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Design and synthesis of novel hydrophilic spacers for the reduction of nonspecific binding proteins on affinity resins

Takaaki Shiyama, Minoru Furuya, Akira Yamazaki, Tomohiro Terada and Akito Tanaka*

Chemistry Department, Reverse Proteomics Research Institute Co., Ltd, 2-6-7 Kazusa-Kamatari, Chiba 292-0818, Japan

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Abstract—Tubulin and actin often bind nonspecifically to affinity chromatography resins, complicating research toward identifying the cellular targets. Reduction of nonspecific binding proteins is important for success in finding such targets. We herein disclose the design, synthesis, and effectiveness in reduction of nonspecific binding proteins, of novel hydrophilic spacers (**2**–**5**), which were introduced between matrices and a ligand. Among them, tartaric acid derivative (**5**) exhibited the most effective reduction of nonspecific binding proteins, whilst maintaining binding of the target protein. Introduction of **5** on TOYOPEARL reduced tubulin and actin by almost 65% and 90% compared to that without the hydrophilic spacer, respectively, with effective binding to the target protein, FKBP12.

1. Introduction

Affinity chromatography matrices bearing bioactive compounds play an important role in the discovery of novel drug targets and the elucidation of drug mechanisms. Their effectiveness has been demonstrated by the discovery of FKBP12,[1] HDAC,[2] and cytosolic malate dehydrogenase[3] as the specific binding proteins of FK506, Trapoxin, and E-7070, respectively. The successful isolation of target proteins by affinity chromatography depends on the synthesis of polymeric resins that can bind to the cellular target with maximum selectivity and efficiency. Nonspecific binding of cellular proteins to affinity matrices is therefore a significant limitation to this biochemical approach, and reduction of such binding by chemical modification is an important goal and the responsibility of chemists and chemical biologists. In particular, tubulin and actin are known as representative nonspecific binding proteins because they often interfere with affinity chromatography studies, due to their high abundance and the similarity of their molecular weights (50 and 42 kDa, respectively) to many putative target proteins.

There are now several commercially available matrices for preparation of affinity resins. AffiGel, a polymer of sugar derivatives, is one of the most popular resins for preparation of affinity matrices.[4] However AffiGel is not suitable for organic synthesis, because it is easily denatured under organic synthesis conditions, and becomes denatured even in *N*,*N*-dimethylformamide (DMF).[5] Thus, chemical approaches using this resin are limited. For example, immobilization of ligands on AffiGel is usually carried out under aqueous conditions, which usually requires use of excess amounts of ligand. This limitation restricts opportunities to find target proteins using affinity resin technology because bioactive derivatives from the bait compound bearing a linker moiety for immobilization are usually synthesized through multi-steps and sample amount is not large. In contrast, TOYOPEARL, a poly(methacrylate) derivative, is stable under most synthetic conditions, which allow the synthesis of more effective affinity resins. This is attractive for chemists since the recent development of combinatorial chemistry allows synthesis of a variety of compounds on functional polymers.[6–8] However, methacrylate polymers bearing bioactive compounds often show high levels of nonspecific protein binding with the target protein, FKBP12, in comparison to AffiGel with the same ligands (Fig. 1B).[9]

Keywords: Nonspecific binding proteins; Affinity chromatography; Hydrophilic spacer; FK506.
* Corresponding author. Tel.: +81-0438523990; fax: +81-0438523986; e-mail: tanaka-a@reprori.jp

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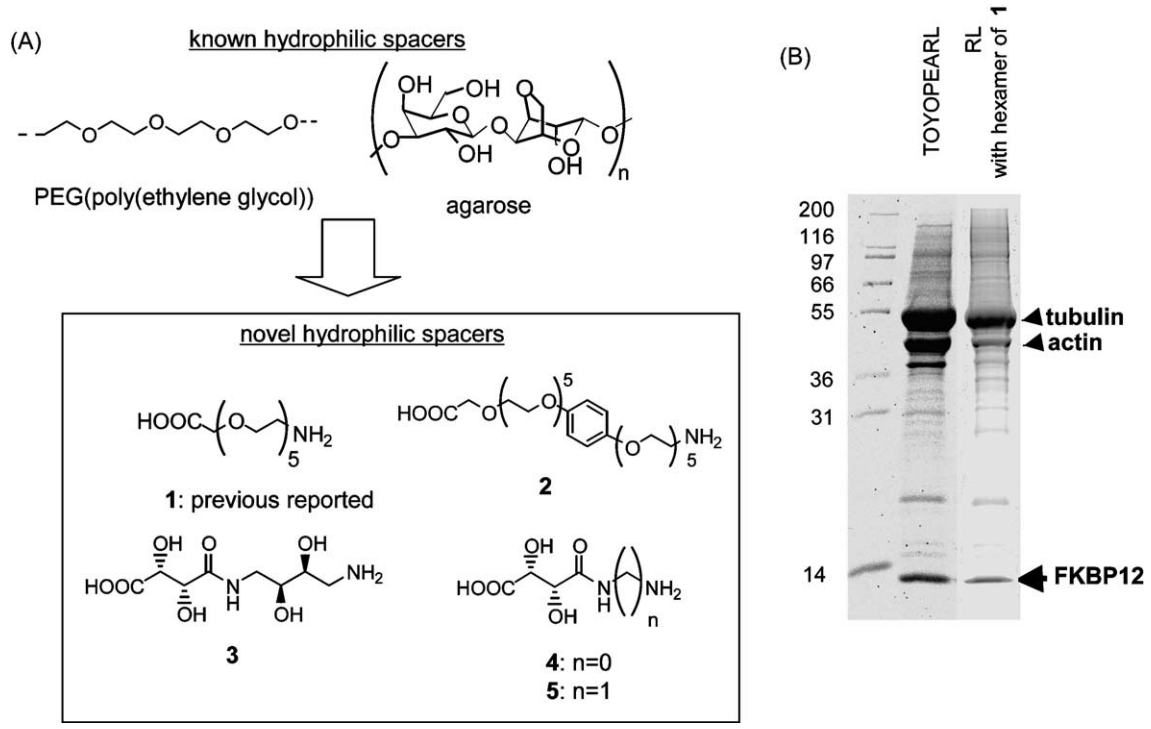


Figure 1. (A) Design of hydrophilic spacers studied in this study. (B) Binding proteins on TOYOPEARL bearing FK506 with and without previous reported hydrophilic spacer **1**. FKBP12 is known as the target protein of FK506, and other proteins such as tubulin and actin are thought to be nonspecific binding proteins.

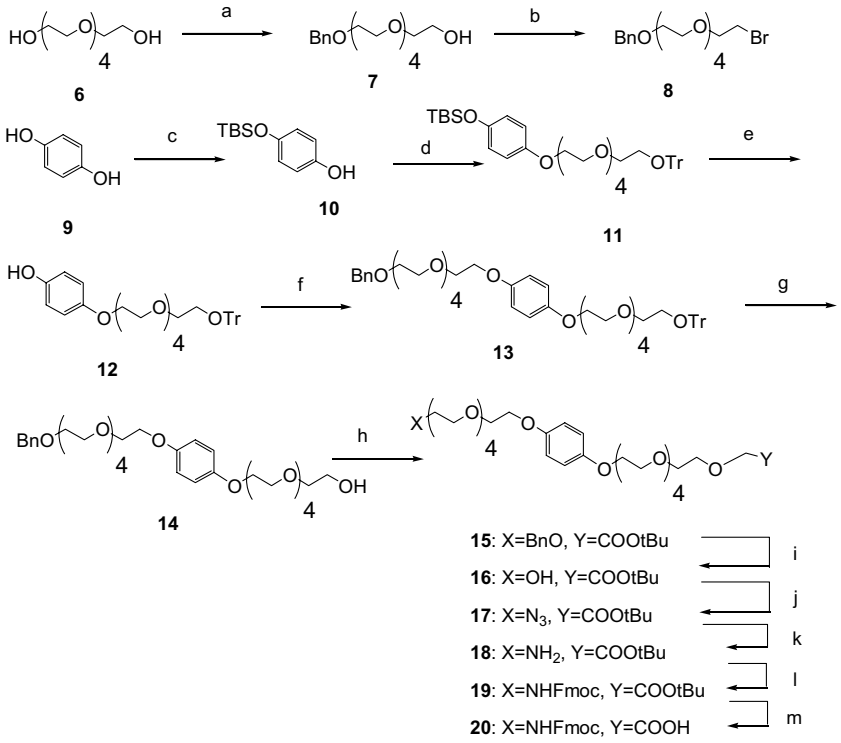
Therefore, reduction of nonspecific binding proteins to methacrylate derivatives is an important goal.

