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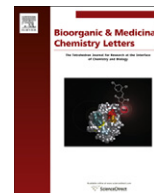

The high performance of 3XFLAG for target purification of a bioactive metabolite: A tag combined with a highly effective linker structure

Minoru Ueda *, Yoshiyuki Manabe, Makoto Mukai

Department of Chemistry, Tohoku University, 6-3 Aramaki-Aza Aoba, Sendai 980-8578, Japan

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ABSTRACT

Affinity purification using immunoprecipitation (IP) is an extremely useful method for target profiling of bioactive natural products. We examined IP purification of CMetE, which is a molecular target for potassium isolespedezate (**1**), a leaf-opening factor of *Cassia* plant. We studied IP efficiency using a panel of FLAG-connected molecular probes (**2**–**8**), including probes with varying structures and lengths of the linker moiety. The results suggest that not only the length, but the chemical nature of the linker moiety, strongly affect the IP efficiency. 3XFLAG, a tag combined with a linker moiety of charged amino acids, gave the best results and was most useful for IP purification of the molecular target.

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Target identification of bioactive natural products that induce physiologically intriguing phenomenon would launch a new field of chemical biology due to the fact that most targets remain unknown.[1] Chemical tagging combined with immunoprecipitation (IP) is a most promising method to purify molecular targets of a natural product. Recently, we succeeded in the extremely effective IP purification of a target protein for isolespedezate (**1**),[2] which is the leaf-opening factor of *Cassia* plants. The identified molecular target, CMetE (*Cassia* 5-methyltetrahydropteroyltriglutamate-homocysteine-methyltrans-ferase), was purified by using 3XFLAG (DYKDHDG-DYKDHDIDYKDDDDK) with no non-specific bindings. In this Letter, we examine the effectiveness of a panel of linkers varying in structure and length, and report that both the structural nature and the length of the linker moiety are controlling factors for successful IP purification.

The panel of FLAG probes used in the IP experiment is shown in Figure 1. It is generally accepted that the role of the linker is to project the large molecular tag away from the pharmacophore, which is essential for binding to a specific target. Thus, the longer the linker, the more effective the projection. Tanaka et al.[3] reported that a long hydrophilic linker is indispensable for the reduction of non-specific bindings and successful IP purification of a molecular target. We examined hydrophilic glycyl and polyethyleneglycol (PEG) linkers. The use of an octapeptidyl FLAG tag also served to increase hydrophilicity of the probes. We prepared a panel consisting of probes possessing a FLAG-tag connected to triglycyl (**3**), hexaglycyl (**4**), pentadecaglycyl (**5**), hexa-PEG (**6**), and octadeca-PEG (**7**) linkers, as well as one without a linker (**2**). Additionally, 3XFLAG, which has high affinity for an anti-FLAG antibody and is widely used as an elution ligand in IP purification, was examined as a tag combined with a linker moiety in probe **8**.

Syntheses of these probes were carried out as shown in Schemes 1, 2, S1 and S2 (for S1 and S2, see Supplementary data). Coupling with the FLAG tag was performed on solid beads. 'Click-made'[4] synthesis of each probe rendered the preparation of the panel compounds quite easy (Scheme 2). For both glycyl and PEG linkers, the solubility in aqueous solution was gradually decreased according to the addition of linker units.

After chemical tagging of the live *Cassia* motor cells with each probe, the cells were disrupted and applied to IP using beads bearing the anti-FLAG antibody according to the procedure in Ref. 2 (Fig. 2). The protein trapped on the beads was eluted using 3XFLAG as a free ligand. The results are summarized in Figure 3. The effectiveness of the IP purification was assessed by the ratio of CMetE in the supernatant to CMetE trapped on the IP beads. This ratio analysis enables the performance of the linker to be assessed regardless of the difference in chemical yield of the affinity labeling among the different chemical probes.

