Structures of the Small-Molecule Bcl-2 Inhibitor (BH3I-2) and Its Related Simple Model in Protonated and Deprotonated Forms

Daisuke Kanamori, Taka-aki Okamura, Hitoshi Yamamoto, Shigeomi Shimizu,[1] Yoshihide Tsujimoto,[1] and Norikazu Ueyama*

Department of Macromolecular Science, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043

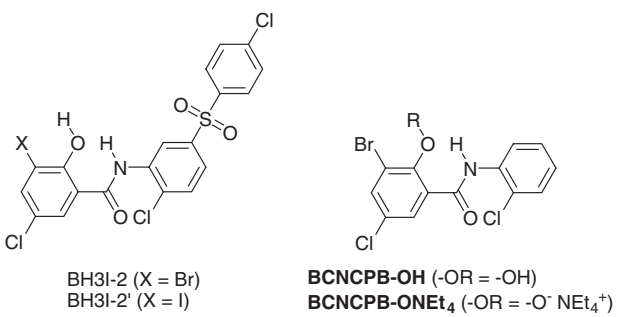
[1]Department of Post-Genomics and Diseases, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871

Received May 24, 2004; E-mail: ueyama@chem.sci.osaka-u.ac.jp

3-Bromo-5-chloro-*N*-(2-chlorophenyl)-2-hydroxybenzamide (**BCNCPB–OH**) was synthesized as a model compound of the small-molecule Bcl-2 inhibitor (BH3I-2) and characterized by ^{1}H NMR and IR measurements. The structures of **BCNCPB–OH** and its deprotonated form (**BCNCPB–O–NEt$_4$**) were determined by X-ray analysis. **BCNCPB–OH** has an intramolecular OH···O=C hydrogen bond and **BCNCPB–O–NEt$_4$** has an intramolecular NH···O(oxyanion) hydrogen bond in the solid and solution states. The solution structures of protonated and deprotonated BH3I-2′ (BH3I-2 analogue) were determined by ^{1}H NMR, NOESY, and IR measurements. They have similar conformations to those of the corresponding model compounds. A pK_a value of **BCNCPB–OH** of 5.1 is considered a very low value for phenol derivatives, suggesting the possibility that BH3I-2 binds to protein in the deprotonated form having an intramolecular NH···O(oxyanion) hydrogen bond.

BH3I-2 is a recently identified small-molecule inhibitor of the Bcl-2 family, which is a regulator of apoptotic pathways (Chart 1).[1,2] A number of inhibitors which have a salicylamide skeleton similar to BH3I-2 have been reported to inhibit various enzymes relating to the respiratory chain,[3–6] the biosynthesis of melanin,[7,8] oxidative phosphorylation,[9–11] bacterial two component systems,[12,13] and so on. Although the precise inhibitory mechanisms in many cases are vague, drug development work has indicated that electron-withdrawing substituents[12,13] and hydrophobicity[13] are needed for a high activity. The importance of the salicylamide skeleton and hydrogen bonds on it have been shown by the following evidence. In X-ray structures of the inhibitor–scytalone dehydratase complex[7] and antimycin A$_1$–cytochrome *bc*$_1$ complex (the chemical structure of the inhibitors are depicted in Chart 2),[3,14] hydrogen bonds between amino acid residues in the binding site and phenolic OH, amide NH, and the carbonyl group on the salicylamide moiety were found. In these structures, intramolecular OH···O=C hydrogen bonding on the inhibitor is suggested by a short distance between the phenolic oxygen and carbonyl oxygen. The analogous inhibitor, antimycin A$_3$, is also represented as an intramolecularly OH···O=C hydrogen-bonded conformation in the proposed structure of antimycin A$_3$/Bcl-xL (one of the Bcl-2 family proteins) complex.[15,16] Modifications to break intra- and/or intermolecular hydrogen bonds such as the deletion of the phenolic OH or carbonyl group,[8] methylation of the phenolic[17] or amide[4] proton, or the insertion of –CH$_2$– between the aromatic ring and carbonyl group[4] reduce the inhibitory activity. These results demonstrate that the importance of the salicylamide skeleton arises from its hydrogen bonding ability.

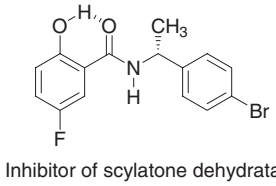
Suezawa et al. has reported that salicylanilide derivatives having a halogen atom (X), a hydrogen bonding acceptor, at the 3-position are in equilibrium between intramolecular



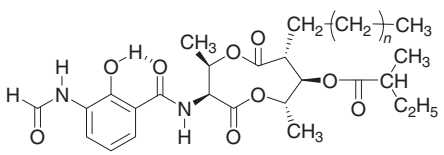
BH3I-2 (X = Br)
BH3I-2' (X = I)

BCNCPB-OH (-OR = -OH)
BCNCPB-ONEt$_4$ (-OR = -O$^-$ NEt$_4^+$)

Chart 1. Chemical structures of BH3I-2, BH3I-2′, **BCNCPB–OH**, and **BCNCPB–O–NEt$_4$**.



Inhibitor of scytalone dehydratase



Antimycin A$_3$ (n = 2), A$_1$ (n = 4)

Chart 2. Other inhibitors having salicylamide moiety.

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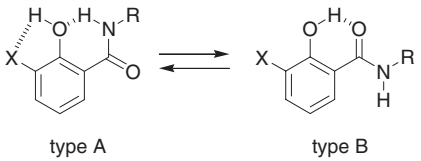


Chart 3. Two hydrogen bonding modes.