The phrase 'nonspecific protein binding' is usually used to represent proteins that bind to affinity resins based on physical adsorption.[10,11] In a previous paper,[9] we carried out a quantitative study and reported a linear relationship between the CLOGP of ligands and the amount of nonspecific binding proteins, and effectiveness of introduction of a chemically stable and hydrophilic spacer (**1**, Fig. 1). Compound **1** was designed based on the structure of poly(ethyleneglycol) (PEG) that is often used as a hydrophilic spacer in biochemistry. However, the nonspecific binding proteins still remained even after introduction of a hexamer of the PEG-type spacer (Fig. 1B). This prompted the present study to develop novel hydrophilic spacers to reduce them more effectively. We herein report the design of novel hydrophilic spacers (**2**–**5**) and their effectiveness on reduction of nonspecific binding proteins.

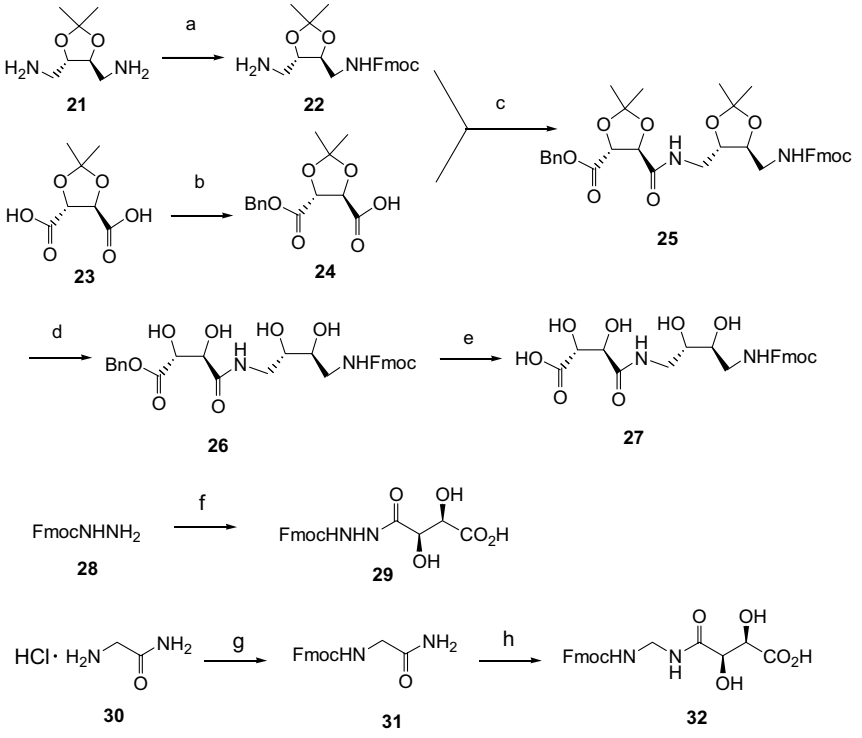
2. Design and synthesis

We designed novel PEG like spacer (**2**, Fig. 1) bearing a phenyl ring to join two PEG moieties. Compound **2** has longer PEG units compared to the previous reported spacer (**1**), aiming at greater hydrophilicity. This novel spacer has an amino group and carboxylic acid moiety at each end, an amino acid like structure, which facilitates the synthesis of polymer on solid phase. Compound **2** was synthesized as an *N*-Fmoc protected derivative (**20**, (Scheme 1), for elongation reactions on resins by the Fmoc strategy. Conversion of the hydroxy group of mono benzyl protected penta(ethyleneglycol) (**7**) to a bromide by carbon tetrabromide and triphenylphosphine gave **8**. Reaction of mono O-TBS protected 1,4-dihydroxybenzene (**10**) with mono trityl protected penta(ethyleneglycol), prepared in a similar manner to **7**, by Mitsunobu reaction afforded **11**. After removal of the TBS group of **11** by tetrabutylammonium fluoride (TBAF), a coupling reaction with **8** gave **13**. Selective deprotection of the trityl group of **13** under acidic conditions, followed by coupling with *tert*-butyl α-bromoacetate, afforded **15**. After removal of benzyl group of **15**, conversion of the hydroxy group to an amino group was performed via azide **17**, and was followed by introduction of an Fmoc group to the amine and subsequent deprotection of the *tert*-butyl ester under acidic conditions, resulting in the synthesis of the desired *N*-Fmoc protected PEG type spacer (**20**).

We next designed other type of spacers based on tartaric acid derivatives, which have an ethylene glycol moiety (**3**–**5**, Scheme 2) because of their hydrophilic properties. These spacers have amino acid-like structures and were synthesized as *N*-Fmoc protected derivatives (**27**, **29**, and **32**, Scheme 2) as well. Mono protected intermediates (**22**, **24**) were synthesized from symmetrical starting materials, (4*S*,5*S*)-4,5-di(aminomethyl)-2,2-dimethyldioxolane **21** and L-(+)-tartaric acid derivative **23**, respectively. After coupling **22** and **24**, deprotection of the acetonide and benzyl groups afforded the desired compound **27** (Scheme 2). Compound **29** was prepared by condensation of *N*-Fmoc hydrazine **28** and L-(+)-tartaric acid (Scheme 2). Compound **32** was synthesized



Scheme 1. Synthesis of *N*-Fmoc protected **2** (**20**): Reagents and conditions: (a) BnCl, KOH; (b) PPh_3, CBr_4/CH_2Cl_2; (c) TBSCl, imidazole/DMF; (d) $TrO(CH_2CH_2O)_5H$, PBu_3, DAMD/toluene; (e) TBAF/THF; (f) **8**, NaH/THF; (g) TFA/CH_2Cl_2, H_2O; (h) $BrCH_2COOtBu$, NaH/THF, DMF; (i) H_2, $Pd(OH)_2$/MeOH; (j) (i) TsCl, DMAP/Py, (ii) NaN_3/DMF, (k) H_2, $Pd(OH)_2$/MeOH; (l) Fmoc-OSu, NEt_3/THF; (m) 95% TFA/H_2O.



Scheme 2. Synthesis of *N*-Fmoc protected **3** (**27**), **4** (**29**), and **5** (**32**): Reagents and conditions: (a) Fmoc-OSu, NEt_3/THF; (b) BnOH, CDI, DBU/CH_3CN; (c) PyBOP, DIPEA/DMF; (d) 80% TFA/CH_2Cl_2; (e) H_2, $Pd(OH)_2$/MeOH, AcOEt; (f) L-(+)-tartaric acid, EDC·HCl/DMF; (g) Fmoc-OSu, Na_2CO_3/acetone, water; (h) (i) $PhI(OCOCF_3)_2$/AcOEt–CH_3CN–H_2O, (ii) L-(+)-tartaric acid, EDC·HCl/DMF.

