Highly Productive Autocondensation and Transglycosylation Reactions with *Sulfolobus solfataricus* Glycosynthase

Antonio Trincone,*[a] Assunta Giordano,[a] Giuseppe Perugino,[b] Mosè Rossi,[b, c] and Marco Moracci[b]

Transglycosylation reactions (autocondensation of the substrate or transfer of the glycon donor moiety to different acceptors) with the hyperthermophilic glycosynthase from Sulfolobus solfataricus *acting in dilute sodium formate buffer at pH 4.0 are reported; the use of 4-nitrophenyl β-glucopyranoside as both donor and acceptor in the self-transfer reaction and a highly productive reaction with 1.1 M 2-nitrophenyl β-glucopyranoside were possible. Interesting effects, governed by the anomeric configuration and lipophilicity of heteroacceptors, on the regioselectivity and yield of reactions were found for the first time with this enzyme and are discussed. The results demonstrate the unexplored synthetic potential of this glycosynthase; the tuning of the reaction conditions and the choice of different donors/acceptors can lead to products of applicative interest.*

Introduction

Glycobiology and related disciplines have received enormous interest in recent years as they shed new light on the functional roles of carbohydrates in biological events, thereby leading to an understanding of mechanisms of important pathologies and to the development of new therapeutics. Enzymatic strategies for the high-yield and stereospecific construction of glycosidic bonds are mainly based on the action of two types of enzymes: glycoside hydrolases (endo- and exo-glycosidases) and glycosyltransferases.[1,2] Recently, glycosynthases were added to the available arsenal of biocatalysts and they emerged as a significant development in this field. These engineered enzymes lack the nucleophile in their active site; they are able to react with glycosyl fluorides or activated aryl glycosides as donors to form transglycosylation products, which can accumulate in the reaction mixture due to the loss of the hydrolytic activity towards nonactivated compounds. The thermophilic representatives of the glycosynthases can be used in inverting (with α-glycosyl fluoride as the donor) or retaining (with aryl β-glycosides as donors) reactions. In the latter case, while mesophilic glycosynthases can react only with highly activated 2,4-dinitrophenyl glycosides in the presence of a high concentration of sodium formate as an external biomimicking nucleophile,[3] stable 2-nitrophenyl glycosides can be also used by the thermophilic biocatalysts.[4]

Several laboratories have put considerable effort into producing glycosynthases with new characteristics to expand their potential applications; biodiversity may expand the repertoir of glycosynthases by using enzymes from different sources. For instance, different regioselectivity has been observed with thermophilic enzymes.[5] To the best of our knowledge, more than ten glycoside hydrolases (GHs), belonging to seven different GH families from bacteria, eukarya, and archaea, have been modified as efficient glycosynthases. (For a review, see ref. [5].)

Glycosynthases are characterized by a certain degree of reactivation compared to the wild-type counterpart when used in the appropriate conditions for synthesis; at pH values below neutrality in diluted sodium formate buffers it has recently been observed that the rate of catalysis (k_{cat}) values of the Ss-β-glyE387G from *Sulfolobus solfataricus* and of two other hyperthermophilic glycosynthases from *Thermosphaera aggregans* and *Pyrococcus furiosus* (Ta-β-glyE386G and CelBE372A, respectively) were 17-fold higher than the values observed at a high concentration of sodium formate and were at levels comparable to the wild-type enzymes.[6] The recently reported reactivation approach improved the efficiency of the reaction in terms of reaction time and the amounts of enzyme used, as established on an analytical scale,[6] and the results were rationalized by hypothesizing that at acidic pH values the acid/base catalytic residue is protonated and can better perform the first step of the reaction. The good stability expressed by the three hyperthermophilic glycosynthases also allowed the synthesis of glycosidic linkages efficiently for long reaction times,[6] as reported mainly for Ta-β-glyE386G[7] in practical applications.

Highly active glycosynthases with multiple mutations were also recently prepared,[8] although, to the best of our knowledge, no actual synthesis by using these enzymes was reported.

In this paper we report on the results obtained for the preparative autocondensation of donor substrates (2- and 4-nitro-

[a] *Dr. A. Trincone, Dr. A. Giordano*
Istituto di Chimica Biomolecolare—Consiglio Nazionale delle Ricerche
Via Campi Flegrei 34, 80072 Pozzuoli, Naples (Italy)
Fax (+39) 81-804-1770
E-mail: antonio.trincone@icb.cnr.it

[b] *Dr. G. Perugino, Prof. M. Rossi, Dr. M. Moracci*
Institute of Protein Biochemistry—Consiglio Nazionale delle Ricerche
Via P. Castellino 111, 80131, Naples (Italy)

[c] *Prof. M. Rossi*
Dipartimento di Chimica Biologica, Università di Napoli "Federico II"
Via Mezzocannone 16, 80134, Naples (Italy)

