Dap-SL: a new site-directed nitroxide spin labeling approach for determining structure and motions in synthesized peptides and proteins

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Abstract **A new approach for site-directed placement of nitroxide spin labels in chemically synthesized peptides and proteins is described. The scheme takes advantage of a novel diaminopropionic acid scaffold to independently control backbone and side chain elongation. The result is a spin-labeled side chain, referred to as Dap-SL, in which an amide bond forms a linker between the nitroxide and the peptide backbone. The method was demonstrated in a series of helical peptides. Circular dichroism and nuclear magnetic resonance showed that Dap-SL introduces only a minor perturbation in the helical structure. The electron paramagnetic resonance spectrum of the singly labeled species allowed for determination of the spin label rotational correlation time and suggests that the Dap-SL side chain is more flexible than the modified Cys side chain frequently used in site-directed spin label studies. Spectra of the doubly labeled peptides indicate a mixture of 3_{10}-helix and α-helix, which parallels findings from previous studies. The scheme demonstrated here offers a fundamentally new approach for introducing spin labels into proteins and promises to significantly extend biophysical investigations of large proteins and receptors. In addition, the technique is readily modified for incorporation of any biophysical probe.**

Key words: Electron paramagnetic resonance; Site-directed spin labeling; Helical peptide; Helix conformation; Synthetic protein

1. Introduction

Nitroxide spin label electron paramagnetic resonance (EPR) is among the leading biophysical techniques for determining structure and structural mobility in proteins [1,2]. To date, there have been two fundamental approaches for attaching nitroxides in a site-specific fashion. The first, and by far most common, uses microbial expression to produce a mutant protein containing a non-native Cys at a chosen location. Treatment with a thiol-specific reagent, such as methanethiosulfonate spin label (MTSSL), then yields a side chain bearing the nitroxide [3]. This approach, often referred to as site-directed spin labeling (SDSL), has been remarkably successful for studying membrane proteins (e.g. [4,5]), and large oligomeric structures such as that formed in the SNARE complex [6] and by α-crystallin [7].

Another approach involves solid-phase protein synthesis (SPPS). Here, organic chemistry is used to add amino acids in a step-wise fashion. An advantage of SPPS is that one may incorporate non-native amino acids with desired biophysical properties. For example, the spin label TOAC attaches with remarkable rigidity and serves as an outstanding reporter of backbone flexibility and local structure [8–11].

Despite the great success enjoyed by these approaches, each possesses limitations. For example, SDSL usually requires a protein sequence free of native Cys residues so that only the chosen location is labeled. In addition, the disulfide bond in the modified Cys side chain is labile and does not stand up to temperatures greater than 60°C [12] or extremes in pH. With regard to TOAC labeling, the bulky nitroxide side chain hinders conventional SPPS chemistry and often leads to low coupling yields [13]. In addition, TOAC is a dialkyl side chain, similar to α-isobutyric acid, and greatly favors helical torsion angles [10]. Thus, TOAC is not readily incorporated into extended strands or β-sheets.

SPPS has advanced significantly in recent years and, with exciting new technologies such as native chemical ligation [14,15], one may now prepare high molecular weight proteins solely by chemical means. Here we present a new approach using SPPS for incorporating nitroxide side chains into proteins. The strategy uses orthogonally protected amines within a single amino acid for individual control of backbone and side chain elongation. This approach has been explored elsewhere for enhancing diversity in bioactive peptide libraries [16,17]. In our application here, the focus is on incorporation of biophysical probes. Although the method is general for attaching a spin label to any amine-bearing side chain, we demonstrate the approach using diaminopropionic (Dap) acid as the scaffold for nitroxide attachment. The result is a peptide containing an enantiomerically pure amino acid possessing a side chain that links to its nitroxide through an amide bond. We refer to this amino acid as Dap-SL. A series of helical peptides containing one or two Dap-SL residues, in conjunction with circular dichroism (CD) and nuclear magnetic resonance (NMR), are used to examine the side chain's influence on peptide conformation. EPR spectra are examined to assess side chain mobility and, in a double label experiment, sensitivity to local structure.

