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COMMUNICATION

Total Synthesis and Conformational Study of The Anti-tubercular Cyclic Peptide Callyaerin A Bearing a Rare Rigidifying (*Z*)-2,3-Diaminoacrylamide Moiety

Shengping Zhang,[a] Luis M. De Leon Rodriguez,[b] Ivanhoe K. H. Leung,[a] Gregory M. Cook,[b,c] Paul W. R. Harris[a,b] and Margaret A. Brimble*[a,b]

Dedication ((optional))

Abstract: The first synthesis of the anti-TB cyclic peptide callyaerin A containing a rare (*Z*)-2,3-diaminoacrylamide bridging motif is reported. Fmoc-formylglycine-diethylacetal was used as a masked equivalent of formylglycine in the synthesis of the linear precursor of callyaerin A. Intramolecular cyclization between the formylglycine residue and the *N*-terminal amine in the linear peptide precursor afforded the macrocyclic natural product callyaerin A. Synthetic callyaerin A possessed potent anti-TB activity (MIC_{100} = 32 μM) while its all-amide congener was inactive. Variable temperature NMR studies of both the natural product and its all amide analogue revealed the extraordinary rigidity imposed by this diaminoacrylamide unit on peptide conformation. The work reported herein pinpoints the intrinsic role that the rare (*Z*)-2,3-diaminoacrylamide moiety confers on peptide bioactivity.

Tuberculosis (TB), a highly contagious and airborne disease caused by *Mycobacterium tuberculosis*, has become one of the leading causes of mortality worldwide. According to the WHO, in 2015 alone an estimated 10.4 million people were diagnosed with new cases of TB infection, out of which 1.8 million people died.[1] The situation is further aggravated by the fast-growing cases of multi-drug-resistant (MDR) and extensively drug-resistant (XDR) strains of *M. tuberculosis*, where currently-used standard treatment with first and second line anti-TB drugs are ineffective or require long treatment durations (>2 years) to achieve therapeutic effect.[2] The development of novel anti-TB drugs, however, has slowed over the past decade, with only one drug, bedaquiline, approved for treatment of MDR-TB in the last 40 years.[3] There is a pressing need for discovery and development of new anti-TB therapeutics.

Peptides from natural sources exhibit a wide spectrum of bioactivities including anti-TB activity. Compared to their small organic molecule counterparts, peptides are attractive drug candidates given their higher target affinities and selectivity and lower off-target effects.[4] Several naturally-occurring peptides which exhibit potent anti-TB activity have been reported, including callyaerin A,[5] trichoderins A[6] and B,[6] lassomycin,[7] ecumicin,[8] wollamide A[9] and teixobactin.[10] Among them, callyaerin A (**1**) (Figure 1), a proline-rich cyclic peptide derived from the Indonesian marine sponge *Callyspongia aerizusa*,[5] is a potent inhibitor of *M. tuberculosis* (MIC_{100} of 6.0 μM) with no cytotoxicity observed in human cells (IC_{50} > 10 μM).[5, 11] Callyaerin A (**1**) features an unusual (*Z*)-2,3-diaminoacrylamide (DAA) unit within the cyclic peptide, which together with the multiple Pro residues in the sequence, influences the topology of the molecule, potentially contributing to its remarkable bioactivity.[11] The frequency of hydrophobic residues may also contribute to the specificity of callyaerin A (**1**) against bacteria.[5] Prompted by its unique structural features and potent anti-TB activity, we embarked on the total synthesis of callyaerin A (**1**) as the first step to explore its potential as a new anti-TB agent.