NH···O hydrogen bonded (type A) and OH···O=C hydrogen bonded conformations (type B) (Chart 3).[18] Although *O*-acetylation removes the stabilization by the intramolecular OH···O=C hydrogen bond, the type B conformation is major in solution. Salicylanilide derivatives, which can form intramolecular OH···O=C hydrogen bonds, including BH3I-2, probably favor a type B conformation. On the other hand, type A is predominant in deprotonated salicylamide derivatives by intramolecular NH···O(oxyanion) hydrogen bonds.[19,20]

The structure of the BH3I-2/Bcl-xL complex has not been determined by X-ray analysis but computationally proposed using the solution structure of the BakBH3–peptide/Bcl-xL complex.[1,21,22] In this structure, BH3I-2 binds to the hydrophobic cleft formed by BH1, BH2, and BH3 regions and takes a type A conformation depicted in Chart 1. Though it was not discussed in detail whether BH3I-2 is protonated or not, we present here the possibility of the deprotonation. The pK_a values of salicylanilide derivatives have been reported below 7.5, and further lowered by electron-withdrawing substituents.[11] The pK_a value of salicylamide derivatives is also reduced by the NH···O(oxyanion) hydrogen bond in its phenolate anion form.[11,19] Such intramolecular NH···O hydrogen bonds are supported in a hydrophobic environment and efficiently lower the pK_a value, as shown previously in our report for thiophenol (benzenethiol) and benzoic acid analogues.[23,24] In the hydrophobic cleft of Bcl-xL, the pK_a value of BH3I-2 is presumably lowered, and its deprotonated form is stabilized.

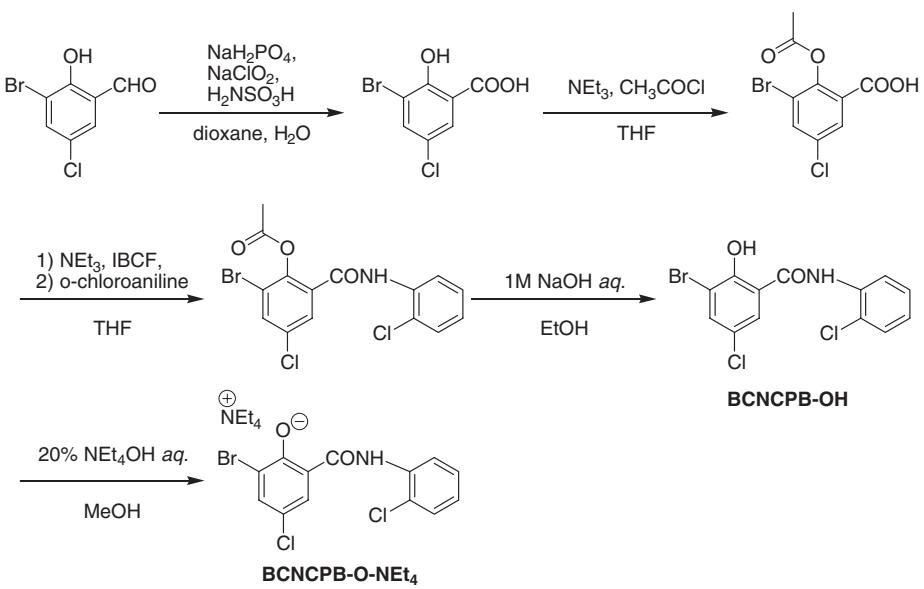
In this paper, we determine the structure of BH3I-2 and its deprotonated form and evaluate the pK_a value in a hydrophobic environment using the model compounds discussed below. To realize a hydrophobic environment where BH3I-2 binds, we use organic solvents and a micellar solution.

The determination of the precise hydrogen bonding mode of BH3I-2 in the complex is important because it will be valuable for understanding inhibitory mechanisms in detail. For this purpose, 3-bromo-5-chloro-*N*-(2-chlorophenyl)-2-hydroxybenzamide (**BCNCPB–OH**) and its deprotonated form (**BCNCPB–O–NEt$_4$**) were synthesized and structurally analyzed as model compounds of protonated and deprotonated BH3I-2 (Chart 1), respectively. Because the replacement of the *N*-substituent does not significantly influence inhibitory activity, the 4-chlorophenylsulfonyl group was removed to improve the crystallinity of the BH3I-2 model compounds.[4] The structures and hydrogen bonding modes were determined by X-ray analysis and IR spectra in the solid state and by ^{1}H NMR and IR spectra in solution. The structures of BH3I-2′ (Chart 1) in protonated and deprotonated forms were determined by ^{1}H NMR and IR spectra in solution. The reason BH3I-2′ was employed is that it probably prefers a similar conformation to BH3I-2, which is not available commercially. To evaluate the tendency to deprotonate the phenolic OH, the pK_a value of **BCNCPB–OH** was determined. The pK_a value of BH3I-2 is considered to be comparable to that of **BCNCPB–OH** because introduction of a stronger electron-withdrawing group (NO_2) had a negligible influence on the pK_a value.[11,25]

Results and Discussion

Synthesis. The synthesis of **BCNCPB–OH** and **BCNCPB–O–NEt$_4$** is shown in Scheme 1. The phenolic hydroxy group was protected by an acetyl group to avoid a side reaction. The deprotonation of the phenolic OH was performed by neutralization with NEt_4OH.

Structures of Model Compound in the Protonated and Deprotonated Forms in the Solid State. The molecular structures of protonated (**BCNCPB–OH**) and deprotonated forms (**BCNCPB–O–NEt$_4$**) were determined by X-ray analy-



Scheme 1. Synthesis of **BCNCPB–OH** and **BCNCPB–O–NEt$_4$**. IBCF = isobutyl chloroformate.

sis. It has been revealed in the solid state that **BCNCPB–OH** prefers the type B conformation and **BCNCPB–O–NEt$_4$** prefers type A.

The molecular structure of **BCNCPB–OH** is shown in Fig. 1a and the selected short contacts, bond lengths, and torsion angles are listed in Table 1. The presence of an intramolecular OH···O=C hydrogen bond is suggested by the short distances between the phenolic OH proton (H1) and the amide carbonyl oxygen atom (O2) (1.91(3) Å) and between the phenolic oxygen (O1) and O2 (2.606(3) Å). The chlorine atom at the 2′-position in Ar_B (Cl2) is located on the opposite side of the carbonyl group to avoid electrostatic repulsion. The location of the chlorine atom suggests the presence of the attractive interaction between the amide NH proton (H2) and Cl2 (Cl2–H2 is 2.51(2) Å). The coplanarity between the amide plane and two aromatic rings is indicated where the torsion angles are

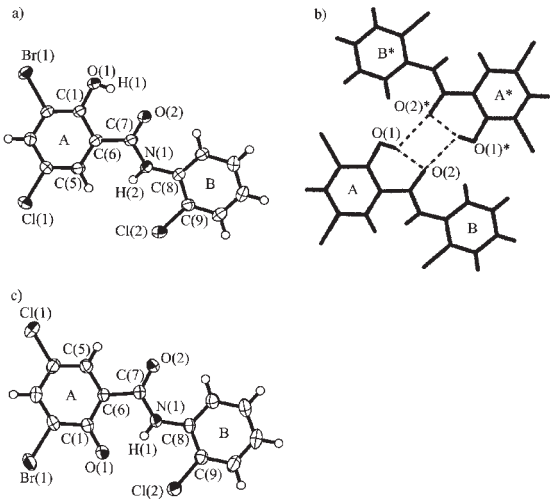


Fig. 1. ORTEP drawing (a) and packing structure (b) of **BCNCPB–OH** and ORTEP drawing of anion part of **BCNCPB–O–NEt$_4$** (c).