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2. Materials and methods

2.1. Peptide synthesis and purification

The peptide sequences were synthesized using standard Boc/Bzl solid-phase peptide synthesis methods. The Dap scaffold to which the spin label was attached, N^{α}-Boc-N^{β}-Fmoc-L-2,3-diaminopropionic acid, was purchased from Peptides International. The spin label was attached to the resin-bound peptide using Fmoc peptide methodology (20% piperidine deprotection), followed by coupling the HOAt/DIC-activated carboxylic acid. Resin cleavage and removal of the side chain protecting groups was achieved by treatment with anhydrous HF with 10% *p*-cresol as scavenger at 0°C for 1 h, followed by cold diethyl ether precipitation and filtration. The solid material was dissolved in aqueous solvent and promptly lyophilized. Purification of the peptides was done using reversed-phase HPLC (pH 2.0–2.5 doubly deionized water with 6 mM NaOH and 0.1% trifluoroacetic acid (TFA) = A; acetonitrile with 0.1% TFA = B), using C18 analytical and semi-preparative columns. Purified material was lyophilized for future use.

2.2. CD and electron spin resonance

All aqueous samples were buffered to pH 7.0 in MOPS. CD spectra were acquired on an Aviv 60DS spectropolarimeter, with temperature regulation provided by a circulating water/ethylene glycol bath attached to the cuvette holder. The CD spectrometer was calibrated using the (+)-10-camphorsulfonic acid standard. For CD measurements, samples contained usually 100 μM peptide in a final volume of 200 μl. EPR signal integration was used to determine peptide concentrations for quantitative CD measurements [12]. All spin concentrations were made relative to a stock solution of TEMPOL in MOPS buffer at pH 7.0 that had been made precisely to 2.00 mM concentration as determined by UV-Vis absorption. The CD samples were acquired at 1.0°C, with sample in a 0.1 cm path length quartz cuvette. All EPR spectra were recorded on a Bruker ESP 380 spectrometer, using methods described previously [12,18]. The temperature was controlled using a Bruker variable temperature unit with liquid nitrogen as the coolant.

2.3. NMR

Methods for determining two-dimensional NMR spectra of spin-labeled peptides have been described previously [19]. Briefly, all NMR spectra were recorded on a Varian Unity+ 500 MHz spectrometer. Peptide concentrations were all approximately 2 mM and determined by the same method as used for the CD samples. The spins of each sample were subsequently reduced using ascorbic acid. The solution containing the reduced peptide was lyophilized, reconstituted in 90/10 H_2O/D_2O and brought to pH 5.0 for acquisition of NOESY and TOCSY ^1H two-dimensional homonuclear spectra. The NOESY spectra were typically acquired with a 400 ms mixing time and 700 increments in the t_1 dimension, whereas the TOCSY spectra were acquired with a 50 ms mixing time and 350–512 increments. Spectra were acquired at 2°C, with temperature controlled by passing dry nitrogen gas through a coil immersed in < -50°C neat isopropanol. Data were processed using nmrPipe [20] and visualized using NMRView (Bruce A. Johnson, Merck Research Laboratories). All spectra were zero-