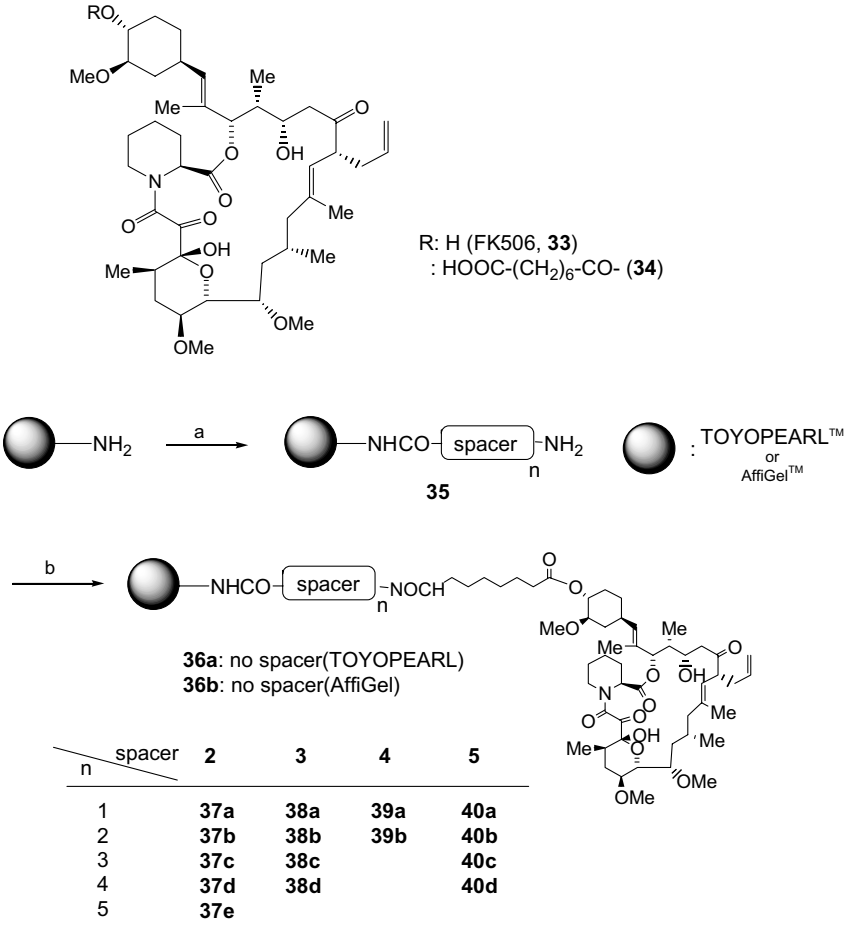
from a mono *N*-Fmoc protected diaminomethane, which was synthesized from Fmoc glycinamide **31** by Hoffman rearrangement using $PhI(OCOCF_3)_2$. This intermediate was condensed with L-(+)-tartaric acid without isolation to afford the desired intermediate **32** because of its instability (Scheme 2).

In order to assess the effectiveness of novel spacers (**2**–**5**) for reduction of nonspecific binding proteins, we synthesized affinity matrices bearing a common ligand, FK506 (**33**) on TOYOPEARL (**36**–**40**, Scheme 3). FK506 is an immunosuppressive drug that targets to FKBP12, the FK506 binding protein, with a Kd of 0.4 nM.[12] The structure and function of the complex of FK506 and FKBP12 have been well characterized at the molecular level by S. L. Schreiber and co-workers.[12–15] Since the molecular weight of FKBP12 (12 kDa) differs significantly from that of actin (42 kDa) and tubulin (50 kDa), it is easy to observe and compare the amounts of specific and nonspecific protein binding. Introduction of a linker moiety onto FK506 to connect it to the resin was carried out on the hydroxyl group at the 32 position (**34**) because crystal structure studies indicate that this position is not involved in the binding of FK506 with FKBP12.[15] Introduction of spacers was carried out by the Fmoc strategy. Immobilization of Fmoc protected spacers **20**, **27**, **29**, **32**, and the bait compound **34** on resins was performed by using EDC·HCl and HOBt.

3. Results and discussion

In order to compare the effect on reduction of nonspecific binding proteins by introduction of the novel hydrophilic spacers, we mixed affinity resins (**36**–**40**) with a common lysate that was prepared from rat brains using a simple buffer (buffer A; 0.25 M Sucrose, 25 mM Tris–HCl, pH 7.4). Rat brain lysate was thought to be suitable for our study since it included both the specific binding protein, FKBP12, and nonspecific binding proteins such as tubulin and actin.[9] Binding proteins on each affinity resin were completely eluted by a SDS sample buffer solution (Nacalai, Sample Buffer Solution with 2-ME(2×) for SDS-PAGE, cat. no 30566-22, including 4%(w/v)-SDS, 20%(v/v)-glycerol, 0.01%(w/v)-bromophenol blue, 10%(v/v)-2-mercaptoethanol, 0.125 M Tris–HCl, pH 6.8) after extensive washes using buffer A. Eluted proteins were resolved by SDS-Polyacrylamide gel electrophoresis, and identification of tubulin, actin, and FKBP12 was performed by MALDI-TOF mass spectrometry peptide mass fingerprinting after in-gel digestion (Fig. 2). As determined by binding competition with soluble FK506, we confirmed that only FKBP12 specifically interacts with the FK506-bearing resins and that other proteins such as tubulin and actin are nonspecific binding proteins.

Both AffiGel and TOYOPEARL matrices bearing FK506 without spacers (**36b**, **36a**) captured the target protein, FKBP12 (lane 3, 4, Fig. 2A), from the complicated protein mixture (lane 2). However, the amount



n \ spacer	2	3	4	5
1	**37a**	**38a**	**39a**	**40a**
2	**37b**	**38b**	**39b**	**40b**
3	**37c**	**38c**		**40c**
4	**37d**	**38d**		**40d**
5	**37e**			

Scheme 3. Synthesis of affinity matrices bearing a FK506 derivative (**34**) with/without hydrophilic spacers (**36**–**40**): Reagents and conditions: (a) (i) *N*-Fmoc protected novel hydrophilic spacer (**20**, **27**, **29**, or **32**), PyBOP, DIPEA/NMP; (ii) piperidine/DMF/CH_2Cl_2 = 1:2:2; (b) **34**, EDC·HCl, HOBt/DMF.

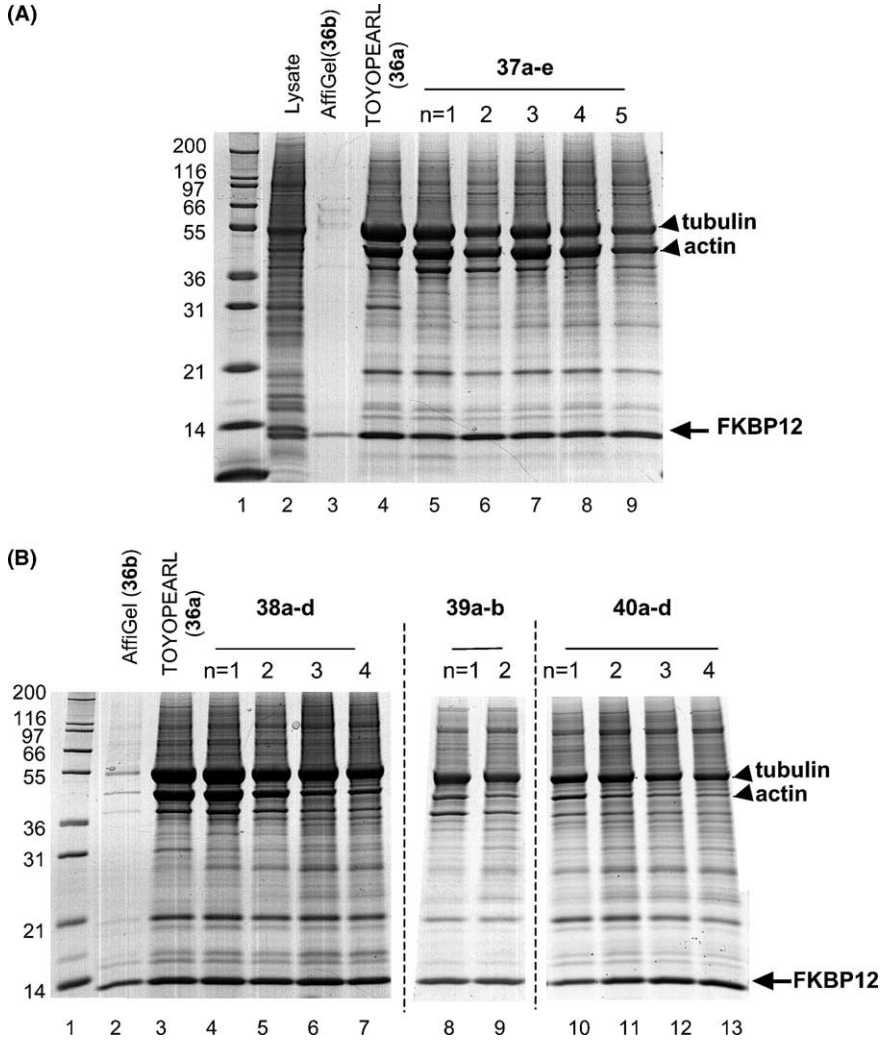


Figure 2. Binding proteins on affinity matrices bearing FK506 with or without hydrophilic spacers (**36**–**40**).