160.0(3)° (C5–C6–C7–O2) and 176.9(3)° (C7–N1–C8–C9). The presence of an intermolecular OH···O=C hydrogen bond is suggested by the short distance between the phenolic hydrogen atom (H1) and amide carbonyl oxygen atom (O2*) of the neighboring molecule (2.50(3) Å) (Fig. 1b). No significant intermolecular interaction around the amide NH exists.

The crystal structure of the deprotonated form (**BCNCPB–O–NEt$_4$**) is shown in Fig. 1c. A different hydrogen bonding mode from that in **BCNCPB–OH** was found. The short distances between the phenoxy oxygen (O1) and the amide proton (H1) (1.87(2) Å) and between O1 and the amide nitrogen (N1) (2.583(2) Å) suggest the presence of an intramolecular NH···O(oxyanion) hydrogen bond. The relative orientation of chlorine at the 2′-position in Ar_B (Cl2) toward the amide NH is similar to that in **BCNCPB–OH**. The coplanarity of **BCNCPB–O–NEt$_4$** is higher than in **BCNCPB–OH**. The torsion angle between Ar_A and amide plane (C5–C6–C7–O2) is −1.2(3)°, and that between the amide plane and Ar_B (C7–N1–C8–C9) is −175.6(2)°. The C1–O1 bond length in **BCNCPB–O–NEt$_4$** (1.282(3) Å) is shorter than that in **BCNCPB–OH** (1.340(3) Å) due to an increase in the double bond character of the C1–O1 bond caused by the delocalization of the negative charge of the phenoxy anion to the aryl ring.

The most significant structural change between **BCNCPB–OH** and **BCNCPB–O–NEt$_4$** is a ca. 180 degree rotation about the C6–C7 bond.

The presence of hydrogen bonds in the solid state was confirmed by IR measurements in a Nujol mull (Fig. 2). In the protonated form, **BCNCPB–OH**, an amide NH stretching band was observed at 3416 cm^{-1}, which is slightly lower than non-hydrogen-bonded ν(NH) (ν(NH) of salicylanilide in dilute solution[20] is 3456 cm^{-1}) and comparable to NH···Cl hydrogen-bonded ν(NH).[26,27] The orientation of Cl toward the carbonyl C=O and the amide NH is controlled by not only the electrostatic repulsion between the carbonyl oxygen and Cl but also the NH···Cl hydrogen bond. In **BCNCPB–O–NEt$_4$**, it is

Table 1. Selected Short Contacts, Bond Distance, and Torsion Angles of **BCNCPB–OH** and **BCNCPB–O–NEt$_4$**

BCNCPB–OH		**BCNCPB–O–NEt$_4$**	
	Short contacts/Å		
H(2)···Cl(2)	2.51(2)	H(1)···Cl(2)	2.54(2)
H(1)···O(2)	1.91(3)	H(1)···O(1)	1.87(2)
O(1)···O(2)	2.606(3)	O(1)···N(1)	2.583(2)
H(1)···O(2)*	2.50(3)		
O(1)···O(2)*	2.921(3)		
	Bond lengths/Å		
C(1)–O(1)	1.340(3)	C(1)–O(1)	1.282(3)
O(2)–C(7)	1.223(4)	O(2)–C(7)	1.239(3)
C(6)–C(7)	1.498(4)	C(6)–C(7)	1.484(3)
N(1)–C(7)	1.353(4)	N(1)–C(7)	1.360(3)
N(1)–C(8)	1.402(4)	N(1)–C(8)	1.400(3)
O(1)–H(1)	0.74(3)	N(1)–H(1)	0.80(2)
N(1)–H(2)	0.87(3)		
	Torsion angles/°		
O(2)–C(7)–C(6)–C(5)	−160.0(3)	O(2)–C(7)–C(6)–C(5)	−1.2(3)
C(7)–N(1)–C(8)–C(9)	176.9(3)	C(7)–N(1)–C(8)–C(9)	−175.6(2)

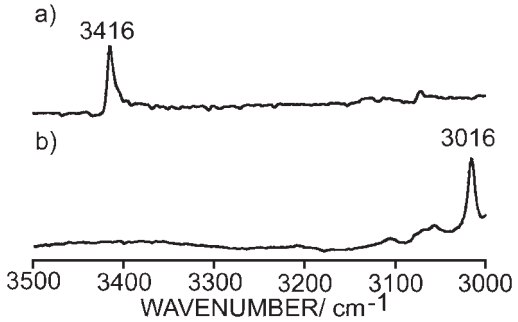


Fig. 2. IR spectra in a Nujol mull of **BCNCPB–OH** (a) and **BCNCPB–O–NEt$_4$** (b).

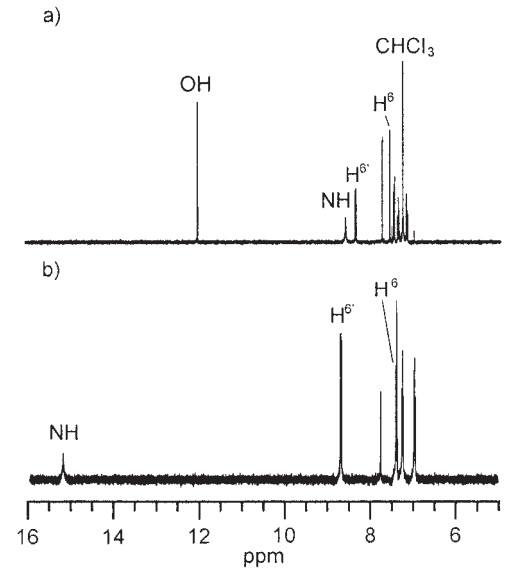


Fig. 3. ^{1}H NMR spectra of **BCNCPB–OH** in $CDCl_3$ (a) and **BCNCPB–O–NEt$_4$** in CD_3CN (b).

