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Metalloform-selective inhibition: Synthesis and structure–activity analysis of Mn(II)-form-selective inhibitors of *Escherichia coli* methionine aminopeptidase

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Abstract—Methionine aminopeptidase (MetAP) is a promising target for development of novel antibacterial, antifungal and anticancer agents. However, its physiologically relevant metal ion remains to be defined, and its inhibitors need to inhibit the in vivo metalloform. Based on the Mn(II)-form-selective inhibitors discovered by high throughput screening as leads, a series of analogs of 5-phenylfuran-2-carboxylic acid was prepared and subsequently evaluated on Co(II)-, Mn(II)-, Ni(II)-, and Fe(II)-forms of *Escherichia coli* MetAP, in order to define the structural elements responsible for their inhibitory potency and metalloform selectivity. Various substitutions on the phenyl ring changed their potency on the Mn(II)-form but not their metalloform selectivity. We conclude that the preferential coordination of the carboxyl group to Mn(II) ions is the major determinant for their superb selectivity toward the Mn(II)-form. Changing the carboxylate to hydroxamate alters its ability to bind and discriminate different metal ions, and the hydroxamate derivative becomes non-selective among the metalloforms tested.

Methionine aminopeptidase (MetAP) is a metalloenzyme responsible for removal of methionine residue from the N-terminus of nascent proteins, which is an important co-translational modification.[1] In eukaryotes, two isozymes (type I and type II MetAPs) catalyze the removal with similar substrate specificities.[2] Fumagillin, ovalicin, and TNP-470 are antiangiogenic and selectively inhibit human type II MetAP.[3,4] Bengamides inhibit the growth of cancer cells and inhibit the two isozymes nonselectively.[5] Therefore, human MetAPs were suggested as the possible molecular targets of these anticancer agents. In contrast, prokaryotes possess only one MetAP, either type I (eubacteria) or type II (archaea), and deletion of the single enzyme was demonstrated to be lethal in *Escherichia coli*[6] and *Salmonella typhimurium*.[7] The essential function of this enzyme in bacteria suggests that it is a promising target for developing antibacterial therapeutics, and MetAP inhibitors could be used as broad-spectrum antibiotics.[8]

Historically, MetAP was considered as a Co(II)-enzyme, because it can be reproducibly activated by Co(II) in vitro. Several X-ray structures of MetAP in the Co(II)-form have been solved, and Co(II) occupies a dinuclear metal binding site.[9] However, other divalent metal ions, such as Mn(II), Fe(II), Ni(II) or Zn(II), can also activate the enzyme in vitro.[10,11] Currently under debate is which metal ion is physiologically relevant. The low cellular concentration of Co(II)[12] and the modest affinity of Co(II) to the enzyme[13] suggest that Co(II) is unlikely the in vivo metal. Under anaerobic conditions, Fe(II) is an excellent activator, and it was suggested that *E. coli* MetAP (*Ec*MetAP) functions as a Fe(II) enzyme.[12,13] Mn(II) is also a possible candidate for the in vivo metal, because Mn(II)-loaded *Ec*MetAP is catalytically competent.[14] Structural studies[9] on the enzymes that share the so-called ''pita bread'' fold indicate that the conserved dinuclear metal site can accommodate either Co(II) or Mn(II), since *E. coli* aminopeptidase P is a Mn(II) enzyme.[15] Based on the inhibitory profile of two MetAP

