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Improvement of solid material for affinity resins by application of long PEG spacers to capture the whole target complex of FK506

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ABSTRACT

Solid materials for affinity resins bearing long PEG spacers between a functional group used for immobilization of a bio-active compound and the solid surface were synthesized to capture not only small target proteins but also large and/or complex target proteins. Solid materials with PEG1000 or PEG2000 as spacers, which bear a benzenesulfonamide derivative, exhibited excellent selectivity between the specific binding protein carbonic anhydrase type II (CAII) and non-specific ones. These materials also exhibited efficacy in capturing a particular target at a maximal amount. Affinity resins using solid materials with PEG1000 or PEG2000 spacers, bear a FK506 derivative, successfully captured the whole target complex of specific binding proteins at the silver staining level, while all previously known affinity resins with solid materials failed to achieve this objective. These novel affinity resins captured other specific binding proteins such as dynamin and neurocalcin δ as well.

Identification of target proteins for bio-active compounds, such as drugs, natural products and toxins, is an essential component of modern pharmaceutical sciences and chemical biology. Phenotype assays of cells or organs without target protein identification have recently been used in screening studies for drug discovery.[1] During these screening studies, the target compounds are unknown, and further studies are necessary to identify them. While affinity chromatography matrices bearing such bioactive compounds are one of the major tools for achieving this purpose,[2,3] it is often difficult to identify specific binding proteins on affinity resins owing to a preponderance of non-specific binding proteins.[4] Therefore, the successful isolation of target proteins by affinity matrices depends on the synthesis of polymeric resins that can capture the cellular target with maximum selectivity and efficiency. Affigel™,[5a] an agarose derivative, is one of the most popular matrices for this purpose, and its hydrophilic character helps reduce non-specific protein absorption.[4] However, Affigel™ becomes easily denatured under organic synthesis conditions; thus, its use in chemical studies is often restricted. Synthetic resins consist of methacrylates or stylenes as the core moiety and are widely known. They are usually synthesized by two steps as follows: first, the hydrophobic core structure is constructed by polymerization of hydrophobic monomers, such as glycidyl methacrylate[5b] or stylene,[5c] and then, the core structure is covered by hydrophilic spacers to reduce non-specific protein absorption. However, the coverage by hydrophilic spacers is usually not sufficient due to difficulty in the additive reaction of the hydrophilic spacers in the second step caused by steric hindrance on the surface. The lack of hydrophilicity of synthetic resins often causes high levels of non-specific binding.[4] In previous a Letter,[6] we reported the development of a novel poly(methacrylate) solid material for affinity resins with a monolithic structure, AquaFirmus™ (**1**), that consists of only hydrophilic monomers and is hydrophilic enough to reduce non-specific protein absorptions (Fig. 1). However, it is still difficult for affinity resins using previously generated solid materials that bear FK506 to identify the whole target complexes of FK506, which include immunosuppressive complexes and complexes with FKBP12, calcineurin A/B and calmodulin.[7] We hypothesize that the inability of affinity resins to capture large-sized target complexes is due to steric hindrance between the captured proteins and the surfaces of the solid material. In this study, we demonstrate the applications and effectiveness of long spacers, such as PEG1000 or PEG2000, for reducing steric hindrance (Fig. 1).

