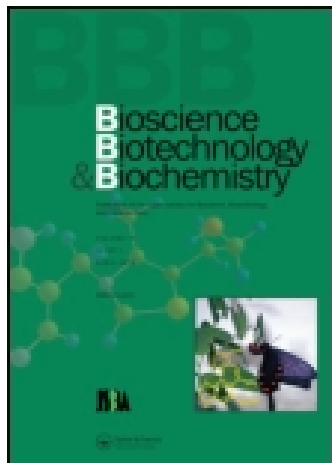




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Preliminary Communication

α -Glucosidase Mutant Catalyzes “ α -Glycosynthase”-type Reaction

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Replacement of the catalytic nucleophile Asp481 by glycine in *Schizosaccharomyces pombe* α -glucosidase eliminated the hydrolytic activity. The mutant enzyme (D481G) was found to catalyze the formation of an α -glucosidic linkage from β -glucosyl fluoride and 4-nitrophenyl (PNP) α -glucoside to produce two kinds of PNP α -diglucosides, α -isomaltoside and α -maltoside. The two products were not hydrolyzed by D481G, giving 41 and 29% yields of PNP α -isomaltoside and α -maltoside, respectively. PNP monoglycosides, such as α -xyloside, α -mannoside, or β -glucoside, acted as the substrate, but PNP α -galactoside and maltose could not. No detectable product was observed in the combination of α -glucosyl fluoride and PNP α -glucoside. This study is the first report on an “ α -glycosynthase”-type reaction to form an α -glucosidic linkage.

Key words: α -glucosidase; catalytic nucleophile mutant; glycosynthase; oligosaccharide production

Glycosynthase is an active site nucleophile mutant enzyme prepared from glucosidase, which is capable of synthesizing oligosaccharide derivatives without hydrolysis of the product.^{1–5} The original glycosynthase has been reported by Withers and co-workers who constructed the catalytic nucleophile mutant of Glu358Ala from *Agrobacterium* sp. β -glucosidase and found a remarkable reaction in which the mutant enzyme missing hydrolytic activity catalyzed the production of β -glycosyl oligosaccharide derivative(s) from two substrates, α -glucosyl (or α -galactosyl) fluoride and 4-nitrophenyl (PNP) glycoside.^{1,2} We tentatively call the mutant enzyme “ β -glycosynthase”. A Ser mutant, Glu358Ser, increased the glycosynthase activity and changed the specificity to PNP glycoside.³ The nucleophile-mutated β -glucosidase from *Sulfolobus solfataricus* also showed

the “ β -glycosynthase”-type reaction.⁴ The second type of β -glycosynthase has been recently reported on an endo-acting β -glucanase from *Bacillus licheniformis*.⁵ The mutant of a catalytic nucleophile (Glu134Ala, β -glucansynthase) produced β -laminaribiosyl saccharide derivatives from α -laminaribiosyl fluoride. Until now glycosynthase research has been limited for β -glycosylase-type enzymes, and there has been no report of any “ α -glycosynthase”-type mutant enzyme from an α -glycosylase so far.

α -Glucosidase (EC 3.2.1.20, α -D-glucoside glucohydrolase) is a typical α -glycosylase which catalyzes exo-type hydrolysis, transglucosylation, condensation, and D-glucal hydration to form α -anomer configuration products.⁶ We reported that three acidic residues (Asp481, Glu484, Asp647) in *Schizosaccharomyces pombe* α -glucosidase (SPG) were essential for the hydrolytic reaction,⁷ in which Asp481 was found to be a catalytic nucleophile.^{7,8} To learn the functions of acidic residues, a series of analyses of mutant enzymes have been done in our laboratory. Recently, the glycosynthase-type reaction was observed in an Asp481Gly mutant of SPG (D481G). This paper describes for the first time the “ α -glycosynthase” reaction.