As shown in Table 1, longer glycyl linkers provided more effective trapping of CMetE on IP beads. Increasing the number of glycyl units, for example, zero in **2** (sup/ppt = 8:2), three in **3** (sup/ppt = 6:4), six in **4** (sup/ppt = 4:6), and 15 in **5** (sup/ppt = 1:9), improved the IP efficiency. However, increasing the number of PEG units did not improve the IP result, for example, zero in **2** (sup/ppt = 8/2), six in **6** (sup/ppt = 7/3), and 18 in **7** (ND in both ppt and sup). It is noteworthy that little improvement was observed by the insertion of a hexa-PEG linker (cf. **2** and **6**). This suggested that the long PEG moiety did not

* Corresponding author.
E-mail address: ueda@m.tohoku.ac.jp (M. Ueda).

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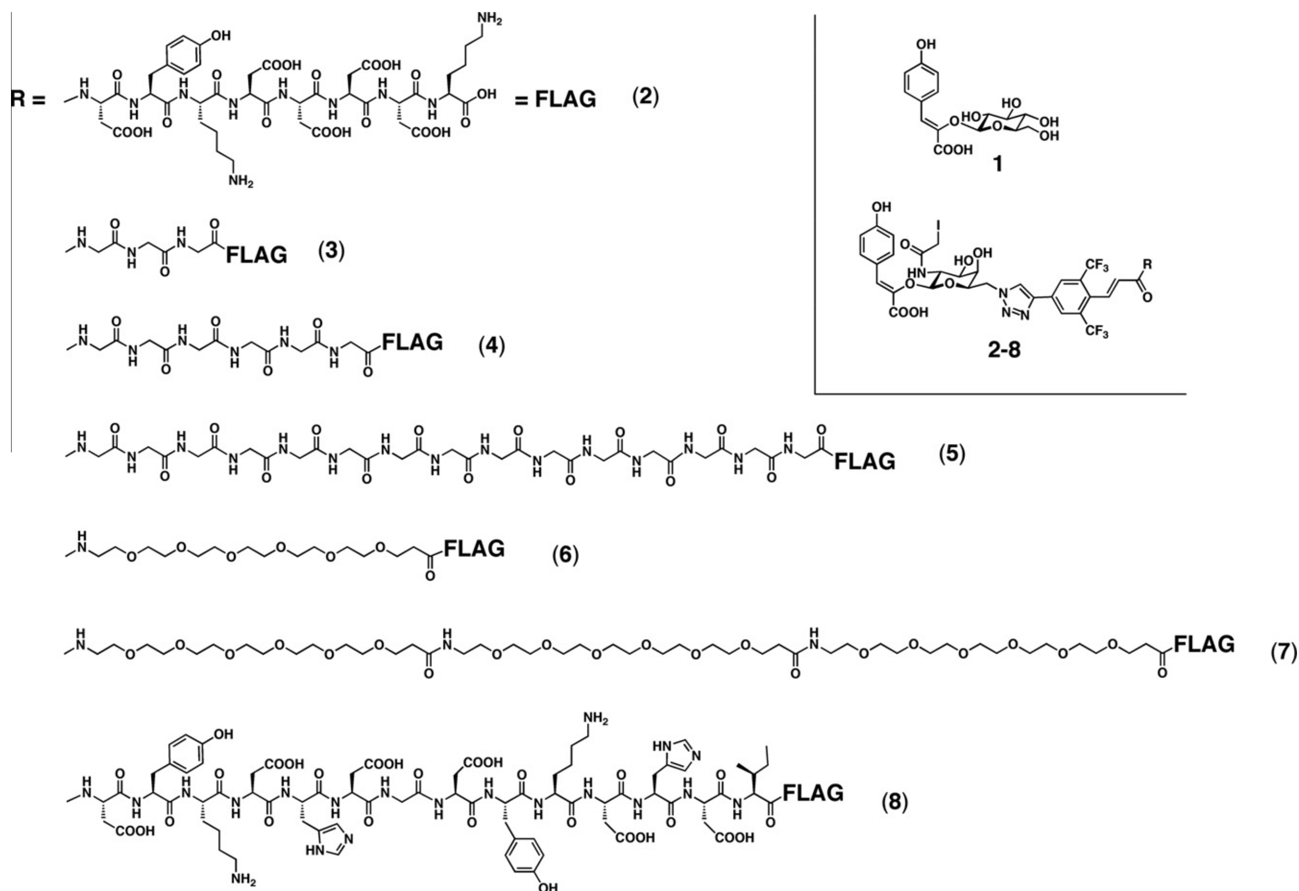
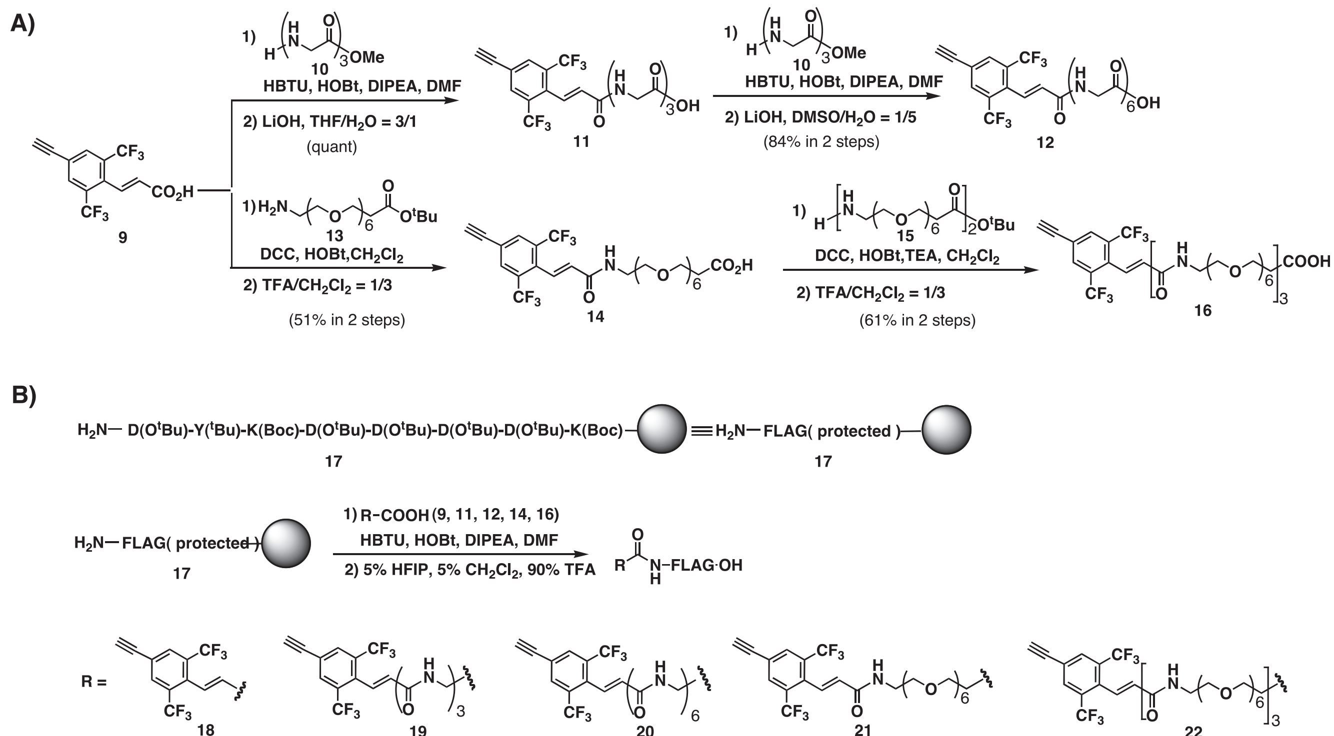
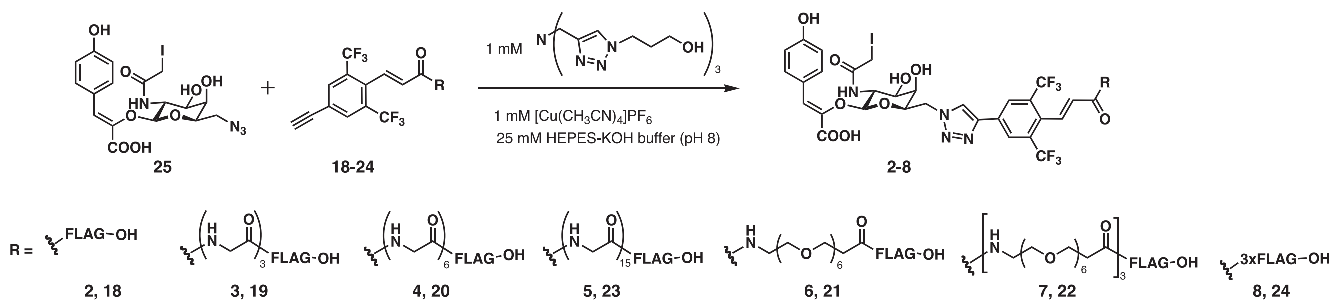


Figure 1. Structures of molecular probes used in the IP purification of CMetE.