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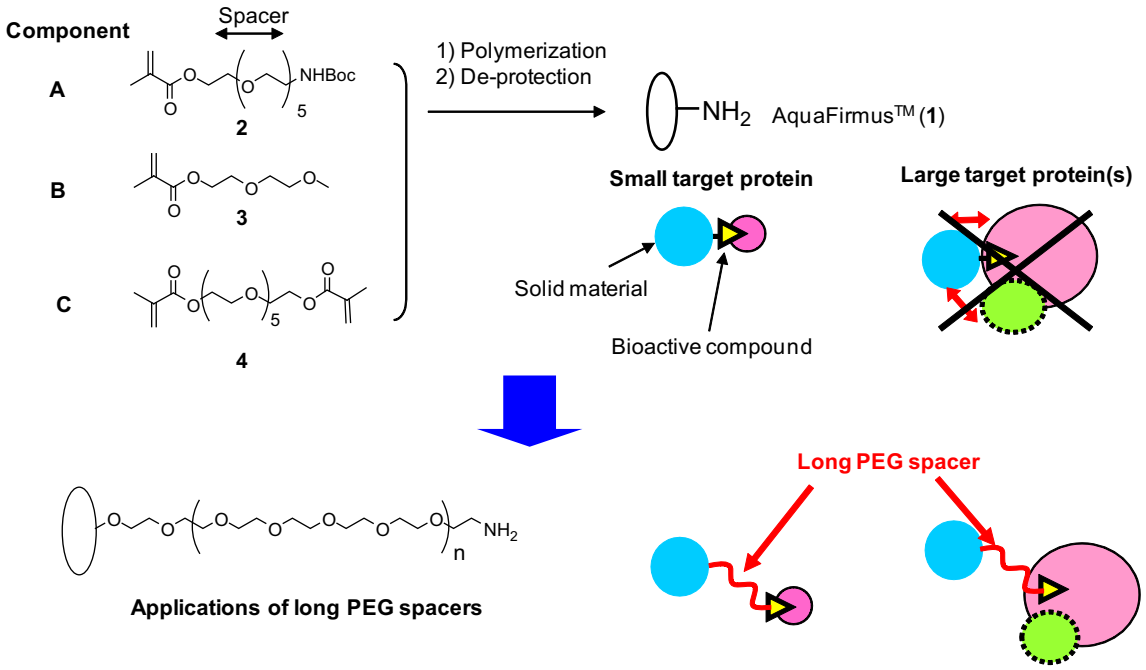

Figure 1. Improvement of affinity resins for capture of large proteins or complexes, **1** was prepared by polymerization of three components (A:B:C = 1:1:9, molar ratio) and subsequent de-protection of the Boc group by 10% H_2O/TFA. **1** contained hexaethylene glycol as a spacer between an amine group for immobilization of bioactive compounds and for backbone structure. We hypothesized that the capture of large proteins and protein complexes was disturbed by the steric hindrances between captured proteins and the surfaces of the solid materials (red arrow). Long PEG spacers were adopted as the spacers in this study to capture both small, large proteins and protein complexes.

Design and synthesis

A previously reported solid material (**1**) was synthesized by co-polymerization of three hydrophilic methacrylates: (a) a derivative with hexaethylene glycol as a spacer bearing a protected amino group for immobilization of the bioactive compound at the terminal position (component A); (b) a co-polymerization monomer for adjusting the conditions on the surface (component B); and (c) a cross-linker monomer (component C) (A:B:C = 1:1:9 in molar ratio). The addition of a large amount of the cross-linker is vital for the polymerization reactions of a wide range of methacrylate monomers bearing flexible PEG spacers.[6b] We designed and synthesized methacrylate monomers bearing long PEG spacers, such as PEG400, PEG1000, PEG2000, PEG4000, PEG8000 and PEG10000 (**5**–**10**, Fig. 2), as component A to reduce the steric interactions between the captured proteins and the surfaces of the solid material. In order to examine effects of components B and C on captures of specific binding protein and non-specific absorbed ones, additive monomers **11**–**12** for component B and cross-linker monomers **13**–**15** for component C were synthesized in similar manners (Fig. 2).

In this study, components A, B and C were co-polymerized under the same conditions as those for **1** and were subsequently de-protected in 10% H_2O/TFA (**16**–**28**, Table 1). The resulting solid materials were stable under acidic conditions. A scanning electromicrograph (SEM) analysis showed that the solid materials consisted of relatively shorter PEG spacers (**16**–**17** and **22**–**26**), which had monolithic structures, similar to **1**, while those with long PEG spacers (**18**–**21** and **27**–**28**) did not (Fig. S1).