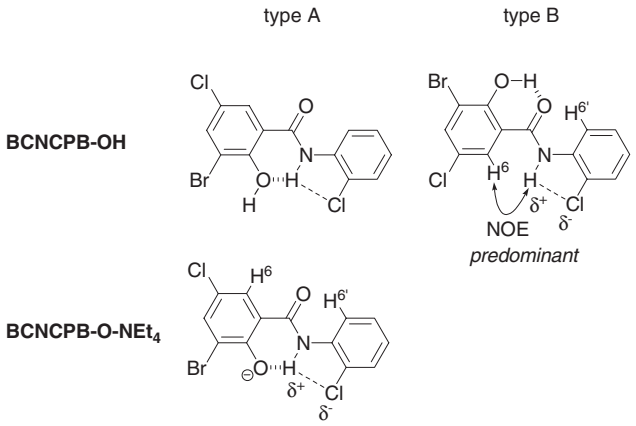


Chart 4. Solution structures of **BCNCPB–OH** and **BCNCPB–O–NEt$_4$**.

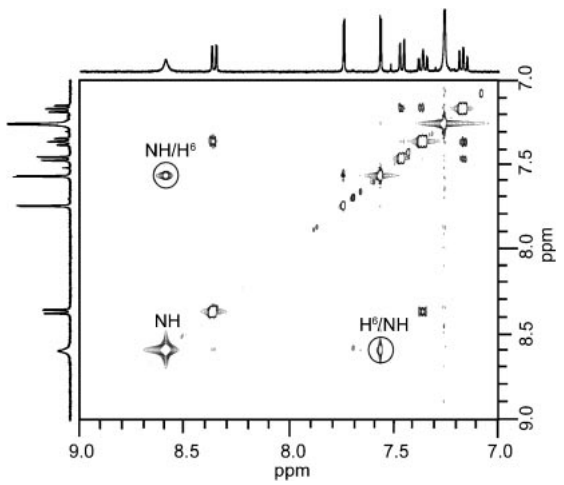


Fig. 4. NOESY spectrum of **BCNCPB–OH** in $CDCl_3$.

thought that the location of Cl2 is controlled by the same interactions. **BCNCPB–O–NEt$_4$** has an amide NH band at 3016 cm^{-1}, which is a very low frequency for ν(NH) even if the NH proton forms a hydrogen bond, (NH···O(H)[28] 3390 cm^{-1}, NH···O(Ac)[29] approximately 3400 cm^{-1}, NH···O(carboxylate)[28] 3024 cm^{-1}, NH···O(phosphate)[30] 3226 cm^{-1}, etc.) and shows that the NH···O(oxyanion) hydrogen bond fomed in **BCNCPB–O–NEt$_4$** is very strong. The ν(NH) shift in **BCNCPB–O–NEt$_4$** is comparable to that in the sodium salt of 3,5-dibromosalicylanilide,[20] which appears at 3000–2900 cm^{-1}.

Solution Structures of BCNCPB–OH and BCNCPB–O–NEt$_4$. Figure 3a shows the ^{1}H NMR spectrum of **BCNCPB–OH** in $CDCl_3$. A phenolic OH signal was observed at 12.0 ppm, which is shifted to a very low field compared to non-hydrogen bonded phenolic OH (4–6 ppm), indicating the formation of a OH···O=C hydrogen bond (type B in Chart 4) similar to the solid state. An amide NH signal was observed at 8.6 ppm, which is slightly downfield compared to δ(NH) in non-hydrogen bonded benzamide (6–8 ppm), showing that the weak NH···Cl hydrogen bond established in the solid state is maintained in solution. A NOE correlation between the NH and the 6-positioned aryl proton (H^6 at 7.6 ppm) was observed, but not between the NH and 6′-positioned aryl proton ($H^{6'}$ at 8.4 ppm) in the NOESY spectrum (Fig. 4). These results also support the type B conformation. On the other hand, a minor conformer in addition to type B was established in the IR results because of its higher time resolution than ^{1}H NMR. **BCNCPB–OH** gives NH stretching bands at 3425 and 3357 cm^{-1} (Fig. 5a). The ratio of each of the conformers was estimated to be about 90 and 10%, respectively, from the ratio of the area of the bands. The predominant band is assigned to the type B conformer (3416 cm^{-1} in the solid state). The NH band at 3357 cm^{-1} is assigned to the NH···O hydrogen bonded conformer (type A).[18,29] The band at high frequency, 3483 cm^{-1}, is assigned to ν(OH) in the type A conformation.[20] The OH band in the type B conformation was not observed in the spectrum because of line broadning.[20]

In the ^{1}H NMR spectrum of **BCNCPB–O–NEt$_4$** (Fig. 3b), an amide NH signal was observed at 15.2 ppm, which is downfield compared to the non-hydrogen bonded NH, indicating the formation of a strong NH···O(oxyanion) hydrogen bond (type A) similar to the solid state. The NH shows no NOE correlation with other protons in the NOESY spectrum (data not shown). It also supports the type A conformation. The IR results (Fig. 5b), where an NH band was observed at 3015

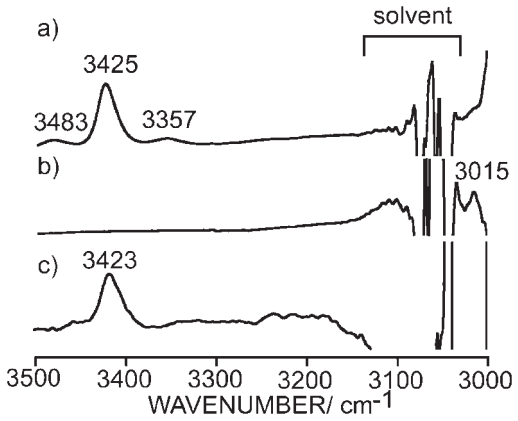


Fig. 5. IR spectra of 5 mM CH_2Cl_2 solution of **BCNCPB–OH** (a), **BCNCPB–O–NEt$_4$** (b), and BH3I-2′ (c).

