1, 77.6, 77.4, 77.1, 75.5, 75.2, 74.4, 73.6, 73.4, 72.7, 71.2, 70.9, 63.8, 63.6, 62.8, 62.4, 58.6, 51.6, 25.9.

Example 28

140 g (107.5 mmol) of the peracetylated tetrasaccharide (example 25) was dissolved in 1.5 L of MeOH, NaOMe-solution (1M) was added until pH 10, and the mixture was stirred at 50 °C overnight. The product crystallized from the reaction mixture. The mixture was allowed to cool to rt., then it was chilled, filtered, the filtrate was washed with cold EtOH, then dried to yield 69 g of white powder (a compound of general formula **1**, wherein R_1= Bn, OR_1 in β; 80 %). ^{13}C NMR (D_2O) δ: 105.6, 105.5, 105.4, 103.6 (anomeric carbons). Mp.: 284-286 °C.

Example 29

150 g of purified amine (compound of general formula **2**, R_1= benzyl, OR_1 in β) were added to a mixture of water (150 mL) and MeOH (200 mL) at room temperature. Acetic anhydride (20 mL) was added in one portion. After 1 h stirring extra 10 mL of acetic anhydride added and the mixture was stirred at r.t. After 1 h stirring extra 10 mL of acetic anhydride added and the mixture was stirred for additional 2 h at r.t. At the end the mixture was a crystalline mass. MeOH (250 mL) was added and the mixture was put to fridge for overnight. The solid was filtered, the cake was washed with cold MeOH (200 mL) to yield 260 g of light brown solid. Drying of this solid at 50 °C at atmospheric pressure (3 days) yielded 118 g of product (compound of general formula **1**, R_1= Bn, OR_1 in β). Recrystallization of 80 g in MeOH/water yielded 72.6 g product with 99.7 % purity according to HPLC.

Example 30

Analogously prepared according to example 29 from compound of example 26: compound of general formula **1**, R_1= 4-methylbenzyl, OR_1 in β.

^1H NMR (D_2O): 7.3 (dd, 4H), 4.88 (d, 1H), 4.7 (m), 4.54 (d, 1H), 4.48 (d, 1H), 4.42 (d, 1H), 4.34 (d), 4.0-3.5 (m), 3.34 (dd, 1H).

^{13}C NMR (D_2O): 184.2, 177.6, 173.7, 141.5, 136.1, 131.9, 131.4, 105.6, 105.5, 105.4, 103.6, 93.2, 84.7, 81.5, 81.0, 80.8, 78.0, 77.6, 77.4, 77.1, 75.5, 75.2, 74.8, 74.0, 73.6, 72.9, 63.7, 62.8, 58.9, 56.4, 25.9, 22.9.

Example 31

Analogously prepared according to example 29 from compound of example 27: compound of general formula **1**, R_1= 4-chlorobenzyl, OR_1 in β.

^{1}H NMR (D_2O): 7.4 (s, 4H), 4.9 (d, 1H), 4.72 (m), 4.52 (d, 1H), 4.8 (d, 1H), 4.42 (d, 1H), 4.16 (d, 1H), 4.0-3.52 (m).

^{13}C NMR (D_2O): 138.9, 177.6, 138.3, 137.9, 136.2, 131.3, 105.6, 105.5, 105.4, 103.7, 93.2, 86.1, 84.7, 81.5, 81.0, 80.8, 78.0, 77.5, 77.4, 77.2, 77.1, 75.5, 75.2, 74.9, 63.7, 58.9, 57.8, 56.4, 24.8.

Example 32

40 g (50.1 mmol) of the benzyl glycoside (example 28 or 29) were dissolved in 200 ml of water, 1.6 g of Pd-C and 400 µl of acetic acid was added, and the mixture was stirred at rt. under H_2-atmosphere (approx. 40 bars) for 2 days. The catalyst was filtered off, the cake was washed with water, and the filtrate was added dropwise to 1.6 l of acetone, then chilled, filtered and the collected solid was dried under vacuum to yield 31.5 g of white powder of LNnT (44.5 mmol, 89 %).

Example 33

Benzyl glycoside (example 28 or 29, 100 g) was dissolved in 300 ml of water, 2 ml of acetic acid and 30 ml of MeOH was added, then 10 g of Pd-C (5 %). The mixture was hydrogenated at r.t. under 5 bar of hydrogen for 2 days. Activated carbon (5 g) was added to the mixture, the catalyst and carbon were filtered off, the filtrate was diluted with water (200 ml), the aqueous solution was heated to 50 °C. The crystallization was performed as above by addition of EtOH (4 L). 65 g of white solid of LNnT was isolated.

Example 34

50 g of the benzyl glycoside (example 28 or 29) were dissolved in 110 mL of water and the solution was diluted with MeOH (120 mL). Pd-C (2 g, 10 % Pd) catalyst was added, and the mixture was hydrogenated at 60 °C under H_2 atmosphere (5 bars). After 7 h the catalyst was filtered off, the cake was washed with approx. 20 mL of water:MeOH mixture (1:1).

