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Enzymatic synthesis of a new inhibitor of α-amylases: acarviosinyl-isomaltosyl-spiro-thiohydantoin

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Dedicated to Professor András Lipták on the occasion of his 70th birthday

Abstract—Synthesis of acarviosinyl-isomaltosyl-spiro-thiohydantoin in yields up to 20%, has been achieved by *Bacillus stearothermophilus* maltogenic amylase (BSMA). BSMA is capable of transferring the acarviosine–glucose residue from an acarbose donor onto glucopyranosylidene-spiro-thiohydantoin. Reactions were followed using HPLC and MALDI-TOF MS. ^{1}H and ^{13}C NMR studies revealed that the enzyme reserved its stereoselectivity. Glycosylation took place mainly at C-6 resulting in α-acarviosinyl-(1→4)-α-D-glucopyranosyl-(1→6)-D-glucopyranosylidene-spiro-thiohydantoin. This compound was found to be a much more efficient salivary amylase inhibitor than glucopyranosylidene-spiro-thiohydantoin with kinetic constants of $K_{EI} = 0.19\ \mu M$ and $K_{ESI} = 0.24\ \mu M$.

Keywords: Enzymatic synthesis; Maltogenic amylase; Acarviosine derivative; MALDI-TOF MS; NMR

1. Introduction

Disorders of carbohydrate uptake may cause severe health problems such as diabetes,[1] obesity[2] or caries[3]—all of which threaten an increasing population. Therefore, there is an obvious need for novel agents or therapeutic strategies that act on the physiological regulation of sugar uptake, blood sugar levels and prevention of oral diseases.

Human α-amylases of both salivary (HSA) and pancreatic origin (HPA) have been studied thoroughly in clinical chemistry because they are important indicators in the diagnosis of pancreatic and salivary gland diseases.[4,5] They have been subjected to extensive biochemical and structural characterisation.[6,7] Furthermore, they are targets of drug design in attempts to treat diabetes, obesity, hyperlipidaemia and dental caries. HSA may play a role in dental plaque formation and the subsequent process of dental caries formation and progression.[6] Four glycone and three aglycone binding sites have been identified as targets for inhibitor binding.[8] Protein crystallography[6] and subsite mapping yielded significant information on the specificity and binding energies of the different binding sites.[9]

Many α-amylase inhibitors of microbial[10] and plant origin[11] have been obtained and found in synthetic

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substances,[12] as well. They have been proven to be of great value because of their various uses as tools for the investigation of the active site for α-amylases, as reagents for the measurement of α-amylase isoenzyme activities by selective inhibition, or as oral agents for the treatment of diabetes, obesity and other sugar metabolic disorders. Acarbose (**1**) is a natural product manufactured by the fermentation of *Actinoplanes* sp. and is a highly effective inhibitor of several carbohydrates such as α-glucosidase, α-amylase, CGTase and glucoamylase.[10] It is often used in crystallographic analyses of protein oligosaccharide interactions.[13] Its structure is shown in Figure 1. The mechanism of inhibition has been attributed to the unsaturated cyclohexene ring and glycosidic nitrogen linkage mimicking the transition state for the enzymatic cleavage of glycosidic linkages.[14] Acarbose is in clinical use in the treatment of diabetes. However, it is degraded by digestive enzymes and/or intestinal microorganisms.[15,16] These degradation products may cause diarrhoea and associated abdominal discomfort. Therefore, there is an obvious need for novel agents or therapeutic strategies capable of acting without degradation. Glucopyranosylidene-spiro-thiohydantoin (G-TH), synthesised by Somsák et al.,[17] and extensively studied as an inhibitor of glycogen phosphorylases[18] was supposed to be the inhibitor of choice to meet our particular needs. G-TH was found to be a mixed non-competitive inhibitor of HSA with the inhibition constants in the millimolar range.[12] Its structure is shown in Figure 2 as compound **2**. Since this inhibitor is a small molecule, the long enough active site is assumed to facilitate its accommodation in different binding modes. Longer G-TH analogues have been presumed to be more effective inhibitors than G-TH itself.