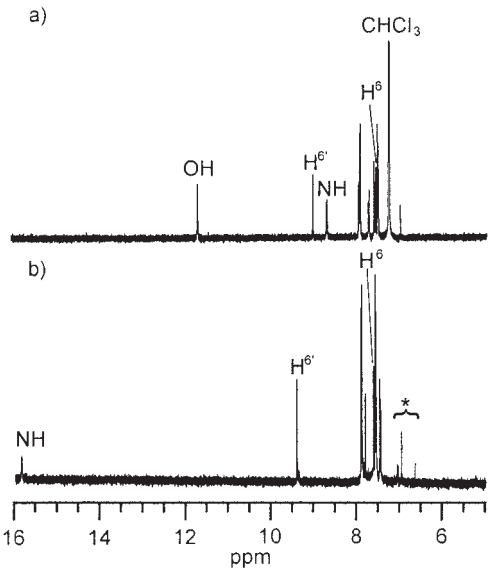


Fig. 6. ^{1}H NMR spectra of BH3I-2′ in $CDCl_3$ (a) and deprotonated BH3I-2′ in CD_3CN (b).

cm^{-1}, also show strong NH···O(oxyanion) hydrogen bonding.

Solution Structures of BH3I-2′. Figure 6a shows the ^{1}H NMR spectrum of BH3I-2′. Phenolic OH and amide NH signals were observed at 11.7 and 8.7 ppm, respectively. These similar chemical shifts to **BCNCPB–OH** show that BH3I-2′ has similar hydrogen bonds (Chart 5). An observed NOE correlation between the NH and the 6-positioned aryl proton (H^6 at 7.5 ppm) supports the type B conformation (Fig. 7). However, BH3I-2′ gives one NH band at 3423 cm^{-1} in the IR spectrum that is different from **BCNCPB–OH** (Fig. 5c). This is because iodine acts as a weaker hydrogen bonding accepter (OH···I) compared to bromine due to a smaller electronegativity. BH3I-2, which has bromine, probably has a minor conformer similar to **BCNCPB–OH**.

In the ^{1}H NMR spectrum of deprotonated BH3I-2′ (Fig. 6b), an amide NH signal was observed at 15.8 ppm, which is extremely downfield, similar to **BCNCPB–O–NEt$_4$**. This indicates NH···O(oxyanion) hydrogen bonding (type A). The type A conformation is also supported by the disappearance of the

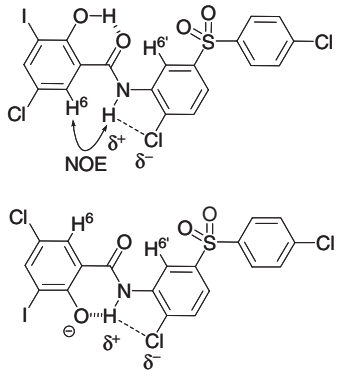


Chart 5. Solution structures of protonated and deprotonated forms of BH3I-2′.

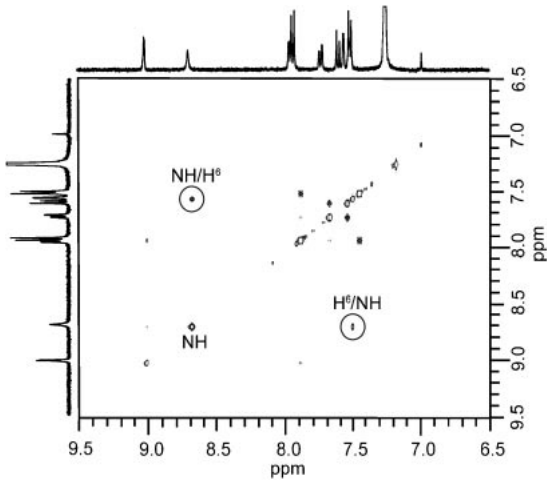


Fig. 7. NOESY spectrum of protonated BH3I-2′ in $CDCl_3$.

NOE correlation regarding NH.

Protonated and deprotonated BH3I-2′ form similar hydrogen bonds to the predominant conformations of **BCNCPB–OH** and **BCNCPB–O–NEt$_4$**, respectively. BH3I-2 probably prefers similar conformations.

pK_a of BCNCPB–OH in an Aqueous Micellar Solution. The pH titration of **BCNCPB–OH** was performed in an aqueous micellar solution. Under these conditions, **BCNCPB–OH** is incorporated into the hydrophobic layer where the hydrogen bonds are supported. These conditions also realize a hydrophobic cleft formed in Bcl-xL where BH3I-2 binds.[1] The titration method using an aqueous micellar solution is adequate to mimic the hydrophobic environments inside of protein.[23,24] Because protons can move inside and outside of micelles freely, the pK_a value of **BCNCPB–OH** is evaluated by pH measurements in the aqueous layer. **BCNCPB–OH** exists inside micelles due to its poor solubility in water during the titration. The pK_a value of **BCNCPB–OH** obtained from the titration was 5.1, which is very low compared with phenol (9.9 in water[31]). In addition to the electron-withdrawing effect of Cl, Br, and the aryl *N*-substituent (Ar_B), the existence of amide NH, which forms an intramolecular NH···O(oxyanion) hydrogen bond in the deprotonated form, lowers the pK_a value.[19,23,28] The pK_a value of BH3I-2 should be equal to or slightly less than 5.1 because an additional electron withdrawing substitu-

ent, the 4-chlorophenylsulfonyl group on the *N*-2-chlorophenyl group, of BH3I-2 has little effect on its acidity.[11]

Proposed Binding Structure of BH3I-2. The structure of the BH3I-2/Bcl-xL complex has been computationally proposed using the solution structure of the BakBH3–peptide/Bcl-xL complex.[1,21,22] This is based on the fact that the structure of Bcl-xL and the inhibitor–binding site are similar to those of the BakBH3–peptide/Bcl-xL complex. In this complex, BH3I-2 binds to a hydrophobic cleft formed by the BH1, BH2, and BH3 regions. The 3-positioned bromine interacts with the side chain of Tyr65 and the 5-positioned chlorine with the side chain of Phe61 and Phe110, etc. The presence of the interactions is demonstrated by a change in the inhibitory activity induced by the substitution of these halogen atoms. In the complex, the structure of BH3I-2 is represented in the protonated form and has an intramolecular NH···O hydrogen bond (type A). Our structural analysis of BH3I-2, however, shows that BH3I-2 forms an intramolecular OH···O=C hydrogen bond (type B) in the protonated form. It is difficult to fit BH3I-2 into the hydrophobic cleft while keeping the intramolecular OH···O=C hydrogen bond and the interactions between bromine–Tyr65, chlorine–Phe110, and chlorine–Phe61, because the chlorine on the *N*-aryl ring would be too close to the side chain of Phe61 or Ala106 under this assumption (Fig. 8b). On the other hand, it is reasonable that BH3I-2 binds in the deprotonated form in which an intramolecular NH···O(oxyanion) hydrogen bond is formed (Fig. 8a). This suggestion is supported by the pK_a value of BH3I-2 of about 5.1, which indicates that BH3I-2 is deprotonated under physiological conditions (pH = 7.2).[2] It is possible that Tyr65 does not interact with bromine but forms an intermolecular OH(Tyr)···O(oxyanion) hydrogen bond.