All chemicals were purchased from Nacalai Tesque Chemical (Kyoto, Japan) unless otherwise stated; α - and β -glucosyl fluoride (α -GF and β -GF, respectively), from Sigma (St. Louis, MO, USA); restriction endonucleases and Klenow fragment of *Escherichia coli* DNA polymerase I, from GIBCO (Rockville, MD, USA). Mutant SPG of Asp481Ala (D481A) was prepared and purified as in our previous paper.⁷ Quantitative analysis and purification of the product diglycosides were done by high performance liquid chromatography (HPLC) under the conditions as follows: column, 4.6 $\times$ 250 mm (5 μ m ODS) YMC

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Abbreviations: SPG, *Schizosaccharomyces pombe* α -glucosidase; D481G and D481A, Asp481Gly and Asp481Ala mutants of SPG, respectively; ESI-MS, electron spray ionization mass spectrometry; α -GF, α -glucosyl fluoride; β -GF, β -glucosyl fluoride; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; PNP, 4-nitrophenyl; PNP-Glc, 4-nitrophenyl α -glucoside; pPICZA, *Pichia pastoris* expression vector; pPICZA/SPG and pPICZA/D481G, expression vectors for wild-type SPG and D481G in *P. pastoris*, respectively

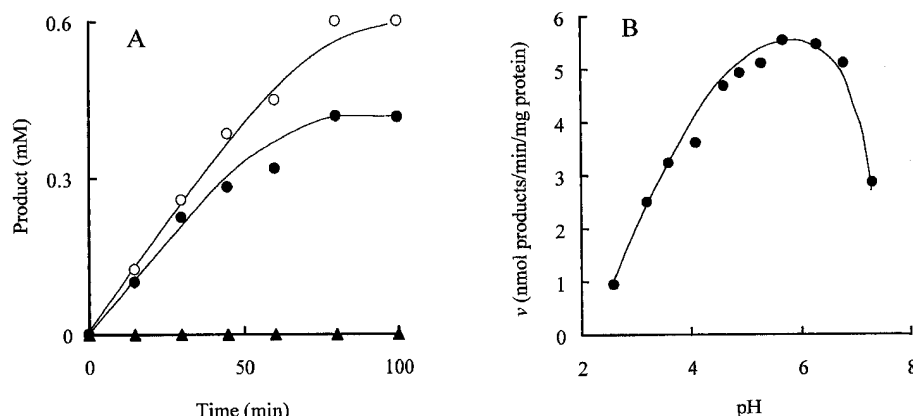


Fig. 1. Courses of Enzymatic Synthesis of PNP α -Maltoside and α -Isomaltoside Catalyzed by D481G and Product by Wild-type SPG (A), and pH Dependence of α -Glycosynthase Reaction by D481G (B).

In panel A, the amounts of PNP α -maltoside (OS-A, $\bullet$) and α -isomaltoside (OS-B, $\circ$) in the 30°C-incubated reaction mixture (0.25 ml) consisting of 20 mM β -GF, 2 mM PNP-Glc, and D481G (230 μ g) in 40 mM sodium acetate buffer (pH 4.5) were measured by HPLC. The symbol of $\blacktriangle$ is the concentration of product formed by wild-type SPG under the same reaction conditions except using wild-type SPG instead of D481G.

In panel B, the reaction mixture (0.25 ml) containing McIlvaine buffer (pH 2.5–7.5, prepared by 0.2 M Na_2HPO_4 and 0.1 M citric acid), 15 mM β -GF, 3 mM PNP-Glc, and 230 μ g D481G was incubated at 30°C.

Packed Column AQ303 (YMC; Kyoto, Japan) incubated at 50°C; mobile phase, 9:91 $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ at the flow rate of 1 ml/min; detection, absorbance at 313 nm. The amount of each diglycoside was calculated from the peak area in the HPLC chromatogram. The purity of isolated products was examined by thin-layer chromatography (TLC; Merck Silica gel 60 plate; development of 1-butanol/2-propanol/ H_2O , 10:5:4). ^1H - and ^{13}C -NMR spectra of digluco-sides were recorded on a Bruker AMX500 at 500 and 125 MHz, respectively, using sodium 3-(trimethylsilyl)propionate-2,2,3,3- d_4 as an external standard. Molecular masses of diglycosides were confirmed by electron spray ionization mass spectrometry (ESI-MS, JEOL JMS-SX102A).