of nonspecific binding proteins such as tubulin and actin were very different, since affinity matrices from AffiGel only captured small amounts of nonspecific binding proteins while the TOYOPEARL ones bound much more. We reported that this difference mainly arose from differences in hydrophilic properties in the previous paper.[9]

The PEG type spacer (**2**) could reduce nonspecific binding proteins whilst binding of the target protein was almost constant in this series, as expected ($n = 1$ to 5, lane 5–9, Fig. 2A). The affinity resin bearing the pentamer of **2** (**37e**, lane 9, Fig. 2A) yielded almost equivalent amounts of FKBP12 compared to that without the spacer (**36a**), while affording lower amounts of nonspecific binding proteins. The amount of these nonspecific binding proteins was lower than that by introduction of the previous PEG type spacer **1** (Fig. 1B, lane 3). The amount decreased in proportion to the number of **2**, indicating a repetition effect of **2**. However reduction of them by introduction of **2** were not sufficient since affinity resins bearing pentamer of **2** still bound some amount of the nonspecific binding proteins.

We next analyzed proteins bound to affinity resins bearing the tartaric acid derivatives as spacers (**38**–**40**, Fig. 2B). All of these spacers also showed reductive effects on nonspecific binding proteins, whilst maintaining capture of the target protein. In particular, introduction of **4** (**39a**–**b**) and **5** (**40a**–**d**) were highly effective compared to **2** (**37a**–**e**), while that of **3** (**38a**–**d**) was almost equivalent to **2**. This difference is interested because both these derivatives are tartaric derivatives and **3** has more hydroxyl groups compared to **4** or **5** and is thought be more hydrophilic. The reason for this difference is, however, not clear. The nonspecific binding proteins were reduced by introduction of **4** or **5** enough to carry out researches on the target finding without being interfered by them, while there were some proteins, which were hardly reduced by introduction of the hydrophilic spacers. We have not identified these proteins yet, and have no information about the reason for not being susceptible to introduction of the hydrophilic spacers.

In order to assess the reductive effects quantitatively, measurements of tubulin and actin were carried out by a

GS-710 Calibrated Imaging Densitometer (BIO-RAD, software; quantity one-4.1.0), and were plotted in Figure 3A and B, respectively. Figure 3A showed that the most effective reduction of tubulin was found by introduction of **5** among hydrophilic spacers employed in this study. Introduction of tetramer of **5** reduced amount of tubulin by 65% almost on the affinity resins. Introduction of **4** also exhibited a good reductive effect on tubulin. Introduction of dimmer of **4** exhibited almost the same reductive effect as that by introduction of the same number of **5**. A plot of the amount of actin was shown in Figure 3B, which showed that the amount of actin was most effectively reduced by introduction of **4** (60% by the monomer, 80% by the dimer) and **5** (70% by the dimmer, 90% by tetramer). While actin was still observed even after introduction of tetramer of **5**, it was thought that the effect reached a maximum. Obvious reductive effect on actin by introduction of **2** was not observed in Figure 3B. The reason for the ineffectiveness of **2** on reduction of actin was not clear.

Among the hydrophilic spacers examined in this study, the introduction of **5** was the most effective. Affinity matrices of FK506 bearing the tetramer of **5** as a spacer captured an equivalent amount of the target protein, FKBP12, with only 35% and 10% of the nonspecific binding proteins, tubulin and actin, respectively, compared to the case without the hydrophilic spacer. These results demonstrated that introduction of **5** can allow us to carry out successful isolation of target proteins by affinity chromatography without interference from nonspecific binding proteins. Moreover, **5** is chemically stable, therefore its use with chemically stable matrices such as TOYOPEARL can allow us to synthesize a wide variety of affinity matrices bearing complexed compounds, for example, using deprotection reactions on the resins after immobilization of bioactive derivatives.

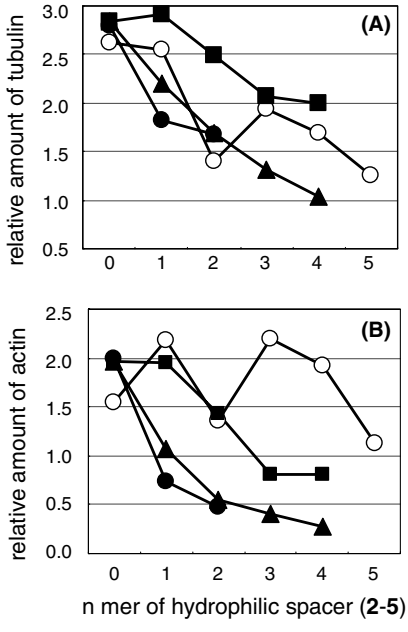


Figure 3. Plots of estimated amount of tubulin and actin with numbers of hydrophilic spacer **2** (○), **3** (■), **4** (●), and **5** (▲). The amounts of tubulin and actin were represented as a ratio compared to amount of FKBP12.

4. Conclusions

Tubulin and actin often bind nonspecifically to affinity chromatography resins, complicating research toward identifying the cellular targets of small molecules. Reduction of nonspecific binding proteins is important for success in discovery of such targets. In order to develop strategies to circumvent this problem, we designed and synthesized novel hydrophilic spacers **2**–**5** (Fig. 1). Effectiveness was compared in experiments using a common lysate prepared from rat brains after a common bait compound, FK506 (**33**), was immobilized on them (Fig. 2). Among spacers examined in this study, the reductive effect of tartaric acid derivative **5** was superior. Introduction of tetramer of **5** on TOYOPEARL reduced tubulin and actin by almost 65% and 90% compared to that without the hydrophilic spacer, respectively, with effective binding to the target protein, FKBP12 (Figs. 2 and 3). While there were still small amounts of nonspecific binding proteins after introduction of tetramer of **5** (lane 13, Fig. 2B), the reductive effect was sufficient for the target finding by affinity chromatography. The use of **5** or other hydrophilic spacers studied in this study with chemically stable matrices such as TOYOPEARL could allow us to synthesize a variety of affinity matrices bearing complexed compounds, while the use of the popular matrix AffiGel is restricted because it is often denatured under various synthetic conditions. We believe that these developments of the novel hydrophilic spacers will accelerate the target-finding research and applications in drug discovery.

5. Experimental

Thin-layer chromatography (TLC) was performed on Merck silica gel 60 F-254 plates. For normal chromatography, Merck silica gel type 60 (size 70–230) was used. All evaporation was performed with a rotary evaporator under reduced atmosphere. The structures of all compounds were confirmed by a LC-MS (Agilent 1100 Series LC/MSD) and 400 MHz proton nuclear magnetic resonance spectrum (Brucker, Advance-series 400). The chemical shift values are reported in parts per million on the δ scale from internal standard tetramethylsilane. No attempt was made to maximize the yields. Compound **34** was synthesized according to Ref. 9.

