Conformational Preferences and Protonation Sequence of *myo*-Inositol Hexaphosphate in Aqueous Solution; Potentiometric and Multinuclear Magnetic Resonance Studies

Cédric Brigando, Jean C. Mossoyan,* Frédéric Favier and David Benlian
Laboratoire de Chimie de Coordination. D22, Université de Provence, Av. Escadrille Normandie-Niemen, 13397 Marseille Cedex 20, France

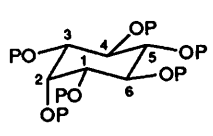
Potentiometric methods and multinuclear NMR spectroscopy have been used to determine the protonation sequence and conformational preferences of *myo*-inositol (cyclohexane-1,2,3,4,5,6-hexol) hexaphosphate over the range pH 0–12. The ^{1}H, ^{31}P and ^{13}C resonances have been assigned from both homo- and hetero-nuclear coupling constants obtained by one- and two-dimensional NMR spectroscopy. The phosphate exists in aqueous solution in either of two conformations, axial and equatorial, as a result of intramolecular hydrogen-bond formation between phosphate groups which leads to stabilization of the equatorial form up to pH 10 and of the axial at pH > 10. Analysis of lanthanide-induced shift data shows unambiguously that the last protonation occurs on the P^2 phosphate group in strongly acidic media.

Inositol (cyclohexane-1,2,3,4,5,6-hexol) phosphates, rather common and ubiquitous molecules, are widely present in many biological systems.[1] Some are active carriers of calcium in cell metabolism, while others act through mobilization of intracellular calcium stores.[2,3] This large family of phosphoesters includes numerous positional isomers the functional and nutritional roles as well as biochemical activities of which depend on the number and relative positions of the phosphate groups. Their reversible protonation and ability to form intramolecular hydrogen bonding and to complex with metals differentiate the specific properties of these polyanions.[4–6]

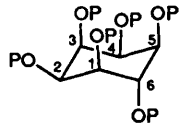
myo-Inositol hexaphosphate (H_{12}inhp), commonly called phytic acid, is a representative example of *myo*-inositol phosphates.[7] It constitutes the essential phosphorus storage in a great variety of seeds used in alimentation.[8,9] Previous studies[10–14] on H_{12}inhp carried out by potentiometric methods and NMR spectroscopy have shown that there is some question as to whether two of the phosphates lie in an axial or in an equatorial plane, and there are some discrepancies in ionization constant values depending on the nature of the cations of the supporting electrolyte and the ionic strength.

myo-Inositol hexaphosphate exists in solution as either of two conformations depending on the pH: equatorial (**a**) or axial (**b**). The axial conformation possesses five phosphate groups axially oriented and one equatorial whereas the equatorial has five equatorial and one axial. Based on NMR spectroscopy, Costello *et al.*[11] conclude that in concentrated NaOH solutions inhp adopts an equatorial conformation. Isbrandt and Oertel[12] through combined use of ^{13}C, ^{31}P NMR and Raman spectroscopic methods assume that below pH 9.4 it possesses an equatorial conformation and an axial one above this pH. Emsley and Niazi[13] through ^{31}P chemical shift analysis suggest an equatorial conformation above pH 12, an axial one between 11 and 5, reverting to equatorial at pH 5 and again to axial below pH 2. On the basis of steric interactions, Bieth and Spiess[14] propound an equatorial form for the entire deprotonated species and an axial one at least for the H_4inhp^{8-} species.

The present work provides results obtained by two separate techniques: NMR spectroscopy and potentiometry. Coupling-constant analysis gives access to assignments of ^{31}P, ^{13}C and ^{1}H resonances and to conformational preferences in solution.



a



b

Chemical shift measurement as a function of pH leads in simple cases to microscopic deprotonation constants, otherwise to apparent microscopic constants the knowledge of which is a prerequisite to an understanding at the microscopic level of the mechanism of action in both chemical and biological processes of each deprotonated group of the molecule. Potentiometric titrations with a standard organic base lead to macroscopic ionization constants. Beyond pH 5, the use of bases containing alkali-metal cations greatly influences macroscopic ionization constants through complex formation between alkali-metal cations and H_{12}inhp.