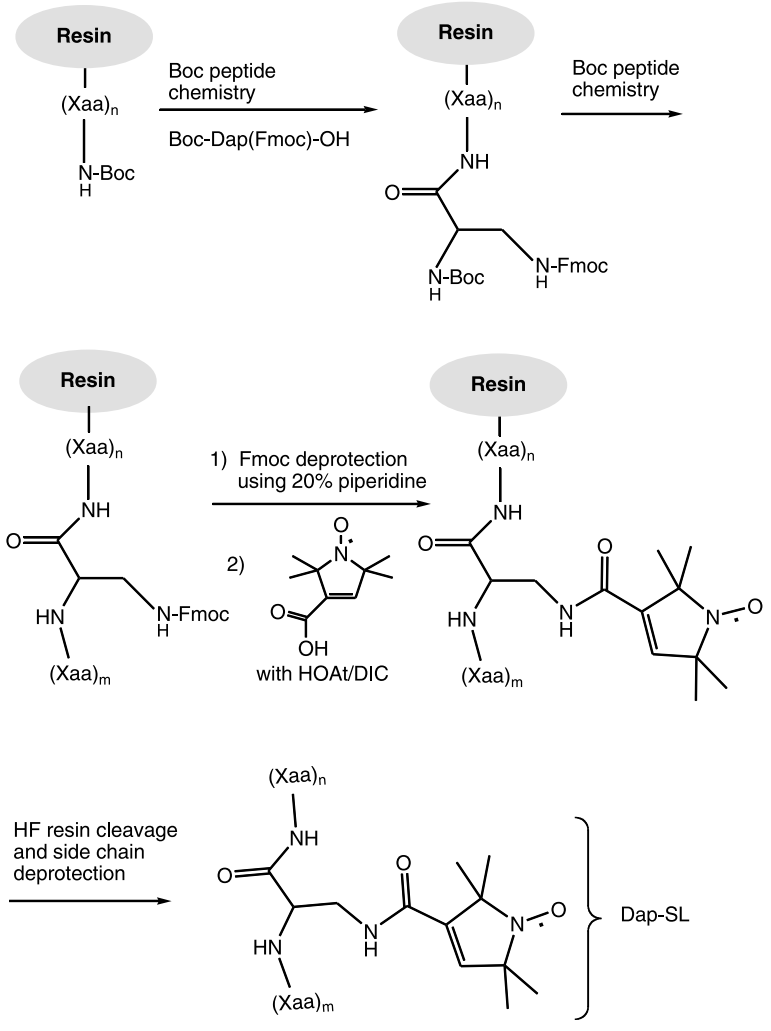

Fig. 1. Synthetic scheme showing how the Dap-SL is incorporated using the orthogonally protected Boc-Dap(Fmoc)-OH scaffold. $(Xaa)_n$ and $(Xaa)_m$ refer to the *n* and *m* amino acids preceding and following the Dap-SL, respectively. This scheme allows for incorporation of different enantiomerically pure scaffolds, as well as different carboxyl-containing nitroxides, for wide-ranging variation of the spin label side chain.

filled to 1024×2048 complex points prior to Fourier transformation. CSIs were tabulated according to the methods of Wishart et al. [21].

3. Results

The synthetic scheme for incorporating Dap-SL into a peptide is shown in Fig. 1. The two amines in Dap are protected using Boc and Fmoc groups that cleave in the presence of acid and base, respectively. Standard Boc chemistry from the resin-attached C-terminal amino acid is used to prepare the peptide sequence. Protected Dap is incorporated at the appropriate position and the Fmoc group remains attached throughout peptide synthesis. When the peptide sequence is complete, but still on the resin, the Fmoc group is removed with base. The carboxylic acid on the nitroxide, activated by a coupling agent, then couples to the unprotected amine. Side chain deprotection and cleavage from the resin in acid produces the final peptide containing the Dap-SL amino acid.

The specific scheme in Fig. 1 was designed to maintain the integrity of the nitroxide group. Using Boc chemistry for chain assembly insures that the only strong acid seen by the nitroxide is HF in the final side chain deprotection and resin cleavage step. Nitroxides are robust in HF and the only side reaction is protonation of the NO group, which is readily reversed with weak base. Alternatively, Fmoc chemistry relies on TFA for the final protection group removal and resin cleavage. Exposure to TFA can result in irreversible destruction of the nitroxide according to some protocols [13]. This destruction is avoided with the scheme outlined here.

The 3K peptide Ac-AAAAKAAAAKAAAAKA-NH_2 was used as a host for exploring the synthetic and biophysical properties of Dap-SL. The 3K sequence is partially helical in aqueous solution and has been exploited in numerous studies for assessing helix stability and folding pathways [22–27]. We prepared the family of three labeled peptides shown below where X is used to indicate Dap-SL.

Ac-AAAAKAA**X**AKAAAAKA-NH_2	3K(Dap)-8
Ac-AAA**X**KA**X**AAKAAAAKA-NH_2	3K(Dap)-4,7
Ac-AAA**X**KAA**X**AKAAAAKA-NH_2	3K(Dap)-4,8

Similar peptide series have been examined previously with both Cys-SL [26,27] and TOAC labels [8,10]. Spin–spin interactions in the −4,7 and −4,8 labeling scheme provide a probe of peptide conformation. The singly labeled 3K(Dap)-8 serves as a control by allowing measurement of the EPR spectrum in the absence of spin–spin interactions.