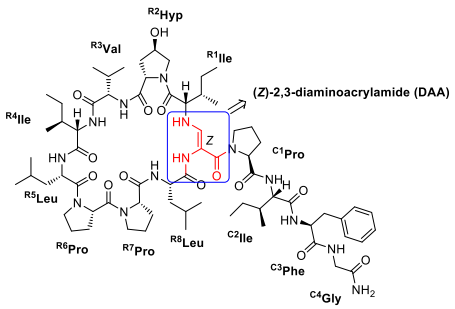

Figure 1. The structure of callyaerin A (**1**)

Inspired by the proposed biosynthesis,[12] we envisaged that the rare DAA moiety could be installed by reacting the aldehyde in an α-formyl glycine (FGly) residue with an *N*-terminal amino group to form an imine, followed by double-bond migration (path A, Scheme 1). However, the synthesis of FGly-containing peptides, such as **2**, is an arduous task as highlighted in a recent review.[13] An amenable approach is to directly oxidize the side chain hydroxyl of Ser to an aldehyde. Unfortunately, oxidation of the Ser residue in protected peptide **3** could not be realized (path A, Scheme 1) possibly due to the chemical lability of FGly caused by the acidic proton α to the carbonyl groups, similar to α-amino aldehydes.[14] Alternatively, Nakazawa *et al.*[15] reported the oxidation of a series of serine-containing peptides

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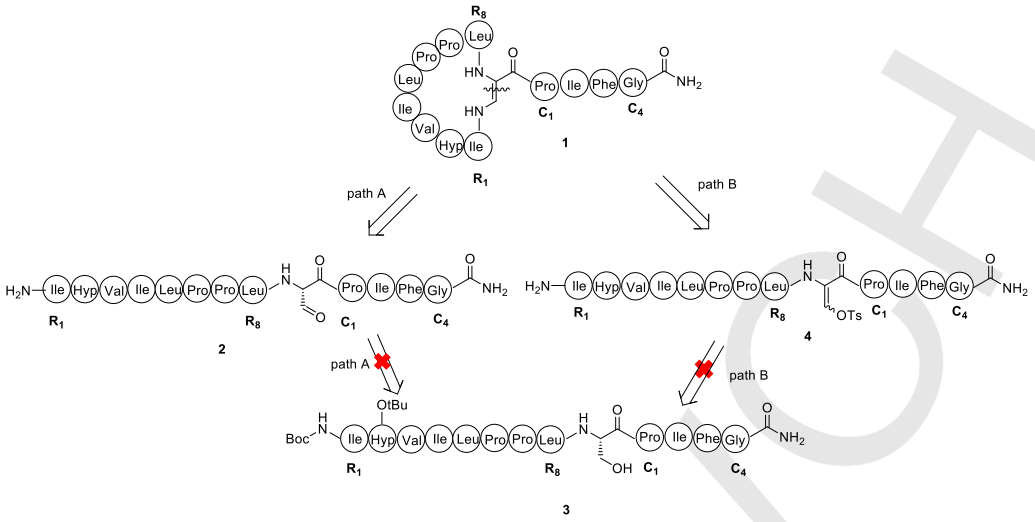


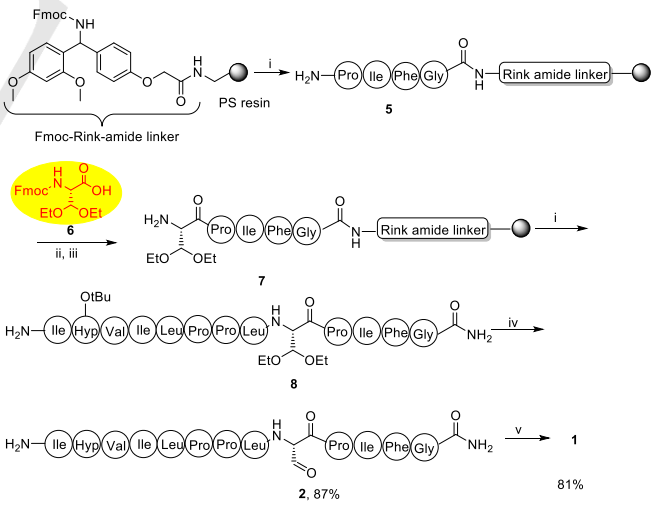
Scheme 1. Initial retrosynthesis of callyaerin A (**1**). R1-R8 indicate residues within the peptide ring while C1-C4 indicate residues in the peptide tail

to the corresponding peptidyl tosyl enolates that could further react with the *N*-terminal amine to furnish a cyclic peptide containing a diaminoacrylamide moiety. Use of this methodology for the attempted oxidation/tosylation of **3** to afford the peptidyl tosyl enolate (**4**) was not successful (path B, Scheme 1).