Different from other salicylamide inhibitors noted above, BH3I-2 binds to the protein in deprotonated form. This is because the pK_a value of BH3I-2 is lower than others due to strong electron-withdrawing substituents (Cl ($\sigma_{para} = 0.23$) and Br ($\sigma_{para} = 0.23$)) compared to others (F ($\sigma_{para} = 0.06$) or NHCHO ($\sigma_{para} = 0.00$)).[25]

Conclusion

In this study, BH3I-2 models, **BCNCPB–OH** and **BCNCPB–O–NEt$_4$**, were synthesized and structurally analyzed. The presence of an intramolecular OH···O=C hydrogen bond in **BCNCPB–OH** and an intramolecular NH···O(oxyanion) hydrogen bond in **BCNCPB–O–NEt$_4$** as established by X-ray analyses and IR spectra. The ^{1}H NMR and IR results of **BCNCPB–O–NEt$_4$** have revealed that the structure in the solid state is maintained in dilute solution with similar hydrogen bonds. **BCNCPB–OH** prefers a similar conformation to the solid state predominantly, but has a minor conformer having an NH···O hydrogen bond. ^{1}H NMR and IR spectra show that the protonated and deprotonated BH3I-2′ form intramolecular hydrogen bonds in the same manner as the corresponding models except for no evidence of the minor conformers in the protonated form. The deprotonation of BH3I-2 in the BH3I-2/Bcl-xL complex is suggested by the low pK_a value of **BCNCPB–OH**. It is also supported by the possible OH-(Tyr)···O(oxyanion) hydrogen bond formation between the OH group of Tyr65 and the phenoxy oxygen atom of BH3I-2.

Experimental

Materials. BH3I-2′ was purchased from CALBIOCHEM. Other reagents were purchased from Nacalai Tesque or Tokyo Chemical Industry and used without further purification. All organic solvents were dried over CaH_2 and distilled under an Ar atmosphere before use.

Synthesis. 3-Bromo-5-chloro-2-hydroxybenzoic Acid (1): This compound was prepared using a modified method reported in the literature.[32]

To a solution of 3-bromo-5-chloro-2-hydroxybenzaldehyde (278 mg, 1.3 mmol) in dioxane (15 mL) were added NaH_2PO_4 (800 mg, 5.1 mmol) in water (5 mL) and sulfamic acid (186 mg, 1.92 mmol). The reaction mixture was cooled to 7 °C and then NaClO2 (186 mg, 1.7 mmol) in water (0.7 mL) was added gradually while keeping the temperature below 10 °C. The reaction mixture was stirred for 30 min below 10 °C. Sodium sulfite (195 mg, 1.5 mmol) was added with stirring for 15 min below 10 °C. Hydrochloric acid was added until a pH of 1 was obtained. Volatile materials were evaporated, and the resulting solution was cooled to deposit a white precipitate, which was collected by filtration and washed with water (309 mg, 94%).

Anal. Found: C, 33.37; H, 1.63%. Calcd for $C_7H_4BrClO_3$: C, 33.43; H, 1.60%. Mp: 238–239 °C. ^{1}H NMR (DMSO-d_6; TMS) δ 7.91 (1H, m, Ar–H), 7.74 (1H, m, Ar–H). ^{13}C NMR (DMSO-d_6): δ 170.3, 156.7, 137.3, 128.7, 122.8, 115.3, 111.3. ESI-MS:

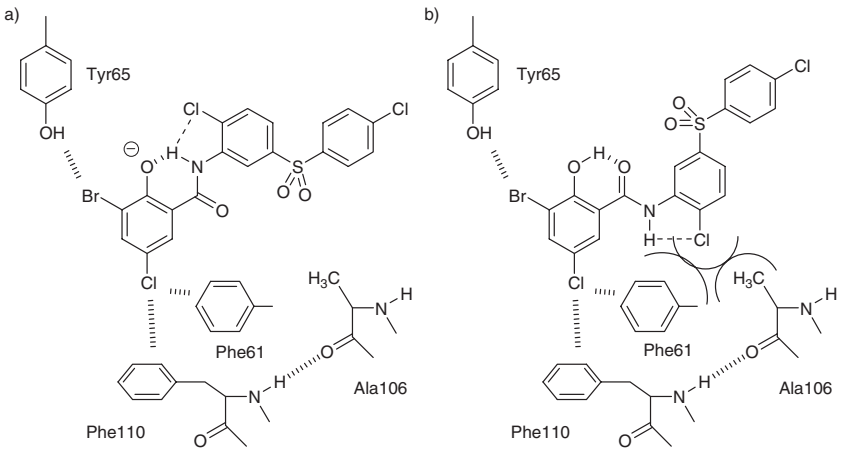


Fig. 8. Proposed binding structure in type A conformation (a) and type B conformation (b).

$(M - H^+)^-$, 249.1 (calcd for $M - H^+$; 248.90).

2-Acetoxy-3-bromo-5-chlorobenzoic Acid (2): 3-Bromo-5-chloro-2-hydroxybenzoic acid (519.3 mg, 2.1 mmol) was dissolved in 10 mL of THF. To the solution was added acetyl chloride (0.15 mL, 2.1 mmol), followed by triethylamine (0.3 mL, 2.2 mmol). The reaction mixture was stirred overnight, and the white precipitate was then filtered out. The filtrate was concentrated under reduced pressure. The resultant white powder was used without further purification (555 mg, 92%).

