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Authors
Denish, Pamela R
Fenger, Julie-Anne
Powers, Randall
et al.

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Discovery of a natural cyan blue: A unique food-sourced anthocyanin could replace synthetic brilliant blue

Pamela R. Denish 1,2,†, Julie-Anne Fenger 3,†, Randall Powers 4,†, Gregory T. Sigurdson 5, Luca Grisanti 6,7, Kathryn G. Guggenheim 8, Sara Laporte 6, Julia Li 9, Tadao Kondo 9, Alessandra Magistrato 10, Micheál P. Moloney 3, Mary Riley 11, Mariami Rusishvili 12, Neda Ahmadiani 13, Stefano Baroni 6,9, Olivier Dangles 3, Monica Giusti 5, Thomas M. Collins 4, John Didzbalis 14, Kumi Yoshida 9, Justin B. Siegel 1,2,8,15,†, Rebecca J. Robbins 16,†

The color of food is critical to the food and beverage industries, as it influences many properties beyond eye-pleasing visuals including flavor, safety, and nutritional value. Blue is one of the rarest colors in nature’s food palette—especially a cyan blue—giving scientists few sources for natural blue food colorants. Finding a natural cyan blue dye equivalent to FD&C Blue No. 1 remains an industry-wide challenge and the subject of several research programs worldwide. Computational simulations and large-array spectroscopic techniques were used to determine the 3D chemical structure, color expression, and stability of this previously uncharacterized cyan blue anthocyanin-based colorant. Synthetic biology and computational protein design tools were leveraged to develop an enzymatic transformation of red cabbage anthocyanins into the desired anthocyanin. More broadly, this research demonstrates the power of a multidisciplinary strategy to solve a long-standing challenge in the food industry.

INTRODUCTION

Despite a long history of exploration, blue remains one of the most challenging colorants to obtain from any source and even more so from natural, edible sources (1–7). Blue is critical as it is necessary to produce other colors across the palette. Furthermore, the subtleties of color differences in different blues are of great importance in the area of blending colors (8). The two main artificial blue food colorants are brilliant blue (FD&C Blue No. 1) and indigotine (FD&C Blue No. 2), providing cyan ($\lambda_{max} = 630$ nm) and indigo ($\lambda_{max} = 608$ nm) hues, respectively. Naturally occurring food-based blues are limited and can be sourced from anthocyanins and a limited set of other blue colorants including phycocyanins (from Spirulina spp.) ($\lambda_{max} = 615$ to 620 nm) (9) and iridoid derivatives from huito (or gardenia) ($\lambda_{max} = 590$ to 610 nm) (10, 11). However, to date, all known natural colorants have either a $\lambda_{max}$ less than 630 nm, a large violet color equivalent, or both (Fig. 1A, method S1.1, and figs. S1.1 and S1.2). Violet contributions affect the final color in blended colorants, e.g., blending natural blues with yellow generally results in a muddy green (fig. S2.1 and discussion S2.1).

Although green is abundant in nature, the chlorophyll chromophore is not stable or water soluble (section S2.1) and, therefore, has limited application (12). Achieving a cyan blue from natural sources that could be used as replacement for FD&C Blue No. 1 enabling a broader color palette has been a long-standing challenge to the food industry. Anthocyanins display a large versatility in color expression because of a complex chemical equilibrium of colored and colorless forms (fig. S3.1 and discussion S3.1) (13–17). These naturally occurring chromophores are generally intensely red-colored under acidic conditions based on the flavlyium cation (Fig. 1B) and change toward violet and blue as the pH increases. Concurrently, the colored forms are susceptible to fading by a combination of water addition (reversible) and autoxidation (irreversible), limiting their stability over time (18). A challenge with anthocyanins is the plethora of different molecules found in a single crop source, such as red cabbage (Fig. 1B) or purple sweet potato (19, 20). Both well-established commodity crops generate blue colors at pH 7 to 9. However, it is a composite color because of the numerous individual anthocyanin molecules present and the various equilibrium forms at a given pH for each individual anthocyanin molecule (10, 19, 20). While the combined red cabbage anthocyanins (RCAs) will produce a vibrant and attractive blue color in pH-neutral solution, there is still a relatively large violet color contribution, limiting their utility as a natural FD&C Blue No. 1 replacement (Fig. 1A).