The failure of both direct and indirect oxidation protocols to convert Ser into FGly in linear peptide **3** prompted adoption of a new strategy. A rare building block, α-formylglycine diethylacetal ethyl ester, has been used in the synthesis of capreomycin IB, a naturally-occurring antibiotic encompassing an exocyclic enediamino functionality.[16] An N^{α}-Fmoc protected variant of this acetal-protected FGly (**6**) for solid phase peptide synthesis (SPPS) has also been reported, highlighting the simultaneous hydrolysis of the acetal to afford the FGly residue during peptide cleavage.[17] We therefore decided to incorporate **6** into Fmoc-SPPS to furnish the linear precursor of callyaerin A (**2**) bearing an FGly residue after TFA-mediated peptide cleavage. Subsequent cyclization to form the DAA moiety would then afford the natural product directly.

Peptide **2** was thus synthesized on Rink amide aminomethyl polystyrene resin using Fmoc-SPPS (Scheme 2). All the amino acids, except FGly(OEt)$_2$-OH, were coupled using *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HATU) as the coupling reagent. The coupling of FGly(OEt)$_2$-OH, on the other hand, was accomplished employing a mixture of Fmoc-FGly(OEt)$_2$-OH, *N*,*N*'-diisopropylcarbodiimide (DIC) and 6-chloro-1-hydroxybenzotriazole (6-Cl-HOBt) in DMF. These base-free coupling conditions minimized epimerization at the α position of Fmoc-FGly(OEt)$_2$-OH..[16]

After peptide chain assembly, the linear peptide was released by treating the resin with 95% TFA in water (v/v). Commonly-used silanes and thiol-based scavengers, *e.g.* *i*Pr$_3$SiH and 1,2-ethanedithiol, are not compatible with the cleavage of peptides containing FGly due to the reactivity of aldehydes with nucleophiles.[17] Furthermore, we observed that the FGly-containing peptide was surprisingly labile under the acidic conditions used for peptide cleavage, resulting in a significant amount of peptide fragment **10** (Scheme 3, found 877.6 [M+H]$^+$, calcd 877.6) resulting from cleavage at the FGly site. This peptide bond scission may occur by hydrolysis of the active 4,5-dihydrooxazole intermediate (**9**) generated by the nucleophilic attack of the neighbouring carbonyl group on the formyl group of FGly (Scheme 3). This undesirable peptide fragmentation was minimized by reducing the cleavage reaction time (2 × 20 min), affording the desired peptide **2** in high yield (87%) with less than 5% of by-product **10**. The crude product **2** was then directly subjected to the subsequent cyclization.



Scheme 2. Synthetic route to callyaerin A (**1**). Reagents and conditions: (i) iterative Fmoc SPPS (20% piperidine in DMF (v/v), RT, 2 × 5 min; Fmoc-amino acid (4 equiv.), HATU (3.9 equiv.), DIPEA (8 equiv.), DMF, RT, 40 min); (ii) **6** (2 equiv.), DIC (2 equiv.), 6-Cl-HOBt (2 equiv.), RT, 1 h; (iii) 20% piperidine in DMF (v/v), RT, 2 × 5 min; (iv) TFA/H_2O (95:5), RT, 2× 20 min; (v) 1% formic acid (v/v) in acetonitrile, anhydrous $MgSO_4$(100 equiv.), RT, 10 h.

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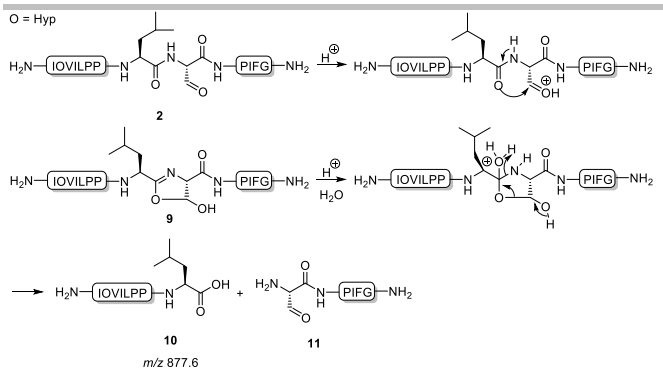


Scheme 3. Proposed mechanism for fragmentation at FGly site.