The original wild-type enzyme was expressed inside and in the surface of the *Saccharomyces cerevisiae* cells that harbored the vector of pYES/SPG carrying SPG cDNA.⁷ We found the *Pichia pastoris* expression system to secrete the wild-type SPG into the culture medium, giving the simple enzyme isolation procedure. For construction of an expression plasmid for the new host cell, the SPG cDNA in the pYES/SPG was cleaved by *Hind*III, blunt-ended with the Klenow fragment of *E. coli* DNA polymerase I, and then digested with *Xho*I. The resulting 3.0-kb DNA fragment containing SPG cDNA was inserted under the *AOX1* promoter in the *P. pastoris* expression vector (pPICZA, Invitrogen; Carlsbad, CA, USA) that had been treated with *Xho*I after being digested with *Eco*RI and blunt-ended. The resulting plasmid was named pPICZA/SPG. Transformation of *P. pastoris* GS155 and expression of recombinant protein were done according to the manufacturer's protocol (Invitrogen).

A "megaprimer" PCR approach⁹ was used to introduce Gly at the position of Asp481 in SPG. The mutagenic antisense primer was 5'-CGCAGAAC-GAGCTCGGTTTCGTTTCATACCAGTCCAAAT-3', in which the underline is the mutated anticodon. The purified megaprimer PCR product was ligated into the pPICZA/SPG, designated pPICZA/D481G. The cloned product was sequenced to verify the introduction of the desired mutation. Transformation of *P. pastoris* and expression of D481G were done basically in the same way as for the wild-type SPG.

Recombinant SPG showing a single band in SDS-PAGE was purified from culture medium of *P. pastoris* GS115 harboring the pPICZA/SPG or pPICZA/D481G by DEAE-Sepharose CL-6B and Sepharose 6B at pH 4.5 (Amersham Pharmacia; Uppsala, Sweden). Enzyme activity, protein concentration and CD spectra of recombinant SPG were measured as in our previous paper.⁷ The properties of the wild-type SPG analyzed were identical to those of native enzyme except for sugar contents, 74% for the wild-type one and 78% for the native one.⁷ There was no difference in CD spectra of native and recombinant SPG.

The recombinant enzymes (D481G and D481A) mutated in the Asp481, which is a nucleophile in the catalytic reaction of the *S. pombe* α -glucosidase,⁷ lost the hydrolytic activity on any carbohydrates, such as PNP α -glucoside (PNP-Glc) and maltose. However, the D481G was found to synthesize the products from a reaction mixture consisting of β -GF and PNP-Glc. A reaction mixture of 0.25 ml containing 20 mM β -GF, 2 mM PNP-Glc, and D481G (230 μ g) in 40 mM sodium acetate buffer (pH 4.5) was incubated at 30°C. Aliquots of reaction mixture were

taken at the indicated reaction times (Fig. 1(A)), and were analyzed by TLC and reverse-phased HPLC. Two new spots were observed on the TLC plate, and each spot size was increased with reaction time. As illustrated in Fig. 1(A), the quantitative HPLC analysis showed that the amounts of two chromogenic compounds (OS-A and OS-B) increased and reached plateaus at 100 min. The spontaneous degradation of β -GF was also observed. In the initial stage of reaction, however, the quantity of fluoride ion released was higher than that in the no-enzyme reaction system (control). The combination of β -GF and maltose did not produce any oligosaccharide.