Although many glycosylation reactions have been based on recent advances in catalyst research for organic synthesis, the perfect control of regio- and stereochemistry of the glycosylating process still remains a difficult and challenging problem for carbohydrate chemists. Enzymes have several remarkable catalytic properties compared with other types of catalysts in terms of their selectivity, high catalytic activity, lack of undesirable side-reactions and operation under mild conditions. Several kinds of oligosaccharides have been synthesised by enzymatic catalysis.[19,20] Considerable effort has been made to elongate this inhibitor with different amylases using maltooligosaccharides and acarbose as donors. In this study we report on the elongation of G-TH catalysed by *Bacillus stearothemophilus* maltogenic amylase (BSMA; EC 3.2.1.133).

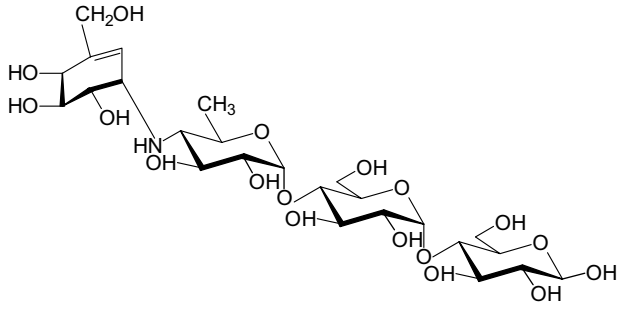

Figure 1. The structure of **1** acarbose.

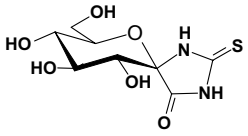

Figure 2. The structure of **2** glucopyranosylidene-spiro-thiohydantoin.

2. Results and discussion

Recently, the gene encoding a new thermostable maltogenic amylase from *B. stearothermophilus* (BSMA) has been cloned and expressed by Park and co-workers.[21] The authors have found that this enzyme hydrolyses starch, cyclodextrins, pullulan as well as acarbose. BSMA binds to acarbose to cleave the first glycosidic linkage that gives a pseudotrisaccharide **3** (PTS). In the presence of various acceptor molecules, BSMA can transfer PTS to the acceptor molecule by forming α-(1→6), α-(1→3) or α-(1→4) glycosidic linkages. It has also been found that the replacement of the maltose unit of acarbose by isomaltose gives an inhibitor more potent than acarbose.[22,23] These observations have encouraged us to study the BSMA catalysed transglycosylation reaction, using acarbose as donor and G-TH as acceptor in the synthesis of a novel amylase inhibitor.

2.1. Monitoring the reaction

Two UV detectable transglycosylated products appeared in the chromatogram after 10 h and their amount increased significantly after 48 h, as seen in Figure 3. The main product **4** (second peak in HPLC chromatogram) was isolated and purified by semipreparative HPLC and used for NMR analysis. Since acarbose and its hydrolysis products, glucose and acarviosinylglucose **3**, do not absorb UV light, they were analysed by TLC using a chromatographic system capable of separating all the compounds of the reaction mixture. The composition of the reaction mixture was also studied by MALDI-TOF MS. Interestingly, the MALDI-TOF spectrum of the reaction mixture (48 h) resulted in a single peak with an *m*/*z* higher than that of the acarbose (Fig. 4) pointing at the that products being isomer compounds. We identified the $(M+Na)^+$ peaks of acarbose, PTS (the hydrolysis product of acarbose) and a newly formed acceptor product with *m*/*z* values of 669, 506,