Selective capture of specific binding protein

To validate novel solid materials (**16**–**28**), we immobilized 4-carboxyl-benzensulfonamide on those solid materials (**29**–**42**, Table 1), and binding proteins on them were analyzed after being mixed with lysate from rat brains. Benzenesulfonamide and its derivatives are known as specific binding inhibitors of carbonic anhydrase type II (CAII) with a Kd of 0.32–1.25 μM.[8] Because the molecular weight of CAII (29 kDa) differs significantly from those of representative non-specific binding proteins, such as actin (42 kDa) and tubulin (50 kDa), it is easy to estimate the amounts of specific nonspecific binding proteins. Binding proteins on **29**–**42** were completely eluted with SDS sample buffer (Fig. 3) after carefully washing the affinity resins with lysate buffer (0.25 M sucrose, 0.3 mM *N,N*-diethylthiocarbamate, 25 mM Tris (pH = 7.6) with/without 0.1% Tween20) (Fig. S2). In this study, we prepared two lysate buffers because the amounts of non-specific absorbed proteins were generally reduced by the addition of detergents, such as Tween20.

AquaFirmus™ (**1**), whose spacer is hexaethylene glycol, which bears the benzenesulfonamide derivative (**29**), successfully captured the specific binding protein CAII with a small amount of non-specific binding proteins found in the lysate buffers with and without 0.1% Tween20. We next examined affinity resins bearing a variety of PEG spacers, from PEG400 to PEG10000, in component A (**30**–**35**). Interestingly, for the affinity resins with PEG1000 (**31**), PEG2000 (**32**), or PEG4000 (**33**), the amount of CAII was greatly increased compared to that on the affinity resins with **29**, while that on the affinity resin bearing PEG400 as a spacer (**30**) was almost same as that with **29**. These results indicate that, at a minimum, PEG1000 was required as the spacer moiety for the maximum capture of the target protein, even for capture of the relatively smaller protein CAII. On the other hand, the amount of CAII on affinity resins with PEG8000 (**34**) and PEG10000 (**35**) was lower than on those with **29**. We hypothesize that this decrease was due to the relatively low density of the amino group, which is necessary for the immobilization of the compound (Table 1).

Next, we analyzed the binding of proteins on solid materials that did not contain component B after immobilization of the

