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Deacetoxycephalosporin C synthase (expandase): Research progress and application potential

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ABSTRACT

Cephalosporins play an indispensable role against bacterial infections. Deacetyloxycephalosporin C synthase (DAOCS), also called expandase, is a key enzyme in cephalosporin biosynthesis that epoxides penicillin to form the hexavalent thiazide ring of cephalosporin. DAOCS in fungus *Acremonium chrysogenum* was identified as a bifunctional enzyme with both ring expansion and hydroxylation, whereas two separate enzymes in bacteria catalyze these two reactions. In this review, we briefly summarize its source and function, improvement of the conversion rate of penicillin to deacetyloxycephalosporin C through enzyme modification, crystallography features, the prediction of the active site, and application perspective.

1. Introduction

Penicillin came into people's sight in 1928 and save millions of peoples' lives from bacterial infections in the later period. However, beneath its ever supreme antibacterial glory, penicillin also encounters the severe development of bacterial resistance due to its massive use, furthermore, penicillin itself has the disadvantages of narrow antibacterial spectrum and easy to cause anaphylactic shock. At this expecting time, a new kind of antibiotics came into being–cephalosporin. In 1945, Italian scientists discovered a spectral antibiotic with strong inhibition against both Gram-positive and Gram-negative bacteria when doing a drug study on the offshore sewage of Sardinia, Italy. In 1955, the first cephalosporin antibiotic, cephalosporin C, was isolated and identified, and is rapidly expanding as an antibacterial chemotherapy drug in a family of cephalosporins, which was widely prepared and used in clinic. In 1976, Kohsaka and Demain first reported the ring expansion reaction of penicillin N to deacetyloxycephalosporin (DAOC) in the cell-free reaction system of *Cephalosporium aeremonium* [1]. In 1983, the DAOCS in *C. aeremonium* was firstly purified by Kupta et al. [2]. In 1994, the cepharosporin sales volume exceeded penicillin, and the number of its varieties ranked the first among all kinds of antibiotics.

β-Lactam antibiotics have been widely used to treat various bacterial infections for over half a century. This kind of antibiotics has the advantages of strong bactericidal activity, low toxicity, broad indications and good clinical efficacy [3]. To date, penicillin and cephalosporin are the most characteristic β-lactam antibiotics. Both act as inhibitors of bacterial peptidoglycan biosynthesis, the advantage of cephalosporin over penicillin lies in their resistance to penicillin β-lactamase. Although cephalosporin has overcome some of the drawbacks of penicillin, there are still some problems to be solved in its production process.

At present, a third of cephalosporins are synthesized from penicillin in industry [4], the industrial production of cephalosporin includes chemical synthesis and potential enzymatic synthesis. The chemical synthesis process is complicated and accompanied with the production of by-products. Enzymatic synthesis has more obvious advantages and is an effective method for the production of cephalosporin. The key enzyme in enzymatic synthesis is deacetoxycephalosporin C synthase (DAOCS) (EC 1.14.20.1), but its natural substrate, penicillin N, is not easily obtained artificially. Penicillin G is the earliest clinical antibiotic due to its strong bactericidal force, easy industrial availability, low toxicity, and environmentally friendly [5]. Therefore, in order to facilitate the acquisition of cephalosporin, the research greatly focuses on

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improving the conversion rate of penicillin G to DAOCS.

2. The origin and function of DAOCS

After decades of efforts by many researchers, 9 DAOCS genes have been cloned and identified. To date, the bifunctional DAOCS/deacetylcephalosporin C synthase (DACS) has been isolated from 1 fungal strain (*Acremonium chrysogenum*) and DAOCS has been isolated from 8 bacterial strains (*Amycolatopsis lactamdurans, Pochonia chlamydosporia, Streptomyces clavuligerus*, *Streptomyces chartreusis*, *Streptomyces ambofaciens*, *Streptomyces jumonjinensis*, *Nocardia lactamdurans*, *Lysobacter lactamgenus*) (Table 1). Here, *A. chrysogenum* DAOCS and *S. clavuligerus* DAOCS/DACS are under overview as typical examples.

