

Development of a Screening Process from
Virtual Mirror-image Library of Natural
Products Using D-Protein Technology

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Preface

Natural products and their derivatives have been valuable resources for drug discovery. Natural products are originally produced by plants, fungi, bacteria, or others as secondary metabolites for defensive and reproductive behaviors, and some of them are used in clinical practice, especially as anticancer, antibacterial, and immunosuppressive agents.¹ For example, fidaxomicin, which was isolated from the fermentation broth of *Dactylosporangium aurantiacum* subspecies *hamdenensis*, is a bactericidal agent against *Clostridium difficile*.² Romidepsin, isolated from the bacterium *Chromobacterium violaceum*, has been used as an anticancer agent for T-cell lymphoma.³ Cyclosporin A, isolated from *Trichoderma polysporum* as an antibiotic, is currently used to prevent rejection following organ transplantation.⁴ The desirable biological activities are attributed to the unique, complex, and sp³-carbon-rich scaffolds (Figure 1).

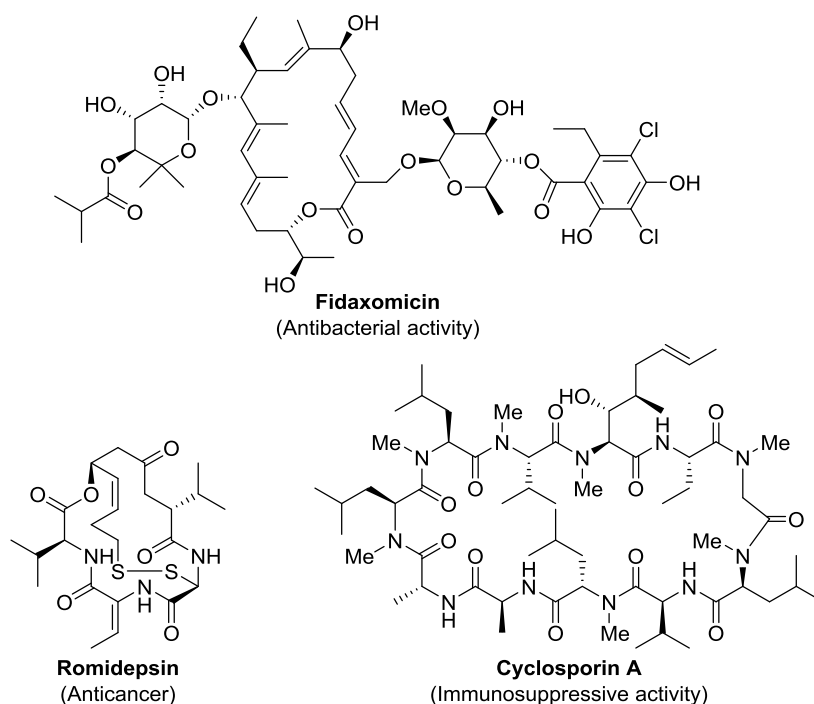


Figure 1. Structures of natural products in clinical practice.

To expand the library of natural product-like scaffolds to provide lead compounds for drug discovery and tool compounds for basic scientific research, a variety of methods have been developed. Recently, using complex natural product structures as starting materials, a variety of natural product-like scaffolds were prepared via a ring-distortion strategy using chemical modifications.⁵ Similarly, the component

sesquiterpenes in the extract from *Curcuma zedaria* were converted into a variety of derivatives with unprecedented scaffolds by treatment with oxidizing agents.⁶ Manipulation of the biosynthetic pathway by modification of culture conditions or co-cultivation of two or more microorganisms increases the chemical diversity of metabolite products.⁷ Epigenetic remodeling *via* activation of silent gene cluster(s) by cultivation in the presence of epigenetic regulatory agents also leads to the production of unprecedented natural products from fungi.⁸

These chemistry- or biology-based approaches are useful to construct novel natural product-based scaffolds. However, the stereochemical outcomes of the newly obtained scaffolds from these approaches are highly dependent on those of the parent substrates. While many natural products are produced as a single enantiomer when bearing a chiral structure(s), in some limited cases, two enantiomeric forms of chiral natural products are produced by a single or various species.⁹ The two mirror-image isomers with identical chemical and physicochemical properties often exhibit different functions and/or biological activities (Figure 2). For example, L-serine is a proteinogenic amino acid, while D-serine, which is produced from L-serine by serine racemase (SR), works as a neuromodulator for NMDA receptors in brain.¹⁰ The insect pheromones (*R*)-(-)-olean and (*S*)-(+)-olean are selective sex attractants for male and female olive fruit flies (*Dacusoleae*), respectively.¹¹ Therefore, mirror-image natural products and their derivatives are expected to have potentially bioactivities as valuable resources in drug discovery.

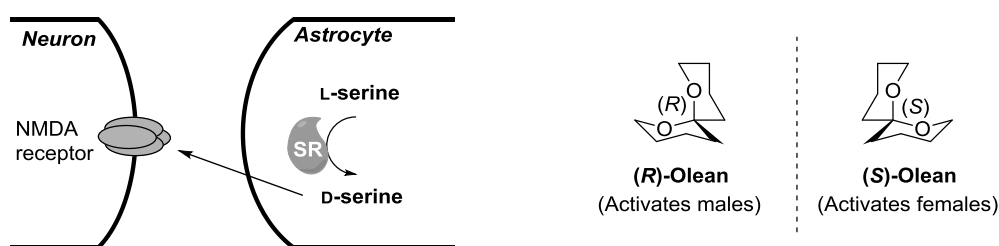


Figure 2. Different biological activities of chiral natural products between their two mirror-image isomers.