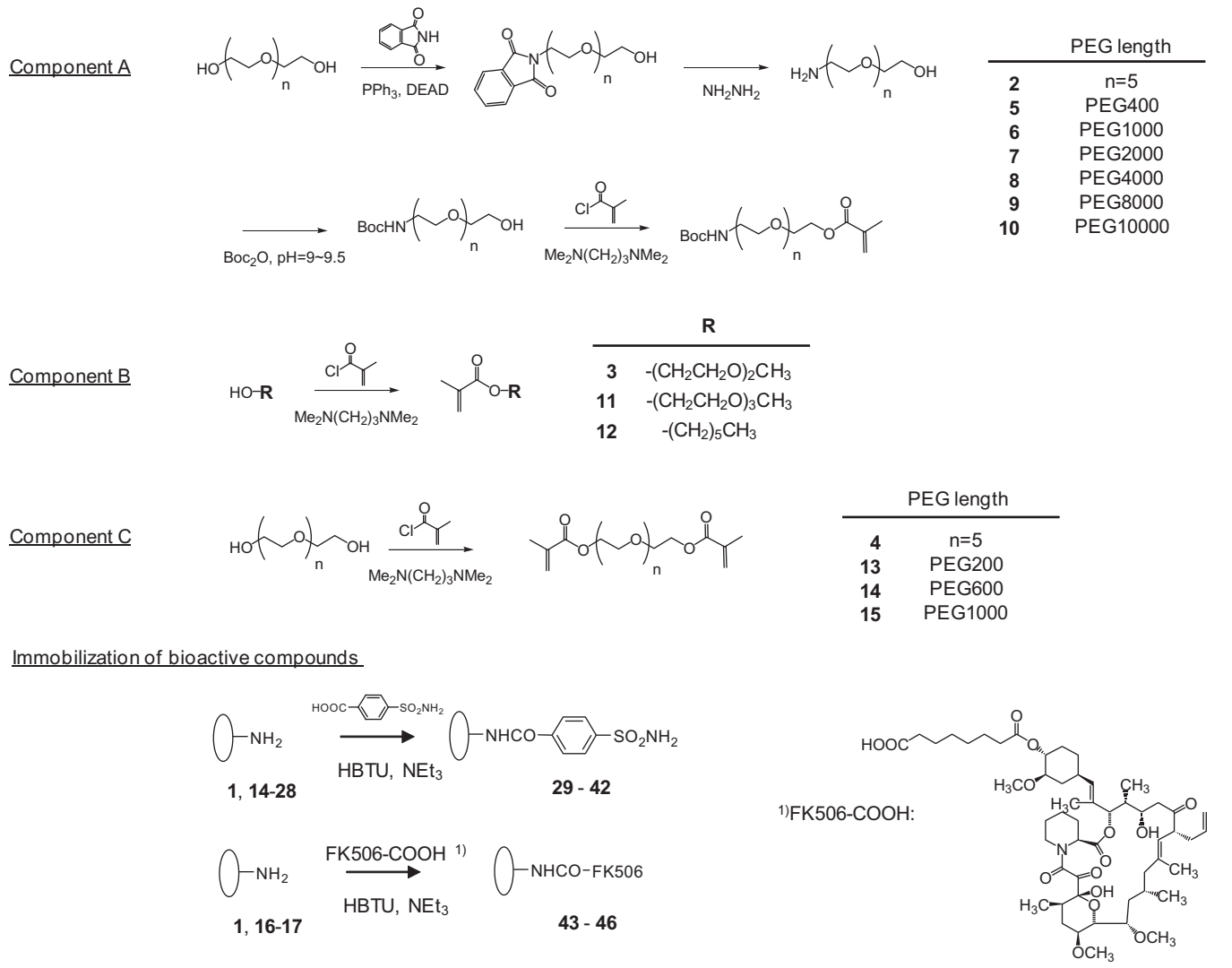

Figure 2. Synthesis of monomers and affinity resins in this study. Synthetic conditions are described in detail in the Supplemental materials.

benzenesulfonamide derivative (**36**, **37**). The amount of CAII on solid materials with **36** was lower than for those with **29** and shows a great amount binding of non-specific proteins, and the amount of CAII on solid materials with **37** was almost the same as on those with **29**. These results suggest that component B is necessary for the specific capture of the target protein (**36**) and that increasing the amine density on the solid surface is not important for capturing target proteins because component A was used twice for the synthesis of **37** and no increase in captured CAII was observed. Solid material with one additional PEG unit (**24**) and a hydrophobic hexyl (**25**) instead of hexaethylene glycol in component B in **1** were prepared, and the benzensulfonamide derivative was immobilized on that solid material (**38**, **39**). The amounts of CAII on solid materials with **38** and **39** were almost the same as for those with **29**. On the other hand, there was a great amount of non-specific binding proteins on solid materials with **39**. We thought this increases of non-specific absorption was due to an increase of hydrophobicity on the surface of the solid material[4] and/or hyper-density of the amino group on them (50.3 μmol/mL, Table 1).

Finally, component C was modified (**40**–**41**). Interestingly, the use of a longer PEG unit for the spacer adversely affected the capture of specific binding proteins (Fig. 3). We currently have no explanation for this result.

Capturing the whole target complex of FK506

Based on the results described above, we selected **17** and **18** for further study of solid materials with an immobilized FK506 derivative (**45**, **46**). Some affinity resins with **1**, **16**, AffiGel™, and Toyopearl™ bearing the FK506 derivative were also prepared. FK506 is an immunosuppressive drug that targets FKBP12 with a Kd of 0.4 nM.[9] The structure and function of the complex of FK506 with its targets, including FKBP12, have been well characterized at the molecular level[9], and a linker moiety was introduced at the hydroxyl group at the 32 position of FK506 to connect it to the resin because crystal structure studies have indicated that this position is not involved in the binding of FK506 with FKBP12 or calcineurin A/B (Fig. 2). If affinity resins bearing FK506 can capture the whole target complex, they are valuable as the standard solid material for affinity resins because it is difficult for previously used solid materials to capture the whole complex at the silver staining level. After being mixed with lysate from rat brains, the binding proteins on affinity resins were analyzed. Affinity resins **45** and **46** successfully captured the whole proteins of the FK506 target complex at the silver staining level with a small amount of non-specific binding proteins, and this was difficult to achieve using other affinity resins (Fig. 3). The amounts of specific binding proteins (band 1–5) were much smaller than that of FKBP12 in