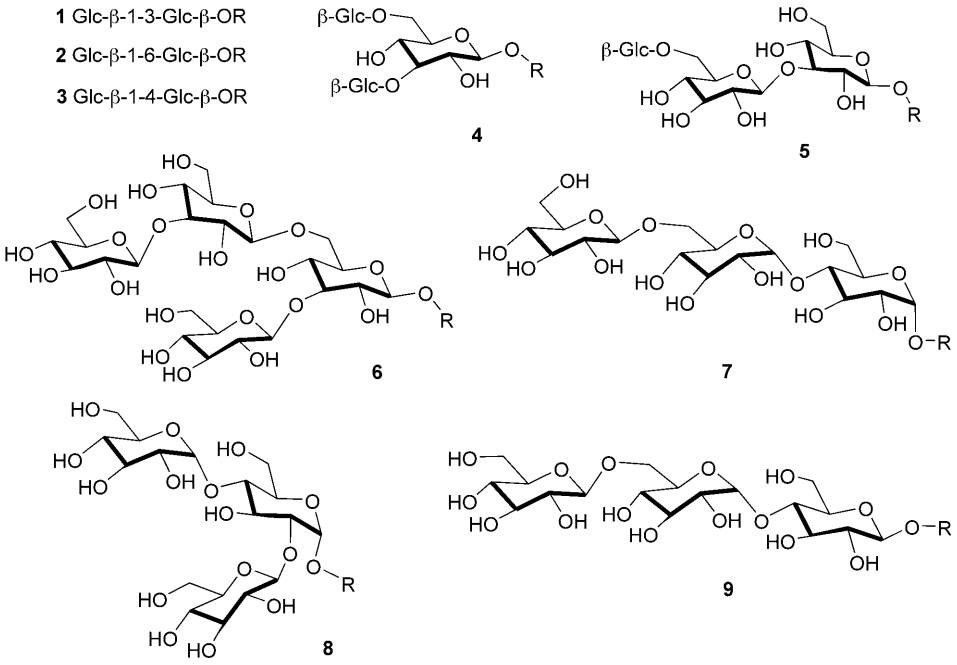
 DOI: 10.1002/cbic.200400430

Table 1. Oligomerization of nitrophenyl β-D-glucopyranosides and direct synthesis of trisaccharides by Ss-β-glyE387G.

	[Products] [μmol mL^{-1}]			C[b]	Glc[c]	[substrate]	E[d]	t [h]	Conditions
	2-nitrophenyl β-D-laminaribioside (**1**) 2-nitrophenyl β-D-gentiobioside (**2**) 2-nitrophenyl β-D-cellobioside (**3**)	trisaccharides **4** and **5** and others	tetrasaccharides **6** and others	[%]	[%]	[μmol mL^{-1}]			
1	8.0	4.3	0.8	90	15	38.5	1103	1	4 M sodium formate
2	33.3	9.6	–	94	6	166	481	6.5	50 mM sodium formate (pH 4.0)[e]
3	260	96	10.8	92	10	1100	300	16	50 mM sodium formate (pH 4.0) with 50% acetonitrile
4	traces of **2** and **3**	17.4	–	72	–	45	2600	8	50 mM sodium formate (pH 4.0)
5[a]	32.1	10.7[a]	–	98	56	125	1200	24	50 mM sodium formate (pH 4.0)

[a] Oligomerization of 4-nitrophenyl β-D-glucopyranoside. (For structures, see Scheme 1 where R = 4-nitrophenyl.) [b] Conversion of the substrate at the end of the reaction indicating the completeness of reaction (translycosylation+hydrolysis). [c] Glc = glucose content. Values are calculated gravimetrically on mmolar scale reactions and represent the percentage of the theoretical glucose value that would be obtained at total hydrolysis. No significant chemical hydrolysis was detected in appropriate blank experiments.[6] [d] Enzyme quantity in μg of protein per mmol of substrate. [e] The substrate is added portionwise to increase the selectivity towards the class of disaccharide compounds.

phenyl β-D-glucopyranosides) and for a series of transglycosylation reactions with Ss-β-glyE387G. In the first case, branched oligoglucosides of up to four sugar units were formed with high productivity. In the transglycosylation reactions, 2-nitrophenyl β-D-glucopyranoside was used as the donor and different aryl and alkyl mono- and disaccaharide substrates containing α- and β-glycosidic linkages were used as acceptors. Some interesting effects governed by the anomeric configuration and lipophilicity of heteroacceptors on the regioselectivity and yield of the reactions were found for the first time with this enzyme and are discussed.



Scheme 1. Oligosaccharides obtained with the hyperthermophilic glycosynthase Ss-β-glyE387G. Compounds **1**–**6** were obtained by using the autocondensation reactions. When R = 2-nitrophenyl the product mixture includes 50% of disaccharides (compounds **1**–**3**), 40% of trisaccharides, and 10% of tetrasaccharides. Within the class of disaccharides, compound **1** is the most abundant (80%), followed by **2** (18%) and **3** (2%). The two major trisaccharides obtained are **4** and **5** (70% and 14%, respectively), while the remaining 16% is composed of at least four trisaccharides. The tetrasaccharides are a mixture of five compounds; however, 54% of this class is constituted by the single component **6**. The structures of these compounds were established by two-dimensional NMR spectroscopy and by carefully kinetically monitored enzymatic hydrolysis reactions with the Ss-β-gly wild-type enzyme and analysis of the products obtained, as previously reported.[9] Compounds **1**–**5**: R = 2-nitrophenyl or 4-nitrophenyl; compound **6**: R = 2-nitrophenyl; compounds **7**–**9**: R = 4-nitrophenyl.

Results and Discussion

Oligomerization of nitrophenyl β-D-glucopyranosides

The general features of the autocondensation reaction used for the synthesis of oligosaccharides of 2-nitrophenyl β-D-glucopyranoside in 4 M sodium formate are described in Table 1, reaction 1, and the structures of the products obtained are shown in Scheme 1. The disaccharides formed (compounds **1**–**3**) were elongated by acting in turn as acceptors, thereby forming the trisaccharides; the latter compounds, after a further elongation step, produced the tetrasaccharides.[9]

A great improvement in this synthetic procedure by using 50 mM sodium formate buffer (pH 4.0) is reported here on the preparative scale (reactions 2–5) in Table 1.