Experimental

Hydrated Na_{12}(inhp) obtained from Sigma Chemicals was used without further purification. A 0.006 06 mol dm^{-3} solution of it was converted into phytic acid by ion exchange using a Dowex 50 W 4 resin. Three successive passages were necessary to obtain a sodium concentration of 0.002 mol dm^{-3}. The sodium concentration was analysed by using an atomic absorption spectrometer, Varian model AA775. The phosphorus concentration, used to determine the concentration of H_{12}inhp, was analysed by a Jobin Yvon model JY38 sequential plasma-emission spectrometer. Praseodymium chloride hexahydrate (Aldrich) was recrystallized twice from D_2O before use.

A Metrohm E 605 pH-meter coupled with a EA 120 Metrohm combined glass microelectrode was used for potentiometric titrations under an argon atmosphere. The electrode was calibrated on the pH scale by means of standard buffers. Prior to making measurements, the performance of the electrode was tested at extremes of pH by adding to standard HCl (10^{-2} mol dm^{-3}) increments of standard base solution (0.2 mol dm^{-3}) up to a meter reading of about 11.5. In a first experiment KOH was

Table 1 ^1H-{^{31}P} NMR data for H_{12}inhp at 25 °C and pD 0.672 without and with Pr^{3+}

				Lanthanide-induced	
Proton	Intensity	δ	^3J(H–H)/Hz	δ	^3J(H–H)/Hz
H^2	1	4.73 (t)	2.0	6.51 (t)	2.0
$H^{4,6}$	2	4.31 (t)	9.5	5.33 (t)	9.5
$H^{1,3}$	2	4.15 *	2.0 and 9.5	5.17 (dd)	2.0, 9.5
H^5	1	4.12 *		4.98 (t)	9.5

H_{ax}–H_{ax} corresponds to a coupling constant of 9.5 Hz and H_{ax}–H_{eq} to 2.0 Hz. * Overlapped lines.

used as titrant (electrolyte cation K^+, 0.2 mol dm^{-3}) and in a second experiment NBu_4OH (electrolyte cation NBu_4^+, 0.2 mol dm^{-3}). Plots of pH meter reading *vs.* pH showed that the performance of the electrode is not sensitive to the presence of NBu_4^+. The titrations were carried out on phytic solutions [20 cm^3; 0.0175 (concentrated under vacuum from H_{12}inhp at 0.006 06 mol dm^{-3}) and 0.006 06 mol dm^{-3}] at 25 ± 0.1 °C with NBu_4OH. The ionic strength was held constant with NBu_4Br (0.54 mol dm^{-3}).

For NMR experiments, lyophilized phytic acid was used in D_2O. The ^{31}P, ^1H and ^{13}C NMR spectra, from 0.08 mol dm^{-3} solutions containing 98% D_2O, were recorded on a Bruker HX Fourier-transform spectrometer operating at 400 MHz for ^1H, ^{31}P chemical shifts were measured relative to an external 85% orthophosphoric acid reference. For ^1H and ^{13}C, MeOH was used as an internal reference. The ^1H and ^{13}C chemical shifts were recalculated for $SiMe_4$ as reference. Decoupling of the phosphorus resonances from those of protons was achieved by continuous irradiations of the latter. All pD values (pD = pH + 0.4)[15] of sample solutions were adjusted by means of DCl and NBu_4OD under argon N55 to prevent carbonate formation.

Results and Discussion

Assignment of ^1H *Resonances and Conformational Analysis.*—The first assignment at pH 5.5 of ^1H NMR signals of H_{12}inhp was made by Johansson *et al.*[16] from two-dimensional ^1H–^1H homonuclear shift correlation experiments and analysis of ^1H spin systems. The one-dimensional ^1H NMR spectra of H_{12}inhp at pD 0.68, decoupled from ^{31}P, consists of three groups of signals: two triplets respectively at δ 4.73 and 4.31 and a poorly resolved multiplet at 4.15. Addition of a paramagnetic cation to the sample (one Pr^{3+} per 10 H_{12}inhp) transforms the multiplet into a doublet of doublets and a triplet. Integration of the NMR peaks is then consistent with one (triplet), two (triplet), two (doublet of doublet), and one (triplet) protons (Table 1).

