Enzymatic Polymerization of α-D-Maltosyl Fluoride Utilizing α-Amylase as the Catalyst: A New Approach for the Synthesis of Maltooligosaccharides

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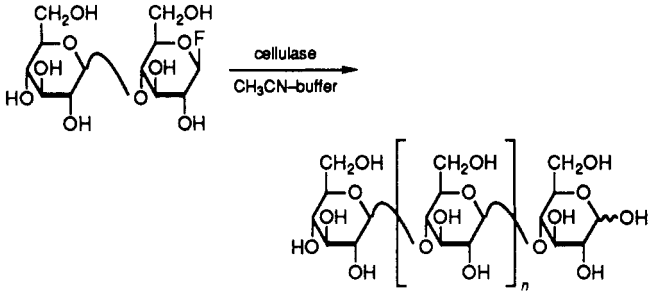
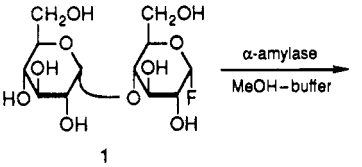
ABSTRACT: Maltooligosaccharides have effectively been prepared by polycondensation of α-D-maltosyl fluoride (1) using α-amylase as the catalyst in a mixed solvent of methanol–phosphate buffer (pH 7) (*enzymatic polymerization*). The structure of the resulting oligomers was confirmed by means of ^{13}C NMR spectroscopy as well as by an enzymatic hydrolysis experiment. The most appropriate reaction conditions for the production of the higher oligomers were to use methanol/buffer = 2/1 as solvent and to employ an amylase enzyme from *Aspergillus oryzae* as the catalyst. The stereo- and regioselective formation of an α(1→4)-glycosidic bond is explained by a mechanism involving "double inversion" of the C1 carbon configuration of monomer 1. Other substrates such as D-maltose, β-D-maltosyl fluoride, and α-D-glucosyl fluoride gave no condensation products.

Introduction

Recent developments of new synthetic methodologies strongly enhanced the production of various useful oligosaccharides and polysaccharides in material science, pharmaceutical science, and the food industry. Chemical approaches, however, require complicated procedures including a regioselective blocking and deblocking of a hydroxy group in the sugar moieties. In addition, complete stereocontrol of the glycosidic bond-forming reactions has not been achieved in spite of numerous efforts to establish a stereoselective glycosylation reaction,[1] a polycondensation reaction of partially protected sugar derivatives,[2] and a ring-opening polymerization of anhydrosugars.[3] The use of an enzyme in the glycosylation process has, therefore, become one of the most promising synthetic tools for construction of stereoregular oligo- or polysaccharides.[4] In the previous paper, we have reported the first in vitro synthesis of cellulose, a stereoregular polysaccharide having a β(1→4) linkage, by utilizing β-D-cellobiosyl fluoride as the substrate monomer for a cellulase enzyme catalyst in an acetonitrile–buffer system (*enzymatic polymerization*).[5]



The results strongly suggest that the polycondensation reaction of a sugar fluoride catalyzed by a hydrolytic enzyme in an organic–water mixed solvent provides a general, efficient method for the construction of stereoregular oligo- and polysaccharides under mild reaction conditions. The present paper deals with a synthesis of maltooligosaccharides by a transglycosylation reaction (enzymatic condensation polymerization) catalyzed by α-amylase, a hydrolysis enzyme of amylose, with use of α-D-maltosyl fluoride (1) as a glycosyl donor (substrate monomer).[6] Maltooligosaccharides are useful substances



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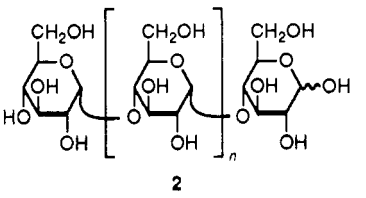

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as food additives, medicines, and enzyme substrates for clinical research. Generally, they are produced by a degradation reaction of polymers such as amylose, amylopectin, and glycogen,[7] whereas only a few studies on their production from monomers, e.g., the condensation of a glucose derivative or a maltose derivative, have been reported so far.[8]