Reaction 2 (Table 1) is used for the synthesis of 2-nitrophenyl laminaribioside; portionwise addition of the substrate resulted in a preference for the synthesis of the disaccharides (3.4:1 ratio to trisaccharides), with respect to the ratios observed for reactions 3 (2.7:1) and 1 (1.8:1).

In reaction 3, the concentration of the products obtained is greatly enhanced. Disaccharides **1**–**3** (Scheme 1) still predominate as products, in the proportions previously observed. In this reaction, the millimolar concentration of sodium formate and the addition of cosolvent (50% acetonitrile) allowed us to use a molar concentration of substrate; oligomers longer than tetrasaccharides were not obtained, a result suggesting that this is the upper molecular dimension allowed by the active site architecture. It is also worth noting that, with respect to the previous conditions (reaction 1, Table 1), we employed one third less enzyme per mmol of substrate and that portionwise addition of biocatalyst to the reaction mixture in 3 aliquots over 16 h of reaction time drove the reaction to almost total conversion of the donor.

Reaction 4 (Table 1) was aimed at the direct synthesis of trisaccharide(s) by starting with 2-nitrophenyl β-D-glucopyranoside as the donor and 2-nitrophenyl laminaribioside as the acceptor in a 1:3 molar ratio. The regioselectivity of the reaction was the same as in the previous cases, with compound **4** being the most abundant trisaccharide formed. However, the presence of other trisaccharides was noticed by TLC and HPLC; in addition, trace amounts of disaccharides **2** and **3** were also formed.

In addition to 2-nitrophenyl β-D-glucopyranoside, in the new reaction conditions adopted,[6] the increase in reactivation rates allowed the use (reaction 5, Table 1) of 4-nitrophenyl β-D-glucopyranoside as a donor/acceptor substrate and the formation of disaccharides with different selectivity (**2** > **1** > **3**, Scheme 1) and a mixture of trisaccharides, as established by NMR spectroscopy and mass spectrometry.[9] The branched nature (due to the presence of β-1,3/1,6 glycosidic linkages) of the two most abundant trisaccharides formed was also noticed in these products by the analysis of DEPT experiments.

Tri- and tetrasaccharides are of particular interest: glucans with a β-1,3/1,6 glycosidic linkage pattern have shown particularly strong immunomodulatory activity as biological response modifiers.[10] In a recent report, a free trisaccharide with the carbohydrate domain of compound **4** (Scheme 1) was synthesized by using β-glycosidases from *Sclerotinia sclerotiorum* and *Aspergillus niger*.[11]

The importance of the branched structure of the compounds in Scheme 1 and the presence of chromophoric residues makes these compounds useful as substrates for new enzymes and in the study of the structure of the active sites.

Effect of added heteroacceptors on activity

In the synthesis with highly active glycosynthases such as Ss-β-glyE387G in 50 mM sodium formate at pH 4.0, the choice of the substrate acting as donor is important. A certain degree of reactivation is observed with galactose- and xylose-based substrates. 4-nitrophenyl β-glucopyranoside is also a substrate for the enzyme (Table 2, upper section) and a very low enzymatic activity was observed on cellobiose.[6] On the other hand, the ideal acceptor is not a substrate for the hydrolysis and its affinity for the donor active site must be as low as possible to avoid any interfering action; in contrast, the affinity for the proper enzymatic acceptor site should be as high as possible compared to water (hydrolysis) and donor (autocondensation), which may act as alternative acceptors.

Table 2. Kinetic constants for different nitrophenyl substrates (upper part) and the effects of the presence of different acceptors on the kinetic constants of Ss-β-glyE387G with 2-nitrophenyl β-D-glucopyranoside (bottom part).

Substrates	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [$s^{-1}mM^{-1}$]
2-nitrophenyl β-glucopyranoside	7.45 ± 0.68	330.0 ± 8.9	44.2
2-nitrophenyl β-galactopyranoside	4.57 ± 0.95	23.4 ± 1.6	5.1
2-nitrophenyl β-xylopyranoside	18.45 ± 1.97	39.0 ± 2.1	2.1
4-nitrophenyl β-glucopyranoside	9.06 ± 1.31	84.0 ± 4.8	9.3

Acceptors	c [mM]	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [$s^{-1}mM^{-1}$]
4-nitrophenyl	1	8.66 ± 0.92	315.5 ± 10.4	36.4
β-glucopyranoside	4	15.27 ± 1.61	322.6 ± 12.6	21.1
	8	26.41 ± 5.07	338.7 ± 29.4	12.8
4-nitrophenyl	1	7.44 ± 0.63	333.3 ± 6.1	44.8
α-glucopyranoside	4	7.40 ± 0.59	321.5 ± 7.6	43.4
	8	7.13 ± 0.50	316.7 ± 6.6	44.4
4-methyl umbelliferyl	1	19.70 ± 2.47	297.0 ± 9.9	15.1
β-glucopyranoside	4	39.17 ± 5.80	255.4 ± 19.9	6.5
	8	46.47 ± 4.31	208.6 ± 17.2	4.5
methyl β-	8	6.77 ± 0.75	310.7 ± 9.9	45.9
glucopyranoside	16	6.71 ± 0.69	314.0 ± 9.4	46.8
methyl α-	8	6.97 ± 0.69	312.7 ± 9.0	44.9
glucopyranoside	16	7.21 ± 0.74	305.9 ± 9.3	42.5
2-nitrophenyl	1	10.88 ± 0.80	310.3 ± 7.8	29.2
β-laminaribioside	4	21.32 ± 3.02	336.1 ± 20.2	15.8
	8	29.91 ± 3.09	327.2 ± 16.2	10.9

Despite the high amount of enzyme used, reaction 4 in Table 1 was noticed to be very slow and it reached only 72% substrate conversion. This observation may suggest that the acceptor competes with the donor in the binding pocket for the donor in the active site of the enzyme. In fact, since access of the disaccharide acceptor into the donor active site of the glycosynthase is unproductive, it could slow down the reaction, thus lowering the conversion of the donor. Due to the high activity of this glycosynthase in acidic conditions, the hydrolysis of the β-1,3 interglycosidic linkage of the acceptor (producing 2-nitrophenyl glucopyranoside) cannot be completely excluded (see also Table 3).