5.1. 2-(2-{2-[2-(2-Benzyloxyethoxy)ethoxy]ethoxy}ethoxy)ethanol (7)

A mixture of potassium hydroxide (1.2 g, 21.4 mmol) and penta(ethylene glycol) (**6**, 5.2 g, 21.8 mmol) was stirred at 130 °C for 15 min. After cooling to room

temperature (rt), benzyl chloride (2.7 g, 21.3 mmol) was added slowly, and the mixture was stirred at 130 °C for 2 h. After cooling to rt, water was added, and the mixture was extracted with dichloromethane (CH_2Cl_2). The separated organic layer was dried over sodium sulfate (Na_2SO_4), and the filtrate was evaporated in vacuo and purified by column chromatography on silica gel (elution; 5% methanol (MeOH) in ethyl acetate (AcOEt)). Fractions containing product were collected and evaporated in vacuo to give **7** (light yellow oil, 2.3 g, 32%). ^{1}H NMR ($CDCl_3$) δ 3.76–3.84 (m, 20H), 4.13 (s, 2H), 7.26–7.35 (m, 5H); MS m/z 329 (MH^+).

5.2. [2-(2-{2-[2-(2-Bromoethoxy)ethoxy]ethoxy}ethoxy)ethoxymethyl]benzene (8)

Carbon tetrabromide (4.12 g, 12.4 mmol) was added to a mixture of **7** (2.04 g, 6.21 mmol) and CH_2Cl_2 (20 mL). After cooling to 0 °C, triphenylphosphine (2.51 g, 12.4 mmol) was added. This mixture was stirred at room temperature (rt) for 1 h, and evaporated in vacuo. The resulting residue was purified by column chromatography on silica gel (elution; 50% AcOEt in *n*-hexane). Product fractions were collected and evaporated in vacuo to give **8** (light yellow oil, 2.1 g, 88%). ^{1}H NMR ($CDCl_3$) δ 3.46 (t, 2H, $J = 6.4$ Hz), 3.62–3.69 (m, 16H), 3.80 (t, 2H, $J = 6.4$ Hz), 4.57 (s, 2H), 7.26–7.35 (m, 5H); MS m/z 391 (MH^+).

5.3. 4-*tert*-Butyldimethylsilyloxyphenol (10)

A mixture of imidazole (3.1 g, 45 mmol), *tert*-butyldimethylsilyl chloride (4.5 g, 30 mmol), and DMF (50 mL) was added to a mixture of hydroquinone (**9**, 17.3 g, 30 mmol) and DMF (30 mL), and the mixture was stirred at rt overnight. This mixture was poured into a mixture of water and diethyl ether. The separated organic layer was washed with brine, and dried over Na_2SO_4. After filtration, the filtrate was evaporated in vacuo and purified by column chromatography on silica gel (elution; 10% AcOEt in *n*-hexane). Product fractions were collected and evaporated in vacuo to give **10** (white crystals, 3.6 g, 53%). ^{1}H NMR ($CDCl_3$) δ 0.16 (s, 6H), 0.97 (s, 9H), 4.43 (s, 1H), 6.70 (d, 4H, $J = 2$ Hz); MS m/z 225 (MH^+).

5.4. *tert*-Butyldimethyl-{4-[2-(2-{2-[2-(2-trityloxyethoxy)ethoxy]ethoxy}ethoxy)ethoxy]phenoxy}silane (11)

A mixture of tributylphosphine (PBu_3, 1.17 g, 5.8 mmol) and toluene (2 mL) was added to a mixture of 2-(2-{2-[2-(2-trityloxyethoxy)ethoxy]ethoxy}ethoxy)ethanol (2.8 g, 5.8 mmol) and toluene (10 mL) and stirred at rt for 1 h. This solution was slowly added to a mixture of **10** (1.1 g, 4.9 mmol), 1,1′-azo-bis-*N*,*N*-dimethylformamide (DAMD, 1.0 g, 5.8 mmol), and toluene (10 mL), and stirred at rt overnight. This mixture was poured into AcOEt (20 mL) and the resulting precipitates removed by filtration, and washed with AcOEt. The collected filtrate was evaporated in vacuo and purified by column chromatography on silica gel (elution; 33% AcOEt in *n*-hexane) to give **11** (colorless oil, 2.03g, 60%). ^{1}H NMR ($CDCl_3$) δ 0.16 (s, 6H), 0.97 (s, 9H), 3.23 (t, 2H, $J = 5$ Hz), 3.64–3.69 (m, 14H), 3.80 (t, 2H, $J = 5$ Hz), 4.04 (t, 2H, $J = 5$ Hz), 6.69–6.75 (m, 5H), 7.23–7.30 (m, 8H), 7.45–7.47 (m, 6H).

5.5. 4-[2-(2-{2-[2-(2-Trityloxyethoxy)ethoxy]ethoxy}ethoxy)ethoxy]phenol (12)

TBAF (1 M) in tetrahydrofuran (THF, 4 mL) was added to a mixture of **11** (1.88 g, 2.74 mmol) and THF (20 mL) at 0 °C, and then stirred at rt for 10 min. The reaction mixture was poured into a mixture of water and AcOEt. The separated organic layer was washed with brine and dried over Na_2SO_4. After filtration, the filtrate was evaporated in vacuo, and purified by column chromatography on silica gel (elution; 10% MeOH in AcOEt). Production fractions were collected and evaporated in vacuo to give compound **12** (light yellow oil, 875 mg, 85%). ^{1}H NMR ($CDCl_3$) δ 3.23 (t, 2H, $J = 5$ Hz), 3.64–3.69 (m, 14H), 3.79 (t, 2H, $J = 5$ Hz), 4.04 (t, 2H, $J = 5$ Hz), 4.68 (s, 1H), 6.71–6.79 (m, 4H), 7.20–7.30 (m, 9H), 7.45–7.47 (m, 6H).

5.6. 1-[2-(2-{2-[2-(2-Benzyloxyethoxy)ethoxy]ethoxy}ethoxy)ethoxy]-4-[2-(2-{2-[2-(2-trityloxyethoxy)ethoxy]ethoxy}ethoxy)ethoxy]benzene (13)

A mixture of **12** (1.2 g, 2.1 mmol) and THF (20 mL) was slowly added to a mixture of sodium hydride (NaH, 800 mg, 20 mmol; 60% in mineral oil) and THF (10 mL) at 0 °C, and stirred for 20 min under the same conditions. A mixture of **8** (1.7 g, 4.3 mmol) and THF (20 mL) was added, and the mixture stirred for 20 min at 0 °C and at rt for 2 h. After addition of water (1 mL), the resulting mixture was concentrated at reduced pressure. After extraction with CH_2Cl_2, the combined organic layers were washed with brine and dried over Na_2SO_4. After filtration, the filtrate was evaporated in vacuo and purified by column chromatography on silica gel (elution; 50% AcOEt in *n*-hexane). Production fractions were collected, and evaporated in vacuo to give **13** (light yellow oil, 1.56 g, 84%). ^{1}H NMR ($CDCl_3$) δ 3.23 (t, 2H, $J = 5$ Hz), 3.61–3.72 (m, 30H), 3.78–3.82 (m, 4H), 4.03–4.07 (m, 4H), 4.56 (s, 2H), 6.82 (s, 4H), 7.09–7.34 (m, 14H), 7.44–7.47 (m, 6H).

5.7. 2-(2-{2-[2-(2-{4-[2-(2-{2-[2-(2-Benzyloxyethoxy)ethoxy]ethoxy}ethoxy)ethoxy]phenoxy}ethoxy)ethoxy]ethoxy}ethoxy)ethanol (14)

A solution of 10% trifluoroacetic acid (TFA) in CH_2Cl_2 (10 mL) was added to a mixture of **13** (1.43 g, 1.62 mmol), water (1 mL), and CH_2Cl_2 (20 mL) at 0 °C, and stirred at rt for 2.5 h. The reaction was poured into saturated $NaHCO_3$ and extracted with CH_2Cl_2 and chloroform ($CHCl_3$). The combined organic layers were washed with brine and dried over Na_2SO_4. After filtration, the filtrate was evaporated in vacuo and purified by

column chromatography on silica gel (elution; 10% MeOH in AcOEt). Production fractions were collected and evaporated in vacuo to give compound **14** (light yellow oil, 875 mg, 85%). ^{1}H NMR ($CDCl_3$) δ 3.59–3.74 (m, 32H), 3.80–3.84 (m, 4H), 4.05–4.08 (m, 4H), 4.56 (s, 2H), 6.83 (s, 4H), 7.26–7.34 (m, 5H); MS m/z 641 (MH^+).