Crystallization: the solution obtained above was warmed (50-55 °C) to which hot MeOH (600 mL) was added in 3-5 portions under gradual chilling to 40 °C. Crystallization started immediately. After addition of the methanol the crystalline mixture was slowly (3 h) cooled to r.t., then put to the fridge for overnight. Filtered on glass filter, the solid was washed with cold MeOH. and dried at 60 °C in a vacuum drying oven for 2 days to yield 37.4 g of white solid. HPLC purity: 100 %, mp.: 226-230 °C. ^{13}C-NMR (600 MHz, D_2O:CD_3OD = 3:2) δ: Glc (α) C-1 93.2 C-2 72.5 C-3 72.7 C-4 79.5 C-5 71.3 C-6 61.2, Glc (β) C-1 97.2 C-2 75.2 C-3 75.7 C-4 79.5 C-5 76.1 C-6 61.3, Gal C-1 104.3 C-2 71.4 C-3 83.1 C-4 69.7 C-5* 76.2 C-6 62.3, GlcNAc C-1 104.1 C-2 56.4 C-3 73.4 C-4 79.2 C-5 75.8 C-6 61.0 $NCOCH_3$ 23.3 174.8, Gal C-1 104.2 C-2 72.3 C-3 73.9 C-4 69.9 C-5* 76.6 C-6 62.3 (* interchangeable assignments).

CLAIMS

1. A method for the preparation of Galpβ1-4GlcNAcpβ1-3Galpβ1-4Glc (LNnT) comprising the steps of:

a) reaction of a donor characterized by general formula **5**

general formula **5**

wherein R_4 is optionally substituted acyl,

$-NR_5R_6$ is selected from $-NAc_2$, -NH-haloacyl, phthalimide, tetrachlorophthalimide, 2,3-diphenylmaleimide and 2,3-dimethylmaleimide,

X is selected from halogen, $-OC(=NH)CCl_3$, -OAc, -OBz and $-SR_7$, wherein R_7 is selected from alkyl and optionally substituted phenyl,

with an acceptor of general formula **6**

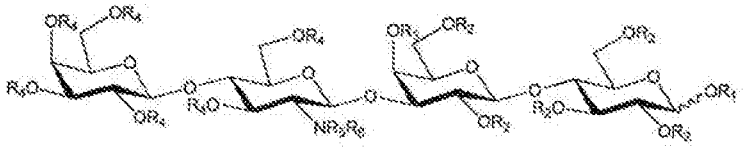
general formula **6**

wherein R_1 is a group removable by catalytic hydrogenolysis,

R_2 is optionally substituted acyl and

R_3 is selected from optionally substituted acyl or H,

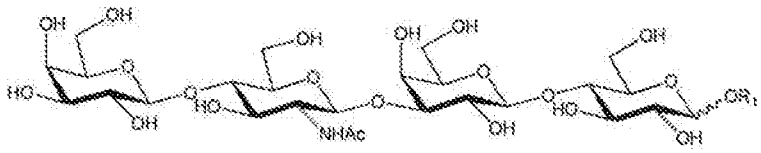
to yield a compound of general formula **4**



general formula **4**

wherein R_1, R_2, R_3, R_4 and $-NR_5R_6$ are as defined above,

b) converting the compound of general formula **4** into a compound of general formula **1**



general formula **1**

wherein R_1 is as defined above,

c) crystallizing the compound of general formula **1**, and

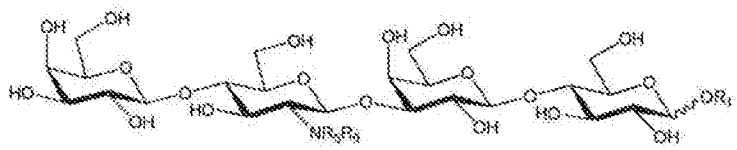
d) subsequently subjecting the compound of general formula **1** to catalytic reduction.

2. The method according to claim 1, wherein in step a) in the donor of general formula **5**, $-NR_5R_6$ is –NH-haloacyl and X is $-SR_7$, wherein R_7 is selected from optionally substituted alkyl or optionally substituted phenyl; and in compound acceptor of general formula **6**, R_1 is optionally substituted benzyl and R_3 is selected from H and optionally substituted benzoyl.