Previous efforts to identify the chemical structures and spectral properties of RCAs uncovered an interesting structural homology where the common building blocks are a cyanidin chromophore, a sophorose moiety bound at position 3, and a glucose bound at position 5 (Fig. 1B) (21–25). The key differentiation factors are the presence of hydroxycinnamic acids and their substitution patterns on sugar 1 (Glc-1) or sugar 2 (Glc-2) of the sophorose (Fig. 1B, table inset). Remarkably, one of the minor mono-acylated anthocyanins, Peak 2 (P2) was found to have a particularly high $\lambda_{max}$ of 640 nm at pH 7 (20). While only a minor component within the RCA mixture, this unique compound warranted further investigation to understand the structural factors conferring its desirable spectral properties.

1Biophysics Graduate Group, University of California, Davis, Davis, CA, USA. 2Genome Center, University of California, Davis, Davis, CA 95616, USA. 3Avignon University, INRAE, Avignon, France. 4Mars Wrigley, Hackettstown, NJ 07840, USA. 5Department of Food Science and Technology, The Ohio State University, Columbus, OH 43210, USA. 6Scuola Internazionale Superiore di Studi Avanzati, Trieste, Italy. 7Division of Theoretical Physics, Institut Ruder Bošković, Zagreb, Croatia. 8Chemistry Department, University of California, Davis, CA 95616, USA. 9Graduate School of Informatics, Nagoya University, Chikusa, Nagoya, Japan. 10Consiglio Nazionale delle Ricerche, Istituto Officina dei Materiali, Scuola Internazionale Superiore di Studi Avanzati, Trieste, Italy. 11Microbiology Graduate Group, University of California, Davis, Davis, CA 95616, USA. 12Pritzker School of Molecular Engineering, The University of Chicago, Chicago, IL, USA. 13Centre d’Innovació, Recerca I Transferència en Tecnologia Aliments, Universidad Autònoma de Barcelona, Bellaterra, Spain. 14Mars Advanced Food Science and Technology, University of California, Davis, Sacramento, CA 95616, USA. 15Mars Wrigley Global Innovation Center, Chicago, IL 60642, USA. 16Consortium for Learning, Research, Innovation, and Development, University of California, Davis, CA 95616, USA. 17Rebecca Robbins, effem.com (R.J.R.)

†These authors contributed equally to this work.
RESULTS AND DISCUSSION

Structural analysis of P2

Previous efforts relied on mass and retention time or established standards to make predictions of the P2 structure (20). Before conducting an in-depth investigation of the structural factors, which impart the unique spectral properties to P2, it was prudent to obtain a complete structural assignment of the compound. The structural assignment for P2 was determined by high-resolution electrospray ionization–time-of-flight mass spectrometry (ESI-TOF-MS) and a combination of $^1$H and $^{13}$C nuclear magnetic resonance (NMR), one-dimensional total correlation spectroscopy (1D TOCSY), correlation spectroscopy (COSY), nuclear Overhauser effect spectroscopy (NOESY), heteronuclear single-quantum correlation spectroscopy (HSQC), and heteronuclear multiple bond correlation (HMBC) experiments and proven to be 3-O-(2-O-(2-O-(E)-sinapoyl-β-D-glucopyranosyl)-β-D-glucopyranosyl)-5-O-β-D-glucopyranosyl-cyanidin (discussion S5.1, table S5.1, and figs. S5.1 to S5.9) (26, 27). The results were consistent with the previously predicted structure, supporting that P2 is a regio-isomer of P5 where only the sinapoyl residue resides on a different sugar (Fig. 1B).

It is well established that cyanidin-based chromophores, such as P2, require a combination of neutral pH and complexation to a metal ion, such as an Al$^{3+}$, at the ortho-dihydroxyphenyl moiety (Fig. 1B, positions 3' and 4') of the cyanidin chromophore to produce a blue color (15, 17, 27, 28). To evaluate the effect of metal ion complexation with specific RCA components, P2, P5, and P8 were incubated with Al$^{3+}$. The only difference between P2, P5, and P8 is the number and placement of sinapoyl moieties on the sophorose, enabling a direct comparison of the primary structural feature differentiating RCAs and their effect on color formation.