To address this question in general, the effects of the presence of different acceptors on the kinetic constants of Ss-β-glyE387G were studied (Table 2, lower part). 4-nitrophenyl β-D-glucopyranoside, 4-methyl umbelliferyl β-D-glucoside, and 2-nitrophenyl β-D-laminaribioside at different concentrations (1–8 mM) affected, to different extents, the rate of hydrolysis of 2-nitrophenyl β-D-glucopyranoside (in the 0.5–60 mM range

studied) by lowering the values of k_{cat}/K_M (K_M = Michaelis constant). Remarkably, the K_M value increased linearly while the k_{cat} values were unaffected by the increasing concentration of the acceptors; this indicates that these compounds compete with the substrate in the donor active site of Ss-β-glyE387G. From these data, the calculated inhibition constants (K_i) for 4-methyl umbelliferyl β-D-glucoside and 2-nitrophenyl β-D-laminaribioside were 0.98 ± 0.15 and 2.64 ± 0.23 mM, respectively.

It is worth noting the effect of the anomeric configuration of the substrate acceptors on this interfering action; the α anomers are not as able to access the active site for the donor as the β anomers, even in the presence of an aglycon with a suitable lipophilic nature, as observed for 4-methyl umbelliferyl α-glucoside which is not a disturbing substrate (data not shown). However, a certain amount of lipophilic character is needed also for β-anomers (see the null effect of methyl β-glucoside).

Transglycosylation reactions to heteroacceptors

Synthesis of disaccharides: The data in Table 2 paralleled very well with the results of the synthetic reactions conducted by using 2-nitrophenyl β-D-glucopyranoside as the donor and different acceptors.

Different aryl monosaccharides (Table 3) were used as acceptors for the synthesis of disaccharide derivatives. As can be seen from the analysis of products, the β anomer of 4-methyl umbelliferyl glucoside behaves in different manner with respect to its α anomer (Table 3, entries 1 and 2). The former revealed a strong interfering action on the donor active site (Table 2), thus leading to long reaction times and low donor conversions. Although the α anomer does not possess any interfering action and the conversion of the donor in this case is almost quantitative, the yield of the disaccharide is low; this is probably due to the low affinity of this molecule for the acceptor site of the enzyme. Nevertheless, the yield of the disaccharide formed with respect to the converted material is still reasonable for these interesting chromophoric glycosides (Table 3).[12] Interestingly, 4-methyl umbelliferyl α-gentiobioside (β-1,6) is the only regioisomer formed by transglycosylation, thus indicating a different spatial approach of the α-glucoside in the acceptor site of the enzyme to that of the β anomer.

When different 2- and 4-nitrophenyl α-glycosides are used as acceptors, high conversion of the donor and high yields of disaccharide products were obtained (Table 3, entries 3–6); these results provide strong evidence that 1) α-anomeric linkages abolish any interfering action of these molecules toward donor active site, 2) the lipophilic nature and the dimension of the nitrophenyl ring are suitable characteristics to enhance the affinity for the acceptor site, and 3) the general lipophilic nature of the aglycon plays a relevant role for the interfering action in the donor site of the enzyme. Confirmation for the latter point is provided by comparison of the action of the two α- and β-4-nitrophenyl glucosides (Table 2) and by the poor yield of products when poor competitors such as alkyl α- and β-glucosides are used (Table 3, entries 8 and 9). Furthermore, it is of interest that selective β-1,6 glycosylation is obtained with all aryl α-glycosides, despite the nature (2- and 4-nitrophenyl) of the aglycons, a result indicating the strong influence of the anomeric linkage of the acceptor on the accomodation of the molecule in the acceptor active site. β-1,6 products were also obtained in control experiments with wild-type enzyme, although in very low yield (10–15%).

Remarkably, with our glycosynthase a quantitative yield of one regioisomer, Glc-β-(1–6)-Gal, is obtained when 4-nitrophenyl α-galactopyranoside is used as the acceptor (Table 3, entry 4); the carbohydrate moiety of the compound prepared is part of a cell-wall component of *Haemophilus* (*Actinobacillus*) *pleuropneumoniae* type 2.[13]

The terminal disaccharide unit of a glycopeptide with phytoalexin elicitor activity,[14] Glc-β-(1–6)-Man, is also prepared in good yield (Table 3, entry 5).

Table 3. Transglycosylation reactions catalyzed by Ss-β-glyE387G.