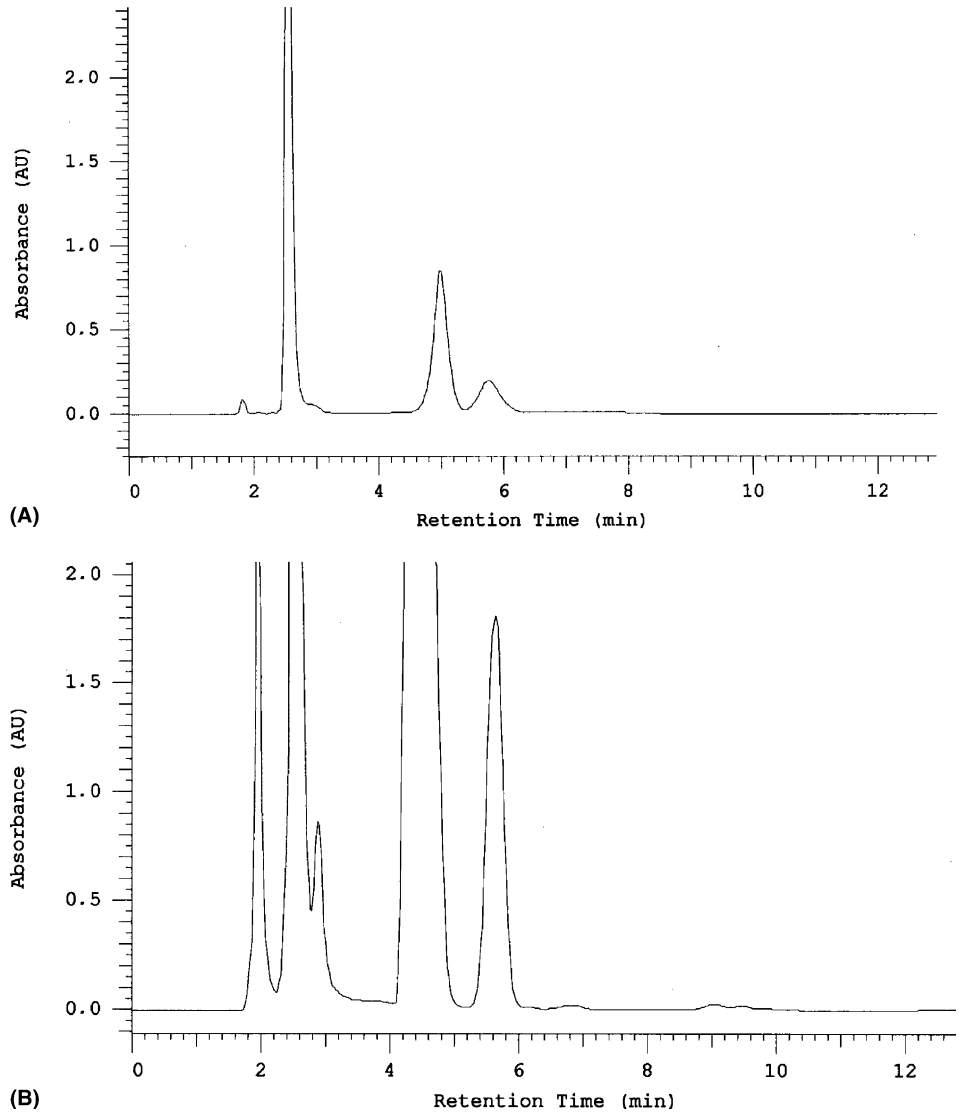

Figure 3. HPLC separation of transglycosylation products after 10 h (A) and 48 h (B). Peaks with retention time of 2.6, 4.8, 5.7 belong to **2** G-TH, **4**, **5** acceptor products, respectively. Reaction conditions and chromatographic conditions found in Section 3.

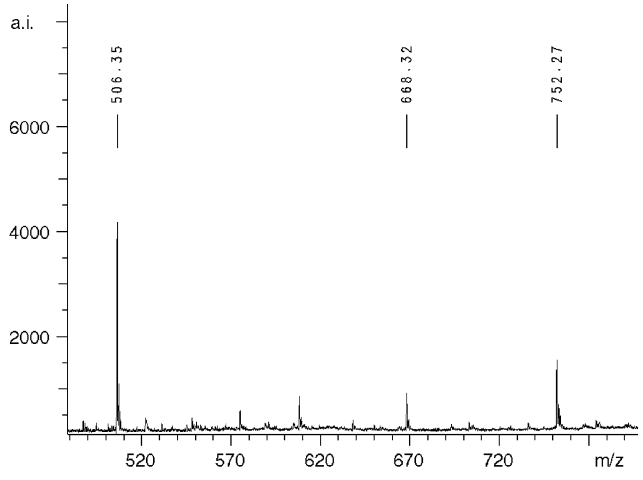

Figure 4. MALDI-TOF spectrum of reaction mixture of 48 h. Peaks of *m/z* 668.32, 506.35, 752.27 belong to **1** acarbose, **3** PTS and **4**, **5** acceptor products, respectively.