With all three peptides, synthesis was straightforward, and purification followed standard protocols. The Boc and Fmoc couplings were efficient and only trace side products were identified by HPLC or mass spectrometry. Of note is that the 3K sequence contains amines at each of the three Lys residues, yet only the desired Dap amine was labeled.

CD was used to probe helix content in all peptides in pH 7 aqueous solution at 274 K; the spectra are shown in Fig. 2. All three spectra exhibit minima at 208 nm and 222 nm characteristic of polypeptide helices. Comparison of the spectra to that from the original 3K peptide suggests a decrease in apparent helix content. Estimates based on the mean residue ellipticity at 222 nm for each peptide place the helix content at ~50% for 3K(Dap)-8 and 3K(Dap)-4,8, and 30% for 3K(Dap)-4,7 [28]. This is compared to the 80% helix for the unmodified 3K sequence [23]. Thus, CD suggests that Dap-SL is somewhat less helix-favoring than Ala. The peptides were also examined in 1:1 (v/v) water/TFE at 274 K, which is a standard solvent system for promoting helical structure. The three resulting spectra are almost superimposable with negative minima exhibiting approximate equal intensity consistent with a well-structured helix.

The nitroxide and amide bond in the Dap-SL side chain both absorb in the CD active ultraviolet region and thus may interfere with quantitative helix determinations using the spectra in Fig. 2 [29]. In addition, the 3K(Dap)-4,7 appears to be less helical than the remaining peptides. To investigate further, we used NMR to assess relative structure in this family of peptides. Chemical shifts, referenced against established random coil values, are extremely sensitive to secondary structure [21]. A conformational shift (experimental chemical shift minus random coil chemical shift) of −0.1 ppm indicates helix and a conformational shift of 0.1 ppm indicates β-strand. We have shown previously that gentle ascorbic acid reduction of the nitroxide to the hydroxylamine allows for detailed NMR analysis of spin-labeled peptides without interference from the paramagnetic center [19]. Using two-dimensional NOESY and TOCSY, the conformational shifts were determined for all three peptides and the results are compared to that of the original 3K sequence in Fig. 3. Consistent with the findings from CD, all three Dap-SL peptides give strongly

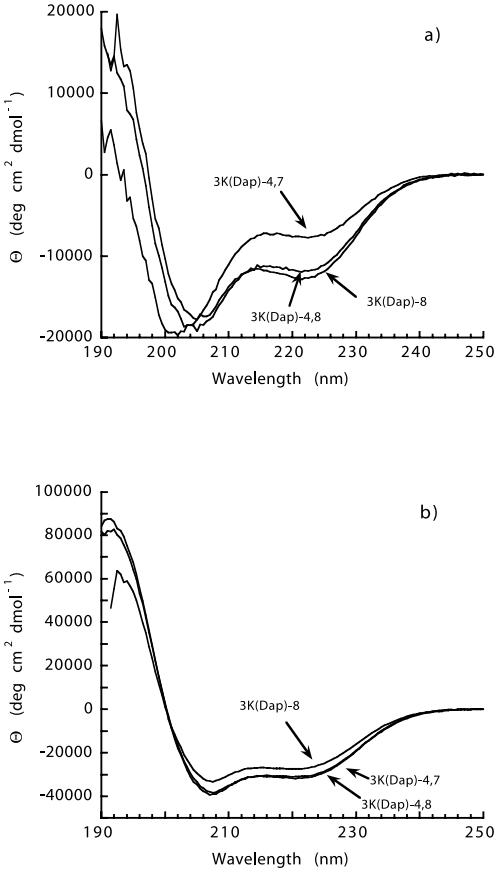

Fig. 2. CD spectra of the family of Dap-SL-containing peptides in (a) MOPS buffer pH 7.0 and (b) 1:1 (v/v) water/TFE. These spectra demonstrate that the spin-labeled peptides adopt helical structure similar to the unlabeled 3K; however, in aqueous solution alone the helix content may be somewhat reduced.