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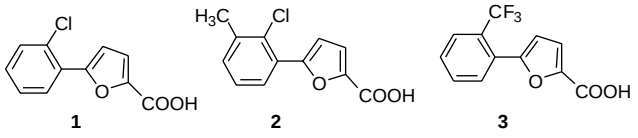
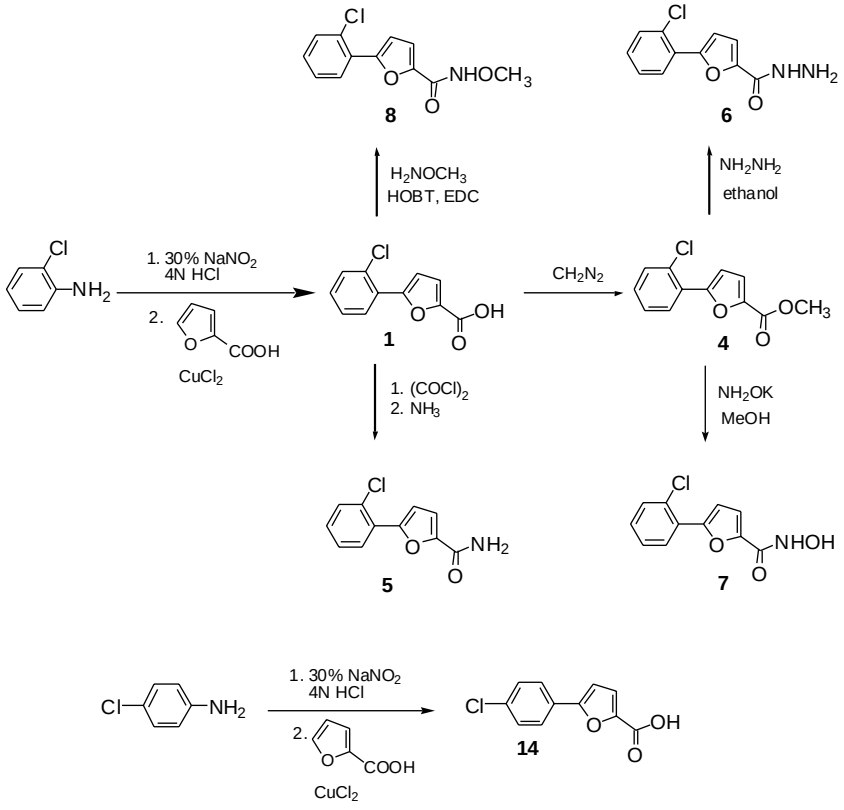
inhibitors, human type II MetAP was suggested to be a Mn(II) enzyme.[16]

Assignment of the correct in vivo metal for MetAPs becomes important considering the fact that MetAP inhibitors discovered and characterized on the Co(II)-form enzyme may or may not inhibit MetAP in other metalloforms.[10,17] It is apparent that MetAP inhibitors have to show potency to the physiologically relevant metalloform to be useful therapeutically. By high throughput screening of a chemical library of 43,736 small organic compounds, we have identified several novel non-peptidic inhibitors with selectivity toward either Mn(II)-form or Co(II) form of *Ec*MetAP.[18] Among them, inhibitors **1**–**3** show remarkable selectivity toward Mn(II)- *Ec*MetAP, and an X-ray structure of *Ec*MetAP complexed with inhibitor **1** provides a detailed view of its binding mode at the active site of *Ec*MetAP. Here, we report the chemical synthesis of a series of analogs of **1**–**3** and their biological evaluation with the intention to reveal the structural elements that contribute to their inhibitory potency and selectivity against different metalloforms.