Scheme 1. Preparation of a panel of alkyne-connected FLAG tags bearing various linkers. (A) Syntheses of alkyne units bearing various linkers; (B) solid phase synthesis of FLAG-connected alkyne units.



Scheme 2. Preparation of a panel of probes bearing various linkers.

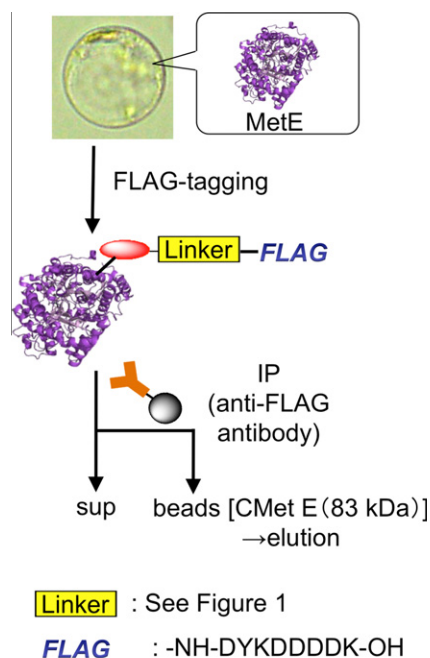


Figure 2. Procedures for IP purification of CMetE from motor cells of *Cassia octadentalis*.

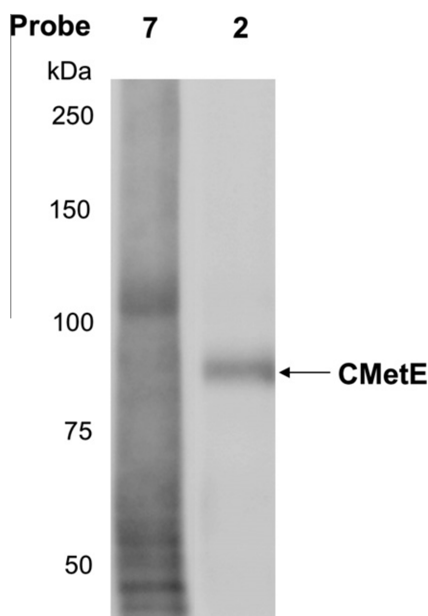


Figure 3. SDS–PAGE analysis of proteins trapped on IP beads using probes **2** and **7**.

work as well as the glycyl linker. Moreover, the effectiveness of the linker cannot be explained only by its length since the lengths of the hexa-PEG linker in **6** and the hexaglycyl linker in **4** are almost the same. However, a distinct difference was found between the hexaglycyl (**4**: sup/ppt = 4:6) and hexa-PEG (**6**: sup/ppt = 7:3) probes. The superiority of the glycyl linker over the PEG linker is also confirmed by this result, suggesting that the chemical nature of the linker structure is also important for high performance.

The calculated log *P* values[5] of probes of various linker moieties (**26**–**31**) are shown in Table 2. The log *D*[6] value was also examined for **31** in which many ionized functionalities are embedded. Comparison of calculated log *P* values for the series of glycyl-linked probes (**26**: log *P* = −1.9; **27**: log *P* = −3.6; **28**: log *P* = −8.5) suggested that longer linkers were more hydrophobic. Thus, the projection worked well and longer linkers gave better IP results. The extremely high performance of 3XFLAG can be explained by the log *D* value of **31** (**31**: log *D* = −10.2) instead of the log *P* value (**31**: log *P* = −6.9), because log *D* gives a better prediction of hydrophobicity for highly ionized compounds.[6] On the other hand, the calculated log *P* value for the ineffective hexa-PEG **29** (log *P* = −1.3) was as high as triglycyl **26** (log *P* = −1.9), and much higher than that of hexaglycyl **27** (log *P* = −3.6), whereas little difference in IP results was observed among them. Additionally, CMetE could not be recovered in either sup or ppt with octadeca-PEG **30** (log *P* = −3.9) possessing a longer linker, and a substantial amount of nonspecific binding was obtained (Fig. 3), even though the log *P* value of **30** (log *P* = −3.9) was as low as that of hexaglycyl **27** (log *P* = −3.6), which gave a moderate IP result (sup/ppt = 4/6). In general, the increase in nonspecific binding of proteins can be attributed to the increase in the hydrophobicity.[7] However, these results revealed that hydrophobicity alone cannot explain the performance of the linkers.