3. The method according to any one of the claims 1-2, wherein the conversion of the compound of general formula **4** into the compound of general formula **1** in step b) comprises the steps of:

ba) base catalyzed transesterification deprotection or basic hydrolysis of the compound of general formula **4**, wherein $-NR_5R_6$ is $-NAc_2$, to give a compound of general formula **1** or

bb) base catalyzed transesterification deprotection of the compound of general formula **4**, wherein $-NR_5R_6$ is selected from -NH-haloacyl, phthalimide, tetrachlorophthalimide, 2,3-diphenylmaleimide and 2,3-dimethylmaleimide, to give a compound of general formula **3**



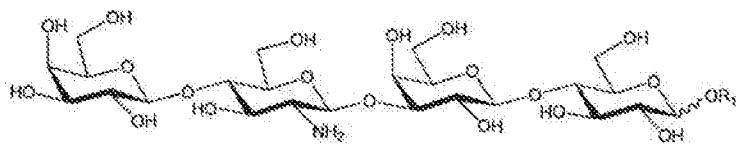
general formula 3

wherein R_1 and $-NR_5R_6$ are defined as above, which compound of general formula **3** is subjected to basic hydrolysis or aminolysis to give rise to a compound of general formula **2**

general formula **2**

wherein R_1 is as defined above, which compound of general formula **2** is converted into the compound of general formula **1**, or

bc) basic hydrolysis of the compound of general formula **4**, wherein $-NR_5R_6$ is selected from -NH-haloacyl, 2,3-diphenylmaleimide and 2,3-dimethylmaleimide, to give a compound of general formula **2**



general formula **2**

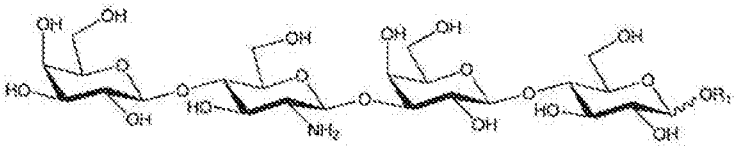
wherein R_1 is as defined above, which compound of general formula **2** is converted into the compound of general formula **1**, or

bd) basic hydrolysis of the compound of general formula **4**, wherein $-NR_5R_6$ is selected from phthalimide and tetrachlorophthalimide, followed by aminolysis to give a compound of general formula **2**

general formula **2**

wherein R_1 is defined as above, which compound of general formula **2** is converted into the compound of general formula **1**, or

be) aminolysis of the compound of general formula **4**, wherein $-NR_5R_6$ is selected from $-NAc_2$, -NH-haloacyl, phthalimide, tetrachlorophthalimide, 2,3-diphenylmaleimide and 2,3-dimethylmaleimide, to give a compound of general formula **2**



general formula **2**

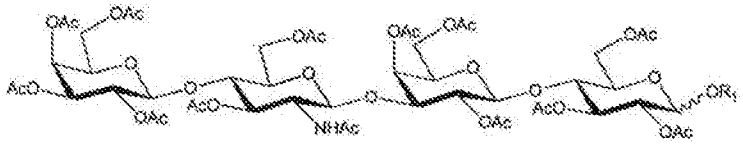
wherein R_1 is defined as above, which compound of general formula **2** is converted into the compound of general formula **1**.

4. The method according to claim 3, wherein the compound of general formula **2** is

a) *N*-acetylated to obtain compounds of general formula **1**,

or

b) peracetylated to compounds of general formula **4a**



general formula **4a**

wherein R_1 is defined as above,

followed by based catalyzed transesterification reaction or basic hydrolysis to obtain compounds of general formula **1**.

5. The method according to any one of the claims 1-4, wherein the crystallization in step c) is carried out in a solvent comprising one or more C_1-C_6 alcohols, preferably in aqueous methanol or aqueous ethanol, and R_1 is benzyl.

6. The method according to any one of the claims 1-5, wherein the compound of general formula **1**, wherein R_1 is benzyl, is subjected to catalytic hydrogenation.

7. A polymorph of Galpβ1-4GlcNAcpβ1-3Galpβ1-4Glc (LNnT), characterized in that it displays X-ray powder diffraction reflections, based on a measurement using CuKα radiation, at 20.32±0.20 2Θ angle, preferably at 20.32±0.20 and 19.10±0.20 2Θ angles, more preferably at 20.32±0.20, 19.10±0.20 and 7.98±0.20 2Θ angles, even more preferably at 20.32±0.20, 19.10±0.20, 7.98±0.20 and 21.03±0.20 2Θ angles, most preferably 20.32±0.20, 19.10±0.20, 7.98±0.20, 21.03±0.20 and 20.95±0.20 2Θ angles, in particular 20.32±0.20, 19.10±0.20, 7.98±0.20, 21.03±0.20, 20.95±0.20 and 5.66±0.20 2Θ angles.

8. The polymorph of Galpβ1-4GlcNAcpβ1-3Galpβ1-4Glc according to claim 7, which displays a melting point between 226-230 °C.

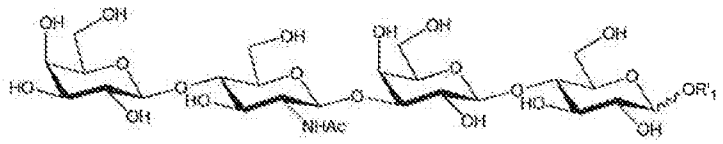
9. The polymorph of Galpβ1-4GlcNAcpβ1-3Galpβ1-4Glc according to any one of claims 7 and 8 for use as pharmaceutical agent.