Table 1
Solid materials studied in this study

Component for polymerization[a]			Synthesized solid material		Affinity resins	
A	B	C	NH_2		Immobilized bioactive compound	
PEG–NHBoc	O-R	PEG	NO	NH_2 density[b]	BSA	FK506
2	**3**	**4**	**1** (AquaFimus™)	18.8	**29**	**43**
5	**3**	**4**	**16**	9.8	**30**	**44**
6	**3**	**4**	**17**	12.3	**31**	**45**
7	**3**	**4**	**18**	6.3	**32**	**46**
8	**3**	**4**	**19**	7.2	**33**	
9	**3**	**4**	**20**	0.56	**34**	
10	**3**	**4**	**21**	0.31	**35**	
2	None	**4**	**22**[c]	21.7	**36**	
2	None	**4**	**23**[d]	28.0	**37**	
2	**11**	**4**	**24**	28.6	**38**	
2	**12**	**4**	**25**	50.3	**39**	
2	**3**	**13**	**26**	1.6	**40**	
2	**3**	**14**	**27**	11.4	**41**	
2	**3**	**15**	**28**	5.18	**42**	

[a] Molar ratio were A:B:C = 1:1:9 (except for those of **22** and **23**).
[b] Density of NH_2 on solid material were estimated by the ninhydrin test (mmol/mL, UV absorption at 570 nm of **1** was the reference value.
[c] Molar ratio for synthesis of **22** was **2**:**4** = 1:9 (no component B).
[d] Molar ratio for synthesis of **23** was **2**:**4** = 2:9 (no component B).