Results and Discussion

Enzymatic Polymerization of α-D-Maltosyl Fluoride (1). In the presence of a catalytic amount (5 wt % for 1) of α-amylase, α-D-maltosyl fluoride (1) was condensed in a mixture of an organic solvent and 0.05 M phosphate buffer (pH 7)[9] at room temperature for 1 h. Then, the reaction mixture was heated at 100 °C for 10 min in order to inactivate the enzyme catalyst. During the procedure, the carbon–fluorine bond at the reducing end of the product oligomers was cleaved to the hydroxy group by a nucleophilic attack of a water molecule to the anomeric carbon atom. After removing the solvent the maltooligosaccharide mixture was dissolved in water and was analyzed by means of high-performance liquid chromatography (HPLC). When a methanol–buffer (2:1) solution of α-D-maltosyl fluoride without α-amylase was heated at 100 °C for 10 min, no polymerized product was obtained.

Table I summarizes the results of the solvent effect on the polymerization of 1 catalyzed by α-amylase. The G_1 to G_7 and G_4F denote glucose to maltoheptaose and maltotetraosyl fluoride, respectively. The enzymatic polycondensation reaction of 1 took place effectively when the reaction was carried out in an organic-water mixed solvent system. Of the organic solvents screened, methanol and

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Table I
Enzymatic Polymerization of 1 Catalyzed by α-Amylase in Various Solvents[a]

entry	solvent	monomer conv (%)	product distribution (%)[c]							
			G_1	G_2	G_3	G_4	G_5	G_6	G_7	G_4F
1	buffer[b]	87	0	48	49	3	0	0	0	0
2	MeOH–buffer (2:1)	quant	0	12	25	28	13	14	5	4
3	EtOH–buffer (2:1)	quant	0	71	21	3	5	0	0	0
4	acetone–buffer (2:1)	73	0	37	24	26	7	2	0	3
5	THF–buffer (2:1)	quant	0	68	25	3	4	0	0	0
6	1,4-dioxane–buffer (2:1)	quant	0	86	11	3	0	0	0	0
7	CH_3CN–buffer (2:1)	quant	0	37	46	17	0	0	0	0

[a] Polymerized at room temperature for 1 h: [1] = 5×10^{-2} mol/L; α-amylase (*A. oryzae*) 5 wt % for 1. [b] Phosphate buffer (0.05 M, pH 7). [c] Determined by HPLC.

Table II
Effect of Methanol Concentration on Enzymatic Polymerization of 1 Catalyzed by α-Amylase[a]

entry	solvent	monomer conv (%)	product distribution (%)[c]						
			G_2	G_3	G_4	G_5	G_6	G_7	G_4F
1	buffer[b]	87	48	49	3	0	0	0	0
2	MeOH–buffer (1:1)	quant	21	33	22	11	5	0	7
3	MeOH–buffer (2:1)	quant	12	25	28	13	14	5	4
4	MeOH–buffer (5:1)	27	43	40	11	0	0	0	5
5	MeOH–buffer (10:1)	11	72	0	0	0	0	0	28

[a] Polymerized at room temperature for 1 h. [1] = 5.0×10^{-2} mol/L, α-amylase (*A. oryzae*) 5 wt % for 1. [b] Phosphate buffer (0.05 M, pH 7). [c] Determined by HPLC.

Table III
Enzymatic Polymerization of 1 with α-Amylase from Different Origin[a]

entry	α-amylase[b]	monomer conv (%)	product distribution						
			G_2	G_3	G_4	G_5	G_6	G_7	G_4F
1	*A. oryzae* (24 units/mg)	quant	26	10	21	11	16	7	11
2	*B. subtilis* (2080 units/mg)	30	17	4	12	34	29	0	4
3	*B. subtilis* (740 units/mg)	35	15	6	12	37	28	0	2
4	*B. subtilis* (22.5 units/mg)	42	17	5	16	41	13	2	2
5	*B. subtilis*[c] (0.226 units/mg)	52	22	8	23	42	6	0	0
6	*B. licheniformis* (765 units/mg)	39	27	14	16	19	3	2	3
7	porcine pancreas (25 units/mg)	23	41	3	21	12	8	12	3