10. The polymorph of Galpβ1-4GlcNAcpβ1-3Galpβ1-4Glc according to any one of claims 7 and 8 for use as nutritional additive.

11. A pharmaceutical composition comprising the polymorph of Galpβ1-4GlcNAcpβ1-3Galpβ1-4Glc as defined in any one of claims 7 and 8.

12. A nutritional composition comprising the polymorph of Galpβ1-4GlcNAcpβ1-3Galpβ1-4Glc as defined in any one of claims 7 and 8.

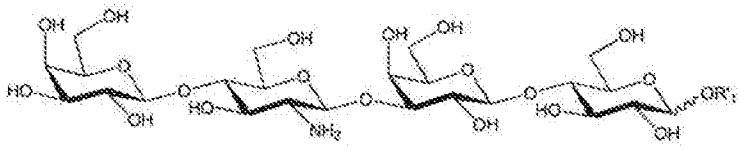
13. A compound of general formula **1'**



general formula **1'**

wherein R'_1 is selected from substituted benzyl, optionally substituted benzhydryl, optionally substituted trityl and optionally substituted naphthylmethyl.

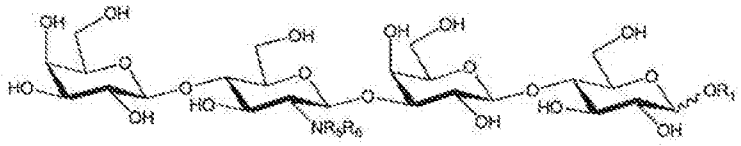
14. A compound of general formula **2'**



general formula **2'**

wherein R'_1 is selected from substituted benzyl, optionally substituted benzhydryl, optionally substituted trityl and optionally substituted naphthylmethyl.

15. A compound of general formula **3**

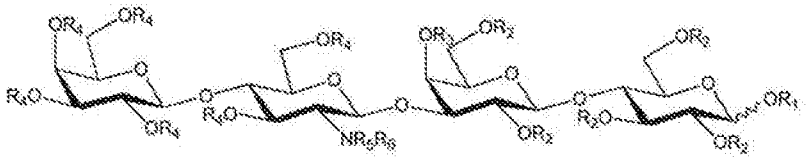


general formula **3**

wherein R_1 is selected from optionally substituted benzyl, optionally substituted benzhydryl, optionally substituted trityl and optionally substituted naphthylmethyl, and $-NR_5R_6$ is selected from -NH-haloacyl, phthalimide, tetrachlorophthalimide, 2,3-diphenylmaleimide and 2,3-dimethylmaleimide.

16. The compound according to claim 15, wherein R_1 is selected from benzyl, 4-methylbenzyl and 4-chlorobenzyl, preferably benzyl and $-NR_5R_6$ is -NH-haloacyl, preferably –NH-trichloroacetyl.

17. A compound of general formula **4'**

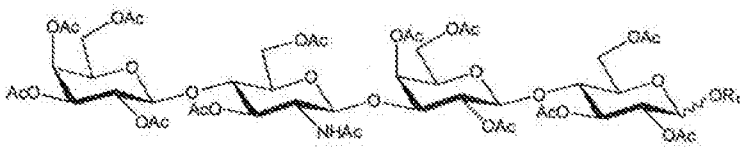


general formula **4'**

wherein R_1 is selected from optionally substituted benzyl, optionally substituted benzhydryl, optionally substituted trityl and optionally substituted naphthylmethyl, and each of R_2 and R_4 are independently optionally substituted acyl, R_3 is selected from optionally substituted acyl and H, $-NR_5R_6$ is selected from $-NAc_2$, -NH-haloacyl, tetrachlorophthalimide, 2,3-diphenylmaleimide and 2,3-dimethylmaleimide.

18. The compound according to claim 17, wherein R_1 is selected from benzyl, 4-methylbenzyl and 4-chlorobenzyl, preferably benzyl, R_2 is benzoyl optionally substituted by chloro, R_3 is optionally substituted benzoyl, preferably benzoyl, R_4 is acetyl and $-NR_5R_6$ is –NH-haloacyl, preferably –NH-trichloroacetyl.

19. A compound of general formula **4a**



general formula **4a**

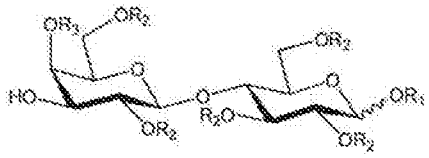
wherein R_1 is selected from optionally substituted benzyl, optionally substituted benzhydryl, optionally substituted trityl and optionally substituted naphthylmethyl.