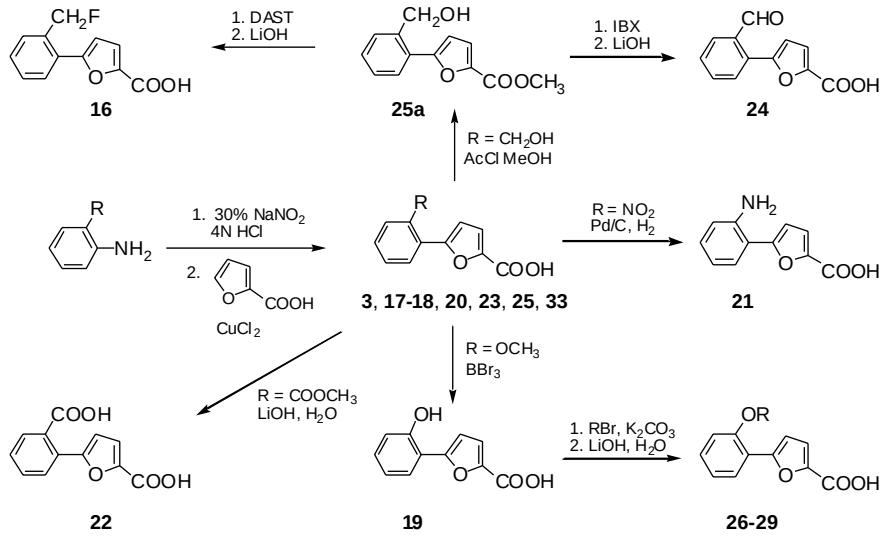


With inhibitor **1** as a lead compound, our syntheses focused on derivatives with modifications on its carboxylate group and on its phenyl ring. The compounds **4**–**8**, **14** were synthesized by the routes outlined in Scheme 1. The compounds **1** and **14** were prepared by a modified Meerwein arylation of pyromucic acid. The methyl ester **4** was prepared by reaction of the acid with diazomethane. The acid hydrazide **6** was prepared by refluxing the methyl ester with an excess of 85% hydrazine hydrate, either with or without the use of ethanol as an additional solvent. We prepared amide **5** by treatment of the corresponding acid chloride with ammonia. The compound **7** was obtained by a two-step preparation of the potassium salt of hydroxylamine followed by the addition to the ester **4** in alcohol.

Compounds with substitutions on the phenyl ring (**3**, **16**–**29**, and **33**) were synthesized as shown in Scheme 2. A series of 2-substituted phenylfuran-2-carboxylic acids **3**, **17**–**18**, **20**, **23**, **25**, and **33** were prepared according to the above method. The methyl carboxylate derivative **25a** was prepared in HCl/MeOH system that was produced by dropping AcCl into MeOH solution in situ, while the hydroxyl was not affected. In the process of synthesizing compound **24**, we chose the IBX oxidation instead of traditional Swern oxidation, which could also obtain the product in high yield. The fluorination of compound **25a** with DAST afforded compound **16**. During reduction of the nitro compound, we found hydrogenation with 10% Pd/C could obtain the corresponding amino product **21**, while there was no reaction with 5% Pd/C. After cleaving aryl methyl ether **18** with boron tribromide at −78 °C, various substitutions were carried out by reacting with different bromide. Although these reactions occurred on not only hydroxyl but also carboxyl, hydrolysis of their corresponding products afforded desired acids **26**–**29**.



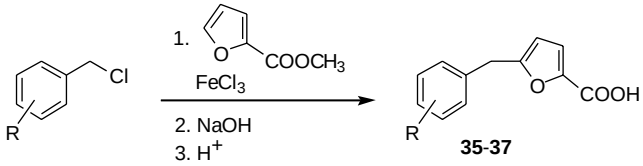
Scheme 1.



Scheme 2.

Compounds **35**–**37** were obtained by Friedel–Crafts reaction from methyl 2-furoate and $ArCH_2Cl$. The corresponding carboxylate derivatives were obtained by a well-precedented course (Scheme 3). During our process of synthesizing these compounds, we found it was not feasible to use furan-2-carboxylic acid undergoing Friedel–Crafts reaction, because it produced a more resinous by-product. Using methyl 2-furoate and moderate Lewis acid such as $FeCl_3$, we obtained the desired compounds after subsequent hydrolysis. The hydroxamate **38** was prepared the same way as for compound **7** shown in Scheme 1. The synthesis of compounds **9**, **10**, and **39** was accomplished according to the reported procedure.[19] The rest of the compounds were purchased from ChemBridge (San Diego, CA).

Lead compounds **1**–**3** displayed not only sub-micromolar inhibitory potencies but also superb selectivity among the four metalloforms of *Ec*MetAP.[18] These inhibitors were hundred-fold more active against the Mn(II)-form than Co(II)-, Ni(II)-, and Fe(II)-forms. The X-ray structure of the Mn(II)-form of *Ec*MetAP complexed with inhibitor **1** clearly showed that it binds to the enzyme active site pocket with the carboxyl group coordinating with the Mn(II) ions and the phenyl and furan rings in a non-coplanar confirmation (Fig. 1). With the dozens of analogs synthesized, along with analogs purchased, we attempt to understand the relationship between their structure and activity, and to define the structural elements that contribute to their unique potency and selectivity.



Scheme 3.