Mp: 153–155 °C. ^{1}H NMR ($CDCl_3$; TMS) δ 8.01 (1H, d, J = 2.4 Hz, Ar–H), 7.83 (1H, d, J = 2.4 Hz, Ar–H), 2.38 (3H, s, CH_3). ^{13}C NMR ($CDCl_3$): δ 168.0, 167.6, 147.3, 137.5, 132.1, 131.2, 125.1, 119.5, 20.7. ESI-MS: $(M - H^+)^-$, 290.9 (calcd for $M - H^+$; 290.91).

2-(2-Chlorophenylcarbamoyl)-6-bromo-4-chlorophenyl Acetate (3): 2-Acetoxy-3-bromo-5-chlorobenzoic acid (319 mg, 1.1 mmol) and triethylamine (0.20 mL, 1.4 mmol) were dissolved in 40 mL of THF. The solution was cooled to −20 °C. To the solution was added isobutyl chloroformate (IBCF) (0.18 mL, 1.4 mmol) gradually, keeping the temperature under −15 °C. After stirring for 5 min, *o*-chloroaniline (0.15 mL, 1.4 mmol) in THF (4 mL) was dropped into the reaction mixture. The reaction mixture was stirred for 1 h at −15 °C, and then 2 days at room temperature. The solvent was removed, and the residue was extracted with ethyl acetate and washed with 2% HCl aq., 4% $NaHCO_3$ aq., and sat. NaCl aq., and then dried over anhydrous sodium sulfate. The removal of the solvent gave a brown oily residue. The reprecipitation from diethyl ether/hexane gave a white powder. Recrystallization from ethyl acetate/hexane gave colorless needles (169 mg, 62%).

Mp: 128–129 °C. ^{1}H NMR ($CDCl_3$; TMS) δ 8.54 (1H, s, NH), 8.49 (1H, d, J = 7.6 Hz, Ar_B–H), 7.85 (1H, d, J = 2.4 Hz, Ar_A–H), 7.76 (1H, d, J = 2.4 Hz, Ar_A–H: protons on phenolic moiety), 7.41 (1H, dd, J = 8.4, 1.6 Hz, Ar_B–H: protons on *N*-aryl group), 7.33 (1H, t, J = 8.4 Hz, Ar_B–H), 7.09 (1H, dt, J = 7.6, 1.6 Hz, Ar_B–H), 2.41 (3H, s, CH_3). ^{13}C NMR ($CDCl_3$) δ 167.6, 161.1, 144.3, 135.6, 134.1, 132.8, 131.2, 129.4, 129.1, 127.9, 125.4, 122.9, 121.8, 118.9, 21.0. ESI-MS: $(M - H^+)^-$, 399.7 (calcd for $M - H^+$; 399.91).

3-Bromo-5-chloro-*N*-(2-chlorophenyl)-2-hydroxybenzamide (BCNCPB–OH): 2-(2-chlorophenylcarbamoyl)-6-bromo-4-chlorophenyl acetate (169 mg, 0.42 mmol) was dissolved in a mixture of MeOH (2 mL) and 1 M NaOH aq. (1 mL). The solution was stirred for two days and acidified by 2% HCl aq. The precipitated white powder was collected by filtration and recrystallized from THF/hexane. Colorless needles were obtained (34 mg, 23%).

Anal. Found: C, 43.23; H, 2.20; N, 3.83%. Calcd for $C_{13}H_8BrCl_2NO_2$: C, 43.25; H, 2.23; N, 3.88%. Mp: 157–160 °C. ^{1}H NMR ($CDCl_3$; TMS) δ 12.02 (1H, s, OH), 8.59 (1H, s, NH), 8.36 (1H, dd, J = 8.4, 1.6 Hz, Ar_B–H), 7.74 (1H, d, J = 2.4 Hz, Ar_A–H), 7.56 (1H, d, J = 2.4 Hz, Ar_A–H), 7.46 (1H, dd, J = 7.6, 1.6 Hz, Ar_B–H), 7.36 (1H, dt, J = 8.4, 1.6 Hz, Ar_B–H), 7.16 (1H, dt, J = 7.6, 1.6 Hz, Ar_B–H). ^{13}C NMR ($CDCl_3$) δ 165.9, 156.4, 137.2, 133.0, 129.2, 127.8, 126.0, 124.9, 124.2, 121.1, 122.4, 116.4, 113.4. ESI-MS: $(M - H^+)^-$, 358.1 (calcd for $M - H^+$; 357.90).

Tetraethylammonium 2-Bromo-4-chloro-6-[*N*-(2-chlorophenyl)carbamoyl]phenolate (BCNCPB–O–NEt$_4$): 3-Bromo-5-chloro-*N*-(2-chlorophenyl)-2-hydroxybenzamide (44.9 mg, 0.12 mmol) was dissolved in 8 mL of methanol. To the solution was added a 25% tetraethylammonium hydroxide aqueous solution (0.09 mL). The solution was stirred for 1 h, and solvents were removed under reduced pressure. The residue was washed with 10 mL of diethyl ether twice. The resultant powder was recrystallized from THF/diethyl ether to give **BCNCPB–O–NEt$_4$** as hygroscopic yellow crystals.

Anal. Found: C, 50.45; H, 5.52; N, 5.56%. Calcd for $C_{21}H_{27}BrCl_2N_2O_2 + (H_2O)_{0.5}$: C, 50.52; H, 5.65; N, 5.61%. Mp: 108–111 °C. ^{1}H NMR (CD_3CN; TMS) δ 15.22 (1H, s, NH), 8.70 (1H, dd, J = 8.0, 1.6 Hz, Ar_B–H), 7.77 (1H, dd, J = 3.2, 0.8 Hz, Ar_A–H), 7.40 (1H, dd, J = 7.6, 1.6 Hz, Ar_B–H), 7.39 (1H, d, J = 3.2 Hz, Ar_A–H), 7.25 (1H, tt, J = 8.0, 1.6 Hz, Ar_B–H), 6.96 (1H, tt, J = 7.6, 1.6 Hz, Ar_B–H), 3.14 (8H, q, J = 7.6 Hz, 4 × CH_2CH_3), 1.19 (12H, tt, J = 7.6, 2.0 Hz, 4 × CH_2CH_3). ^{13}C NMR (CD_3CN) δ 167.5, 167.2, 139.2, 134.7, 130.1, 129.4, 128.0, 123.8, 123.7, 123.1, 120.1, 119.1, 112.5, 53.1, 7.6.