20. A compound of general formula **5'**

general formula **5'**

wherein R_4 is optionally substituted acyl, preferably acetyl, $-NR_5R_6$ is –NH-haloacyl, preferably –NH-trichloroacetyl or –NH-trifluoroacetyl, and R_7 is optionally substituted phenyl, preferably phenyl.

21. A compound of general formula **6'**



general formula **6'**

wherein R_1 is selected from optionally substituted benzyl, optionally substituted benzhydryl, optionally substituted trityl and optionally substituted naphthylmethyl, R_2 is optionally substituted benzoyl, and R_3 is selected from optionally substituted acyl and H.

22. The compound according to claim 21, wherein R_2 is optionally substituted benzoyl, preferably benzoyl or 4-chlorobenzoyl, and R_3 means benzoyl or H.

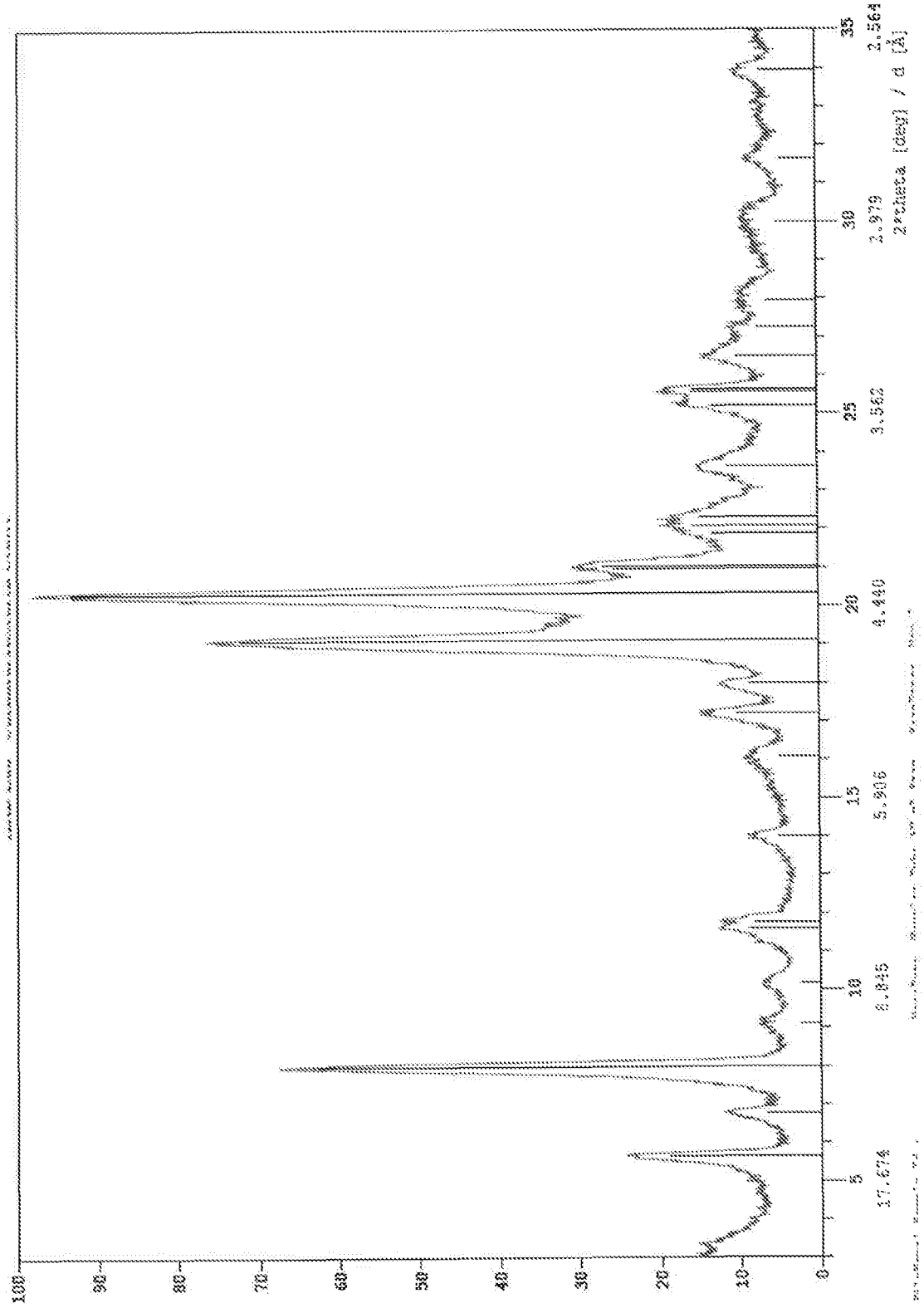

100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
35
17.674
8.845
5.906
4.440
3.562
2.979
2.564
2*theta [deg] / d [Å]