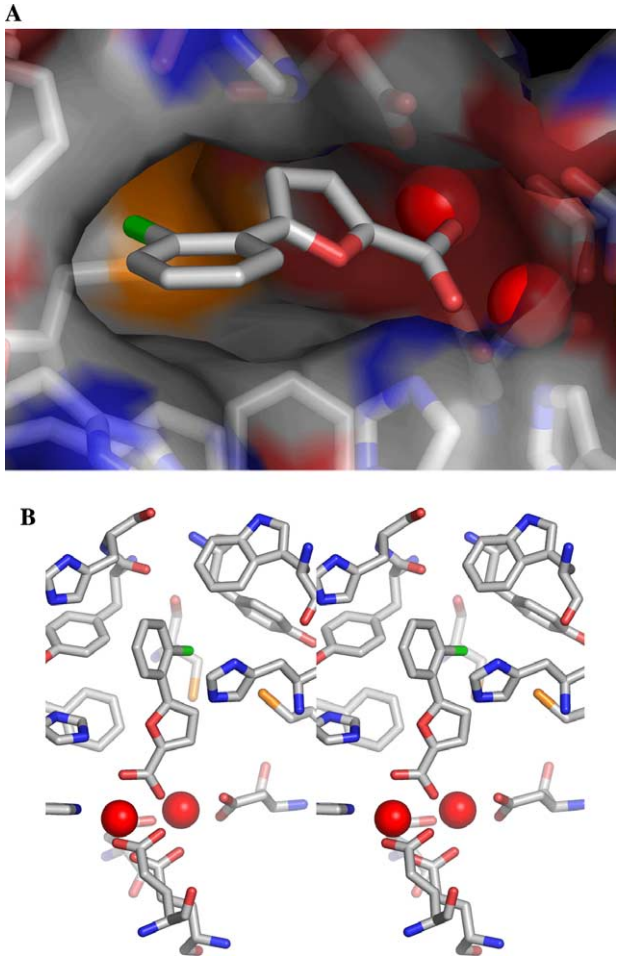
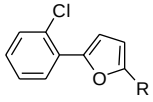


Figure 1. (A) Close-up view of inhibitor **1** occupying the binding pocket of *Ec*MetAP (PDB code: 1XNZ). (B) Stereo view of inhibitor **1** surrounded by residues at the binding site. Mn(II) ions are shown as red spheres, and the inhibitor and the surrounding residues are shown as sticks (carbon gray, oxygen red, nitrogen blue, sulfur yellow, and chlorine green).

Table 1. Inhibition of *Ec*MetAP by compounds with modifications at the carboxyl group



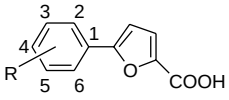
Compound	R	IC_{50}[a] (μM)			
		Co(II)	Mn(II)	Ni(II)	Fe(II)
1	COOH	138	0.511	141	116
4	$COOCH_3$	>200	166	>200	>200
5	$CONH_2$	71.4	15.9	137	>200
6	$CONHNH_2$	77.1	6.56	5.78	58.0
7	CONHOH	4.61	0.469	5.90	0.793
8	$CONHOCH_3$	57.5	40.0	51.2	178
9	CH_2OH	84.2	28.1	55.0	>200
10	CH_2CN	57.3	47.6	56.3	97.7

[a] Values were from several determinations with relative standard deviations <19%.

The carboxyl group interacts directly with two Mn(II) ions according to the X-ray structure. We have evaluated a series of derivatives with modifications at the carboxyl group on four metalloforms of *Ec*MetAP (Table 1). It is conceivable that coordination of the carboxyl group with the Mn(II) ions provides major contribution for its potency. Derivatizing this group to ester **4**, amide **5**, or hydrazide **6** resulted in reduction in potency to different degrees. Hydroxamate derivative **7** was an exception but understandable because of the known strong chelation of this group to metals. Inhibitors **1** and **7** were equally potent for the Mn(II)-form. However, **1** was very selective for the Mn(II)-form enzyme, while in contrast, **7** was non-selective against the four metalloforms tested. The great difference in selectivity may be due to the ability of hydroxamate group to ligate effectively with any of the four metals. In contrast, the carboxyl group ligates preferentially with the Mn(II) ion. We are in the process of getting the X-ray structure of *Ec*-MetAP complexed with **7** to find out which of the hetero atoms interacts with the metal ions and what differences between **1** and **7** exist in binding at the active site. Apparently, a free hydroxyl in the hydroxamate **7** is important for potency, because its methylation (compound **8**) reduced its potency on all of the metalloforms. This importance of a free carboxyl in **1** was further confirmed by the much weaker activities of hydroxymethyl and cyanomethyl derivatives **9** and **10**.