Deprotonation of BH3I-2′. BH3I-2′ (1.5 mg, 2.8 μmol) was dissolved in 2 mL of THF. To the solution was added one drop of 25% tetraethylammonium hydroxide aqueous solution. The solution was then concentrated in vacuo. The residue was washed with 2 mL of diethyl ether and dried in vacuo.

^{1}H NMR Results of BH3I-2′ and Deprotonated BH3I-2′. BH3I-2′: ^{1}H NMR ($CDCl_3$; TMS) δ 11.70 (1H, s, OH), 9.01 (1H, d, J = 2.0 Hz, Ar_B–H), 8.69 (1H, s, NH), 7.96 (1H, d, J = 2.4 Hz, Ar_A–H), 7.93 (2H, d, J = 8.8 Hz, Ar_C–H: protons on *p*-chlorophenyl group), 7.73 (1H, dd, J = 8.8, 2.0 Hz, Ar_B–H), 7.60 (1H, d, J = 8.8 Hz, Ar_B–H), 7.56 (1H, d, J = 2.4 Hz, Ar_A–H), 7.52 (2H, d, J = 8.8 Hz, Ar_C–H).

Deprotonated BH3I-2′: ^{1}H NMR (CD_3CN; TMS) δ 15.85 (1H, s, NH), 9.41 (1H, d, J = 2.4 Hz, Ar_B–H), 7.90 (2H, d, J = 9.2 Hz, Ar_C–H), 7.82 (1H, d, J = 2.8 Hz, Ar_A–H), 7.63 (1H, d, J = 2.8 Hz, Ar_A–H), 7.58 (1H, d, J = 8.4 Hz, Ar_B–H), 7.57 (2H, d, J = 9.2 Hz, Ar_C–H), 7.46 (1H, dd, J = 8.4, 2.4 Hz, Ar_B–H), 3.14 (8H, q, J = 7.6 Hz, 4 × CH_2CH_3), 1.19 (12H, tt, J = 7.6, 2.0 Hz, 4 × CH_2CH_3).

Physical Measurements. ^{1}H NMR spectra in a $CDCl_3$ or CD_3CN solution were recorded on a JEOL GSX 400 spectrometer and a JEOL JNM EX 270 at 30 °C. NOESY spectra were recorded on a Varian UNITYplus 600 MHz spectrometer at 30 °C. Tetramethylsilane was used as a standard (0 ppm). ^{13}C NMR spectra in $CDCl_3$, CD_3CN, and DMSO-d_6 were recorded on a JEOL JNM EX 270 and Varian UNITYplus 600 MHz at 30 °C. Signals of the solvent were used as a standard {77.0 ppm ($CDCl_3$), 118.2 ppm (CD_3CN), and 39.7 ppm (DMSO-d_6)}. ESI-MS measurements were performed on a Finnigan MAT LCQ ion trap mass spectrometer in a methanol solution. IR spectra were recorded on a Jasco FT/IR 8300 spectrometer. Samples were prepared as Nujol mulls and in CH_2Cl_2 solution (1 and 5 mM).

pH Titration. The pH of a 10 mM **BCNCPB–OH** solution was determined using a Metrohm 716 DMS titrino, which is combined with a Metrohm 728 stirrer and a saturated calomel LL micro pH glass electrode. The saturated calomel micro glass electrode was calibrated with a 0.05 M $KHC_6H_4(COO)_2$ buffer (pH = 4.01), and a 0.025 M KH_2PO_4–Na_2HPO_4 buffer (pH = 6.86) at 30 °C. **BCNCPB–OH** was dissolved in a small amount of THF, and to this solution was added Triton X-100. The solution was concentrated to remove THF. The obtained residue was diluted with degassed water to give a micellar solution. The final concentration was a 10% Triton X-100 aqueous solution containing 10 mM of **BCNCPB–OH**. The solution was titrated with 0.1 M NaOH aq. at 30 °C. The pK_a value was estimated by the following equation:[23,24] $pK_a = pH - \log[Na^+] + \log\{[phenol]_0 - [Na^+]\}$.

X-ray Structure Determination. Each single crystal of **BCNCPB–OH** and **BCNCPB–O–NEt$_4$** was mounted in a loop

with Nujol. The X-ray data were collected at 200 K on a Rigaku Raxis-RAPID Imaging Plate diffractometer with graphite monochromated Mo Kα ($\lambda = 0.71075$ Å). The basic crystallographic parameters are listed in Table S1 (See Supporting Information). A symmetry-related absorption correction using the program ABSCOR[33] was applied, which resulted in transmission factors ranging from 0.43 to 0.49 for **BCNCPB–OH** and 0.42 to 0.53 for **BCNCPB–O–NEt$_4$**. The structure was solved by the direct method (SHELXS-97[34] for **BCNCPB–OH** and SIR92[35] for **BCNCPB–O–NEt$_4$**) and expanded using Fourier techniques using *teXsan* crystallographic software[36] and SHELXL-97.[37] Non-hydrogen atoms were refined anisotropically. The positions of OH and NH were refined using fixed thermal parameters, and the other hydrogen atoms were placed at calculated positions.

Crystal Data. **BCNCPB–OH:** $C_{13}H_8BrCl_2NO_2$, $M = 361.02$, orthorhombic, $a = 23.15(2)$, $b = 15.73(2)$, $c = 7.126(7)$ Å, $U = 2594(10)$ Å^3, $T = 200$ K, space group *Pccn* (no. 56), $Z = 8$, $\mu = 35.84$ cm^{-1}, 22578 reflections measured, 2836 unique ($R_{int} = 0.096$), $R_1 = 0.036$ ($I > 2\sigma$), $wR_2 = 0.070$ (all data).

BCNCPB–O–NEt$_4$: $C_{21}H_{27}BrCl_2N_2O_2$, $M = 490.27$, orthorhombic, $a = 7.307(4)$, $b = 16.18(1)$, $c = 18.99(1)$ Å, $U = 2245(8)$ Å^3, $T = 200$ K, space group $P2_12_12_1$ (no. 19), $Z = 4$, $\mu = 20.93$ cm^{-1}, 21267 reflections measured, 2935 unique ($R_{int} = 0.045$), $R_1 = 0.028$ ($I > 2\sigma$), $wR_2 = 0.048$ (all data).

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