5.8. *tert*-Butyl [2-(2-{2-[2-(2-{4-[2-(2-{2-[2-(2-benzyloxyethoxy)ethoxy]ethoxy}ethoxy)ethoxy]phenoxy}ethoxy)ethoxy]ethoxy}ethoxy)ethoxy]acetate (15)

A mixture of **14** (817 mg, 1.28 mmol) and THF (6 mL) was slowly added to an ice-cooled mixture of NaH (180 mg, 4.5 mmol; 60% in mineral oil), THF (5 mL) and DMF (1 mL), and was stirred under the same conditions for 30 min. A mixture of *tert*-butyl bromoacetate (1.0 g, 5.1 mmol) and THF (7 mL) was added and the mixture stirred at rt for 2 h. This mixture was poured into water and extracted with AcOEt, and washed with brine, then dried over Na_2SO_4. After filtration, the filtrate was evaporated in vacuo, and purified by column chromatography on silica gel (elution; 5% MeOH in AcOEt). Production fractions were collected, and evaporated in vacuo to give **15** (light yellow oil, 655 mg, 68%). ^{1}H NMR ($CDCl_3$) δ 1.47 (s, 9H), 3.61–3.73 (m, 32H), 3.81–3.84 (m, 4H), 4.02 (s, 2H), 4.05–4.08 (m, 4H), 4.56 (s, 2H), 6.83 (s, 4H), 7.26–7.24 (m, 5H).

5.9. *tert*-Butyl [2-(2-{2-[2-(2-{4-[2-(2-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}ethoxy)ethoxy]phenoxy}ethoxy)ethoxy]ethoxy}ethoxy)ethoxy]acetate (16)

A mixture of **15** (655 mg, 0.868 mmol), palladium hydroxide on activated carbon ($Pd(OH)_2$–C, 145 mg), and MeOH (6 mL) was stirred under an hydrogen atmosphere at rt for 4 h. After filtration, the filtrate was evaporated in vacuo to give crude **16** (575 mg). ^{1}H NMR ($CDCl_3$) δ 1.47 (s, 9H), 3.59–3.74 (m, 32H), 3.81–3.84 (m, 4H), 4.02 (s, 2H), 4.07–4.09 (m, 4H), 6.84 (s, 4H).

5.10. *tert*-Butyl [2-(2-{2-[2-(2-{4-[2-(2-{2-[2-(2-azidoethoxy)ethoxy]ethoxy}ethoxy)ethoxy]phenoxy}ethoxy)ethoxy]ethoxy}ethoxy)ethoxy]acetate (17)

4-Dimethylaminopyridine (DMAP, 11 mg, 0.09 mmol) was added to a mixture of **16** (254 mg, 0.451 mmol) and pyridine (1.5 mL). After this mixture was cooled at 0 °C, *p*-toluenesulfonyl chloride (TsCl, 130 mg, 0.677 mmol) was added and the mixture stirred at rt for 2 h, then poured into a mixture of ice, water, and AcOEt. The organic layer was washed with saturated $KHSO_4$, saturated $NaHCO_3$, and brine, and dried over Na_2SO_4. After filtration, the filtrate was evaporated in vacuo to give crude *tert*-butyl [2-(2-{2-[2-(2-{4-[2-(2-{2-[2-(2-tosyloxyethoxy)ethoxy]ethoxy}ethoxy)ethoxy]phenoxy}-ethoxy)ethoxy]ethoxy}ethoxy)ethoxy]acetate, which was used in the following step without further purification. Sodium azide (NaN_3, 26.4 mg, 4.1 mmol) was added to a mixture of the above intermediate and DMF (1 mL), and was stirred at 60 °C for 90 min. After cooling to rt, this mixture was poured into a mixture of AcOEt and saturated $NaHCO_3$. The organic layer was then washed with brine and dried over Na_2SO_4. After filtration, the filtrate was evaporated in vacuo and purified by column chromatography on silica gel (elution; 10% MeOH in AcOEt). Product fractions were collected, and evaporated in vacuo to give *tert*-butyl [2-(2-{2-[2-(2-{4-[2-(2-{2-[2-(2-azideethoxy)ethoxy]ethoxy}ethoxy)ethoxy]-phenoxy}-ethoxy)ethoxy]ethoxy}ethoxy)ethoxy]acetate (**17**, colorless oil, 195 mg, 73%). ^{1}H NMR ($CDCl_3$) δ 1.47 (s, 9H), 3.38 (t, 2H, $J = 5$ Hz), 3.66–3.72 (m, 30H), 3.81–3.84 (m, 4H), 4.02 (s, 2H), 4.06–4.08 (m, 4H), 6.84 (s, 4H).

5.11. *tert*-Butyl [2-(2-{2-[2-(2-{4-[2-(2-{2-[2-(2-aminoethoxy)ethoxy]ethoxy}ethoxy)ethoxy]phenoxy}ethoxy)ethoxy]ethoxy}ethoxy)ethoxy]acetate (18)

A mixture of **17** (190 mg, 0.323 mmol), $Pd(OH)_2$–C (18 mg), and MeOH (1 mL) was stirred under an hydrogen atmosphere at rt for 1 h. After filtration, the filtrate was evaporated in vacuo to give crude **18** (175 mg). MS m/z 664 (MH^+). This compound was used in the following step without further purification.

5.12. *tert*-Butyl {2-[2-(2-{2-[2-(4-{2-[2-(2-{2-[2-(9*H*-fluoren-9-yl-methoxycarbonylamino)ethoxy]ethoxy}ethoxy)ethoxy]ethoxy}phenoxy)ethoxy]ethoxy}ethoxy)ethoxy]ethoxy}acetate (19)

A mixture of 9-fluorenylmethylsucinimidyl carbonate (Fmoc-OSu, 115 mg, 0.34 mmol), triethylamine (NEt_3, 62 mg, 0.62 mmol), and THF (0.5 mL) was added to a mixture of **18** (175 mg) and THF (1.2 mL) at 0 °C, and was stirred under the same conditions for 45 min. This mixture was poured into a mixture of water and AcOEt. The separated organic layer was washed with brine and dried over Na_2SO_4. After filtration, the filtrate was evaporated in vacuo and purified by column chromatography on silica gel (elution; 10% MeOH in AcOEt). Product fractions were collected and evaporated in vacuo to give compound **19** (colorless oil, 196 mg, 84%). ^{1}H NMR ($CDCl_3$) δ 1.47 (s, 9H), 3.37–3.41 (m, 2H), 3.55–3.84 (m, 34H), 4.01 (s, 2H), 4.01–4.07 (m, 4H), 4.20–4.23 (m, 1H), 4.40 (d, 2H, $J = 7$ Hz), 6.81 (s, 4H), 7.29–7.33 (m, 2H), 7.39–7.41 (m, 2H), 7.60 (d, 2H, $J = 7$ Hz), 7.76 (d, 2H, $J = 7$ Hz).

5.13. {2-[2-(2-{2-[2-(4-{2-[2-(2-{2-[2-(9*H*-Fluoren-9-yl-methoxycarbonylamino)ethoxy]ethoxy}ethoxy)ethoxy]-ethoxy}phenoxy)ethoxy]ethoxy}ethoxy)ethoxy]ethoxy}-acetic acid (20)

A mixture of **19** (196 mg, 0.25 mmol), water (0.075 mL), and TFA (1.5 mL) was stirred at rt for 5 min. After evaporation, the residue was purified by column chromatography on silica gel (elution; 10% MeOH in

$CHCl_3$). Fractions including the target were collected, and evaporated in vacuo to give compound **20** (colorless oil, 175 mg, 96%). ^{1}H NMR ($CDCl_3$) δ 3.37–3.39 (m, 2H), 3.56–3.84 (m, 34H), 4.02–4.07 (m, 4H), 4.12 (s, 2H), 4.22–4.23 (m, 1H), 4.40 (d, 2H, $J = 7$ Hz), 5.47 (br s, 1H), 6.81 (s, 4H), 7.29–7.33 (m, 2H), 7.38–7.41 (m, 2H), 7.60 (d, 2H, $J = 7$ Hz), 7.76 (d, 2H, $J = 7$ Hz); MS m/z 830 (MH^+). Anal. Calcd for $C_{43}H_{59}NO_{15}/1.5H_2O$: C, 60.27; H, 7.29; N, 1.63. Found: C, 60.09; H, 7.11; N, 1.85.