Cephalosporin biosynthesis is mediated by DAOCS, which is mainly responsible for the ring expansion of the five-member thiazole ring of the penicillin nucleus to form the six-member dihydrothiazide ring of the cephalosporin nucleus, namely the ring expansion reaction, which is also a rate-limiting step in the process of cephalosporin biosynthesis. There is no doubt that DAOCS is the bridge between penicillin and cephalosporin [11].

In both fungi and bacteria, the key enzymes for the biosynthesis of penicillin and cephalosporin are isopenicillin N synthase (IPNS) and DAOCS, respectively [12]. First, three amino acids, L-α-aminoadipic acid, L-cysteine and L-valine, were condensed to form LLD-ACV (d-L-α-aminoadipyl-L-cysteinyl-D-valine) tripeptide under the catalysis of non-ribosomal peptide LLD-ACV synthase [13]. IPNS catalyzes the cyclic production of isopenicillin N from ACV tripeptide, which is then isomerized to penicillin N by isopenicillin N isomerase. DAOCS catalyzes the five-member thiazole of penicillin N to oxidative cyclization to form a six-member dihydrothiazide ring of cephalosporin, which will generate DAOC. Next, DAOC hydroxylation leads to deacetylation of cephalosporin C (DAC), which is formed by DAC acetylation [14]. The following Fig. 1 depicts this specific process of cephalosporin biosynthesis.

In the above-mentioned cephalosporin biosynthesis, penicillin ring expansion reaction and DAOC hydroxylation reaction are slightly different between fungi and bacteria. In this review, the discussion of DAOCS from *S. clavuligerus* (scDAOCS) and *A. chrysogenum* (acDAOCS) was evaluated as examples. In *A. chrysogenum*, the dual-functional enzyme DAOCS/DACS encoded by *cef* gene not only catalyzes the expansion of penicillin N to produce DAOC, but also catalyzes the hydroxylation of DAOC to produce DAC [16]. However, in the case of *S. clavuligerus*, the expansion ring of penicillin N towards DAOC and the hydroxylation of DAOC are independently catalyzed by DAOCS encoded by *cefE* gene and DACS encoded by *cefF* gene [17]. Although all the known DAOCS exhibits highly specificity of the natural penicillin N substrate, but their substrate spectra are quite narrow. Since penicillin G is a cheap industrial antibiotics but with much low conversion rate to DAOC by DAOCS, therefore, the directional modification of DAOCS to improve the conversion rate of other penicillin is extremely important.

Table 1
DAOCS orthologues cloned and characterized.

Microorganism	GenBank ID	Peptide length
Amycolatopsis lactamdurans [6]	Q03047	314 aa
Acremonium chrysogenum (*A. chrysogenum*) [7]	P11935	332 aa
P. chlamydosporia	XP018144307	313 aa
N. lactamdurans	Z13974	314 aa
L. lactamgenus YK90 [8]	CAA39984	319 aa
S. clavuligerus NRRL 3585 [9]	P18548	311 aa
S. jumonjinensis NRRL 5741	AF317908	311 aa
S. chartreusis 102SH3 [10]	AY318743	311 aa
S. ambofaciens 29SA4 [10]	AY318742	311 aa

3. DAOCS as an Iron(II) and 2-oxoglutarate dependent enzyme

DAOCS belongs to the family of non-heme iron (II) and 2-oxoglutarate (2OG or α-ketoglutarate) dependent oxygenases [18]. Most of the enzymes in this family use iron (IV) and 2-oxygenated [19] intermediates to initiate different oxidative transformations [20] and play an indispensable role in biochemical reactions [21]. DAOCS forms a ternary complex with α-ketoglutarate and substrate, which then react with oxygen to form ferriyl intermediates, initiating the following series of reactions [22].

In the study of DAOCS, reductants other than α-ketoglutaric acid and Fe^{2+} were found to be required for its activity [23], such as vitamin C, molecular oxygen, ATP and DTT (dithiothreitol) [24] as cofactors to participate in the reaction of cephalosporin generated by penicillin expansion [25]. However, Cu^{2+}, Co^{2+} and Zn^{2+} have a stronger inhibitory effect on DAOCS [26]. Besides, parahydroxymercurate and 5,5′-dithiobis (2-nitrobenzoic acid) also inactivate the native scDAOCS. Not only the cofactors affect enzyme activity, but also the order in which cofactors and substrates are added [27]. DAOCS exists in solution as a balanced mixture of monomers and oligomers, whereas DTT addition partially reverses the aggregation.