OS-A and OS-B were purified by HPLC in order to analyze the structures. The isolated OS-A and -B were homogeneous on TLC. The R_f and retention time of OS-A were identical to those of authentic PNP α -maltoside. The partial acid hydrolysis using 0.1% trifluoroacetic acid at 100°C for 3 h released maltose from OS-A and isomaltose from OS-B, implying the formation of an α -anomeric configuration in the reaction. NMR and MS analyses of OS-A and OS-B were done. PNP α -maltoside (OS-A); ESI-MS: m/z $[M + Na]^+$ 486, NMR δ_H and δ_C (D_2O): being completely identical to authentic PNP α -maltoside. PNP α -isomaltoside (OS-B); ESI-MS: m/z $[M + Na]^+$ 486, NMR δ_H (D_2O): 3.39 (1H, dd, $J=9.5, 9.5$ Hz, 4'-H), 3.47 (1H, dd, $J=9.8, 6.7$ Hz, 2'-H), 3.58 (1H, dd, $J=9.3, 9.4$ Hz, 3'-H), 3.59 (1H, dd, $J=9.6, 9.5$ Hz, 4-H), 3.65 (1H, m, 5'-H), 3.66 (2H, m, 6a-H), 3.72 (2H, dd, $J=12.2, 8.7$ Hz, 6'a-H), 3.79 (1H, m, 2-H), 3.80 (1H, m, 6'b-H), 3.88 (1H, m, 5-H), 3.89 (1H, dd, $J=11.8, 5.3$ Hz, 6b-H), 3.93 (1H, dd, $J=9.5, 9.5$ Hz, 3-H), 4.86 (1H, d, $J=3.5$ Hz, 1'-H), 5.82 (1H, d, $J=3.7$ Hz, 1-H), 7.3 (2H, d, $J=9.2$ Hz, PNP-3,5-H), 8.26 (2H, d, $J=9.2$ Hz, PNP-2,4-H); NMR δ_C (D_2O): 61.2 (6'-C), 66.2 (6-C), 70.1 (4-C), 70.2 (4'-C), 71.6 (2-C), 72.1 (2'-C), 72.3 (5-C), 72.5 (5'-C), 73.8 (3'-C), 73.9 (3-C), 97.3 (1-C), 98.5 (1'-C), 117.7, 117.7, 126.9, 126.9, 143.3, 162.1 (PNP-C). ESI-MS data indicated that two compounds were PNP diglucosides. The NMR data of OS-A agreed completely with those of authentic PNP α -maltoside. OS-B was determined to be a PNP α -isomaltoside, in which two-dimensional NMR and HMBC analysis also supported the occurrence of α -1,6-linkage. A possible reaction is shown in Scheme 1(B). D481G of the catalytic nucleophile mutant could not hydrolyze two products (PNP α -maltoside and α -isomaltoside), meaning that the product only accumulated in the reaction system. Under the same reaction conditions, D481A gave no detectable product. The mutant enzyme in which Asp481 was replaced by an amino acid of small size catalyzed the formation of PNP α -diglucosides. The wild-type SPG showing no hydrolytic activity on β -GF did not synthesize any product (Fig. 1(A)) and the enzyme hydrolyzed the PNP-Glc only.

Table 1. PNP Diglycoside Formation by D481G from β -GF and PNP Glycoside

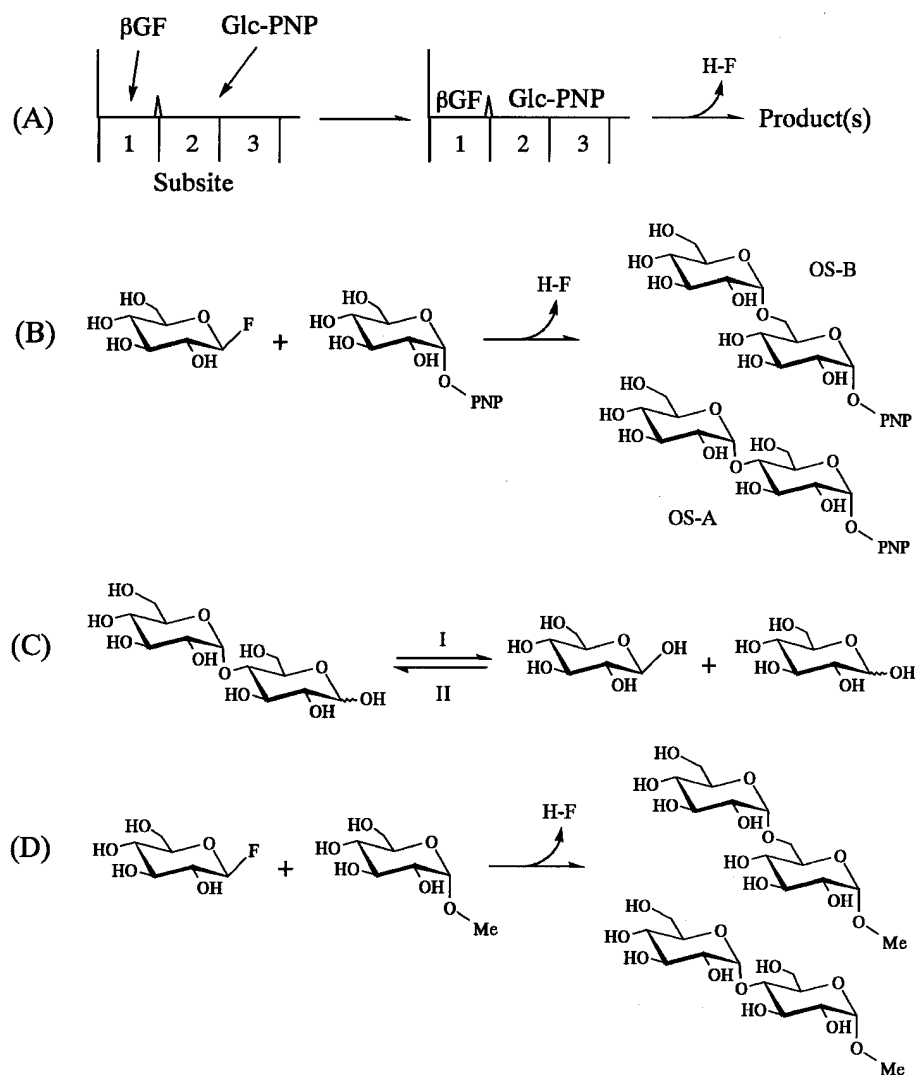
Substrate (PNP monoglycoside)	Yield of PNP diglycoside ^a (%)
PNP α -glucoside	41 (PNP α -isomaltoside), 29 (PNP α -maltoside)
PNP α -xyloside	82
PNP β -glucoside	13
PNP α -mannoside	10
PNP α -galactoside	0