Formation of the rare DAA unit in the cyclic peptide is the key step to access callyaerin A (**1**). Traditionally, formation of an enamine functionality can be achieved through acid-catalysed reaction of an amine with an aldehyde in water.[18] Moreover, the cyclization needs to be performed under high dilution in order to prevent the intermolecular reaction. We therefore investigated this reaction using a mixture of linear peptide and anhydrous $MgSO_4$ in acetonitrile at a peptide concentration of 0.75 mM with several organic acids at different concentrations. Peptides containing FGly were not stable in the presence of strong acid (TFA, pKa = 0.3) or moderately strong acid (camphorsulfonic acid (CSA, pKa = 1.2) as varying levels of peptide fragment (**10**) were obtained (Figure 2). A lower concentration of acid (1% TFA *vs* 10% TFA) mitigated this undesirable fragmentation, but the amount of peptide fragment (**10**) still increased over an extended reaction time. Fortunately, use of weaker 1% formic acid (pKa = 3.7) showed favourable catalysis, shortening the reaction time to 10 h and effecting almost quantitative peptide cyclization with little peptide fragmentation. Pleasingly, solution-phase peptide cyclization performed using 1% formic acid (v/v) afforded callyaerin A (**1**) in 81% yield after isolation by HPLC.

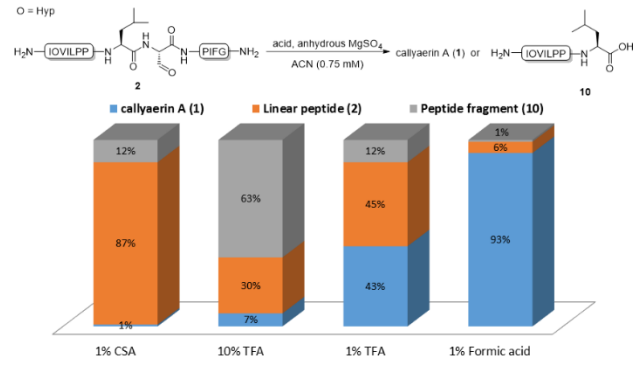
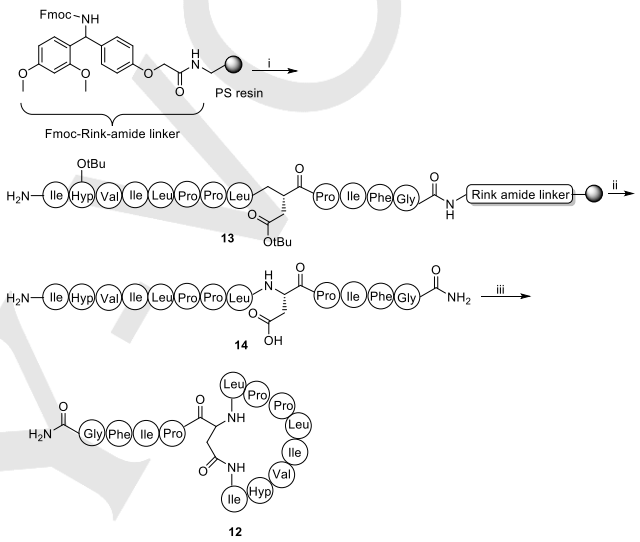


Figure 2. Composition of peptide cyclization reaction using acids with different pKa and concentration. Data was determined by integration of the HPLC peaks of the corresponding reaction mixture at a wavelength of 210 nm; linear gradient of 5%B-95%B over 30 min (*ca.* 3%B/min), 1 mL•min^{-1}, using an XTerra MS C_{18} column (4.6 mm × 150 mm, 5 µm). A = 0.1% TFA in H_2O and B = 0.1%TFA in MeCN. Reagents and conditions: **2** (2 mg, 1.4 µmol), anhydrous $MgSO_4$ (17.5 mg, 140.5 µmol), MeCN (1.9 mL), acids (19 or 190 µL), RT, 10 h.