	Acceptor	C[a] [%]	m.e.[b]	*t* [h]	E[c]	Products[d]
1	4-methyl umbelliferyl β-glucopyranoside	45	2	24	903	4-methyl umbelliferyl β-laminaribioside[e] (60% yield)
2	4-methyl umbelliferyl α-glucopyranoside	95	3	2.5	2100	4-methyl umbelliferyl α-gentiobioside[f] (33% yield)
3	4-nitrophenyl α-glucopyranoside	100	3	1	1807	Glc-β-(1–6)-Glc α-PNP[f] (61% yield)
4	4-nitrophenyl α-galactopyranoside	100	3	1.5	2100	Glc-β-(1–6)-Gal α-PNP[f] (quantitative yield)
5	4-nitrophenyl α-mannopyranoside	100	3	1.5	2100	Glc-β-(1–6)-Man α-PNP[f] (72% yield)
6	2-nitrophenyl α-2-deoxy-*N*-acetyl glucopyranoside	100	3	2	2100	Glc-β-(1–6)-GlcNAc α-ONP[f] (75% yield)
7	allyl α-galactopyranoside	100	3	1	1800	total donor hydrolysis
8	methyl α-glucopyranoside	100	2	1	2000	trace of expected products; oligomerization of donor
9	methyl β-glucopyranoside	100	2	1	2000	trace of expected products; oligomerization of donor
10	2-nitrophenyl β-laminaribioside	72	3	8	2600	branched trisaccharides **4** and **5** (53.3% yield)
11	4-methyl umbelliferyl β-laminaribioside	40	2	24	2941	acceptor hydrolysis, donor oligomerization, trace of acceptor trisaccharides
12	4-methyl umbelliferyl β-cellobioside	85	2	8	2000	mixture of linear trisaccharides of acceptor (9% yield)[g]
13	4-nitrophenyl β-lactopyranoside	90	2	24	1923	branched trisaccharide of acceptor (8% yield)[g]
14	4-nitrophenyl α-maltoside	100	3	1.5	2100	**7** and **8**[g] (6:4, 40% yield)
15	4-nitrophenyl β-maltoside	95	3	1.5	2100	oligomerization of donor; approximetly 5% yield of **9**

[a] Percentage of substrate conversion. [b] Molar excess of acceptor with respect to the donor (2-nitrophenyl β-D-glucopyranoside). [c] Enzyme quantity in μg of protein per mmol of substrate. [d] The presence of oligomers of the donor was analyzed by TLC with the use of authentic standards. The structures of the main transglycosylation products are established as follows: [d] as previously described,[7] [e] by using DEPT experiments and two-dimensional NMR spectroscopy, [f] by using DEPT experiments and/or mass spectra, and [g] by using two-dimensional NMR spectroscopy.

The disaccharide unit Glc-β-(1–6)-GlcNAc, also obtained in interesting yield and purity (Table 3, entry 6), is present in the antibiotic moenomycin A.[15]

Synthesis of trisaccharides

A series of disaccharide templates were used as acceptors for the synthesis of trisaccharide derivatives (Table 3, entries 10–15).

With both substrate compounds possessing β-1,3 interglycosidic linkages (2-nitrophenyl β-laminaribioside, entry 10, and 4-methyl umbelliferyl β-laminaribioside, entry 11), the results are in accordance. *Thermosphaera aggregans* glycosynthase, however, produced 16% yield of two trisaccharide compounds in the same reaction to that in entry 11 (Table 3).[7]

It is of interest that the β-1,4 interglycosidic linkage in 4-methyl umbelliferyl β-cellobioside, (Table 3, entry 12) allowed the formation of trisaccharides not glycosylated at the C-6 positions (linear compounds as observed by DEPT experiments) albeit in modest yields.

4-Nitrophenyl β-D-lactopyranoside (Table 3, entry 13), shows a result similar to the one observed under 4 M sodium formate conditions.[9]

Encouraged by the excellent results obtained with α acceptors for the synthesis of disaccharides, we tested two examples possessing α-1,4 interglycosidic linkages, 4-nitrophenyl α- and β-maltoside, for the synthesis of trisaccharide derivatives. The former acceptor (Table 3, entry 14) produced two out of seven possible regioisomers, namely, compounds **7** and **8** (Scheme 1). The yield of these trisaccharides was approximately 40% and their ratio was 6:4; moreover, the conversion of the donor substrate was 100% in approximately 1.5 h, thus indicating that the acceptor does not have any disturbing effect in the active site for the donor, as was also observed for the other α-glycosides.

The presence of β-1,6 glycosylation in the product **7** (Scheme 1) is in accordance with the results for disaccharide formation from α acceptors. The formation of the other trisaccharide with a β-1,2-linked glucose as the internal carbohydrate unit (**8**, Scheme 1) is interesting and paralleled with a similar result observed for the transferring of the glucal moiety on 4-nitrophenyl α-glucopyranoside as operated by the wild-type enzyme.[16]

The trisaccharide moiety of compound **7** (Scheme 1) is present in a recently synthesized β-glucosyl derivative of maltopentaoside used for the differential analysis of human α-amylase isozymes for the diagnosis of pancreatitis and parotisis.[17]

The use of the disaccharide 4-nitrophenyl β-maltoside (Table 3, entry 15) as an acceptor resulted in different behavior; donor oligomerization was primarily noticed, which paralleled with poor yield of acceptor glycosylation product (5%).

It is clear from this result that the β-aryl-linked group in this acceptor is responsible for the poor binding capacity of this molecule for the acceptor site. As in the case of entry 14, the α-interglycosidic linkage is responsible for the complete lack of interfering effect in the donor site and governs the regioselectivity of the reaction to give, as the overall result, a poor yield of a β-1,6 linkage containing trisaccharide (**9**, Scheme 1) and the total conversion of donor.

Conclusion

In this article a clear pattern for the glycosylation conducted by the hyperthermophilic glycosynthase from *S. solfataricus* was reported.