The aforementioned results suggested that the long linker moiety with its low log *P* value does not work well and can also detract from the selectivity of IP purification due to the increase in nonspecific bindings. This could imply that the long and comparatively hydrophobic linker structure predominantly adopts a folded conformation, and thus cannot separate both ends effectively. The poor IP result using probe **7** could be attributed to such nature of long PEG linker. Charged oligopeptides, such as 3XFLAG, which possesses as many as 11 aspartate residues in the 22 amino acid sequence, are expected to be useful in improving both efficiency and selectivity in IP purification because they can predominantly adopt extended conformations in aqueous solution due to the electronic repulsion between charged aspartate residues. Thus, significant improvement was achieved when 3XFLAG was used. 3XFLAG is a large molecular tag combined with a linker unit, and is expected to be of high affinity to anti-FLAG antibodies immobilized on IP beads. The ratio of CMetE in sup to CMetE on IP beads was as high as 1/99. Considering that the length of 3XFLAG is similar to that of the pentadecaglycyl FLAG, the extremely high performance of 3XFLAG could be attributed to higher affinity to the anti-FLAG antibody as well as to the difference in structures be-

Table 1
Comparison of efficiencies in IP purification by a panel of probes (**2**–**8**)

Linker + Tag	Bands @ 83 kDa		$MetE_{sup/ppt}$	
	Sup	Beads	Sup	Beads
-FLAG (**2**)			8	2
-(Gly)$_3$-FLAG (**3**)			6	4
-(Gly)$_6$-FLAG (**4**)			4	6
-(Gly)$_{15}$-FLAG (**5**)			1	9
-(PEG)$_6$-FLAG (**6**)			7	3
-(PEG)$_{18}$-FLAG (**7**)			–	–
-3×FLAG (**8**)			<1	>99

Table 2
Calculated log *P* (log *D*) values for various linker structures (**26**–**31**) which correspond to linker moieties of probes **3**–**8**

Linker	log *P* (or log *D*[*])
Ac-(Gly)$_3$-OMe (**26**)	−1.9
Ac-(Gly)$_6$-OMe (**27**)	−3.6
Ac-(Gly)$_{15}$-OMe (**28**)	−8.5
Ac-(PEG)$_6$-OMe (**29**)	−1.3
Ac-[(PEG)$_6$]$_3$-OMe (**30**)	−3.9
Ac-DYKDHDGDYKDHDI-OMe (**31**)[*]	−6.9 (−10.2)

[*] log D was calculated for 31.

tween the 15-glycyl and aspartate-rich 14-aminoacyl (DYKDHDG-DYKDHDI) moieties.

Our present studies suggest that the nature of the linker structure strongly affects the IP purification of molecular targets for a small bioactive molecule. The use of good linkers improves the efficiency and selectivity in IP purification by decreasing nonspecific binding of proteins, which often causes significant obstacles for target identification. The performance of the linker was not only determined by its length but also by its nature, including hydrophobicity and conformation in an aqueous solution. Our study using a panel of probes with various linkers concluded that the 3XFLAG as a tag combined with a linker provided the best result. To the best of our knowledge, this study is the first example to use 3XFLAG as a tag for affinity purification of a target protein for a small molecule. This result will assure the promising performance of 3XFLAG as a high-performance molecular tag for IP purification of targets for small bioactive molecules.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.01.038. These data include Schemes S1 and S2, experimental method, and HRMS as well as LC–MS/MS data of alkyne units **18**–**22**, **28**, **30**, and probes **2**–**8**.

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