The ^{1}H and ^{13}C NMR data for synthetic **1** agreed with those reported for the natural product[5] (Table S1 in the Supporting Information). Moreover, the successful establishment of the *Z* configuration for the DAA unit was confirmed by the identical chemical shift (7.35 ppm) and coupling constant (13.2 Hz) for the β proton in the DAA moiety (Table S1 in the supporting information).[5]

For comparison purposes, we also prepared a homodetic analogue of callyaerin A (**12**), in which the FGly residue was substituted with an Asp enabling formation of a lactam linkage with the *N*-terminus (Scheme 4). The linear precursor of **12** (**14**) was prepared using Fmoc-SPPS and a solution-phase cyclization under high dilution (0.37 mM) then gave **12** in good yield (68%) (Scheme 4).



Scheme 4. Synthetic route to the homodetic analogue of callyaerin A (**12**). Reagents and conditions: (i) iterative Fmoc SPPS (20% piperidine in DMF(v/v), RT, 2 × 5 min; Fmoc-amino acid (4 equiv.), HATU (3.9 equiv.), DIPEA (8 equiv.), DMF, RT, 40 min); (ii) TFA/H_2O/*i*Pr_3SiH (95:2.5:2.5, v/v/v), RT, 1 h; (iii) HBTU (3 equiv.), 6-Cl-HOBt (3 equiv.), DIPEA (5 equiv.), CH_2Cl_2/DMF (9:1) RT, 1 day.

Cyclic peptides **1** and **12** were tested *in vitro* for their anti-TB activity in order to elucidate the role of the (*Z*)-2,3-diaminoacrylamide on bioactivity. Callyaerin A (**1**) exhibited inhibitory activity against *M. tuberculosis* (MIC_{100} = 32 µM), which was 5-fold higher than the reported MIC_{100} data (6 µM).[11] The homodetic analogue (**12**) did not exhibit any inhibitory activity up to a concentration of 512 µM. This dramatic difference in biological activity may be attributed to subtle variations in peptide conformation, thus prompting further investigation of their conformational properties.

Using variable temperature ^{1}H NMR, we established a comprehensive profile of the temperature-chemical shift coefficients (T_{coeff}) for all the amide protons in callyaerin A (**1**) from which the intramolecular hydrogen-bonding pattern was deduced. Figure 3a shows the aligned ^{1}H NMR spectra of callyaerin A (**1**) recorded from 20 °C to 45 °C and the corresponding T_{coeff} value calculated for each amide proton. Four amide protons exhibit a T_{coeff} value between −4 and −1 ppb/K (DAA, ^{R4}Ile, ^{R5}Leu and ^{C2}Ile), thus indicating a high tendency to participate in H-bond interactions.[19] Notably, the amide proton of DAA seems to participate in H-bond formation, however, the relatively more positive value of T_{coeff} for the amide proton of DAA can also be attributed to the deshielding effect of the neighbouring double

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bond, which is similar to amide protons influenced by an adjacent aromatic ring.[19] In this case, an increase in thermal motion does not cause meaningful chemical shift changes hence the obtained T_{coeff} may no longer serve as a marker of H-bonding.[19] Additionally, the unusual positive T_{coeff} values for the amide protons of the Phe and ^{R8}Leu provide evidence for the strong impact of the neighboring aromatic ring on the amide proton temperature shift. The amide proton of ^{R8}Leu is possibly oriented above the side chain of ^{C3}Phe, thus forming an pseudo H-bonding interaction with the π-electron cloud of aromatic ring.[20]