Subtle changes in the nature of the aglycon, other than its lipophilic character, are issues for acceptors containing β anomeric groups; the formation of the β-1,3 linkage is observed with acceptors containing the 2-nitrophenyl group, while β-1,6-disaccharides are predominant with 4-nitrophenyl β-D-glucoside (Table 1). When a β linkage of the acceptor is between two sugars, as for the formation of trisaccharides, the β-1,3 acceptors possess affinity for the donor active site and are also partially hydrolyzed. Alternatively, the β-1,4-based acceptor (Table 3, entry 12) furnished only linear trisaccharides, albeit in very poor yield.

The presence of α linkages in the acceptors induces β-1,6 glycosylation despite the nature of the aglycons (Table 3, entries 2–6 and 14–15). In these cases, the yield is higher with the increasing lipophilic nature of the aglycon; however, the yield depends on the dimensions and nature of this group. In fact, while maltose as free disaccharide gave very poor yields of trisaccharide products (data not shown), we reached 33% yield with 4-nitrophenyl α-D-glucopyranoside (Table 3, entry 2) and a quantitative yield in the reaction shown in entry 4 (Table 3).

An interesting functionalization on the O-2 position was noticed with 4-nitrophenyl α-maltoside as the acceptor. Both of the easily separable trisaccharides **7** and **8**, formed in 40% yield, add molecular diversity to the library of possible compounds that can be formed by our hyperthermophilic glycosynthase and new structures to the arsenal of substrates for enzymologists.

Experimental Section

General: Nitrophenyl glycosides were obtained from Sigma (St. Louis, MO) and reversed-phase silica gel and TLC silica gel plates were obtained from Merck (Darmstadt, Germany). The protein concentration was determined by using the Bradford assay system (Biorad, USA). Compounds were visualized (TLC) under UV light or by charring with α-naphthol reagent. Acetylation of compounds was performed with pyridine/Ac_2O at room temperature; the solvents were removed by N_2 stream, and the reaction mixture was purified by silica gel chromatography or preparative TLC. Chromatographic purifications were performed by using methanol/water or EtOAc/MeOH gradients for Lobar reversed-phase and silica gel chromatography, respectively. HPLC was performed on a Milton Roy apparatus equipped with a Waters UV detector as previously reported.[9] NMR spectra were recorded on Bruker instruments at 400 or 300 MHz. Samples for NMR analysis were dissolved in suitable solvents and the signal of the solvent was used as an internal standard. ESI-MS spectra were obtained on a Micromass Q-Tof mass spectrometer, while FAB-MS spectra were obtained as previously described.[9]

Enzyme and kinetic studies: Wild-type Ss-β-gly and mutant Ss-β-glyE387G enzymes were expressed and purified as previously described.[6] The enzymes used for all subsequent reactions and kinetic studies were >95% pure by sodium dodecylsulfate (SDS) PAGE. The samples (2.1 mg mL^{-1}) are stable for several months when stored at 4°C in sodium phosphate 20 mM (pH 6.5) buffer. The procedures utilized for the kinetic characterization of the hyperthermophilic glycosynthase from *S. solfataricus* have been described previously.[6]

Oligosaccharide synthesis: All details regarding each synthetic reaction are described in Table 1 and Table 3. Total amounts (0.02–1 g) or portions of substrates were dissolved at 65°C in the proper amount of 50 mM sodium formate buffer at pH 4.0 and the enzyme was added in one or multiple aliquots to the reaction mixture; the reactions were monitored by TLC developed in EtOAc/MeOH/H_2O (70:20:10). At the end of the reaction, usually after total donor conversion, the reaction mixture was cooled, lyophilized, and subjected to different chromatographic purification procedures depending on the nature of products.

Compounds in Scheme 1 and the branched trisaccharide reported in entry 13 of Table 3 were purified as previously described.[9] The products of the transglycosylation reaction reported in entries 1 and 12 of Table 3 were purified as previously described;[7] the products reported in entries 2–6 and 14–15 in Table 3 were purified by preparative TLC (EtOAc/MeOH/H_2O, 70:20:10) and acetylated, then the peracetylated derivatives were used for NMR spectroscopy.

Structural assignment: The structures of compounds **1**–**6** (Scheme 1) were established both by two-dimensional NMR spectroscopy and by carefully kinetically monitored enzymatic hydrolysis reactions with the Ss-β-gly wild-type enzyme for which the products obtained were analyzed as previously reported.[9]

The structures of compounds reported in Table 3 were determined by NMR spectroscopy by comparison with literature-reported values[7,9] or two-dimensional NMR spectra (COSY, ^{1}H–^{13}C correlation).

Glc-β-(1–6)-GlcNAc α-ONP: ^{1}H NMR: δ(β-1,6-linked glucose unit) = 4.51 (J = 7.6 Hz, H-1; ^{13}C NMR: δ = 100.8 ppm (C-1)), 4.98 (H-2), 5.18 (H-3), 4.99 (H-4), 3.66 (H-5), 4.10–3.66 ppm (H-6); δ(α-aryl-linked glucosamine unit) = 5.52 (J = 3.5 Hz, H-1; ^{13}C NMR: δ = 99.0 ppm (C-1)), 4.50 (H-2), 5.37 (H-3), 5.03 (H-4), 4.14 (H-5), 3.90–3.60 ppm (H-6).

Glc-β-(1–6)-Gal α-PNP: ^{1}H NMR: δ(β-1,6-linked glucose unit) = 4.44 (J = 7.9 Hz, H-1; ^{13}C NMR: δ = 100.1 ppm (C-1)), 4.83 (H-2), 5.13 (H-3), 4.95 (H-4), 3.65 (H-5), 4.07–4.15 ppm (H-6); δ(α-aryl-linked galactose unit) = 5.82 (J = 3.5 Hz, H-1; ^{13}C NMR: δ = 95.2 ppm (C-1)), 5.29 (H-2), 5.56 (H-3), 5.49 (H-4), 4.26 (H-5), 3.65–3.77 ppm (H-6).