Inspection of the values of the vicinal coupling constants from the ^1H-{^{31}P} NMR spectra indicates an equatorial position for proton H^2 and axial ones for others. Such a position is consistent with an equatorial conformation of H_{12}inhp. Assignment of proton resonances can then be achieved *via* analysis of coupling constant patterns (Table 1). The results agree with those of Johansson *et al.*[16] obtained at a higher pH value. At the same pD value, all ^1H NMR resonances not decoupled from ^{31}P show a ^3J(HCOP) coupling constant of 9.5 Hz.

Up to pH 10 no change in both proton–proton coupling constant values [$^3J(H_{ax}–H_{ax})$ = 9.5 and $^3J(H_{ax}–H_{eq})$ = 2.0 Hz] and in ^3J(HCOP) (=9.5 Hz) is observed. In the range pH 10–12 the ^1H NMR spectra are complex (as are the ^{13}C signals, see below), but above pH 12 to some extent the ^1H NMR shifts are resolved. The ^1H assignments at pD 12.8 were made by ^1H–^{31}P heteronuclear correlation. While ^3J(HCOP) is well resolved (12.2 for H^5 and 11.1 Hz for other protons) (see Table 2), the one-dimensional ^1H-{^{31}P} NMR spectrum of H_{12}inhp recorded at 25 °C and at pD 12.8 does not show the $^3J(H_{ax}–H_{ax})$ coupling patterns. It consists of four resolved signals but no measurement of $^3J(H_{eq}–H_{eq})$ and $^3J(H_{eq}–H_{ax})$ was possible,

Table 2 Proton NMR data for H_{12}inhp at 25 °C and pD 12.87

Proton	δ	^3J(HCOP)/Hz
H^2	4.21	11.1
$H^{1,3}$	4.25	11.1
$H^{4,6}$	4.35	11.1
H^5	4.06	12.2

All doublets, but singlets in ^1H-{^{31}P} spectrum.

Table 3 Phosphorus-31, ^{13}C and ^1H NMR chemical shifts at pD 0.68 and at 25 °C

	Position			
	1,3	2	4,6	5
δ(^{31}P)	−0.632	−1.39	−0.087	0.149
δ(^{13}C)	76.69	76.37	73.58	77.54
δ(^1H)	4.15	4.73	4.31	4.12

the maximum value of any splitting (2.5 Hz) due to these couplings being of the order of the linewidths. On the basis of these results we can conclude that at pH < 10 H_{12}inhp is in an equatorial conformation, at pH > 12 it is present in an axial conformation, and at 10 ⩽ pH ⩽ 12 there is an intermediate dynamic exchange process on the NMR time-scale between the axial and equatorial conformations.

Assignment of ^{31}P *and* ^{13}C *NMR Signals.*—The ^{31}P and ^{13}C resonances were first assigned at pD 0.68 by performing ^{31}P–^1H and ^{13}C–^1H heteronuclear correlations (Table 3). Unequivocal assignment of ^{31}P is then possible throughout the pH range considered, by following the variations of the chemical shifts of this nucleus as a function of pH (Fig. 1).

In the range pD 4.2–5.8 the ^{31}P NMR signals are poorly resolved even when NBu_4OH or NaOD is used as titrant, whereas ^{13}C spectra are always well resolved with both alkali-metal and organic titrants. The complex ^{31}P spectral patterns, which may be indicative of some exchange process, do not simplify upon temperature variation between 298 and 338 K. As ^3J(H–H) and ^3J(POCH) stay constant throughout the temperature variation there is no conformational change of the *myo*-inositol ring.

According to Lankhorst *et al.*,[17] ^3J(POCH) is dependent on the dihedral angle φ = H–C–O–P, following Karplus-type curves based on equations (1) and (2). For ^3J(POCH) = 9.5 Hz,

$$^3J(\text{POCH}) = 18.1\cos^2\varphi - 4.8\cos\varphi \quad \text{for } 0 < \varphi < 90° \quad (1)$$

$$^3J(\text{POCH}) = 15.3\cos^2\varphi - 6.1\cos\varphi + 1.6 \quad \text{for } 90 < \varphi < 180° \quad (2)$$

equations (1) and (2) yield the φ values 30 and 120°. Based on these results we have suggested that internal rotation occurs by means of movement of phosphate groups about the C–O single bonds, between the two typical dihedral angles, with an intermediate exchange rate on the NMR time-scale. This