[a] Polymerized in methanol–phosphate buffer (0.05 M, pH 7) at room temperature for 10 days. [1] = 5.0×10^{-2} mol/L; amylase, 60 units for 0.15 mmol of 1. [b] The unit numbers of the commercial enzyme are given in parentheses. [c] Fixed on a polyacrylamide gel (5.7 units for 0.15 mmol of 1).

acetone were found to be effective for promotion of the polycondensation reaction. Especially, the use of methanol as a cosolvent gave the best result with respect to the formation of maltooligosaccharides (G_3 to G_7 and G_4F) (entry 2). In the absence of an α-amylase catalyst in this solvent system, no condensation products were obtained. When ethanol, tetrahydrofuran (THF), 1,4-dioxane, and acetonitrile were used as cosolvents, good results could not be obtained in terms of the formation of higher maltooligosaccharides (entries 3 and 5–7). When 1 was treated with a catalytic amount of α-amylase in phosphate buffer without using an organic solvent, the formation of the oligosaccharides (G_3–G_5) was observed at the early stage of the reaction[10] and they were finally converted to D-glucose (G_1) and D-maltose (G_2).

The formation of the odd-numbered maltooligosaccharide fragment may be explained by an enzymatic hydrolysis of an oligomer having glucose units of even number during the reaction. When maltose was used instead of maltosyl fluoride (1) as the substrate, only a trace amount of condensation products could be detected by HPLC even under a condition of high substrate concentration. This result indicates that the introduction of the fluorine atom into the anomeric position of maltose is essential for the production of the oligosaccharides.

Table II shows the effect of the methanol concentration on the polycondensation of 1. The best result in terms of the maltooligosaccharide formation was obtained when the ratio of methanol–buffer was 2:1 (entry 3). Under the reaction conditions of a higher methanol concentration (entries 4 and 5), the reaction rate decreased markedly and consequently the monomer conversion was very low (27 and 11%, respectively). This result shows that the enzyme was considerably inactivated in the solvent system of high methanol concentration. In a solvent of low methanol concentration (entries 1 and 2), the hydrolysis of 1 to maltose (the elimination of anomeric fluorine atom) occurred predominantly compared with the transglycosylation reaction because of the more preferable attack of a water molecule to a reactive intermediate than that of 4′-hydroxy group of another molecule of monomer 1.

Table III shows the results of enzymatic polymerization catalyzed by amylase from various origins. Amylase produced from *Aspergillus oryzae* was found to be most effective for the synthesis of maltooligosaccharides (entry 1). It is to be noted that a small amount of maltooligosaccharides of a higher degree of polymerization (G_8 and G_9) was also detected when the catalyst of *A. oryzae* origin was used, whereas other amylases from *Bacillus subtilis*, *Bacillus licheniformis*, or porcine pancreas gave maltooligomers smaller than heptamer (G_7). In addition the reaction rate decreased remarkably when these amylases were used as catalysts, and consequently the monomer conversions became low even after 10 days (entries 2–7).

Structure Determination of the Products. The structures of the resulting maltooligosaccharides have been

Table IV
^{13}C NMR Chemical Shifts of Maltooligosaccharide in D_2O Solution[a]

residue	C1	C2	C3	C4	C5	C6
reducing end unit	α 92.4	72.3	73.4	77.3	70.5	61.3
	β 96.3	74.6	76.8	77.5	75.1	61.1
internal unit	100.2	72.2	73.4	77.4	71.8	61.1
nonreducing end unit	100.1	73.2	73.8	69.9	72.3	61.1

[a] Chemical shift (ppm) relative to TMS by setting the shift of 1,4-dioxane at δ 67.4.