Glc-β-(1–6)-Man α-PNP: ^{1}H NMR: δ(β-1,6-linked glucose unit) = 4.45 (J = 7.6 Hz, H-1; ^{13}C NMR: δ = 100.4 ppm (C-1)), 5.04 (H-2), 5.19 (H-3), 5.02 (H-4), 3.67 (H-5), 4.19–4.12 ppm (H-6); δ(α-aryl-linked mannose unit) = 5.62 (J = 1.9 Hz, H-1; ^{13}C NMR δ = 96.1 ppm (C-1)), 5.40 (H-2), 5.53 (H-3), 5.34 (H-4), 3.97 (H-5), 3.96–3.49 ppm (H-6).

Glc-β-(1–6)-Glc α-PNP: ^{1}H NMR: δ(β-1,6-linked glucose unit) = 4.47 (J = 7.8 Hz, H-1; ^{13}C NMR: δ = 104.6 ppm (C-1)), 4.94 (H-2), 5.16 (H-3), 5.01 (H-4), 3.64 (H-5), 4.18–4.07 ppm (H-6); δ(α-aryl-linked glucose unit): 5.79 (J = 3.6 Hz, H-1; ^{13}C NMR: δ = 99.3 ppm (C-1)), 4.98 (H-2), 5.64 (H-3), 5.08 (H-4), 3.98 (H-5), 3.88–3.50 ppm (H-6).

For the precise structural assignment of chemical shifts in compounds **7** and **8** (Scheme 1), we firstly needed to assign all the signals for the acceptor as an acetylated derivative in C_6D_6 by COSY and ^{1}H–^{13}C correlation spectra; good signal separation was observed in this solvent.

The structure of compound **7** (Scheme 1) has been assigned from the COSY spectrum of the acetylated derivative in the same solvent by following the correlation for each single glucose unit; for the α-aryl-linked unit, in fact, only one glucosylated signal has been found at δ = 4.10 ppm, while the H-6 protons for the central glucose unit are found at δ = 3.95–3.45 ppm, typical values for a glycosylated position. Finally, the DEPT spectrum confirmed the structure by showing a methylene signal at δ = 67.80 ppm, which corresponds to the C-6 atom of the central glucose molecule. For compound **8** (Scheme 1), with the same reasoning, the β-Glc unit has been found attached to the internal aryl-linked glucose of the acceptor. The H-2 proton of this moiety in fact resonates at δ = 3.41 ppm, thus indicating it to be on the glycosylated position; in the same pyranosidic ring, another glycosylated position is found at δ = 4.07 ppm (H-4). The H-2 proton also correlates in the long range spectrum with the anomeric signal of the β-Glc unit and the H-1 proton of this latter moiety correlates with the C-2 atom of the aryl-linked glucose unit. Finally, TOCSY experiments were in accordance with the proposed structures.

Acceptor Glc-α-(1–4)-Glc α-PNP: ^{1}H/^{13}C NMR (400 MHz, C_6D_6): δ(external glucose unit) = 5.62/96.1 (H/C-1), 5.09/70.3 (H/C-2), 5.83/69.7 (H/C-3), 5.40/68.5 (H/C-4), 4.13/69.0 (H/C-5), 4.41–4.36/61.6 ppm (H/C-6); δ(internal aryl-linked glucose unit) = 5.31/94.2 (H/C-1), 4.92/70.6 (H/C-2), 5.95/72.3 (H/C-3), 4.00/72.4 (H/C-4), 3.62/69.3 (H/C-5), 4.23–3.96/62.2 ppm (H/C-6).

Compound 7: ^{1}H NMR (400 MHz, C_6D_6): δ(external β-1,6-linked glucose) = 4.48 (H-1), 5.04 (H-2), 5.23 (H-3), 5.08 (H-4), 3.69 (H-5), 4.15–4.25 ppm (H-6); δ(central α-1,4-linked glucose unit) = 5.45 (H-1), 4.81 (H-2), 5.34 (H-3), 4.95 (H-4), 3.87 (H-5), 3.95–3.45 ppm (H-6); δ(internal aryl-linked glucose unit) = 5.73 (H-1), 5.02 (H-2), 5.72 (H-3), 4.10 (H-4), 4.04 (H-5), 4.24–4.35 ppm (H-6).

Compound 8: ^{1}H/^{13}C NMR (400 MHz, C_6D_6): δ(β-1,2-linked glucose unit) = 4.24/101.4 (H/C-1), 5.15/71.1 (H/C-2), 5.37/73.2 (H/C-3), 5.03/68.1 (H/C-4), 3.29/72.1 (H/C-5), 4.02–3.88/61.0 ppm (H/C-6); δ(external α-1,4-linked glucose unit) = 5.67/95.7 (H/C-1), 5.16/70.6 (H/C-2), 5.88/69.5 (H/C-3), 5.47/68.8 (H/C-4), 4.15/69.2 (H/C-5), 4.42/62.0 ppm (H/C-6); δ(internal aryl-linked glucose unit) = 5.57/97.1 (H/C-1), 3.41/77.4 (H/C-2), 5.94/73.3 (H/C-3), 4.07/71.8 (H/C-4), 3.65/68.8 (H/C-5), 4.23–4.05/62.0 ppm (H/C-6).

The COSY spectrum of the acetylated derivative of the new trisaccharide **9** confirmed the structure reported. Signal assignment was possible after complete assignment of the signals of the acceptor.