The discrimination between the spectral properties of P2, P5, and P8 in the presence of Al$^{3+}$ is highly unexpected (Fig. 2A). With only one-third equivalent of Al$^{3+}$, the P2 solution gave the desired blue colored complex with a large bathochromic shift of >40 nm ($\lambda_{\text{max}}$ at 640 nm; Figs. 1A and 2A, figs. S6.1 to S6.4, and discussion S6.2). In contrast, P8 and P5 had only modest shifts of <20 nm. This provides direct evidence that the sinapoyl moiety and its position (on Glc-2 in P2), despite its intrinsic inability to bind metal ions, play a critical role in the structure of the metal complex, leading to a very strong bathochromic shift.

To elucidate this uniquely colored self-assembled structure of the three P2 molecules coordinated to aluminum metal (29), the circular dichroism (CD) spectrum of P2 and Al$^{3+}$ was recorded in buffered solutions. With the addition of Al$^{3+}$, P2 showed an exciton-type positive Cotton effect around $\lambda_{\text{max}}$ without Al$^{3+}$. These results indicate that Al$^{3+}$ is critical to the coordination and building of the tertiary structure that exhibits a chiral arrangement of three P2 monomers around the Al$^{3+}$ ion (Fig. 3, A and B, and section S8). By contrast, P5 displayed very minor Cotton effects (section S7.1, figs. S7.1 to S7.3, and table S7.1). Furthermore, negative-mode high-resolution ESI-TOF-MS analysis of the P2/Al solution at pH 7 provided evidence for molecular ion peaks at mass/charge ratio ($m/z$) = 1478.8629 and at $m/z = 985.5760$, which can be respectively attributed to $[3\times P2+Al^{3+}]^{2+}$ and $[3\times P2+Al^{3+}]^{-}$, indicating the formation of a Al$^{3+}$-P2 complex of 1:3 stoichiometry (Fig. 3A, section S7.2, and figs. S7.4 to S7.6). P5 did not give such multivalent molecular ion peaks by addition of Al$^{3+}$ (fig. S7.5 to S7.7); however, P8 showed the similar divalent and trivalent ion peaks of $[3\times P8+Al^{3+}]^{2+}$ and $[3\times P8+Al^{3+}]^{3+}$ (figs. S7.8 to S7.10).
$^1$H NMR measurement of the trimeric complex of Al$^{3+}$P$_2$$_3$ in D$_2$O gave a very broad spectrum. However, numbers of signals in the lower field were relatively simple, indicating that the arrangement of the three P$_2$ ligands within the Al complex might be highly symmetrical. Several signals were assignable, but analysis of NOE correlations was difficult (discussion S7.3, figs. S7.11 to S7.15, and table S7.2). Therefore, a structural analysis of Al$^{3+}$P$_2$$_3$ was undertaken by computational modeling. After extensive classical and first-principles molecular dynamics (MD) (9) simulations of P$_2$, plausible geometries were constructed for threefold symmetric propeller-like structures of three P$_2$ molecules around a single aluminum ion and minimized with first-principles calculations (discussion S8.1, method S8.1, fig. S8.1, and table S8.1). In all proposed configurations, the structure of the P$_2$ cyanidin chromophore is distorted by a torsional displacement...
of the bond between the B and C rings (Fig. 3A, θ angle, and fig. S8.2, inset). A very interesting and remarkable result from the MD simulations, coupled with first-principles evaluation of excited states, is that torsional displacement of this bond causes the greatest change in the simulated color expression (30, 31). The π–π electronic interaction between acyl and cyanidin motifs is not the main cause of the large color shift, as typically suggested. In this case, the HOMO and LUMO energies are more impacted by distortion of the cyanidin nucleus and the interactions among the ones coordinated to the same metal (excitonic effects) than by acyl-cyanidin interactions (discussions S8.1 and S8.2). However, the π–π interaction between the hydrophobic aromatic rings of the acyl and cyanin groups has a critical influence on the conformations: While intramolecular π–π stacking dominates in isolated P2 and P5 (discussion S8.3), the proposed Al3+(P2−)3 complex manifests intermolecular π–π stacking between adjacent P2 moieties (interlocking between P2 units shown in figs. S8.2 to S8.4), which is consistent with the strong Cotton effect observed by CD and the enhanced stability of the complex discussed below.