^a Reaction mixture (0.25 ml) containing 20 mM of β -GF, 2 mM of PNP monoglycoside, and D481G (230 μ g) was incubated at pH 5.7 and 30°C for 80 min. Yield was calculated from the conversion of each PNP monoglycoside into PNP diglycoside. Molecular mass of each product was analyzed by ESI-MS.

Under the conditions shown in Fig. 1(A), about 50% of the PNP-Glc was converted to PNP oligosaccharide derivatives. We examined the effects of pH on the glycosynthase reaction to obtain a high yield of oligosaccharide synthesis. The optimal pH for α -glycosynthase activity of D481G was around pH 5.7 (Fig. 1(B)), at which the 41 and 29% yields of PNP α -isomaltoside and of PNP α -maltoside, respectively, were obtained (Table 1). Table 1 also shows the substrate specificities for PNP monoglycosides. PNP α -xyloside was found to be more efficient than PNP-Glc. PNP α -mannoside and β -glucoside were poor substrates, and PNP α -galactoside was not.

Wild-type enzyme, which was expressed in *P. pastoris*, catalyzed α -1,4- and α -1,6-glucosyl transferring reactions to produce PNP α -maltoside and PNP α -isomaltoside from 20 mM α -GF and 2 mM PNP-Glc. Native SPG, which was isolated from *S. pombe*, catalyzed the α -1,6-transglucosylation to produce mainly isomaltose from maltose of high concentration,¹⁰ in which there was a possibility of maltose formation (α -1,4-transglucosylation), but it was difficult to find the newly synthesized maltose in this reaction. Therefore, the regiospecificity in D481G reaction to produce PNP α -isomaltoside and α -maltoside was maintained even after replacement of Asp481 by Gly, implying that the subsites 2 and 3 in active center of D481G were unchangeable in recognition of glucosyl and PNP groups in PNP-Glc, respectively (Scheme 1(A)). At the middle and final reaction stages of native and wild-type enzymes, the transglucosylation products formed were subsequently hydrolyzed and decreased, as the isomaltose and two PNP diglucosides were favorable substrates for both enzymes. The best advantage of the D481G reaction, therefore, is the accumulation of product without further degradation, providing an efficient approach to synthesize oligosaccharide.