5.14. (5*S*-Aminomethyl-2,2-dimethyl-[1,3]dioxolan-4*S*-ylmethyl)carbamic acid 9*H*-fluoren-9-ylmethyl ester (22)

Fmoc-OSu (2.1 g, 6.25 mmol) in THF (10 mL) and NEt_3 (1.26 g, 12.5 mmol) were added to an ice-cooled solution of **21** (1.0 g, 6.25 mmol) and THF (20 mL), and the mixture stirred for 30 min under the same conditions. The reaction mixture was then poured into a mixture of water and AcOEt, and the separated organic layer was washed with brine and was dried over anhydrous magnesium sulfate ($MgSO_4$). After filtration, the filtrate was evaporated in vacuo and purified by column chromatography on silica gel (elution; 5% ethanol (EtOH) in AcOEt) to give **22** (1.22 g, 53%). ^{1}H NMR (DMSO-d_6) δ 1.30 (s, 6H), 2.68 (d, 2H), 3.07 (m, 2H), 3.63–3.69 (m, 1H), 3.74–3.80 (m, 1H), 4.19–4.23 (m, 1H), 4.29 (m, 2H), 7.33 (m, 2H), 7.42 (t, 2H), 7.58 (m, 1H), 7.70 (d, 2H), 7.89 (d, 2H); MS m/z 383 (MH^+).

5.15. 5*R*-Hydroxycarboyl-2,2-dimethyl-[1,3]dioxolane-4*R*-carboxylic acid benzyl ester (24)

A solution of **23** (740 mg, 3.89 mmol), 1,1′-carbonyldiimidazole (CDI, 1.26 g, 7.78 mmol) and acetonitrile (CH_3CN, 50 mL) was stirred at rt for 30 min. Benzyl alcohol (420 mg, 3.89 mmol) and 1,8-diazabycyclo[5.4.0]undec-7-ene (DBU, 887 mg, 5.84 mmol) were added and the mixture stirred at rt overnight. The reaction mixture was poured into a mixture of diluted HCl and AcOEt and the separated organic layer was washed with brine and dried over $MgSO_4$. After filtration, the filtrate was concentrated in vacuo to give crude **24**, which was used in the next reaction without further purification.

5.16. 5*R*-({5*S*-[(9*H*-Fluoren-9-ylmethoxycarbonylamino)methyl]-2,2-dimethyl-[1,3]dioxolane-4*S*-ylmethyl}-carbamoyl)-2,2-dimethyl-[1,3]dioxolane-4*R*-carboxylic acid benzyl ester (25)

A mixture of crude **24**, **22** (1.2 g, 3.14 mmol), bromotris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP, 3.37 g, 6.48 mmol), *N*,*N*′-diisopropylethylamine (DIPEA, 1.25 g, 9.74 mmol), and DMF (10 mL) was stirred at rt for 17 h. The mixture was poured into a mixture of water and AcOEt and the separated organic layer was washed with brine, and dried over $MgSO_4$. After filtration, the filtrate was evaporated in vacuo and purified by column chromatography on silica gel (elution; 50% AcOEt in *n*-hexane) to give **25** (0.49 g, 24%). ^{1}H NMR ($CDCl_3$) δ 1.33–1.39 (m, 6H), 1.43 (s, 3H), 1.50 (m, 3H), 3.42 (br s, 2H), 3.53 (br s, 2H), 3.75 (m, 2H), 4.21 (m, 1H), 4.37–4.48 (m, 2H), 4.76–4.82 (m, 2H), 5.25 (s, 2H), 5.29 (m, 1H), 6.93 (m, 1H), 7.28–7.41 (m, 9H), 7.59 (d, 2H, $J = 7.5$ Hz), 7.75 (d, 2H, $J = 7.5$ Hz); MS m/z 645 (MH^+).

5.17. *N*-[4-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-2*S*,3*S*-dihydroxybutyl]-2*R*,3*R*-dihydroxysuccinamic acid benzyl ester (26)

A mixture of **25** (0.46 g, 0.71 mmol), TFA (8 mL), and CH_2Cl_2 (2 mL) was stirred at rt for 5 h. The reaction mixture was concentrated in vacuo, and the resulting residue recrystallized from *n*-hexane/AcOEt to give **26** (220 mg, 55%). ^{1}H NMR (DMSO-d_6) δ 2.99–3.06 (m, 2H), 3.09–3.19 (m, 2H), 3.44 (m, 2H), 4.18–4.23 (m, 1H), 4.26–4.27 (m, 3H), 4.47 (d, 1H),4.63 (m, 2H), 5.15 (m, 2H), 5.40 (d, 1H), 5.88 (t, 1H), 7.14 (m, 1H), 7.28–7.42 (m, 9H), 7.64 (m, 1H), 7.70 (d, 2H), 7.88 (d, 2H); MS m/z 565 (MH^+).

5.18. *N*-[4-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-2*S*,3*S*-dihydroxybutyl]-2*R*,3*R*-dihydroxysuccinamic acid (27)

A mixture of **26** (200 mg, 0.35 mmol), 10% palladium on carbon (Pd-C, 50 mg), AcOEt (50 mL), and MeOH (50 mL) was stirred under a hydrogen atmosphere at rt for 2 h. After filtration, the filtrate was evaporated in vacuo to give **27** (165 mg, 100%). ^{1}H NMR (acetone-*d*6) δ 3.2–3.4 (m, 3H), 3.49–3.70 (m, 3H), 4.23 (m, 1H), 4.33–4.35 (m, 2H), 4.46 (m, 1H), 4.60 (m, 1H), 6.50 (m, 1H), 7.33 (t, 2H), 7.41 (t, 2H), 7.68 (m, 1H), 7.71 (d, 2H), 7.86 (d, 2H); MS m/z 475 (MH^+). Anal. Calcd for $C_{23}H_{26}N_2O_9/1.5H_2O$: C, 55.08; H, 5.82; N, 5.59. Found: C, 54.88; H, 5.53; N, 5.91.

5.19. 4-[*N*′-(9*H*-Fluoren-9-ylmethoxycarbonyl)-hydrazino]-2*R*,3*R*-dihydroxy-4-oxo-butyric acid (29)

A mixture of Fmoc-hydrazine hydrochloride (**28**, 500 mg, 1.72 mmol), (+)-tartaric acid (634 mg, 4.22 mmol), DIPEA (0.300 mL, 1.72 mmol), EDC·HCl (485 mg, 2.53 mmol), and DMF (10 mL) was stirred overnight at rt. After concentration in vacuo, the resulting residue was dissolved in saturated $NaHCO_3$ and ethyl ether. The water layer was acidified with 2 N $KHSO_4$ and extracted with AcOEt. The organic solution was washed with brine and dried over Na_2SO_4. After filtration, the filtrate was evaporated in vacuo, and recrystallized from MeOH/ethyl ether to give **29** (413 mg, 63%). ^{1}H NMR (acetone-d_6) δ 4.29 (t, 2H, $J = 7.1$ Hz), 4.35 (m, 1H), 4.60 (s, 1H), 4.63 (s, 1H), 7.33 (t, 2H, $J = 7.4$ Hz), 7.42 (t, 2H, $J = 7.4$ Hz), 7.76 (d, 2H, $J = 7.4$ Hz), 7.85 (d, 2H, $J = 7.4$ Hz); MS m/z 387 (MH^+). Anal. Calcd for $C_{19}H_{18}N_2O_7/0.2H_2O$: C, 58.52; H, 4.76; N, 7.18. Found: C, 58.42; H, 4.69; N, 7.37.