Among all RCAs, P2 uniquely displays two remarkable long-range NOE correlations between the cyanidin and sinapoyl residue in a solvent that does not favor π-stacking interactions (MeOH), indicating a folding of the molecule (figs. S5.3 and S5.4) (21). The disaccharide in the β-β-β-Glc(2-sinapoyl)-1,2-β-β-β-Glc-3-cyanidin sequence within monoacylated P2 likely provides optimal intramolecular spacing for enhanced conformational stability. Protection against hydration (water addition at position C2) was modest with monoacylated P5 (pK′ h = 2.7, versus 2.1 for nonacylated P1) but more pronounced with diacylated P8 (pK′ h = 3.7) (15, 21, 28). P2 deviates from this trend and is exceptionally resistant to water addition (pK′ h = 4.4) despite its monoacylated status (section S9.1, fig. S9.1, and table S9.1) (14, 15). This resistance to hydration is reflected by the superior long-term stability of P2 compared to P5 and P8 and Al3+(P2−)3 compared to the P5 and P8 mixtures (Fig. 2B and fig. S6.3). In addition, although MD simulations of P2 and P5 show that, in both cases, folded conformations are favored, P2 displays a higher percentage of closed conformations than P5 (method S10.1, fig. S10.1, and discussion S10.1). It is hypothesized that the “tightly closed” P2 structure enables a closer approach to Al3+ (fig. S10.1c) and a trimeric arrangement around the metal ion. This arrangement causes an increase in the torsional distortion (angle θ) of the B-ring to C-ring bond, which enhances the bathochromic shift of the visible absorption band (Fig. 3A, inset). Although P8 is closely related to P2, its additional sinapoyl residue might provide steric hindrance limiting the simultaneous interaction of three P8 molecules with Al3+. As for P5, its more open structure (cyanidin-sinapoyl interactions weaker than in P2) might prevent the arrangement of anthocyanin molecules around Al3+, precluding formation of the Al3+(P5−)3 complex.

**Enzymatic enrichment of P2**

While the Al3+(P2−)3 complex has the unique chemical conformation that provides the spectral properties desired for replacing FD&C Blue No. 1, P2 represents <5% of the total anthocyanin content naturally occurring in red cabbage. Hence, an ambitious challenge was obtaining sufficient quantities of P2 at high purity for the present research program and for potential use as a food coloring agent. To that end, the homology of structures in red cabbage was leveraged and a hydrolytic enzyme capable of a highly selective catalytic decylation was found and further developed. The enzyme selectively removes any acyl group bound to Glc-1 of the sophorose moiety while leaving the sinapoyl group on Glc-2 intact, thus converting P6, P7, and P8 into P2 (Fig. 4, A and B). Initial database mining efforts comprised screening a broad range of genes encoding hydrolytic enzymes, including lactonases, esterases, lactamases, and hydrolases, curated from the BRENDA database across 26 enzyme classifications (ECs) (Fig. 4C and tables S11.1 and S11.2) (32). Over three rounds of genomic mining, a total of 46 genes were tested, of which 17 were active on P6 to P8, converting them to P2 (method S11.2). The median sequence identity of the total set of tested enzymes is 7.0%, and the median sequence identity of active enzymes is 13.0% (Fig. 4C), highlighting the plasticity of functionality within the esterase family through the diversity of sequences capable of catalyzing this reaction.

The most active of these enzymes was 1AUR, which in its native form hydrolyzes longer fatty acid chains but has demonstrated broad specificity toward esters (fig. S11.1) (33). We subsequently redesigned the pocket of the 1AUR protein (24) using a combination of Rosetta Design and FoldId to introduce new favorable interactions with P8 (the largest and most complex of the RCAs) (34, 35). Across multiple rounds of design, the mutant with the best conversion of P6, P7, and P8 was M73H, which was able to drive the transformation of RCAs 3 to 8 to P1 and P2 to completion in a time frame and enzyme concentration viable for the gram-scale production of P2 needed for food-product prototyping (Fig. 4B, table S11.3, and figs. S11.2 and S11.3). On the basis of the modeling and design efforts, we hypothesize that the two additional hydrogen bonds that are developed by the histidine residue substituted at position 73, which are both exposed at the active cleft, strengthen the enzyme-substrate interaction (Fig. 4D).