Initial testing on a few lead compounds suggested a requirement of non-coplanar conformation between the two aromatic rings for inhibitory activity, and indeed, inhibitor **1** binds at the active site in a non-coplanar conformation (Fig. 1). A series of compounds with

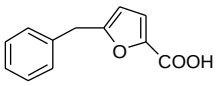
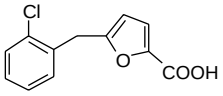
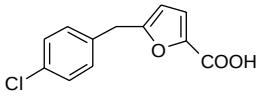
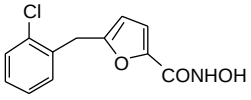
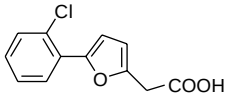
Table 2. Inhibition of *Ec*MetAP by compounds with substitutions at the phenyl ring



Compound	R	IC_{50}[a] (μM)			
		Co(II)	Mn(II)	Ni(II)	Fe(II)
11	H	188	16.3	195	>200
12	2-F	>200	13.6	>200	>200
1	2-Cl	138	0.511	141	116
13	3-Cl	199	11.7	>200	>200
14	4-Cl	180	12.1	>200	>200
15	2-Cl, 5-Cl	126	0.693	173	84.0
16	2-CH_2F	55.4	0.893	68.0	85.2
3	2-CF_3	101	0.290	165	158
17	2-OCF_3	170	0.368	197	191
18	2-OCH_3	120	0.558	>200	>200
19	2-OH	127	6.92	153	190
20	2-NO_2	120	1.08	175	178
21	2-NH_2	56.6	12.5	94.5	95.8
22	2-COOH	>200	23.5	>200	>200
23	2-$COOCH_3$	160	3.47	194	>200
24	2-CHO	82.6	7.17	67.5	69.2
25	2-CH_2OH	189	10.0	190	>200
26	2-OCH_2CH_3	147	1.13	>200	>200
27	2-$OCH_2CH_2OCH_3$	103	9.45	163	169
28	2-OCH_2CCH	98.9	4.71	171	193
29	2-$OCH_2CH_2CH{=}CH_2$	93.5	3.65	113	111
30	2-Cl, 4-NO_2	38.3	1.56	42.9	48.6
31	2-Cl, 5-NO_2	44.3	1.32	38.4	31.1
32	2-CH_3, 4-NO_2	59.5	1.11	28.5	150
33	2-OCH_3, 5-NO_2	24.1	0.642	34.5	36.2
34	2-F, 5-NO_2	157	14.9	193	199
2	2-Cl, 3-CH_3	187	1.71	188	196

[a] Values were from several determinations with relative standard deviations <23%.

Table 3. Inhibition of *Ec*MetAP by compounds with methylene insertions

Compound	Structure	IC_{50}[a] (μM)			
		Co(II)	Mn(II)	Ni(II)	Fe(II)
35		42.5	33.5	67.5	33.7
36		158	1.22	191	199
37		90.5	44.6	89.8	138
38		119	9.84	107	122
39		77.3	0.784	117	177

[a] Values were from several determinations with relative standard deviations <20%.

different substitutions on the phenyl ring have been either synthesized or purchased to confirm this non-coplanar requirement and to probe the substrate and inhibitor binding pocket. Their activities on the four metalloforms are summarized in Table 2.

Requirement of a non-coplanar conformation for activity is clearly supported by inactivity of the unsubstituted compound **11** or compounds with a fluorine at the 2-position (**12** and **34**). Activities of monochlorine-substituted isomers (**1**, **13**, and **14**) are also in agreement with this conclusion. Hydrogen or fluorine at the 2-position is just not big enough to force the non-coplanar conformation. X-ray structure of the *Ec*MetAP/inhibitor **1** shows that the 2-Cl fits to the hydrophobic area created by the residues Cys-59, Tyr-62, Tyr-65, and Cys-70 in the binding pocket. This hydrophobic cavity is shallow and can accommodate a small group such as Cl (**1**), CH_2F (**16**), CF_3 (**3**), OCF_3 (**17**), and OCH_3 (**18**). However, the much reduced activity displayed by compounds with substitution at this position [OH (**19**), NH_2 (**21**), or COOH (**22**)] clearly indicates that polar groups are not compatible with this cavity. The overall hydrophobic nature of the binding pocket is understandable considering the substrates for this enzyme usually with a methionine or norleucine side chain.[20] Substitution with a longer hydrophobic chain may be accommodated, such as **26**–**29**. However, they did not show increased potencies at all.