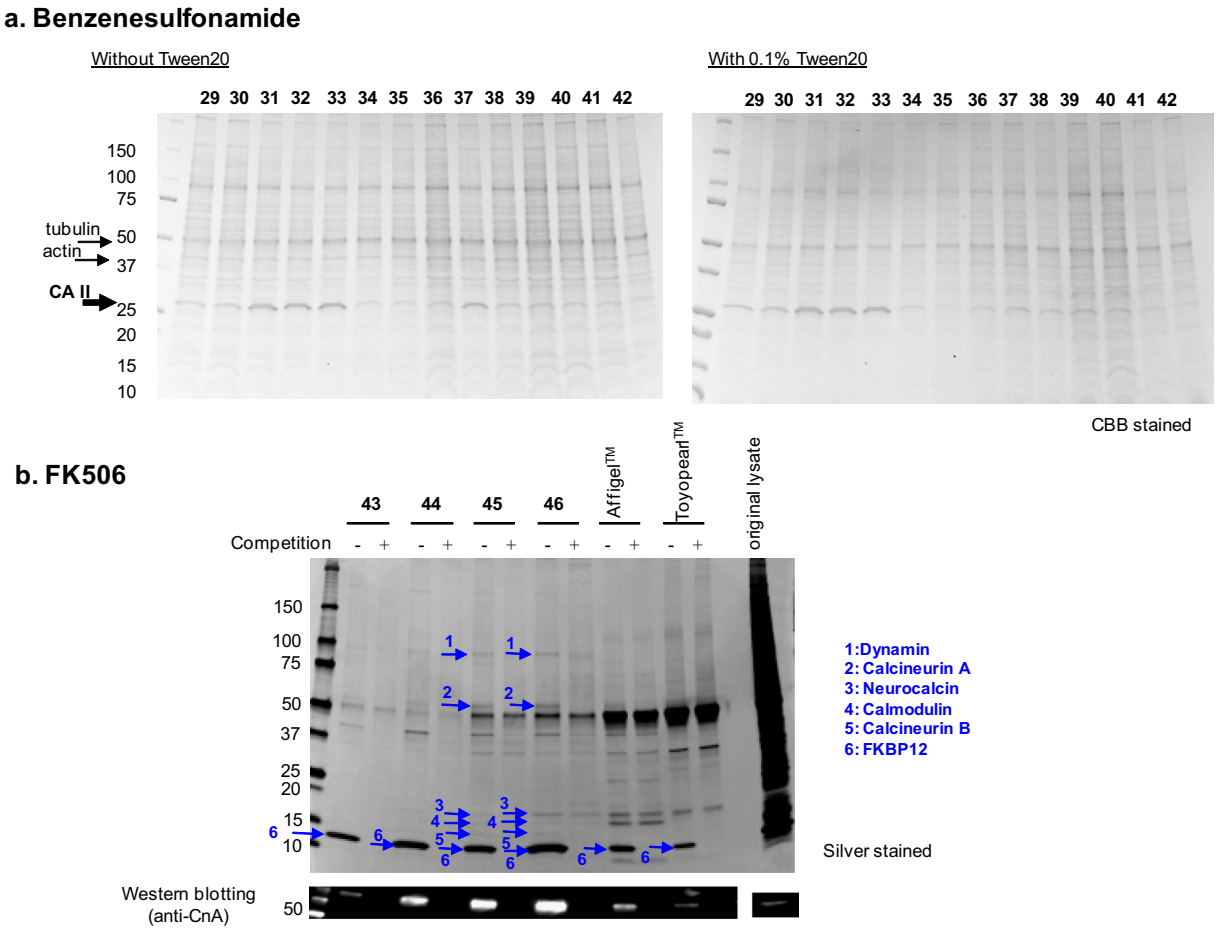

Figure 3. Binding proteins on solid materials bearing (a) benzensulfonamide and (b) FK506. Each resin bearing *p*-carboxyl-benzensulfonamide (a) or FK506 (b) was mixed with lysate from rat brains, and binding proteins were eluted with SDS sample buffer (Nacalai #30566-22) after carefully being washed with lysate buffer (0.25 M sucrose, 0.3 mM *N*,*N*-diethylthiocarbamate, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM Tris (pH = 7.6)) with or without 0.1% Tween20. Specific binding proteins, CA II (carbonic anhydrase type II) for **29**–**42** and FKBP12 (band 6) and other proteins (bands 1–5) for **43**–**46**, and nonspecific absorbed proteins were analyzed.

Figure 3. We thought the reason for this stoichiometrical difference due to their population in cells and the strong affinity between FK506 and FKBBP12 compared to the others.

Interestingly, dynamin and neurocalcin δ were also identified as specific binding proteins on **45** and **46**. Dynamin is a large protein (97 KDa) and known as an associated protein of calcineurin in synaptic cells and this interaction is vital for exocytosis of synaptic vesicles.[10] Neurocalcin δ is a neuronal calcium-binding protein like the calmodulin in mammalian brains.[11] Roles of these specific bindings in bioactivities of FK506 were now unclear while neuroprotective effect in vivo of FK506 has been reported.[12] The specificities of each binding protein were confirmed by the competition method, and identifications of FKBP12, calmodulin calcineurin A, Calcineurin B, dynamin, and neurocalcin δ were carried out by a MS/MS ion search method based on ESI ion trap mass spectrometry after in-gel digestion (Figs. S3–S4).

In conclusion, some solid materials for affinity resins were synthesized for the target identification of bio-active compounds and were intended to be effective at identifying both small target proteins and large target complexes (Fig. 1). Solid materials with PEG1000 (**17**) or PEG2000 (**18**) spacers, which both bear a benzensulfonamide derivative (**31**, **32**), captured the target protein, CAII, at a maximal amount with low amounts of non-specific binding proteins (Fig. 3a). These results indicated that, at a minimum, PEG1000 was required to reduce the steric hindrance between the captured proteins and the solid surfaces, even to capture the small target protein CAII (29 KDa). Affinity resins bearing a FK506 derivative (**45**, **46**) successfully captured the whole target complex, while all of the commercially available solid materials were unable to do so (Fig. 3). On **45** and **46**, two novel specific binding proteins of FK506, dynamin and neurocalcin, were also identified while their role were unclear. Based on these results, we believe that **17** and **18** were effective solid materials for target identification studies examining a wide range of bioactive compounds.

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Supplementary data

Supplementary data (experimental procedures and Figs. S1–S4) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.05.014.

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