**Color expression and application of P2**

Following chromatographic purification of the enzymatic transformation and complex formation with Al3+ (methods S11.3 to S11.4 and section S12), gram quantities of the Al3+(P2−)3 complex were obtained, enabling detailed investigations of the color expression and stability of the novel colorant. In any food product application, color stability is critical. While many naturally sourced colorants, including anthocyanins, have limited stability over time, the Al3+(P2−)3 complex at pH 7 showed remarkable stability in sugar syrup over 55 days with only a 14% loss of color (Fig. S5A) (19). Its performance as a colorant was also demonstrated in application to create blue and green colors (hue angle match to a Christmas green shade; fig. S2.1) in several food and confectionery products (Fig. 5, B and C, figs. S13.1 to S13.4, and table S13.1 to S13.4). The stability of this novel colorant in these product applications is excellent as well, with no notable color decay over a 30-day period when stored at ambient conditions (fig. S13.5 and table S13.5). Storage under acidic conditions further enhances the stability of the colorant by reducing fraction of hydrolyzed species, although neutral pH must be able to be restored for integration into product (figs. S3.1 and S9.1) (19). While these initial studies provide a clear starting point for the development of a natural FD&C Blue No. 1, future efforts will be critical to evaluate both stability and color in a wide variety of applications to define usage limitations (discussion S12.1) and appropriate colorant and food safety precautions (36–38).

In summary, combining modern techniques from analytical chemistry, food science, biochemistry, synthetic biology, color science, and computational chemistry, we found, characterized, and defined a path to production for a naturally sourced cyan blue colorant whose
color properties are nearly identical to those of the industry standard brilliant blue FCF (FD&C Blue No. 1). This colorant is also capable of producing superior green colors compared to many existing natural blue colorants. This discovery requiring a global collaboration provides a solution to a long-standing food color need, potentially fulfilling the growing consumer demands for utilization of more natural ingredients in food while keeping a vibrant color palette.

**MATERIALS AND METHODS**

Detailed methods for the experiments conducted in this study can be found in the Supplementary Materials.

**Violet contribution calculation**

The violet contribution of a blue colorant was defined as the area under the visible absorption curve (AUC). The area was calculated...
using the left Riemann sum for integration, where the height is the absorbance and the width is the spectral resolution (for more specific details, see method S1.1).

**Ultraviolet-visible spectrometry**

Solutions were poured into relevant cuvette of 10-, 1-, and 0.1-mm cell length, and then ultraviolet (UV)–visible absorption spectra (200 to 800 nm) were recorded on a JASCO V-560 spectrophotometer. The solutions were kept at room temperature and protected from light (method S6.1).

**Measurement of CD**

CD was measured from 200 to 800 nm with a JASCO J-720 spectrometer using the same solution for the measurement of stability described in section S6.1. To increase signal/noise ratio, the scan was repeated four times, and the data were averaged (method S7.1) (21).

**ESI-TOF-MS of aluminum complexes**

ESI-MS spectra were recorded with a Bruker COMPACT instrument and analyzed with the application purchased from Bruker Daltonics (method S7.1).

**NMR measurement of aluminum complex of P2**

NMR spectra were obtained with Bruker Daltonics AVANCE III HD 600 with a TCI cryoprobe and BBO cryoprobe (1H: 600 MHz and 13C: 150 MHz) in a 5-mm–inside diameter tube at variable temperatures in D2O. Chemical shifts were recorded as parts per million (ppm) using the proton resonance in the semi-heavy water (HDO) as a standard (4.67 ppm). Various 1D and 2D measurements were carried out (method S7.3).