Figure 1

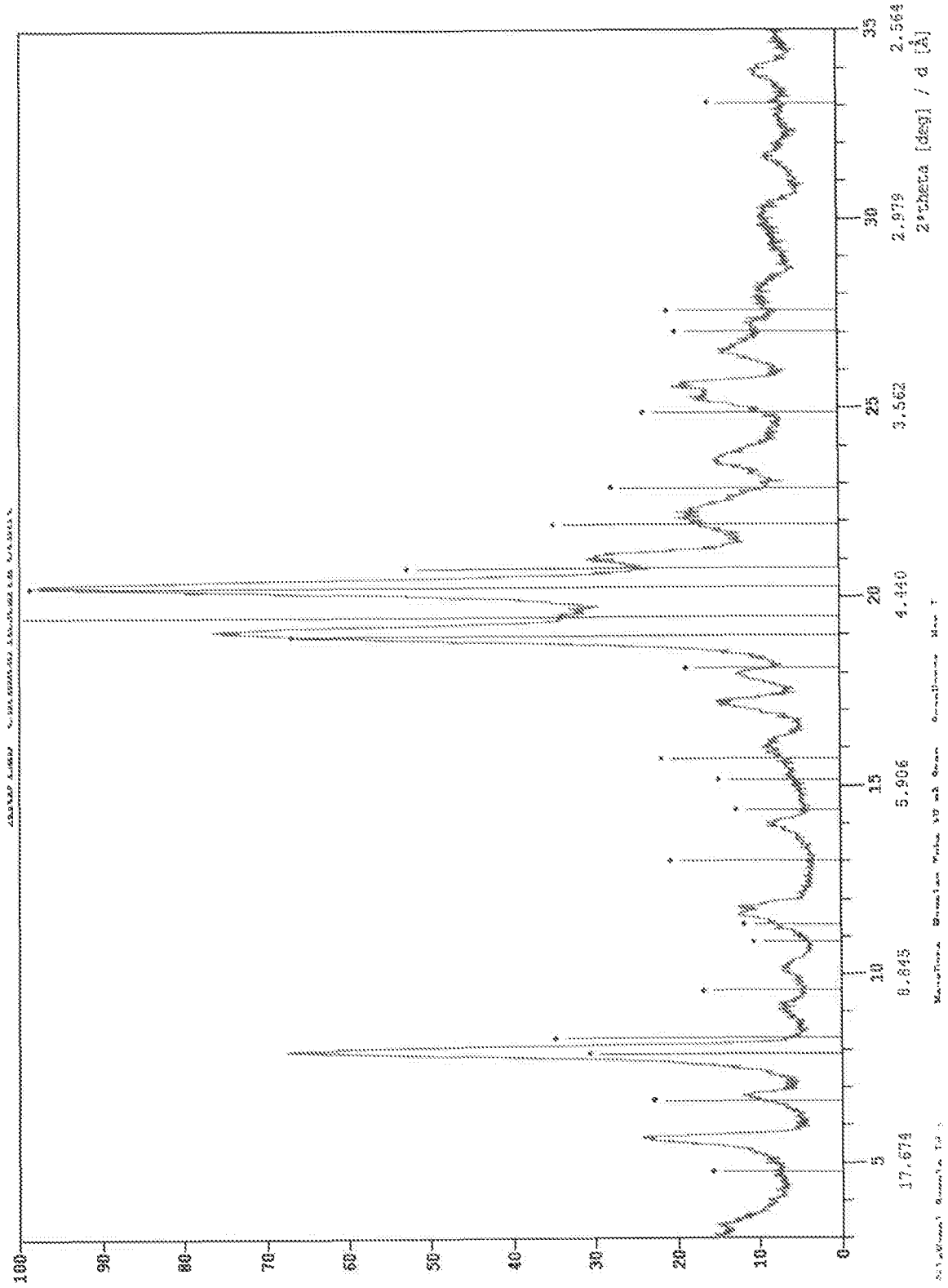

100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
35
17.674
8.843
5.906
4.440
3.562
2.979
2.563
2*theta [deg] / d [Å]