D481G synthesized no product from the mixture of α -GF and PNP-Glc. The synthetic reaction only occurred by using β -GF as a fluoride substrate to produce PNP diglucosides having α -anomeric con-



Scheme 1. Subsite Structure of D481G Recognizing Substrates (A), D481G-Catalyzed α -Glycosynthase-type Reaction with β -GF and PNP-Glc (B), Hydrolytic (I) and Reverse (II) Reactions of Glucoamylase (C), and Glucoamylase-catalyzed Formation of Methyl α -Maltoside and α -Isomaltoside from β -GF and Methyl α -Glucoside (D).¹¹⁾

Glc-PNP, PNP α -glucoside; Me, methyl; wedge between subsite 1 and 2 (panel A), catalytic position.

figuration, meaning that the anomeric configuration is opposite between the fluoride substrate (β) and the products (α). A similar reaction catalyzed by D481G (Scheme 1(B)) has been reported in the “inverting enzymes” like glucoamylase, trehalase, glucodextranase, and β -amylase,^{11–16)} which catalyzed two inverting-type reactions, such as hydrolysis and oligosaccharide production. In the hydrolysis (Scheme 1(C)-I), the α -glucosidic linkage in each substrate is inverted to release a β -product, namely β -glucose or β -maltose.^{17–20)} In the oligosaccharide production, Hehre and co-workers have shown that glucoamylase, glucodextranase, and trehalase of inverting enzymes synthesized α -configurational products from β -glucose or β -GF,^{11,12)} and that β -amylase synthesized an α -configurational product from β -maltose or β -maltosyl fluoride.¹³⁾ Glucoamylase, for example, catalyzed the formation of maltose

and isomaltose from β -glucose by condensation, that is, the reverse reaction (Scheme 1(C)-II), and the formation of methyl α -maltoside and methyl α -isomaltoside (plus HF also) from β -GF and methyl α -glucoside (Scheme 1(D)).¹¹⁾ Glucodextranase also produced the same methyl α -diglucosides and HF from β -GF and methyl α -glucoside.¹¹⁾ Trehalase formed α,α -trehalose through two kinds of reaction mixtures: the first mixture composed of β -glucose and α -glucose (condensation) and the second one composed of β -GF and α -glucose at which HF was also released.¹²⁾ The last example is a β -amylase to produce maltotetraose from β -maltose by condensation^{15,16)} and to produce β -maltose from β -maltosyl fluoride.¹³⁾ In the latter reaction catalyzed by β -amylase, β -maltotetraosyl fluoride intermediate was expected to be synthesized at first and subsequently to be split to β -maltose and β -maltosyl fluoride by β -

amylase-catalyzed hydrolysis, that is, the whole reaction involved two combined steps of i) β -maltotetraosyl fluoride and HF formation from two molecules of β -maltosyl fluoride and ii) hydrolysis of resultant tetrasaccharide derivative to give β -maltose.¹³⁾ The mechanism in the former step is similar to that of maltotetraose formation in the condensation of β -maltose.^{13,15,16)} Also the other three inverting enzymes, glucoamylase, trehalase and glucodextranase, have been found to catalyze the condensation and the oligosaccharide formation by using a fluoride substrate through the same reaction path,^{11,12)} meaning that the oligosaccharide production from fluoride substrate is the condensation.

The reaction in D481G to form the inverted α -anomer-product from β -GF and PNP-Glc (Scheme 1(B)) is considered to be the condensation itself in the inverting enzymes-catalyzed oligosaccharide production mentioned above, implying that D481G changes from a "retaining enzyme" to an "inverting enzyme". Catalytic nucleophile mutants of some β -retaining enzymes, β -glucosidase and β -glucanase, have been designated glycosynthases thus far.¹⁻⁵⁾ These " β -glycosynthase"-type enzymes catalyze the production of β -anomer oligosaccharide derivatives and HF from α -glycosyl fluoride and suitable PNP (or 4-methylumbelliferyl) glycoside, and the synthetic reaction is considered to be the transglycosylation. It seems, however, to be rational that the glycosynthase reactions involving β -glycosidases and α -glucosidase are interpreted on the basis of the condensation rather than the transglycosylation by the catalytic nucleophile mutant enzyme which are changed from "retaining" to "inverting" enzymes.

In this study, we found that the D481G also showed an " α -glycosynthase"-type reaction with β -GF and PNP-Glc to form α -configurational oligosaccharide derivatives. The D481G enzyme is the first example of an " α -glycosynthase". It is of interest that the anomeric structures of the fluoride substrate and product are completely opposite in α -glycosynthase and β -glycosynthase.¹⁻⁵⁾ Therefore, it is preferable to divide the names of glycosynthase into " α -glycosynthase" and " β -glycosynthase".

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