4. Effect of modification on DAOCS

Since penicillin N is a natural substrate for DAOCS, the modification of DAOCS to improve the conversion rate of other kinds of penicillin is extremely important [28]. But, penicillin N is at a disadvantage compared to other penicillin [29] in terms of economic cost and clinical use. So, in order to improve the conversion rate of DAOCS from penicillin, many related researches have been conducted by changing the enzyme reaction conditions, or by modifying the DAOCS. For example, single mutation, double mutation and combined mutation were carried out on the enzyme, and the mutants that could significantly improve the enzyme activity and the conversion rate of penicillin were screened out. Next, we discuss the modification of DAOCS on its activity and conversion rate.

Based on the study of the crystal structure of DAOCS and the scientific deduction of the experimental results, it is shown that the C-terminal arm of DAOCS plays an important role in assisting the transformation of penicillin substrate, but the specific mechanism remains to be explored. According to the current studies, there are several conjectures as follows: C-terminal may be helpful to introduce the substrate into the active site [30], C-terminal amino acid residues are related to the activity of DAOCS [31], modification of the C-terminal might make the DAOCS more receptive to its unnatural penicillin substrates (penicillin G and penicillin V) [32]. By combining the existing theories with the experimental results, we can purposefully modify the C-terminal to achieve the desired effect.

4.1. Effect of single mutation on DAOCS

4.1.1. Effect of directional modification of acDAOCS

It has been reported that *A. chrysogenum* DAOCS/DACS (acDAOCS/DACS) residue N305 has a significant effect on enzyme activity, and C-terminal residue M306 has substrate selectivity and catalytic specificity [33]. By establishing the DAOCS/DACS model, it was observed that the side chain of C-terminal residue R307 extended from the substrate binding region, while the side chains of N305, M306 and R308 pointed to the substrate. The results of a series of experiments proved this conjecture.

Therefore, the residues N305, R307 and R308 of acDAOCS/DACS located near its C-terminal were respectively replaced by leucine in some experiments. The results showed that the transformation ability of N305L and R308L mutants to penicillin analogue was significantly improved. R308 was identified as a residue with significant influence on the activity of acDAOCS/DACS [34]. The kinetic data demonstrated that