752, respectively. Since α-amylases are retaining enzymes, the products should be α-glycosidic bonded regioisomers. Based on the good agreement between the calculated (752.22) and measured (752.27) molecular masses the isolated products **4** and **5** were identified to be transglycosylation compounds between PTS and G-TH.

2.2. Structural analysis of 4

Full $^{1}H/^{13}C$ assignment was carried out using HSQC (Fig. 5), COSY, TOCSY, HMBC and ROESY methods in order to confirm the structure of the main acceptor product **4**. All interglycosidic through bond connections were detected in an HMBC experiment. In particular, the new C1→O–D6 linkage was unequivocally detected. The site of glycosylation was also corroborated by the ^{13}C glycosylation shift (δ_{D6} = 67.50 ppm). Furthermore,

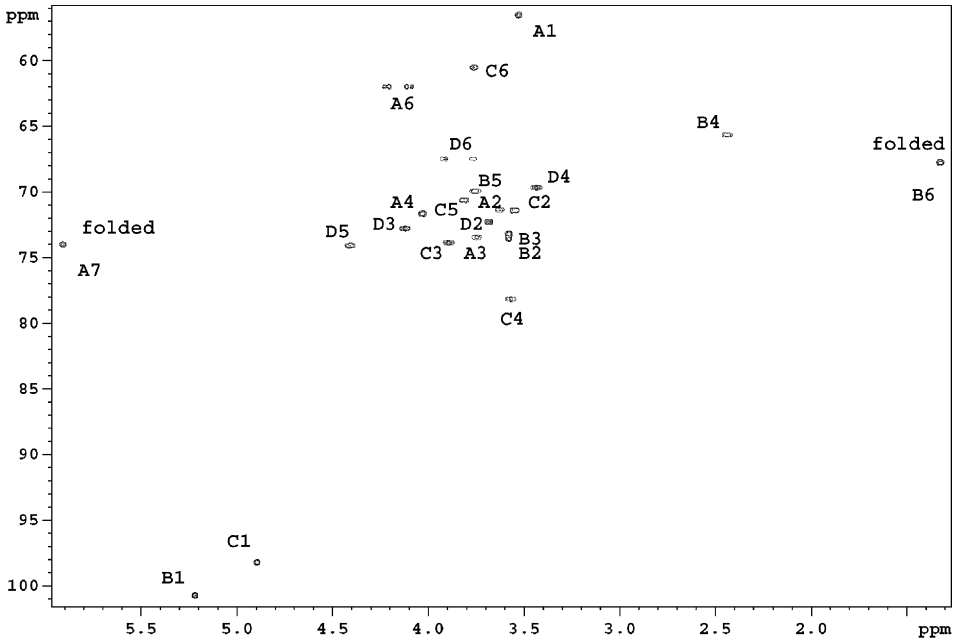

Figure 5. Full two dimensional HSQC NMR spectrum.

all interglycosidic through space connections were also observed in the ROESY experiment. Comparison with the ^{13}C assignment of Bock and Pederson[24] all ^{13}C shift were in reasonable agreement for rings A, B and C. The hydantoin in ring E showed characteristic ^{13}C shifts for the carbonyl and thiocarbonyl groups.

Table 1. ^{1}H and ^{13}C assignments, proton–proton coupling constants and ROESY through-space interactions