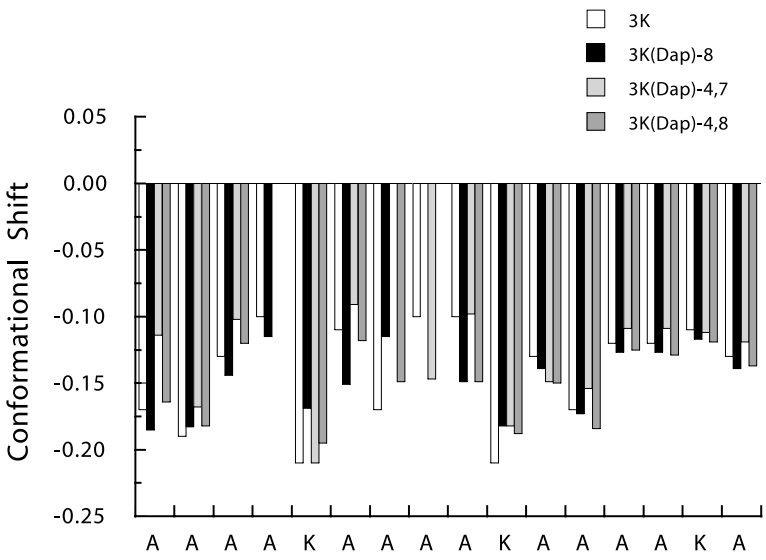

Fig. 3. α-Proton conformational shifts (chemical shift minus random coil chemical shifts) for the peptides in this study. The strongly negative shifts are characteristic of helical structure. All peptides exhibit approximately the same position-dependent pattern suggesting that helix content and structure is preserved in all labeled peptides.

helical conformational shifts. In addition, to within experimental error, the data in Fig. 3 suggest that all of the labeled peptides possess nearly equivalent conformational shifts as the original 3K sequence. Although the CD spectra suggest a decrease in helix content relative to the 3K peptide, especially for the 3K(Dap)-4,7, the NMR conformational shifts suggest that there is little to no perturbation introduced by Dap-SL.

Fig. 4 shows EPR spectra obtained at 274 K from the family of peptides. The 3K(Dap)-8 gives sharp lines consistent with a singly labeled species. The rotational correlation, determined using motional narrowing theory, is 0.20 ns [12]. A homologous peptide labeled with MTSSL at position 8 gives a correlation time of 0.45 ns [30] thereby suggesting that the Dap-SL side chain is more flexible than the Cys-SL side chain.

Consistent with the introduction of a biradical interaction, the doubly labeled peptides give substantially broader lines than the singly labeled peptide. While the detailed line-broadening mechanisms are not known, it is believed that they arise from a combination of residual dipolar coupling, scalar J-coupling and time-dependent modulation of these couplings leading to rapid relaxation [9,31]. Despite the specific interaction, the extent of line broadening correlates inversely with distance between the labels. Inspection of the spectra obtained from the aqueous samples suggests that the 3K(Dap)-4,7 and 3K(Dap)-4,8 line widths are nearly equivalent but with the 3K(Dap)-4,7 being slightly broader. Thus, the distances between the labels in each peptide are approximately equivalent. A similar distance hierarchy was observed previously in homologous Cys-SL containing peptides and was interpreted as arising from an admixture of 3_{10}-helix and α-helix. In a pure α-helix, the −4,8 distance is shorter than the −4,7 and this is indeed observed for longer helical peptides (see [26] for analysis). The doubly labeled peptides were also examined in the helix-promoting solvent system water/TFE. Here the lines are broader reflecting either enhancement of the helical structure, consistent with the CD spectra, or increased viscosity relative to water. The relative line broadening still suggests an admixture of 3_{10}-helix and α-helix.

The broad spectra obtained from the doubly labeled species do not exhibit superimposed narrow line components as might arise from contamination from a monoradical species. To test the robustness of the Dap-SL side chain, aqueous samples of the singly and doubly labeled species were brought to approx-