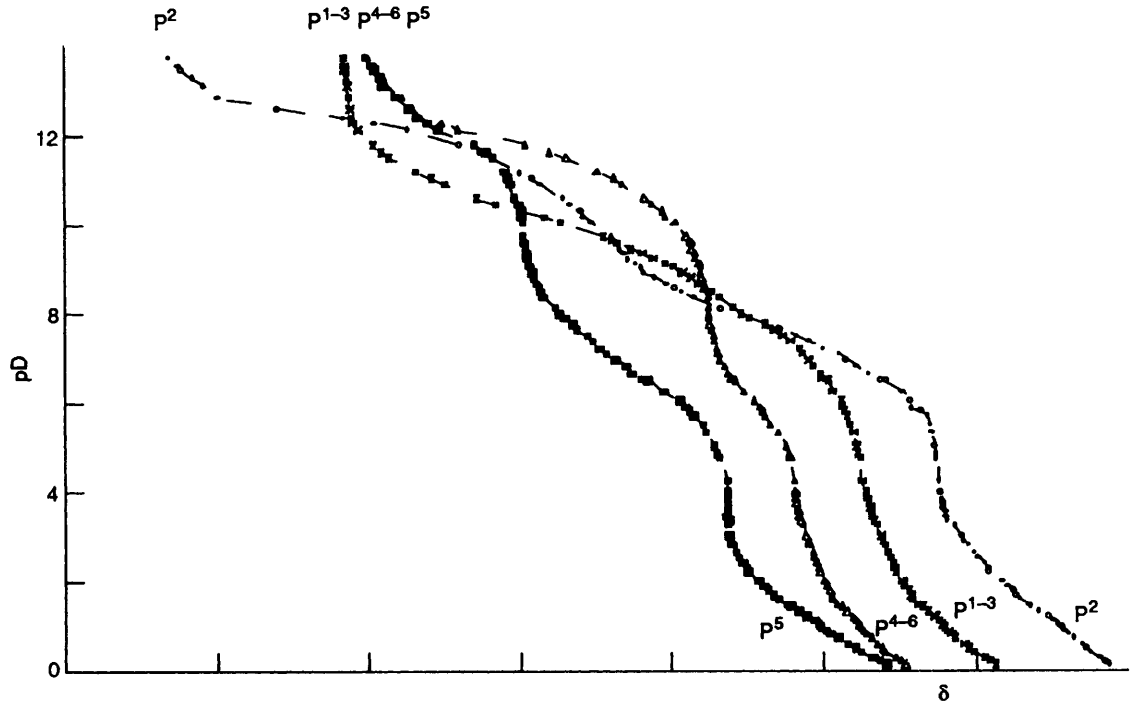


Fig. 1 Phosphorus-31 NMR titration curves of H_{12}inhp; 130 spectra were recorded at various pD. All the curves have been extrapolated between pD 4.2 and 5.8

behaviour may be reflected in proton transfer in intramolecular hydrogen bonds between deprotonated and protonated phosphate moieties. Unfortunately, neither studies by means of Fourier-transform IR spectroscopy on solids nor analysis of isotopic effects on chemical shifts by H–D exchange of substitution-labile protons have allowed us to resolve this problem. Furthermore, when Na^+ and K^+ are present in solution they compete with the protons for complexation and above pH 5 remove and substitute them. Tighter binding of these cations and probably the high degree of electrostatic character in the bonding may hinder or even prevent rotation of the phosphate groups. However, as there is no change in ^3J(POCH) values (=9.5 Hz) in the presence or absence of Na^+ such effects may be disregarded.

Determination of Apparent Microscopic Constants by NMR Spectroscopy.—Fig. 1 shows the ^{31}P NMR titration curves. When the pH is increased all the ^{31}P chemical shifts move downfield. The shape of the curves is largely dependent on the deprotonation state. Progressive deprotonation of the phosphate groups in acidic media results in a rather smooth pattern contrasting with the large variation in chemical shifts in neutral and basic solutions.