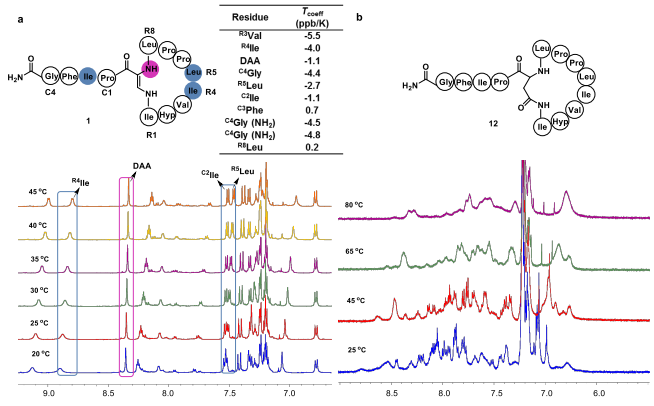

Figure 3. Variable temperature ^{1}H NMR (500 MHz, DMSO-d_6) analysis of callyaerin A (**1**) and its homodetic counterpart (**12**). (a) The ^{1}H NMR spectra of **1** recorded between 20 and 45 °C and the corresponding T_{coeff} values calculated for all the amide protons; (b) The ^{1}H NMR spectra of **12** recorded between 25 and 80 °C. Only the amide region is shown.

By way of contrast, the ^{1}H NMR spectra of the lactam analogue of callyaerin A (**12**) recorded at different temperatures were more complex. The number of amide resonances exceeded the number of amide protons present in **12**, which may be attributed to multiple exchanging rotamers. The peptide adopted multiple conformations that were in slow transition at 298 K (Figure 3b). As the temperature increased, the exchange rate between the different conformers was accelerated and the peaks became broader, indicating that the transition regime switched from slow to slow-intermediate exchange. However, the proton signals remained broad even when the temperature increased to 80 °C. We reasoned that a much higher temperature would be required to force the peptide into fast exchange, which was not available with our instrumentation.

Comparing the spectra of **1** and its lactam analogue **12**, it is clear that **1** was present as a single conformer in solution, stabilized by a 3-4 intramolecular hydrogen bonds, while its lactam counterpart **12** existed as multiple conformations in solution probably due to the lack of appropriate structural constraints. Furthermore, the non-canonical (*Z*)-2,3-diaminoacrylamide linkage in the cyclic peptide **1** conferred extraordinary structural rigidity to the peptide conformation that was not evident in the homodetic analogue **12** and this unusual conformational stabilizing effect may account for the large difference in bioactivity between **1** and **12**.

In summary, we have developed the first efficient route for the synthesis of callyaerin A (**1**), a potent anti-tubercular cyclic peptide featuring a rare (*Z*)-2,3-diaminoacrylamide moiety. Fmoc-FGly(OEt)$_2$-OH was incorporated into SPSS as a masked form of an FGly residue. After careful peptide cleavage using optimized conditions that minimized undesired peptide fragmentation, the linear peptide was successfully cyclized using dilute acid to effect intramolecular acid-catalyzed enamine formation. The spectroscopic data for the synthetic compound was consistent with the reported data for the natural product. The potent anti-TB activity of **1** contrasted with the homodetic analogue **12** which was inactive. Finally, a variable temperature NMR study of **1** and **12** demonstrated the high conformational rigidity imposed by the (*Z*)-2,3-diaminoacrylamide linkage on the peptide structure. The significant influence that peptide conformation confers on observed bioactivity highlights the potential use of the (*Z*)-2,3-diaminoacrylamide unit as a novel cyclic constraint to complement existing conformational constraining methodologies (*e.g.* amide cyclization, disulfide cyclization and stapling) to improve the properties of bioactive peptides.

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Keywords: peptides • diaminoacrylamide • cyclization • formylglycine • conformational constraints

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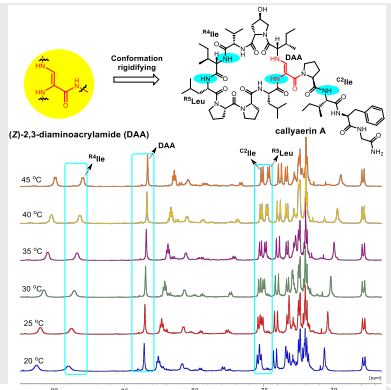
Callyaerin A, an anti-tuberculosis macrocyclic peptide containing a rare (*Z*)-2,3-diaminoacrylamide moiety, was synthesized in high yield using an uncommon building block, Fmoc-formylglycine-diethylacetal. Variable temperature NMR studies revealed the high conformational rigidity the (Z)-2,3-diaminoacrylamide moiety confers on peptide structure, thus highlighting its potential as a novel conformation-constraining structural element.



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Layout 2:

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