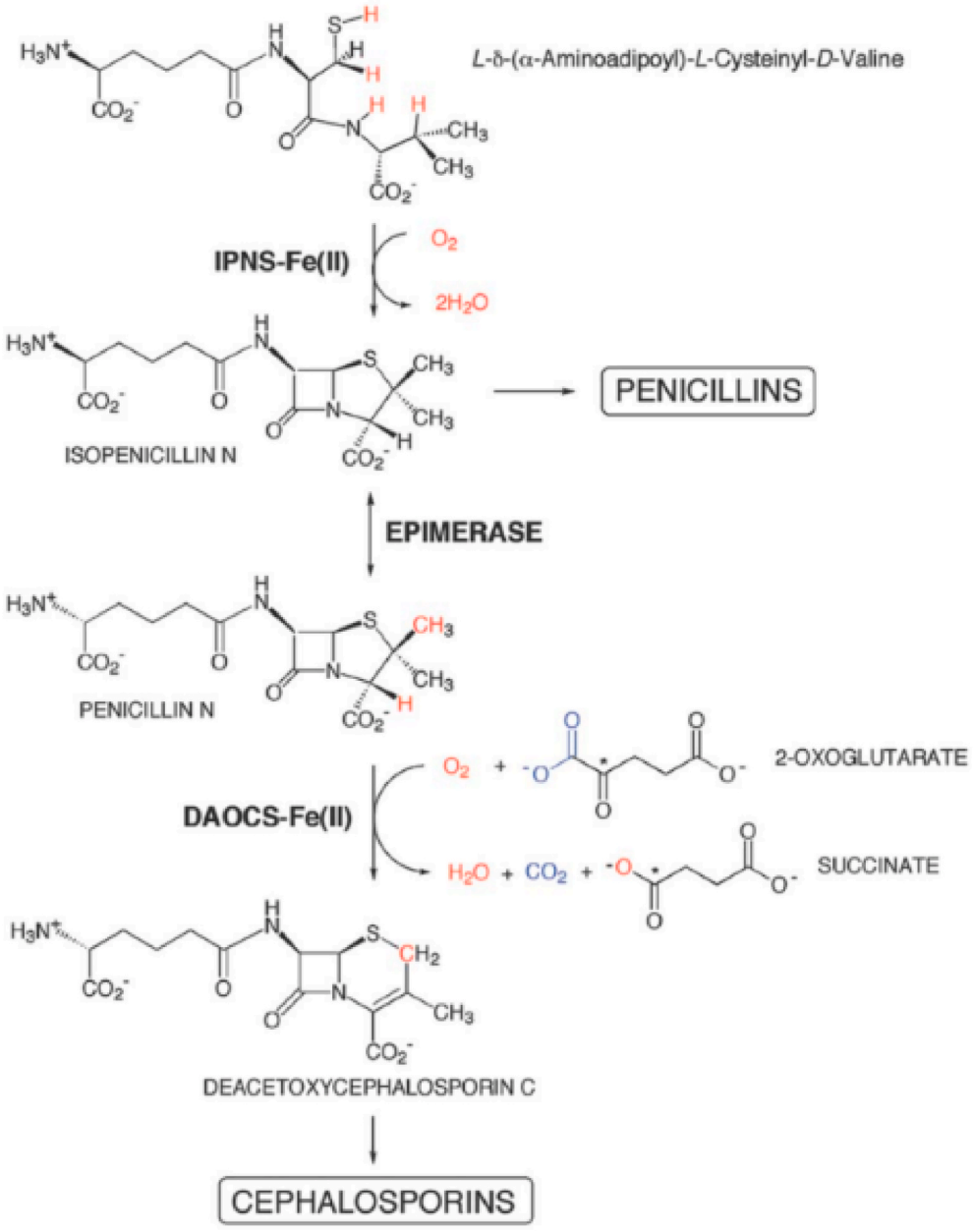

Fig. 1. The biosynthesis process of cephalosporin [15].

R308L and N305L had better affinity for the substrate penicillin G.

After the initial attempts, more mutations were made at the R308 site. Among the numerous R308 mutants, the relative specificity of the transformation of various penicillin analogues was studied, and the specific activity of R308L, R308V, R308I and R308T against penicillin G was increased by 3.46–7.62 fold [35]. R308L and R308I revealed the most meaningful improvement in the conversion of penicillin G at about 520% and 760%, respectively, and showed the widest substrate specificity and improved catalytic activity, capable of converting all penicillin analogues in the tests. These experimental results suggest that R308 occupies the strategic entry and exit channels in DAOCS.

4.1.2. *Effect of modified scDAOCS*

In the construction of mutants of scDAOCS, residues not directly participated in substrate recognition were selected to construct several mutants, such as Q126M, T213V, S261M, S261A and Y184A, all of which exhibited increased activity to penicillin G [36]. In the single round of random mutation, the kcat/Km values of G79E, V275I, C281Y, N304K, I305L and I305M were significantly increased, indicating that the enzyme activity was improved [37]. Q126, T213 and S261 were also identified as the sites with significant effects on enzyme activity [38]. scDAOCS contains 7 cysteine residues, and attempts have been made to mutate 3 cysteine residues at 100, 155 and 197 sites of DAOCS to alanine residues [39]. However, the results were not very satisfactory, in which the property of C100A mutant was similar to that of wild-type enzymes, furthermore, the activities of C155A and C197A mutants on both penicillin N and penicillin G were not improved but decreased.