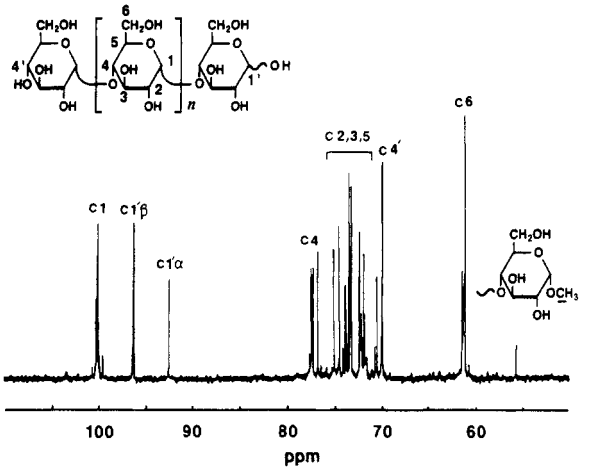


Figure 1. ^{13}C NMR spectrum of maltooligosaccharides (entry 2, Table I) in deuterium oxide.

determined by ^{13}C NMR spectroscopy as well as by its hydrolysis product. Figure 1 shows the ^{13}C NMR spectrum of the mixture of the resulting maltooligosaccharides in deuterium oxide. Each signal can be assigned to the corresponding carbon atom of the glucose unit (C1–C6) of oligosaccharides having an α(1→4) linkage.[11] The signals at δ 92.4, 96.3, 100.1, and 100.2 are assigned to the C1 carbon atom of the reducing end units (α and β forms), the nonreducing end unit, and the internal unit, respectively. The signals at δ 69.9, 77.3, 77.4, and 77.5 are due to the C4 carbon atom of the nonreducing end unit, the reducing end unit (α form), the internal unit, and the reducing end unit (β form), respectively. The assignment of all peaks due to C1–C6 carbon atoms is shown in Table IV.

No signal due to the methylene carbon atom (C6) adjacent to the α(1→6) glycosidic bond was observed around 67 ppm, which is normally detected when an isomeric α(1→6) linkage is formed.[12] A small signal at δ 55.7 is due to the methyl carbon atom of the methyl glycoside at the reducing unit of the oligosaccharide, which indicates that the glycosyl donor intermediate has partly been transglycosylated to the solvent of methanol.

Treatment of the resulting maltooligosaccharides with α-amylase in 0.05 M phosphate buffer afforded hydrolysis products of D-glucose and D-maltose exclusively. These results clearly indicate that the glycosidic bond formation occurred in a regio- and stereoselective manner between maltose units to afford a stereoregular polysaccharide having an α(1→4) linkage.

Reaction Mechanism. The production of the stereoregular oligosaccharide can be explained by assuming the formation of a glycosyl–enzyme intermediate (Figure 2a) or a glycosyl oxocarbenium ion (Figure 2b) at an active site of amylase with the elimination of fluoride ion.[13] This reactive intermediate is then attacked by the 4′-hydroxy group of another molecule of monomer or oligomer, which locates in a subsite of the enzyme, leading to the stereo- and regioselective formation of the α(1→4) linkage. Consequently, the stereochemistry of the product is

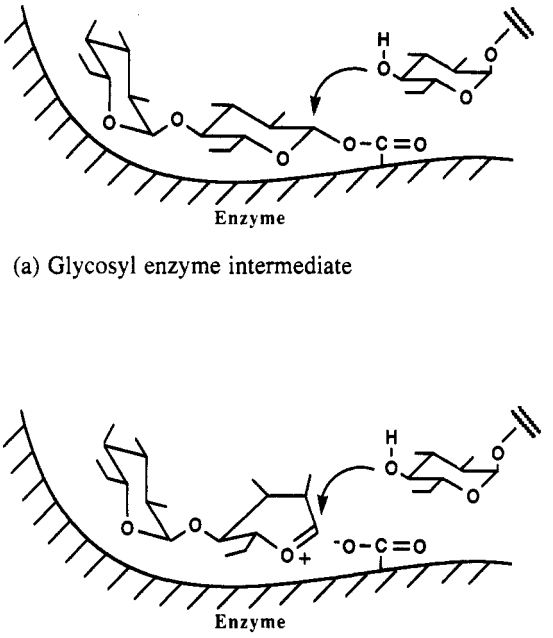
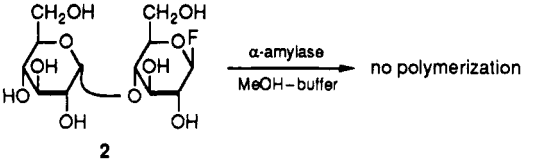


Figure 2. Proposed models in the stereoselective formation of the α(1→4) glycosidic linkage via a substrate–enzyme complex involving double inversion of the configuration at the C1 carbon. (a) Glycosyl enzyme intermediate. (b) Glycosyl oxocarbenium ion intermediate.

retention of the configuration via "double inversion" of the configuration with respect to the C1 carbon atom of α-D-maltosyl fluoride (1).