Assignment	CH_n	^{13}C shifts	^{1}H shift	$^{3}J_{HH}$ (Hz)	^{1}H ROE's to
A1	CH	56.6	3.531	$J_{12} = 5.1$	A2, A7, **B4**, **B6**
A2	CH	71.35	3.63	$J_{23} = 9.5$	
A3	CH	73.49	3.743	$J_{34} = 7.5$	
A4	CH	71.69	4.031		
A5	C	139.68			
A6	CH_2	62.02	4.232(a)		A7
			4.114(b)		
A7	CH	123.97	5.909		B4(w), **B6**
B1	CH	100.74	5.22	$J_{12} = 3.1$	**C4**, **C6**
B2	CH	73.57	3.582		
B3	CH	73.21	3.582		B5
B4	CH	65.67	2.443	$J_{45} = 8.5$	B2/B3, B5, B6
B5	CH	69.93	3.758		B6
B6	CH_3	17.81	1.328		
C1	CH	98.23	4.897	$J_{12} = 3.8$	C2, **D6a**, **D6b**
C2	CH	71.42	3.563		**D6a**
C3	CH	73.89	3.896	$J_{34} = 9.9$	
C4	CH	78.2	3.582	$J_{45} \sim 10$	C5
C5	CH	70.65	3.826		
C6	CH_2	61.42	3.765		
D1	C	89.52			
D2	CH	72.31	3.695	$J_{23} = 9.8$	D3
D3	CH	72.79	4.122	$J_{34} = 9.7$	
D4	CH	69.67	3.439	$J_{45} = 9.7$	
D5	CH	74.17	4.425		D3, D4, D6a
D6	CH_2	67.5	3.931(a)		
			3.779(b)		
E1	C=O	185.74			
E2	C=O	174.79			

(w = weak effect) of compound **4**. Important interglycosidic ROE connections are highlighted.

Table 1 summarises the measured NMR data. Based on the evaluation of homonuclear ^1H coupling constants of **4** we suggest that ring 'A' has half-chair $^2H_5$, rings 'B' and 'C' have $^4C_1$ chair conformations, virtually identical to that of acarbose.[24] Ring 'D' also has $^4C_1$ chair conformation. Furthermore, ROESY exhibits an interglycosidic ROE peak pattern similar to that of the sporadic NOE-s, reported by Bock and Pederson.[24] With respect to acarbose interglycosidic conformation, a slight difference is that the A7–B4 interaction is weak in **4**, while the A7–B6(Me) distance is short according to a strong ROE. It is supposed, therefore, that the overall conformation of the ABC rings in **4** is similar to that of acarbose and maltotetraose, with a separated hydrophobic and hydrophilic side. The new 1→6 interglycosidic linkage preceding the thiohydantoin function might give sufficient conformational freedom to accommodate at a possibly extended binding site in the target proteins.

We tested the effectiveness and specificity of compound **4** inhibition of 2-chloro-4-nitrophenyl-4-*O*-β-D-galactopyranosyl maltoside ($GalG_2CNP$) hydrolysis catalysed by human salivary α-amylase (HSA). The kinetic analyses confirmed the inhibition of hydrolysis being a mixed noncompetitive type, similar to glucopyranosylidene-spiro-thiohydantoin but compound **4** was found to be a much more efficient salivary amylase inhibitor. Inhibition constants are given in Table 2. Detailed kinetic analysis will be published in a separate article.

Compound **5** was a minor component—a regioisomer of **4** according to MS and UV data—probably possessing 1→4 linkage, but the presence of another minor component with 1→3 linkage could not be excluded either. NMR assignments of **5** were ambiguous because of the limited quantity of the available sample.

Table 2. Inhibition constants of inhibitors on $GalG_2CNP$ hydrolysis catalysed by HSA

Inhibitor	K_{EI} (mM)	K_{ESI} (mM)
G-TH[12]	7.3	2.84
Acarbose	0.00070	0.0001
PTS-G-TH	0.00019	0.00024

BSMA exhibits both high hydrolytic and transglycosylation activity. It is capable of hydrolysing acarbose or transferring a nonreducing end product to the G-TH acceptor molecule (Fig. 6). It is well established that the mechanism of action of retaining enzymes, like α-amylases, is a two step one: the first step involves the formation of a glycosyl–enzyme intermediate, then an attack by water or molecule **2** leads to the hydrolysis products glucose and **3** or transglycosylation products **4** or **5**. In the presence of this acceptor, PTS is primarily transferred to the C-6 of the acceptor, which results in the formation of α-acarviosinyl-(1→4)-α-D-glucopyranosyl-(1→6)-D-glucopyranosylidene-spiro-thiohydantoin. Comparison of the conformations of the highly potent major product in free and bond states is in progress.