Similarly, in *S. clavuligerus*, studies have been devoted to directional modification of hydrophilic residues N301, Y302, N304, R306 and R307 near the end of scDAOCS C-terminal, which were replaced by a hydrophobic leucine residue, respectively. Calculation and analysis indicated that N304 was strategically located in the catalytic chamber of scDAOCS [40], it is important to attract different substrates into the center. In the bioassay of penicillin analogue transformation, it is found that the enzyme activities of both mutants N304L and R306L significantly enhanced the conversion rate of penicillin substrate by 145–400% as

compared with WT enzyme. Both N304K and N304R mutants showed a significant increase in enzyme activity of up to 730% [41], the N304A mutant also gave an enzyme activity increase of about 160–330%, but the mutation of N301L, Y302L and R307L around N304 did not work well [42]. In the future, double or triple mutations should be constructed at the C-terminal of scDAOCS, which may contribute to the construction of scDAOCS mutant and further improve enzyme activity.

However, previous studies revealed that the C-terminal of scDAOCS might hinder the transformation of penicillin G [43], because the truncation of the C-terminal of scDAOCS enhanced the enzyme activity and the conversion rate of penicillin [42]. Some experiments also evaluated the involvement of scDAOCS C-terminal by constructing the C-terminal truncated mutants, and demonstrated that truncating the C-terminal of scDAOCS could change its substrate specificity and enzyme activity. In C-terminal truncated mutants, WT-ΔK310 enzyme transformation of penicillin G increased by 124% compared to that of WT, N304L-ΔK310 enzyme activity versus WT scDAOCS significantly enhanced up to 155–400%, and conversion were also increased for other penicillin analogues. These results suggest that it is reasonable to further improve the enzyme activity of scDAOCS and the transformation of penicillin analogues through C-terminal mutation or the combination of mutation and truncation. Unfortunately, the true functionality of the C-terminal in DAOCS remains unclear.

4.2. *Effect of iterative combined mutagenesis on scDAOCS*

In addition to the single round of random mutagenesis, another widely used method is stochastic iterative combined mutagenesis (ICM), which is used to guide the combined mutagenesis route. First, the targeted site mutation at the predetermined mutation site was introduced into the initiation enzyme, and then the most enhanced enzyme was selected as the next round of targeted mutation initiation enzyme [44], Reetz et al. first applied the ICM strategy in combination with saturation mutagenesis [45]. In practical application, ICM significantly reduced the screened number of mutants to reduce the workload of experimental researchers. This is a great strategy to combine effective identification of beneficial combination mutations with minimal effort [46].

In the evolution of scDAOCS, random and directed mutagenesis strategies have been tried. Random double mutant YS67 (V275I, I305M) was screened out from *S. clavuligerus*, kcat/km ratio was significantly increased by 32-fold, and the relative activity of penicillin G was increased by 5-fold. The relative activity of the triple mutant YS81 (V275I, C281Y, I305M) against penicillin G was increased by 13-fold. In the process of random or directed mutations, although there have been many reports of a single beneficial mutation in DAOCS, e.g., M73T, G79E, T91A, C155Y, Y184H, M188V, H244Q, V275I, L277Q, C281Y, N304K, I305L, I305M, etc., the catalytic efficiency of penicillin G [47] was determined to be significantly improved from 1.5 to 117.8 fold. However, the combination of these mutations has not been systematically verified to further improve the activity of scDAOCS, so the combination of mutations is also one of the strategies to improve enzyme activity and penicillin transformation.

5. Prediction of scDAOCS catalytic sites

With the development of protein structure analysis software, it is possible to predict the catalytic site of proteins with similar structure. The active ligands were observed in scDAOCS to be embedded [48] in a colloidal structure and arranged by hydrophobic residues [49], which might help to isolate highly reactive intermediates from the environment to accelerate the catalytic reaction of enzymes with substrates [50]. Experimental results showed that IPNS, DAOCS, DACS and other enzymes share a conserved structural framework in the catalytic center. Therefore, a reliable prediction model can be established according to the structural similarity of these enzymes for further research.

Through amino acid sequence alignment, it is found that DAOCS and IPNS are only 14% similar in amino acid sequence, which indicates that the amino acid similarity ratio of these two substances is not high [51], but they have obvious similarities in secondary and tertiary structures. Successful crystallization of scDAOCS and IPNS can elucidate the spatial organization of these proteins and the function of the substrate and cofactor binding sites [52]. The 3D structure of scDAOCS is shown in Fig. 2. Therefore, it is able to use the computer and its X-ray structure to assist in inferring possible residues near the catalytic center of scDAOCS and model validation [53].