In relation to the reaction mechanism, the effect of the stereochemistry of the anomeric carbon atom of 1 was investigated. The reaction of β-D-maltosyl fluoride (2) having the opposite configuration (the anomer of 1) in the



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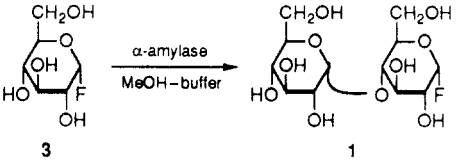
presence of a catalytic amount of α-amylase (5 wt %) in methanol–buffer (2:1) for 1 h at room temperature afforded no product of polysaccharide, 2 being recovered unchanged after the reaction. This result clearly indicates that substrate 2 with the β configuration of the fluorine atom is not recognized by α-amylase, giving rise to none of the reactive intermediates essential for the transglycosylation.

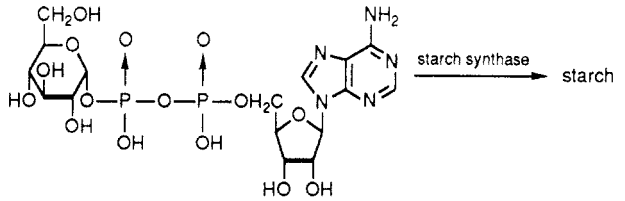
The amylase-catalyzed reaction of α-D-glucosyl fluoride (3) having the same anomeric configuration as 1 in methanol–buffer (2:1) afforded a small amount of α-D-maltosyl fluoride (1), giving no polymeric product. This result implies that α-D-glucosyl fluoride is recognizable to the active site of α-amylase, giving rise to a glycosyl–enzyme complex, and the resulting intermediate can be attacked by another molecule of 3. The concentration of the



3 1

resulting 1 is, however, too low to promote the required polymerization effectively. It is preferable, therefore, to use a maltose (disaccharide) derivative (1) rather than a glucose (monosaccharide) derivative (3) for the maltooligosaccharide synthesis.[14]

The characteristic feature of the present enzymatic polymerization is to utilize a disaccharide derivative whose anomeric carbon is activated by the fluorine atom. This is to be interestingly compared with the construction of the $\alpha(1\rightarrow4)$ glycosidic linkage in the starch biosynthesis which involves the polymerization of a monosaccharide derivative of adenosine diphosphate glucose (ADP-glucose) catalyzed by starch synthase.[15]



ADP–glucose

Further studies concerning the detailed reaction mechanism and inherent properties of the resulting maltooligosaccharides are now in progress.