It is important to note that all of these compounds showed preference for the Mn(II)-form of the enzyme. When X-ray structures of the Co(II)-form (PDB code 3MAT) and Mn(II) form (PDB code 1XNZ) enzymes were compared, alignment of the main chain alpha-carbons gives a rmsd (root mean square deviation) value of 0.422 Å, indicating very similar structures as a whole and at the substrate and inhibitor binding site. There are no major changes in geometry upon the replacement of Co(II) by Mn(II). The parallel shifts in potency among these compounds further confirm the structural similarity among different metalloforms. To explain the superb selectivity of inhibitors **1**–**3** on the Mn(II)-form, it seems clear that coordination by the carboxyl group to the metal ions is discriminative and the carboxyl group is the major determinant for the metalloform selectivity.

Substitution at the 2-position of the phenyl ring forces a rotation around the bond joining the two rings. Insertion of a methylene group between the two rings could also break the conjugation of the two rings and let the two rings to adopt their proper conformations independently. Three compounds (**35**–**37**) were prepared and tested (Table 3). It was surprising to find that the unsubstituted **35** did not show high potency on the Mn(II)-forms, although the phenyl ring can now adopt a conformation that is non-coplanar to the furan ring. Even more puzzling was that the 2-Cl derivative **36** was still better than the 4-Cl derivative **37**. It is possible that the methylene insertion changes the relative positions between the phenyl ring and the carboxylate. It either pushes the phenyl ring toward the opening of the binding pocket or moves the carboxylate to a slightly different place. The hydroxamate **38** showed significantly weaker activity against the Mn(II)-form and was almost inactive against Co(II)-, Ni(II)- or Fe(II)-forms. Comparing this carboxylate-hydroxamate pair **36** and **38** with the similar pair **1** and **7**, it seems that the interaction of the hydroxamate group in **7** with the metal ions has been greatly affected by this methylene insertion. However, the methylene insertion between the furan ring and the carboxyl group (**39**) did not seem to affect the potency and selectivity.

From the above inhibitors, we selected and tested a few typical compounds for antibacterial activity against *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Staphylococcus epidermidis.* All of the compounds tested showed poor activity

(MIC ranged between 32 and >128 μg/ml, data not shown) in comparison with Gatifloxacin. Compound **7** was the better one against Gram-negative bacteria, while compound **1** was slightly better against Gram-positive bacteria, but there was no significance difference ($P > 0.05$) among these compounds.

Chemical modifications of the Mn(II)-form-selective lead compounds **1**–**3** and subsequent biological evaluation of these compounds on Co(II)-, Mn(II)-, Ni(II)- and Fe(II)-forms of *Ec*MetAP have provided valuable information on the structural elements responsible for their potency and metalloform selectivity. A small hydrophobic substitution at the 2-position of the phenyl ring is required for inhibitory activity, consistent with the non-coplanar conformation of the two aromatic rings of inhibitor **1** found at the enzyme active site. However, although various substitutions on the phenyl ring changed their potency, there was no change in their metalloform selectivity. It is likely that the superb metalloform selectivity displayed by inhibitors such as **1** is achieved primarily by coordination of the carboxyl group to the metal ions. Changing the carboxyl to hydroxamate alters the ability to bind and discriminate different metal ions, and the hydroxamate derivative **7**, although it is still potent, becomes non-selective among the metalloforms tested. Although no significant antibacterial activity was observed, the present compounds are MetAP inhibitors with unique metalloform selectivity. At this time when the physiologically relevant metalloform remains to be defined, these metalloform-selective inhibitors, as well as the non-selective inhibitors, will be valuable tools for current research and potential leads for future drug development.

Acknowledgments

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