5.20. Fmoc-glycinamide (31)

A solution of Fmoc-OSu (30.5 g, 90.5 mmol) in acetone (250 mL) was added dropwise to a mixture of glycinamide hydrochloride (**30**, 10.0 g, 90.5 mmol), 10% sodium carbonate solution (600 mL), and acetone (250 mL) at 0 °C. After stirring at rt for 1 h, the reaction mixture was concentrated in vacuo and the residue was dissolved in AcOEt. The solution was washed with 2 N citric acid, saturated $NaHCO_3$, and brine, and dried over $MgSO_4$. After filtration, the filtrate was evaporated in vacuo, and recrystallized from MeOH to give **31** (27.7 g, quant.). ^{1}H NMR ($CDCl_3$) δ 3.87 (d, 2H, $J = 5.6$ Hz), 4.23 (t, 1H, $J = 6.6$ Hz), 4.46 (d, 2H, $J = 6.6$ Hz), 7.30 (t, 2H, $J = 7.5$ Hz), 7.39 (t, 2H, $J = 7.5$ Hz), 7.57 (d, 2H, $J = 7.5$ Hz), 7.75 (d, 2H, $J = 7.5$ Hz); MS m/z 297 (MH^+).

5.21. *N*-[(9*H*-Fluoren-9-ylmethoxycarbonylamino)-methyl]-2*R*,3*R*-dihydroxysuccinamic acid (32)

A solution of **31** (1.50 g, 5.06 mmol), [bis(trifluoroacetoxy)iodo]benzene ($PhI(OCOCF_3)_2$, 2.83 g, 6.58 mmol), AcOEt (15 mL), CH_3CN (15 mL), and water (15 mL) was stirred at rt for 1.5 h. After acidification with 0.2 N HCl, the water layer was washed with a mixture of AcOEt and *n*-hexane (1:1), and basified with saturated $NaHCO_3$ at 0 °C. After extraction with $CHCl_3$, the separated organic layer was washed with brine and dried over $MgSO_4$. After filtration, the filtrate was evaporated in vacuo to give *N*-Fmoc-diaminomethane, which was immediately used in the next reaction.

A mixture of the above *N*-Fmoc-diaminomethane, L-(+)-tartaric acid (1.42 g, 9.45 mmol), EDC·HCl (795 mg, 4.15 mmol), and DMF (100 mL) was stirred at rt overnight. After concentration in vacuo, the resulting residue was dissolved in AcOEt. The organic solution was washed with 2 N citric acid and was extracted with saturated $NaHCO_3$. The water solution was washed with AcOEt, acidified with 2 N citric acid at 0 °C, and extracted with AcOEt. The separated organic layer was washed with brine and dried over Na_2SO_4. After filtration, the filtrate was evaporated in vacuo and the residue recrystallized from MeOH to give **32** (588 mg, 29%). ^{1}H NMR (acetone-d_6) δ 4.24 (t, 1H, $J = 7.0$ Hz), 4.33 (m, 2H), 4.44 (d, 1H, $J = 1.9$ Hz), 4.58–4.71 (m, 3H), 7.33 (t, 2H, $J = 7.5$ Hz), 7.41 (t, 2H, $J = 7.5$ Hz), 7.71 (d, 2H, $J = 7.5$ Hz), 7.86 (d, 2H, $J = 7.5$ Hz); MS m/z 401 (MH^+). Anal. Calcd for $C_{20}H_{20}N_2O_7/0.67H_2O$: C, 58.25; H, 5.21; N, 6.79. Found: C, 58.11; H, 5.05; N, 6.74.

5.22. Representative procedure for introduction of hydrophilic spacer on resins

A mixture of **20** (66.4 mg, 0.08 mmol), TOYOPEARL (200 μL, 0.02 mmol), PyBOP (52 mg, 0.1 mmol), DIPEA (34 μL, 0.10 mmol), CH_2Cl_2 (0.8 mL), and *N*-methyl-2-pyrrolidone (NMP, 0.2 mL) was shaken at rt for 4 h. After filtration, the resin was washed with DMF 5 times. The reaction ratio was determined by the Ninhydrin test (80%). After washing the resin with DMF carefully, 0.5 mL of a mixture (acetic anhydride/CH_2Cl_2/NMP = 1:8:2) was added for acetyl capping of remaining amine groups. The reaction mixture was shaken at rt for 3 h, and then washed with DMF cleaning five more times.

This Fmoc resin was mixed with 0.5 mL of a mixture (piperidine/DMF/CH_2Cl_2 = 1:4:4) at rt for 3 h. After filtration, the resin was washed five times with DMF to afford the objective resins bearing the hydrophilic spacer (**35**).

5.23. Affinity resin bearing FK506 on TOYOPEARL (36a)

A mixture of **34** (38.4 mg, 0.04 mmol), TOYOPEARL™ (TOSOH, AF-Amino-650M, cat. 08002, 100 μL, 0.01 mmol), EDC·HCl (9.2 mg, 0.048 mmol), HOBt (6.5 mg, 0.048 mmol), and DMF (1 mL) was shaken at rt for 6 h. After removal of solvent by filtration, the resin was washed with DMF. The reaction ratio was determined by the Ninhydrin test (82%). The resin was mixed with a 20% DMF solution of acetic anhydride at rt for 1 h, washed with DMF, and 20% aqueous EtOH.

The synthesis of affinity resins bearing FK506 on AffiGel™ (**36b**, BIO-RAD, AffiGel 102 Gel, cat. 153-2401) was carried out in a similar manner. AffiGel was used after washing with DMF five times. Other resins (**37**–**40**) were prepared in a similar manner.

5.24. Preparation of rat brain lysate

Fresh rat brain was homogenized (1:10, w/v) in buffer A (25 mM Tris pH = 7.4, 0.25 M sucrose). The homogenate was centrifuged at 9500 rpm for 10 min. After the supernatant was separated, it was centrifuged again at 50,000 rpm for 30 min. The obtained supernatant was used for lysate and kept at −80 °C before use.

5.25. Binding assay on affinity resins

The lysate as a crude tissue extract was diluted by buffer A and total protein concentration was about 7 mg/mL. This lysate was stirred with affinity resin at 4 °C for about 15 h to adsorb the non specific binding proteins or specific binding proteins. A typical mixture has a total volume of 1.0 mL, consisting of buffer A, 10 μL of beads previously equilibrated by buffer A, and 0.5 mL of tissue extract. After incubation, the resins were precipitated by centrifugation in a microcentrifuge at 12,000 rpm for 1 min. The resins were washed five times with 1.0 mL of buffer A. The washed beads were then resuspended in 20 μL of SDS sample buffer solution (Nacalai, Sample Buffer Solution with 2-mercaptoethanol (2-ME, 2×) for SDS-PAGE, cat. 30566-22, including 4%(w/v)-SDS, 20%(v/v)-glycerol, 0.01%(w/v)-bromophenol blue, 10% (v/v)-2-mercaptoethanol, 0.125 M Tris–HCl, pH 6.8), shaken at 25 °C for 10 min, and centrifuged for 1 min.

The supernatant was subjected to SDS-PAGE followed by CBB staining.

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4. Information on an agarose derivative, 'AffiGel' can be found at the web site of Bio-Rad Laboratories, Inc. (http://www.bio-rad.com).
5. As described in the text, AffiGel is stable in aqueous conditions, and is usually used in aqueous solutions. Immobilization of a ligand on AffiGel is usually carried out in aqueous solution by addition of excess amounts of a bioactive ligand and coupling reagents such as EDC. However, it is impossible for us to use an excess amount of the ligand for immobilization since we usually synthesize the compound with a linker moiety via a number of synthetic steps, and only have small amounts. Thus, immobilization reaction such as amide formation reaction need to be carried out in organic solvent to avoid the presence of water. AffiGel is sometimes irreversibly denatured in organic solvent and sometimes give false-positive targets. For example, we found some novel nonspecific binding proteins when we synthesized affinity material bearing FK506 in acetonitrile (data not shown) whilst they were not detected when prepared in aqueous solution.
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