Deprotonation of the phosphate groups, as observed from inflection points, allows us to deduce the order of removal of protons from H_{12}inhp at 25 °C: P^2, apparent pk 7.5; $P^{1,3}$, 9.6; $P^{4,6}$ 11.5; P^5, 6.4. The apparent microscopic pk values agree to some extent with those reported by Costello[11] and Emsley[13] and co-workers but differ with respect to the sequence of deprotonation of the various phosphate sites. The first pD data point which represents approximately four equivalents of H^+ when the concentration of H_{12}inhp is 6×10^{-3} mol dm^{-3} is indicative of the presence of hydrogen ions released in solution by strongly acidic phosphate groups. From considerations of steric hindrances and coulombic repulsion it may be argued that P^2 and P^5 being more exposed to solvent and hence undergoing the strongest interactions with water molecules each lose one proton. The other two protons lost may be attributed to $P^{1,3}$ as the curve of pD *vs.* δ displays only one inflection point at pD 9.7 over the pD range considered.

As noted by Emsley and Niazi,[13] it is somewhat surprising to find that P^5 and P^2 both behave as three-proton releasing groups. A satisfactory explanation of this abnormal behaviour is to envisage the formation of double hydrogen bonds involving P^2 and $P^{1,3}$, P^5 and $P^{4,6}$, using hydrogens initially linked to $P^{1,3}$ and to $P^{4,6}$. Then, when deprotonation takes place, it affects, by a shielding effect, the chemical shifts of the phosphorus atoms coupled together through the same hydrogen bonds. We can see that for $P^{1,3}$ and P^2, $P^{4,6}$ and P^5 which undergo downfield shifts there are inflection points at approximately the same pD values: $P^{1,3}$, 9.6; P^2, 7.5; $P^{4,6}$, 11.5; P^5, 6.4.

At this stage several general comments can be made. First, as observed the deprotonation of $P^{4,6}$ (*i.e.* the pD) is appreciably higher than that of $P^{1,3}$. This can be ascribed to the fact that hydrogen bonds involving P^2 are not as strong as those of P^5 with $P^{4,6}$. As the inflection points for $P^{1,3}$ and P^2 and $P^{4,6}$ and P^5 respectively occur at the same pD values we can infer that the intramolecular interactions produce a symmetrical delocalization of protons with respect to phosphate groups linked by the same hydrogen bonds.

The use of lanthanide cations to identify deprotonated sites from induced NMR shifts confirms part of the preceding suggestions by showing that the first deprotonation occurs on P^2. Indeed, at pD 0.4, the observed ^{31}P shifts for H_{12}inhp interacting with the paramagnetic Pr^{3+} clearly show that in strongly acidic solution, the binding site of Pr^{3+} is the P^2 phosphate which undergoes the largest induced shifts (Table 4). The lanthanide cation is then located in the symmetry plane H^2, C^2, C^5 as P^2, P^4 on the one hand and P^3, P^6 on the other are still magnetically equivalent. Lanthanide-induced shift values have then been exploited to establish simultaneously the stability constant β and the first pk value by a data-fitting procedure described elsewhere[18] and based on the unique stoichiometry 1:1 for a complex formed in one step. An arbitrary initial value of k_1 is introduced and β values are optimized by linear regression.. The computation from the experimental induced δ values gives the following results for the Pr^{3+}–H_{12}inhp complex at pD 0.4: Δ (co-ordination shift) 41.41 ppm, $\log k_1 = -0.1$ and $\log \beta = 2.54$.

Table 4 Lanthanide-induced downfield shifts of phosphorus in H_{12}inhp (0.08 mol dm^{-3}) at pD 0.4

	δ			
$[H_{12}inhp]/[Pr^{3+}]$	P^2	$P^{1,3}$	$P^{4,6}$	P^5
1/30	1.264	0.783	0.585	0.480
1/15	2.506	1.642	1.174	0.986
1/10	3.673	2.315	1.759	1.504
1/5	7.204	4.652	3.591	3.118
1/3	11.637	7.672	5.988	5.321

Table 5 Logarithms of the protonation constants of H_{12}inhp at 25 °C

y in pK_y	a	b
1	*−0.15*	*−0.14*
2	*0.41*	*0.4*
3	*0.85*	*0.82*
4	1.84 ± 0.01	*1.57*
5	2.61 ± 0.01	2.58 ± 0.03
6	3.72 ± 0.02	3.63 ± 0.01
7	5.99 ± 0.01	5.98 ± 0.01
8	7.11 ± 0.06	7.15 ± 0.09
9	8.96 ± 0.07	9.08 ± 0.05
10	10.43 ± 0.01	10.54 ± 0.01
11	10.98 ± 0.11	11.08 ± 0.05
12	11.76 ± 0.40	11.76 ± 0.05

The values in italics are less than the first pH value. They have been computed from the values of the other constants previously determined. [a] c_L = 0.0175 mol dm^{-3}, 150 experimental points. [b] c_L = 0.006 06 mol dm^{-3}, 101 points.