3. Experimental

3.1. Materials

Buffer chemicals and other reagents (reagent grade) were obtained from Sigma–Aldrich Co., and acetonitrile

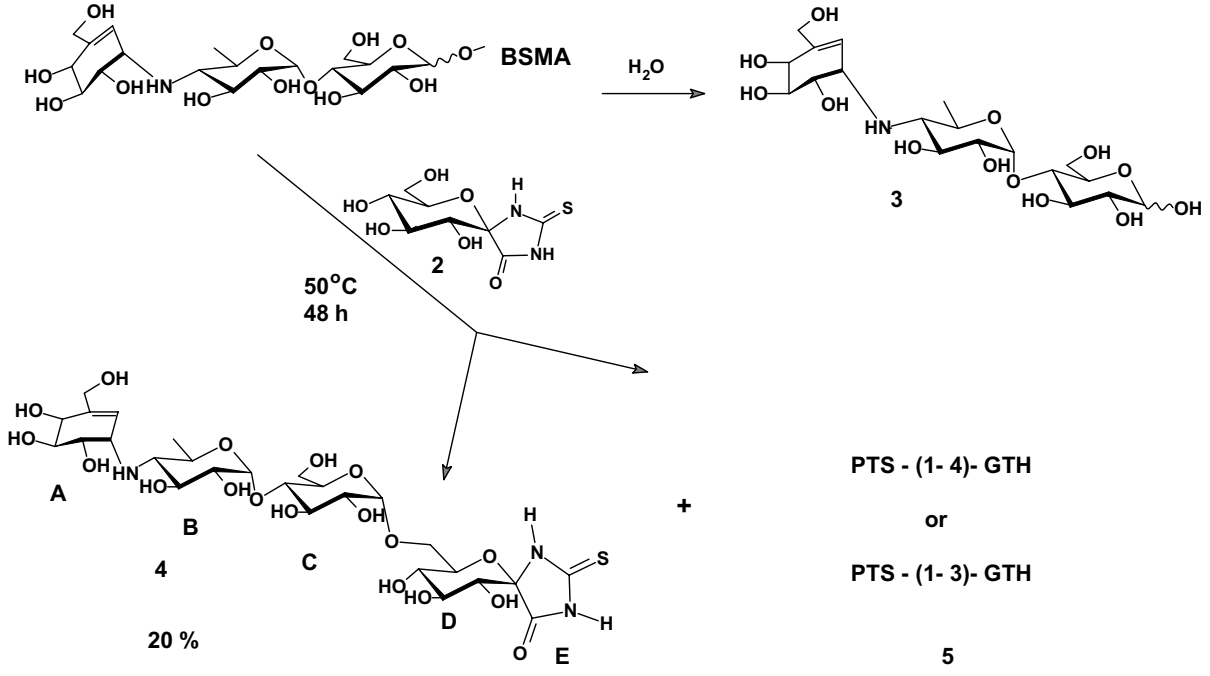

Figure 6. Suggested reaction of acarbose and G-TH catalysed by BSMA.

(HPLC grade) was purchased from Scharlau Chemie S.A., Barcelona, Spain. Purified water was obtained from a laboratory purification system equipped with both ion-exchange and carbon filters (Millipore, Bedford, MA, USA). *B. stearothermophilus* maltogenic amylase, BSMA, was received from the laboratory of Park[21] (activity: 149.1 U mg^{-1}; 1 U: the amount of the enzyme that catalyses the hydrolysis of 1 mmol of maltose equivalent glycosidic bonds per minute from 1% β-cyclodextrin, at pH 6.0, at 55 °C using dinitrosalicylic acid method).[25] G-TH was synthesised chemically.[17] Acarbose was extracted from Glucobay® tablet (Bayer) and its purity was confirmed by NMR and MALDI-TOF MS data.

3.2. General procedure for enzymatic synthesis of G-TH acceptor products

Incubation in trisodium–citrate buffer (50 mM, pH 6.0) containing glucopyranosylidene-spiro-thiohydantoin (10 mM) and acarbose (3 mM) was carried out at 50 °C for different periods of time. The reaction was initiated by the addition of BSMA (20 U). Samples were taken at different time intervals (0–48 h) and the reaction was stopped by boiling for 5 min. After cooling, the precipitated enzyme was removed by filtration through a Millipore 0.2 μm filter. Samples were analysed by TLC, HPLC and MALDI-TOF MS. Preparative scale isolation was carried out by semipreparative HPLC.