Figure 2

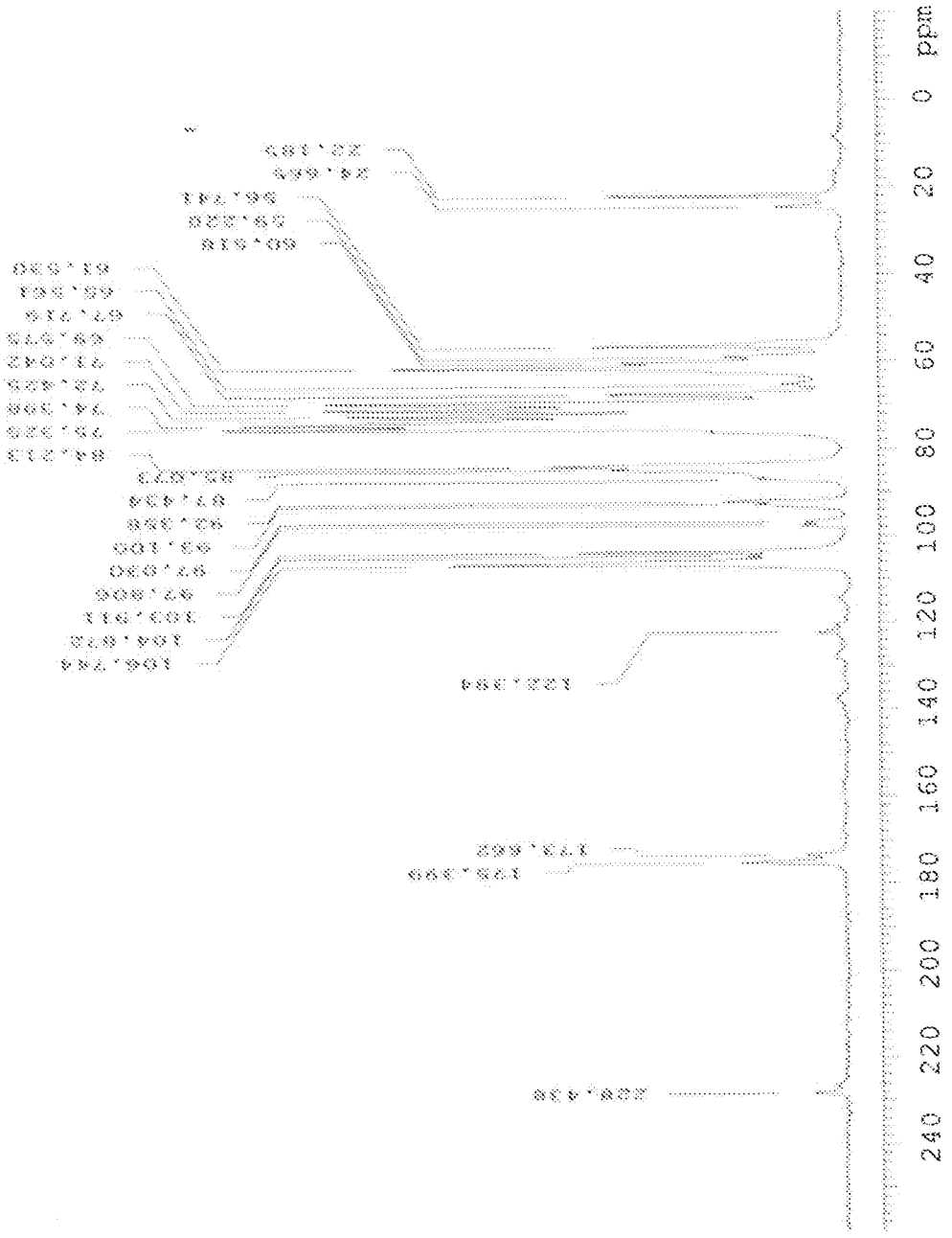


Figure 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK2011/050053

A. CLASSIFICATION OF SUBJECT MATTER
IPC (2006.01): C07H 1/00 (2006.01), C07H 5/04 (2006.01), C07H 15/203 (2006.01), C07H 15/18 (2006.01), A61K 31/702 (2006.01), A23L 1/29 (2006.01)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC (2006.01): C07H, A61K, A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
DK, NO, SE, FI: Classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPODOC, WPI, MEDLINE, REGISTRY, CAPLUS, TXTE, TXTG, TXTF

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PONPIPOM, M.M. et al.: "Synthesis of Paragloboside Analogs", Tetrahedron Lett, 1978, Vol. 19, No. 20, pages 1717-1720 See whole document	1-6
A	Database REGISTRY, RN 13007-32-4, STN entry date 16 November 1984	
A	YAN, F. et al.: "Polymer-supported and chemoenzymatic synthesis of the Neisseria meningitidis pentasaccharide: a methodological comparison", Carbohydr. Res., 2000, Vol. 328, pages 3-16	
A	BRÖDER, W. et al.: "Glycosyl Azides as Building Blocks in Convergent Syntheses of Oligomeric Lactosamine and Lewisx Saccharides", Bioorg. Med. Chem. 1997, Vol. 5, No. 1, pages 1-19	
A	BOMMER, R. et al.: "Glycosyl Imidates, 43. Synthesis of Lactoneotetrasyl Ceramide", Liebigs Ann. Chem. 1989, pages 1107-1111	

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03/05/2011	10/05/2011
Name and mailing address of the ISA/ Nordic Patent Institute, Helgeshøj Allé 81, DK-2630 Taastrup, Denmark Facsimile No. +45 4350 8008	Authorized officer Bülow, Anne Telephone No. +45 4350 8125

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK2011/050053

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

The International Searching Authority found that the application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept. The result is a lack of unity between:

See extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-6

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK2011/050053

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ALY, M.R.E. et al.: "Synthesis of lacto-N-neotetraose and lacto-N-tetraose using the dimethylmaleoyl group as amino protective group", Carbohydr. Res., 1999, Vol. 316, pages 121-132	
A	SHERMAN, A.A. et al.: "Study of glycosylation with N-trichloroacetyl-D-glucosamine derivatives in the syntheses of the spacer-armed pentasaccharides sialyl lacto-N-neotetraose and sialyl lacto-N-tetraose, their fragments, and analogues", Carbohydr. Res., 2001, Vol. 336, pages 13-46	
A	YAMADA, A. et al.: "Syntheses of a series of lacto-N-neotetraose clusters using a carbosilane dendrimer scaffold", Carbohydr. Res.	
A	MALLERON, A. et al.: "Chemo-enzymatic supported synthesis of the 3-sulfated Lewisa pentasaccharide on a multimeric polyethylene glycol", Carbohydr. Res., 2008, Vol. 343, pages 970-976	
A	PAULSEN, H. et al.: "Regioselektive glycosylerung an stellungen 3' und 4' von lactose-derivaten", Carbohydr. Res., 1987, Vol. 169, pages 105-125	
A	MARANDUBA, A., et al.:" Glycosylation of lactose. Synthesis of methyl O-(2-acetamido-2-deoxy-b-D-glucopyranosyl)-(1-3) -O-b-D-galactopyranosyl-(1-4)-b-D-glucopyranoside and methyl O-b-D-galactopyranosyl-(1-4)-O-(2-acetamido-2 -deoxy-b-D-glucopyranosyl)-(1-3)-O-b-D-galactopyranosyl-(1-4) -b-D-glucopyranoside", Carbohydr. Res., 1985, Vol. 135, pages 330 -336	
A	DAHMÉN, J. et al.: "Synthesis of di-, tri-, and tetra-saccharides corresponding to receptor structures recognised by Streptococcus pneumoniae", Carbohydr. Res., 1985, Vol. 138, pages 17-28	
A	MARANDUBA, A. et al.: "Glycosylation of lactose: Synthesis of branched oligosaccharides involved in the biosynthesis of glycolipids having blood-group I activity", Carbohydr. Res., 1986, Vol. 151, pages 105-119	

Continuation of box No. III:

Invention 1 (claims 1-6, 13-22):
A method for preparation of lacto-N-neotetraose and intermediates in the synthetic route

Invention 2 (claims 7-12):
A new polymorph form of lacto-N-neotetraose, pharmaceutical and nutritional compositions comprising said polymorph, and the use of said compositions

The common technical feature linking inventions 1 and 2 is lacto-N-neotetraose (LNnt) which is a known compound as also explained by the applicant (see also REGISTRY, RN 13007-32-4). Therefore, the common technical feature is not patentable and lack of unity arise.

Invention 1 relates to both a method of preparation of LNnt and to various intermediates in the synthesis. The technical interrelation between the method of preparation and the various intermediates is dependent on the patentability of the method of preparation of LNnt.

Ponpipom et al. (D1) describes a method for preparation of 1-O-benzyl-LNnt (a compound of general formula 1) from a glycosyl chloride (6) and a glycosyl acceptor (8) falling within the scope of general formulae 5 and 6, respectively. The resulting acetyl protected N-phthalimido 1-O-benzyl LNnt derivative 9 (falling within general formula 4 of the present application), is deacetylated to give 10 (falling within general formula 2 of the present application), which is further N-acetylated to yield 1-O-benzyl-LNnt (11) i.e. a compound of general formula 1 of the present application. Compound 11 has a melting point of 286-288C. For the identical compound of the present application (see example 28) a melting point of 284-286C is given. It is therefore assumed that compound 11 of D1 must be a crystalline form as well, even though it is not specifically mentioned.

Steps a) and b) of claim 1 are known from D1 and step d) is regarded as a routine reaction for the skilled person. Therefore, the special technical feature of the method of preparation is regarded to be the crystallisation of the compound of general formula 1, i.e. step c) in claim 1. However, it appears that step c) has already been made in D1 based on the melting points.

Therefore, the method of preparation in the present application does not constitute a special technical feature that makes a contribution over the prior art. Hence, the method of preparation (claims 1-6) and the intermediates are not so linked as to form a single inventive concept. As there is no interrelation between the various intermediates, the lack of unity results in a division of above invention 1 into the following 8 inventions:

Invention 1a (claims 1-6): A method for preparation of LNnt

Invention 1b (claim 13): A compound of general formula 1'

Invention 1c (claim 14): A compound of general formula 2'

Invention 1d (claims 15-16): A compound of general formula 3

Invention 1e (claims 17-18): A compound of general formula 4'

Invention 1f (claim 19): A compound of general formula 4a

Invention 1g (claim 20): A compound of general formula 5'

Invention 1h (claims 21-22): A compound of general formula 6'