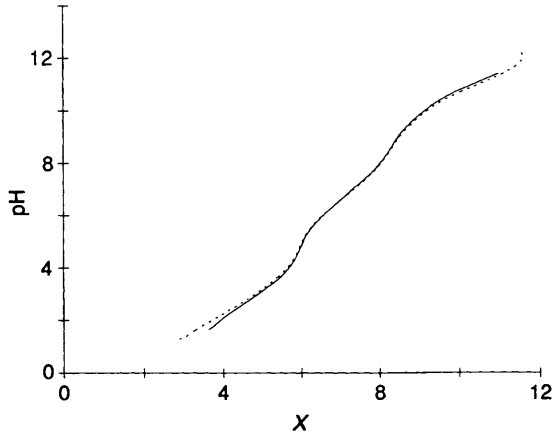


Fig. 2 Potentiometric titration curves where $X = x + ([H^+] - [OH^-])/[H_{12}inhp]$, and x is the ratio of mol of base added to mol of H_{12}inhp. c_L = 0.006 06 (– – –) and 0.017 50 mol dm^{-3} (——)

Determination of Macroscopic Constants.—Protonation constants ($K_i^H = [H_iL]/[H_{i-1}][H]^i$) were calculated by fitting the potentiometric data by use of the pKAS[19] program. This computer program has the ability to evaluate protonation constants under highly acidic conditions. Table 5 shows the set of pK values computed from titration curves at two different concentrations of H_{12}inhp. All constants are consistent. Titration plots at increasing ligand concentration c_L are non-superimposable as polymer appears. In our experiments all plots of pH *vs.* X coincide in a unique neutralization curve (Fig. 2). This is an important point and denotes the absence of polymeric forms. The four first log protonation constants are very high (11.76, 10.98, 10.43, 8.96) and correspond approximately to the protonation of $P^{4,6}$ and $P^{1,3}$. The next four, 7.11, 5.99, 3.72 and 2.61, correspond to the protonation of P^2, P^5 and $P^{4,6}$ and the last four, which are highly acidic, to $P^{1,3}$, P^2 and P^5.

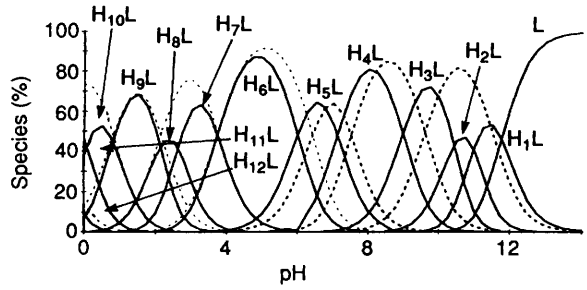


Fig. 3 Relative concentrations of inhp (L) species as a function of pH. Full curves correspond to macroscopic pK values and dashed ones to microscopic pk values. Three apparent microscopic pk values, 0.8 (P^5), 2.6 ($P^{1,3}$) and 3.8 ($P^{4,6}$), estimated from NMR titration curves have also been used

Fig. 3 shows the distribution diagrams for the species in equilibria in the pH range studied, obtained from macroscopic constants and NMR apparent constants. The NMR curves overlap the potentiometric ones. This indicates that the results obtained by the two independent methods are reliable.

The pK values of H_{12}inhp obtained from dilute solutions are very sensitive to the accuracy of the ligand concentration used in parametric computations, probably more than those of weakly complexing cations such as Na^+ or K^+ which may be present in small amounts.

Loss of the last two protons of H_{12}inhp, according to ^{31}P chemical shift variations, occurs at pD 11.5. This result provides important geometric information about the conformation of the molecule. Indeed, it can reasonably be envisaged that when hydrogen bonds are present (up to pD 12) they stabilize the equatorial form, whilst the completely deprotonated species which appears beyond pD 10 adopts the axial conformation in which steric and electrostatic interactions are minimized.

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