**Geometric optimization of putative P2 and P5 3:1 complexes with aluminum**

Optimizations of either P2 or P5 molecules in a 3:1 complex with aluminum were performed, starting from relevant structures of monomers obtained from extensive MD simulations and analysis, as detailed in section S10.1. These were arranged with a C3 symmetry around Al3+ and deprotonated at 3′ and 4′, and with Al3+ coordinated to the oxygens at 3′ and 4′. The two chiral assemblies of the ligands, corresponding to left and right handed (labeled \( \Lambda \) and \( \Delta \), respectively), were tried. Relaxations were then performed in two steps: (i) A pre-optimization was run in vacuum with CP2K (39), with DZVP-MOLOPT-SR-GTH basis set and GTH-BLYP pseudopotential (40–42) corrected with the D3(0) Grimme dispersion (43). (ii) Final optimizations were run in Quantum Espresso (44, 45), using ultrasoft pseudopotentials and the Perdew-Burke-Ernzerhof (PBE) exchange-correlation functional (46) with implicit solvent (Environ module) (47) in periodic cubic box of 80 bohr. The following parameters were adopted for the wavefunction convergence: kinetic-energy cutoff of 35 rydberg (Ry) and charge density cutoff of 320 Ry. The Makov-Payne energy correction was used to remove finite-box effects (method S8.1).

**MD simulations of P2 and P5**

Classical MD simulations of P5 and P2 were run using GROMACS 4.5.5 (48) upon adequate equilibration with all bonds to hydrogen atoms constrained using LINCS (49). General AMBER force field (GAFF) parameters were assigned using the antechamber module of AmberTools13 (50, 51) with RESP charges at the HF/6-31G* level, calculated on DFT-B3LYP optimized geometries, 50-/200-Ry basis set. For each species studied, 10 replicas were simulated using Hamiltonian replica-exchange MD (HREMD) (52) as implemented in the PLUMED plugin (53) (method S10.1).

**Sequence alignment and construction of the phylogenetic tree of esterases of interest**

Sequences tested in this study (table S11.1) were aligned using Geneious 2017.10.1.3 using multiple sequence comparison by log-expectation (MUSCLE) alignment (method S11.1).

The phylogenetic tree in Fig. 4C was constructed using the Geneious Consensus Tree Builder (method S11.1) and visualized using GraPhlAn (https://github.com/siegel-lab-ucd/blue-pigment-publication.git) (54).

**Protein purification and activity screening of esterases**

An *Escherichia coli* codon-optimized gene encoding each protein was purchased from Twist Biosciences and transferred into pET29b+ to encode a C-terminal hexahistidine tag. Mutant plasmids were produced by Kunkel mutagenesis (55). Plasmids were incorporated into *E. coli* BL21(DE3) via electroporation. Cultures were grown in Terrific Broth at 37°C, induced with 1 mM isopropyl-\( \beta \)-d-thiogalactopyranoside, and allowed to express at 18°C for 24 hours, after which cells were lysed, clarified, and protein-purified using gravity columns with immobilized metal affinity chromatography, the details of which are provided in method S11.2. Proteins were screened for activity by combining 10 \( \mu \text{l} \) of RCE (100 mg/ml) with 90 \( \mu \text{l} \) of 50 mM Hepes buffer (pH 7.5) and allowed to proceed at room temperature for 24 hours. Reactions were quenched with 70% methanol and brought to pH 3 with 1 \( \mu \text{l} \) of HCl. Reactions were centrifuged at 4700 rpm for 3 min to remove insoluble protein. Analysis was done using the high-performance liquid chromatography (HPLC) method described in method S11.2.

**Gram-scale protein production**

The M73H point mutant was created from the 1AUR (sequence ID no. 46) wild-type (WT) plasmid via Kunkel mutagenesis (55) and then transformed into chemically competent *E. coli* BLR (DE3) cells (details provided in method S11.3). The supernatant lysate containing active enzyme was collected and used for the reaction. The scaled-up generation of P2 via the enzymatic reaction is described in method S11.4.

**P2 purification from enzyme-treated red cabbage**

The material was purified using several techniques to remove impurities: enzyme precipitation, solid-phase extraction, and preparatory HPLC (more detailed description is found in method S12.1).

**Anthocyanin-metal complex [Al3+(P2−)]3 formation details**

One-third equivalents of AlK(SO4)2 stock solution were added to the aqueous P2 solution, adjusted to 7.0. The P2-Al complex solution was transferred to a polypropylene container, immersed in liquid nitrogen until fully frozen, and then placed into the lyophilizer with vacuum set to 0.03 mbar and the condenser to ~80°C. More detailed description is provided in method S12.2.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/15/eabe7871/DC1

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