3.3. Chromatographic analysis

For HPLC, a Merck–Hitachi LaChrom liquid chromatograph equipped with diode array detector, automatic sampler and HPLC System Manager software, was used. The samples were separated on a Discovery RP Amide C16 column (150 × 4.6 mm, 5 μm), with isocratic elution using MeCN–water = 3:97 eluent, at a flow rate of 1 mL min^{-1}. The effluent was monitored for the thiohydantoin ring at 268 nm.

Kieselgel 60 F_{254} plate was used in MeCN–water = 8:2 eluent for TLC. The compounds were detected under UV light and by charring with 50% aqueous sulfuric acid and heating at 120 °C to follow the conversion of acarbose.

3.4. Mass spectrometry

The MALDI spectra of the compounds were obtained in positive-ion mode using a Bruker Biflex MALDI-TOF mass spectrometer equipped with delayed-ion extraction. Desorption/ionisation of the sample molecules was effected with a 337 nm nitrogen laser. Spectra from multiple (at least 100) laser shots were summarised using 19 kV accelerating and 20 kV reflectron voltage. External calibration was applied using the $[M+Na]^+$ peaks of cyclodextrins DP 6–8, *m/z*: 995, 1157, 1319 Da, respectively. The spectrum was obtained in 2,5-dihydroxy benzoic acid (DHB) matrix using the dry-droplet method.

The negative ion ESI spectra were recorded using a Bruker BioTof electrospray mass spectrometer. External calibration was applied using $[M-H]^-$ peaks of sodium trifluoroacetate cluster ions in mass range of 200–1200 Da. The resolution of measurements was better than 6500, the accuracy of measured mass was below 5 ppm. The concentration of sample was 10 μM using MeOH–water = 1:1 as a solvent.

3.5. ^1H and ^{13}C NMR analyses

^1H (500.13 MHz) and ^{13}C NMR (125.76 MHz) spectra were recorded with Bruker DRX-500 spectrometer in D_2O. Chemical shifts were referenced to external DSS. Full $^1H/^{13}C$ assignment was carried out using HSQC, COSY, TOCSY, HMBC and ROESY methods in order to prove the structure of the main acceptor product. Off resonance ROESY spectra were obtained at 500 MHz, at 300 K in a shigemi tube using ca. 3 mg of **4** in 225 μL D_2O solution. The strength of the spin lock field was γB_1 = 8700 Hz, and it was off set ±5900 Hz from the RF carrier for the 2 × 50 ms = 100 ms mixing time. Sixty four transients were acquired for each of the 340 increments in the 2D experiment. Squared cosine window function was used for data processing.

3.6. α-Acarviosinyl-(1→4)-α-D-glucopyranosyl-(1→6)-D-glucopyranosylidene-spiro-thiohydantoin (4)

Incubation in trisodium–citrate buffer (200 μL, 50 mM, pH 6.0) containing glucopyranosylidene-spiro-thiohydantoin (20 mg, 10 mM) and acarbose (15 mg, 3 mM) was carried out at 50 °C for 48 h. The reaction was initiated by the addition of BSMA (20 U). After cooling the precipitated enzyme was removed by filtration through a Millipore 0.2 μm filter. Purification was carried out by semipreparative HPLC using Discovery RP Amide C16 column (25 cm × 10 mm, 0.5 μm), acetonitrile–water = 3:97 as eluent with flow rate 3 mL/min. After lyophilisation 11.8 mg white powder was obtained, the yield being 20%. UV maxima were 235, 268 nm, for both **2** and **4**. The NMR data are presented in Table 1. UV, ^1H, ^{13}C NMR and ROESY spectra can be found in Supporting data. $[\alpha]_D^{20}$ 100.5 (*c* 0.1, H_2O). MALDI-TOF MS: $[M+Na]^+$, found 752.27. $C_{27}H_{43}O_{18}N_3SNa$ requires 752.22. HRMS (ESI): $[M-H]^-$, found 728.2214. $C_{27}H_{42}O_{18}N_3S$ requires 728.2179.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2005.03.003.

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