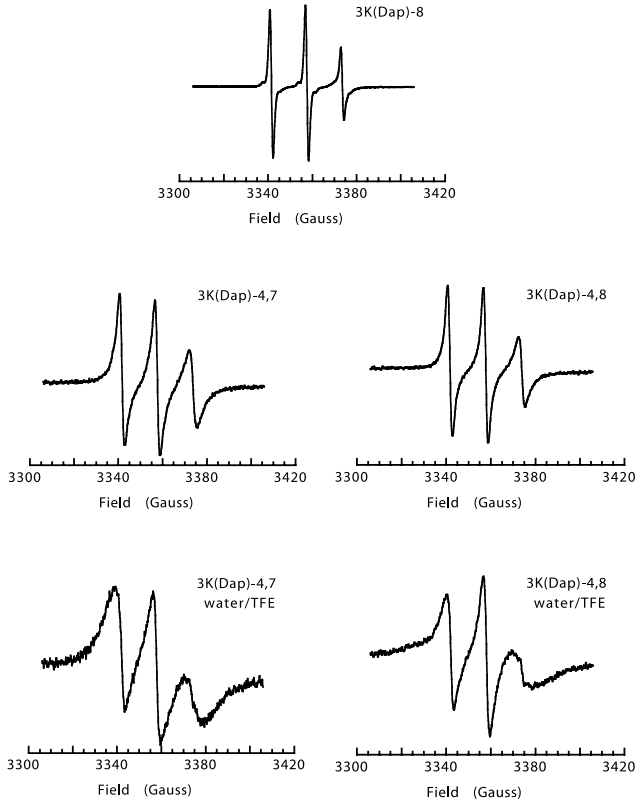

Fig. 4. EPR spectra of Dap-SL-containing peptides. Analysis of the singly labeled 3K(Dap)-8 spectrum yields a rotational correlation time of 0.2 ns. The doubly labeled spectra exhibit broader lines consistent with biradical interactions.

imately 90°C for several minutes. After the samples were cooled, the EPR spectra were reacquired. There were no measurable changes in any of the spectra indicating that the side chains remained intact.

4. Discussion

We have developed a new approach for the site-specific placement of spin labels in chemically synthesized peptides and proteins. The label was tested in a series of helical peptides. Analysis of the CD and NMR data demonstrates that the Dap-SL does not introduce a significant perturbation in the helical structure. However, when considering the CD alone, the Dap-SL side chain appears to be less helix-favoring than Ala. EPR shows that the Dap-SL side chain is more flexible than the widely used Cys-SL. However, EPR of the doubly labeled peptides yielded results similar to that obtained previously from peptides containing Cys-SL side chains and demonstrates that double labeling with Dap-SL serves as a useful probe of secondary structure.

The synthetic scheme in Fig. 1 was designed to provide a convenient means for wide-ranging site-directed spin labeling applications. In principle, one could prepare Fmoc or Boc nitroxide amino acids and incorporate them directly through normal peptide synthesis as demonstrated with TOAC. However, this approach involves synthesis of significant quantities of the protected nitroxide amino acid since these reagents are added in large (usually fourfold) excess to the peptide on the resin. In addition, for chiral nitroxide amino acids, one must have enantiomerically pure materials. Fig. 1 shows that in the scheme here one may use commercially available reagents along with any nitroxide containing a carboxyl group. In addition, by simply varying the orthogonally protected scaffold, one may readily develop a series of nitroxide side chains of varying length, as has been demonstrated in peptide libraries [17].

Dap-SL opens new avenues for preparation of spin-labeled proteins. We consider two examples from our own laboratory. We have recently determined the NMR structure of the agouti-related protein (AGRP), a potent antagonist of a subfamily of G protein-coupled receptors called melanocortin receptors (MCRs) [32]. As technologies evolve for overexpression and biophysical studies of MCRs, having spin-labeled AGRP could be remarkably useful for evaluating receptor binding domains and conformational changes. AGRP contains 10 Cys residues essential for activity and is thus not amenable to MTSSL labeling. In addition, it contains no helical structure and will likely be perturbed by incorporation of TOAC. However, AGRP can be prepared by SPPS [33] and is thus conveniently labeled with the Dap-SL scheme described here.

Another research direction involves the copper binding sites in the octarepeat domain of the prion protein. We have recently used peptide design, EPR and isotopic labeling to determine the molecular features of these metal ion binding sites [34,35]. At this juncture, however, there is little information regarding how these sites organize relative to each other in the full copper binding domain. Spin label technologies could be ideal for determining local motions and organization. Cys-SL labeling is probably not feasible since thiols bind avidly to copper; TOAC labeling is also problematic since there are no helical stretches for incorporation. However, Dap-SL should not interfere with either normal copper binding or native backbone structure and thus may be key for addressing issues of organization in the holo protein. In conclusion, Dap-SL promises to open many new avenues of spin label research.

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