The crystal structure indicates that in a trimer unit, the C-terminal of a molecule is cyclically inserted into the active site of its neighboring molecule. This arrangement impedes the production of the crystalloase-substrate complex [54]. This also explains the rationality of constructing a series of C-terminal modified DAOCS mutants.

6. The application prospect of DAOCS

As described above, the key enzyme DAOCS plays an indispensable role as the rate-limiting step in cephalosporin biosynthesis. A number of attempts, such as random or targeted mutation and genetic engineering, have been continuously carried out to enhance DAOCS activity for higher DAOC production.

Due to the weak antibacterial activity of cephalosporin C, the clinical important cephalosprins are manufactured by ligation of various side chains to the 7-amino residue of the intermediate, 7- amino-deacetoxycephalosporanic acid (7-ADCA). Currently in industrial practice, 7-ADCA is prepared using chemical expansion of penicillin V or penicillin G. This process has many disadvantages, such as strict reaction condition, high cost and serious environmental pollution, etc. To overcome these disadvantages, enzymatic synthesis using DAOCS came into human's sight. However, native DAOCS only recognize penicillin N rather than penicillin G as substrate, the effective modification of native DAOCS for penicillin G is imperative with modern molecular biology and synthetic biology. For example, Lin et al.enhanced the yield of phenylacetyl-7-ADCA (the precursor of 7-ADCA) from penicillin G by 11-fold using engineered *Escherichia coli* as efficient whole cell catalyst by reconstitution of TCA cycle with DAOCS.

In another case, avermectin (AVM) is a kind of 16-membered ring macrolide fermented by *Streptomyces avermitilis*. As a world-famous anthelmintic and pesticide antibiotics, AVM was awarded 2015 Nobel Prize in Physiology or Medicine. However, the AVM yield was very low in the fermentation. Based on decades of related efforts in China, the 5 M strategy (Mine, Model, Manipulation, Measure and Manufacture) was proposed by Zhang et al. [55]. With the aid of synthetic biology principle and technique, intelligent AVM production has been achieved with the titer increase by ~1000-fold and the sale price decreased by ~40-fold, which led China to be the only producer of raw AVM product in the world since late 2015. Therefore, this successful example can draw lessons from for improving DAOCS substrate spectrum and activity,

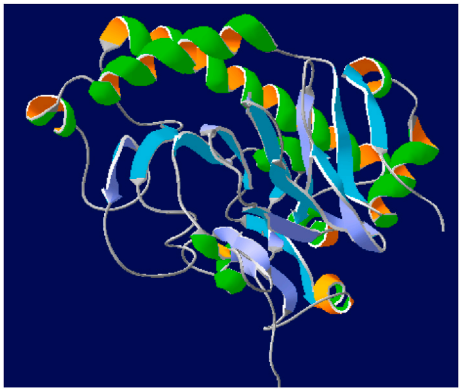

Fig. 2. The 3D structure of scDAOCS.

7-ADCA and cephalosporin production. On the other hand, the success in modification might greatly facilitate efficient and new cephalosporin development as well as for other kinds of antibiotics.

7. Summary

To date, the research progress in DOACS have greatly enhanced our understanding for cephalosporin biosynthesis and potential manufacture application. Since new DAOCS might be still isolated, identified and purified from various strains, we should have been using scientific theoretical knowledge and the latest technology to continuously improve its quality to meet our production needs. Future studies on DAOCS should try to improve the conversion rate of DAOCS to various penicillin substrates. If DAOCS mutants can be constructed to significantly increase the conversion rate, it will mean more efficient and clean production of cephalosporins. At the same time, the catalytic active site of DAOCS should be further explored through the study of its structure, the mechanism of its C-terminal influence on the activity of DAOCS, and the directed modification of DAOCS will finally realize the efficient intelligent bio-manufacture of cephalosporin in industry to contribute to our life and medical cause.

Credit author statement

Xiaofan Niu: Writing – original draft, Methodology, Jian Zhang: Data curation, Validation, Xianli Xue: Resources, Depei Wang: Funding acquisition; Lin Wang: Visualization, Qiang Gao: Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition. All authors read and agreed to the publication of the manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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