Experimental Section

Materials. All the solvents used for the enzymatic polymerization were purified by distillation. Commercial reagents of D-glucose, D-maltose, acetic anhydride, 30% hydrogen bromide–acetic acid solution, and silver fluoride were used without purification. 2,3,6,2′,3′,4′,6′-Hepta-*O*-acetyl-β-D-maltosyl fluoride was prepared by the reaction of 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-α-D-maltosyl bromide with silver fluoride according to the literature.[16] 2,3,6,2′,3′,4′,6′-Hepta-*O*-acetyl-α-D-maltosyl fluoride was prepared by modifying the anomerization procedure of the literature:[17] To a solution of 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-β-D-maltosyl fluoride (8.9 g, 13.8 mmol) in dichloromethane (41 mL) was added a boron trifluoride ether complex (0.45 mL) dropwise at 0 °C, and the reaction mixture was vigorously stirred for 2 h at room temperature. The anomerization process was followed by means of ^{19}F NMR spectroscopy. After the anomerization was completed, the reaction mixture was washed with an ice $NaHCO_3$ aqueous solution. The organic layer was dried on sodium sulfate and the solvent evaporated in vacuo. The residue was recrystallized from diethyl ether–petroleum ether, giving rise to 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-α-D-maltosyl fluoride (46% yield). The deacetylation of the resulting heptaacetate was carried out by the action of sodium methoxide in methanol according to the literature,[16] giving rise to α-D-maltosyl fluoride (1). β-D-Maltosyl fluoride (2) was prepared by deacetylation of the corresponding heptaacetate according to the literature.[18] α-D-Glucosyl fluoride was prepared by the reaction of 2,3,4,6-tetra-*O*-acetyl-α-D-glucosyl bromide with silver fluoride followed by anomerization and deacetylation using procedures similar to those described above. The buffer solution (0.05 M, pH 7) was prepared by mixing a sodium dihydrogen phosphate (0.05 M) solution and a disodium hydrogen phosphate (0.05 M) solution by monitoring the pH value with a pH meter (Toa Denki Kogyo HM-20E). α-Amylase enzymes from *A. oryzae* (Type X-A, fungal, crude), *B. subtilis* (2080 units/mg; Type II-A, crystallized four times, lyophilized), *B. subtilis* 22.5 units/mg; Type XI-A, crude, approximately 25% protein standardized with cornstarch), *B. subtilis* (0.226 units/mg; insoluble enzyme attached to polyacrylamide), *B. licheniformis* (Type XII-A, aqueous solution containing approximately 15% sodium chloride), and porcine pancreas (Type VI-B) were purchased from Sigma Co. *B. subtilis* (740 units/mg; liquefying type) amylase was obtained from Seikagaku Kogyo Co. (Japan).

Measurements. ^1H, ^{13}C, and ^{19}F NMR spectra were recorded on a Bruker AC-250-T spectrometer with tetramethylsilane or 1,4-dioxane as standards. Chromatographic data were obtained by use of a Hitachi 655A liquid chromatograph with a Merck LiChrosorb RP-18 column (water eluent) by comparing the peak positions of the products with those of authentic maltooligosaccharides.

Enzymatic Polymerization of α-D-Maltosyl Fluoride (1). A typical procedure for the polymerization of α-D-maltosyl fluoride (1) is given as follows (entry 3, Table I). A solution of α-D-maltosyl fluoride (100 mg, 0.3 mmol) was dissolved in a mixture of methanol (4 mL) and 0.05 M phosphate buffer (pH 7, 1.3 mL). To this solution was added a 0.05 M phosphate buffer (0.7 mL) solution of α-amylase (5.0 mg, 120 units, 5 wt % for the substrate) from *A. oryzae*, and the mixture was shaken at room temperature for 1 h. The resulting mixture was then heated at 100 °C for 10 min to inactivate the enzyme. After removing the precipitated enzyme by filtration, the filtrate was condensed in vacuo, giving rise to the crude mixture of the reaction products which was subjected to analysis by high-performance liquid chromatography. Reactions with other organic solvents were carried out in a similar manner.

Enzymatic Hydrolysis of the Maltooligosaccharides. To a solution of the maltooligosaccharides mixture (0.3 mmol scale) in 0.05 M phosphate buffer (5.3 mL) was added a 0.05 M phosphate buffer (0.7 mL) solution of α-amylase (5.0 mg; *A. oryzae*), and the mixture was stirred at room temperature for 48 h. After heating the reaction mixture at 100 °C for 10 min, the resulting solution was directly analyzed by HPLC, which showed the exclusive formation of D-glucose and D-maltose in a 1:1.5 ratio.

Enzymatic Reaction of α-D-Glucosyl Fluoride. α-D-Glucosyl fluoride (3) (27 mg, 0.15 mmol) was dissolved in a mixture of methanol (2 mL) and 0.05 M phosphate buffer (pH 7, 0.7 mL). To this solution was added a 0.05 M phosphate buffer (0.3 mL) solution of α-amylase (1.4 mg, 5 wt % for 3) for *A. oryzae*. The reaction mixture was shaken at room temperature for 1 h. After deactivating the enzyme by heating the reaction mixture at 100 °C for 10 min, the formation of a small amount of α-D-maltosyl fluoride (7% yield) was detected by means of HPLC.

Acknowledgment. This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (No. 03453114).

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