Acceptor Glc-α-(1–4)-Glc β-PNP: ^{1}H NMR (400 MHz, C_6D_6): δ(external α-1,4-linked glucose unit) = 5.49 (J = 3.8 Hz, H-1; ^{13}C NMR: δ = 96.5 ppm (C-1)), 5.02 (H-2), 5.81 (H-3), 5.34 (H-4), 4.21 (H-5), 4.39–4.33 ppm (H-6); δ(internal β-aryl-linked glucose unit) = 4.82 (J = 7.6 Hz, H-1; ^{13}C NMR: δ = 97.2 ppm (C-1)), 5.19 (H-2), 5.37 (H-3), 3.85 (H-4), 2.91 (H-5), 4.11–4.30 ppm (H-6).

Compound 9: ^{1}H NMR (400 MHz, C_6D_6): δ(external β-1,6-linked glucose unit) = 4.29 (J = 7.8 Hz, H-1; ^{13}C NMR: δ = 101.1 ppm (C-1)), 5.32 (H-2), 5.42 (H-3), 5.25 (H-4), 3.29 (H-5), 4.05–4.26 ppm (H-6); δ(central α-1,4-linked glucose unit) = 5.45 (J = 3.8 Hz, H-1; ^{13}C NMR: δ = 95.0 ppm (C-1)), 5.01 (H-2), 5.89 (H-3), 5.25 (H-4), 4.21 (H-5), 3.52–4.09 ppm (H-6; ^{13}C NMR: δ = 67.8 ppm (C-6)); δ(internal aryl-linked glucose unit) = 4.80 (J = 7.6 Hz, H-1; ^{13}C NMR: δ = 97.8 ppm (C-1)), 5.37 (H-2), 5.43 (H-3), 4.00 (H-4), 2.94 (H-5), 4.30–4.36 ppm (H-6).

Acknowledgements

The authors wish to thank E. Pagnotta for skillful technical assistance and D. Melck, V. Mirra, and S. Zambardino of the NMR service of ICB-Naples for running NMR spectra. The present research was supported by the project entitled "Nuove attività enzimatiche per la sintesi di oligosaccaridi di interesse biotecnologico" from Regione Campania L.R. (Grant no.: 5–28.03.2002) and by the MIUR project "Folding di proteine: l'altra metà del codice genetico". The Institute of Protein Biochemistry-CNR belongs to the Centro Regionale di Competenza in Applicazioni Tecnologico-Industriali di Biomolecole e Biosistemi.

Keywords: biocatalysis · enzymes · glycosides · glycosynthases · oligosaccharides

[1] K. M. Koeller, C.-H. Wong, *Chem. Rev.* **2000**, *100*, 4465 – 4493.
[2] C. J. Hamilton, *Nat. Prod. Rep.* **2004**, *21*, 365 – 385.
[3] D. L. Zechel, S. G. Withers, *Acc. Chem. Res.* **2000**, *33*, 11 – 18.
[4] M. Moracci, A. Trincone, M. Rossi, *J. Mol. Catal. B Enzym.* **2001**, *11*, 155 – 163.
[5] G. Perugino, A. Trincone, M. Rossi, M. Moracci, *Trends Biotechnol.* **2004**, *22*, 31 – 37.
[6] G. Perugino, A. Trincone, A. Giordano, J. van der Oost, T. Kaper, M. Rossi, M. Moracci, *Biochemistry* **2003**, *42*, 8484 – 8493.
[7] A. Trincone, A. Giordano, G. Perugino, M. Rossi, M. Moracci, *Bioorg. Med. Chem. Lett.* **2003**, *13*, 4039 – 4042.
[8] Y. W. Kim, S. S. Lee, R. A. J. Warren, S. G. Withers, *J. Biol. Chem.* **2004**, *279*, 42 787 – 42 793.
[9] A. Trincone, G. Perugino, M. Rossi, M. Moracci, *Bioorg. Med. Chem. Lett.* **2000**, *10*, 365 – 368.
[10] G. D. Brown, S. Gordon, *Immunity* **2003**, *19*, 3, 311 – 315.
[11] I. M. Smaali, N. Michaud, N. Marzouki, M. D. Legoy, T. Maugard, *Biotechnol. Lett.* **2004**, *26*, 675 – 679.
[12] R. Borriss, M. Krah, H. Brumer, M. A. Kerzhner, D. R. Ivanen, E. V. Eneyskaya, L. A. Elyakova, M. S. Shishlyannikov, A. C. Shabalin, K. N. Neustroevc, *Carbohydr. Res.* **2003**, *338*, 1455 – 1467.
[13] G. H. Veeneman, H. F. Brugghe, H. van den Elst, J. H. van Boom, *Carbohydr. Res.* **1990**, *195*, C1 – 4.
[14] T. Takeda, T. Kanemitsu, M. Ishiguro, Y. Ogihara, M. Matsubara, *Carbohydr. Res.* **1994**, *256*, 59 – 69.
[15] F. J. Witteler, P. Welzel, H. Duddeck, G. Hoefle, W. Riemer, H. Budzikiewicz, *Tetrahedron Lett.* **1979**, *20*, 3493 – 3496.
[16] A. Trincone, E. Pagnotta, A. Giordano, G. Perugino, M. Rossi, M. Moracci, *Biocatal. Biotransform.* **2003**, *21*, 17 – 24.
[17] K. Ozawa, Y. Yamagata, S. Satomura, H. Ishida, M. Kiso, A. Hasegawa, *Carbohydr. Res.* **1994**, *262*, 137 – 145.

Received: December 3, 2004