The major obstacle to utilize enantiomers of chiral natural products as resources is that they are rarely obtained from natural resources. Construction of the corresponding library by chemical synthesis is hardly realized as well. To improve access to mirror-image natural products and their derivatives with potentially desirable bioactivities as valuable resources, a complementary approach needs to be developed. For this purpose, the author designed a new screening strategy for a virtual mirror-image library of chiral natural products, taking advantage of the mirror-image

protein (named D-protein, in which all chirality centers are mirror-inverted) technology. In this strategy, the screening of chiral natural product derivatives using a chemically synthesized D-protein corresponds to that of mirror-image natural products using a native protein (L-protein) in a mirror (Figure 3). This process involves the following: Initially, (1) the D-protein of the target molecule is prepared by chemical protein synthesis; (2) using the synthetic D-protein, chiral natural products are screened to identify hit compound(s); (3) the mirror-image structure(s) of the hit compound(s) is synthesized; and (4) the biological activities for the native target molecule (L-protein) would be assessed. In this strategy, screening of the mirror-image library could be performed only by syntheses of mirror-image substances including a target protein and hit compound(s) without syntheses of numerous mirror-image natural products.

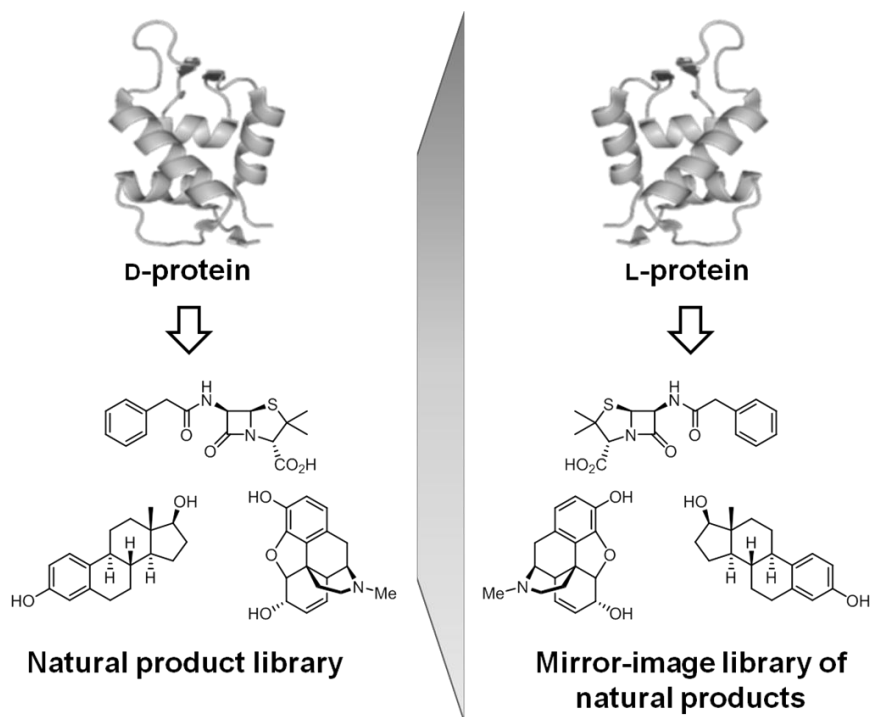


Figure 3. Concept of screening strategy for a virtual mirror-image library of natural products. Screening of chiral natural products using D-protein corresponds to that of mirror-image natural products using L-protein in a mirror.

Recent progress in chemical protein synthesis has facilitated preparation of small to medium-sized biomolecules. Taking advantage of unique solid support materials, longer peptide sequences can be synthesized by stepwise Fmoc-based solid-phase peptide synthesis (SPPS).¹² Additionally, a number of novel methods, including native chemical ligation (NCL), have been established.¹³ Developments of novel strategies for peptide thioester preparations¹⁴ and the desulfurization reaction¹⁵ have also facilitated a wide variety of NCL processes at a preferable position(s). Using

these technologies, a variety of D-proteins have also been synthesized to understand and elucidate biological phenomena. For example, the molecular basis of the antifreeze properties of the snow flea antifreeze protein was elucidated *via* chemical synthesis of both protein enantiomers and racemic protein crystallography.¹⁶ Crystal structures of the poly-ubiquitin-K27 were also obtained by using racemic proteins.¹⁷ Moreover, D-protein technology has been applied for elucidation of molecular recognition of biologically important proteins. Ambidextrous chiral recognition of GroEL/ES for molecular chaperone activity was assessed using a mirror-image protein of DapA.¹⁸ The stereoselectivity of the GroEL–GroES interaction was revealed by using a mirror-image protein of GroES.¹⁹ Flexible chiral recognition of cargo-based transport across the cellular membrane by a protective antigen was also revealed using mirror-image polypeptides combined with the lethal factor in native form.²⁰

The mirror-image protein technology was also used for identification of novel functional molecules (Figure 4). Mirror-image phage display (MIFD) led to identification of antiviral and anticancer D-peptides with resistance against peptidase-mediated degradation.²¹ Schumacher *et al.* identified D-peptide ligands for Src homology 3 (SH3) domain of c-Src in an initial proof-of-concept study.^{21a} HIV-1 entry inhibitors targeting the gp41 coiled-coil pocket were identified in the MIFD method.^{21b,c} Spiegelmers, which are nuclease-resistant L-RNA aptamers, were identified by

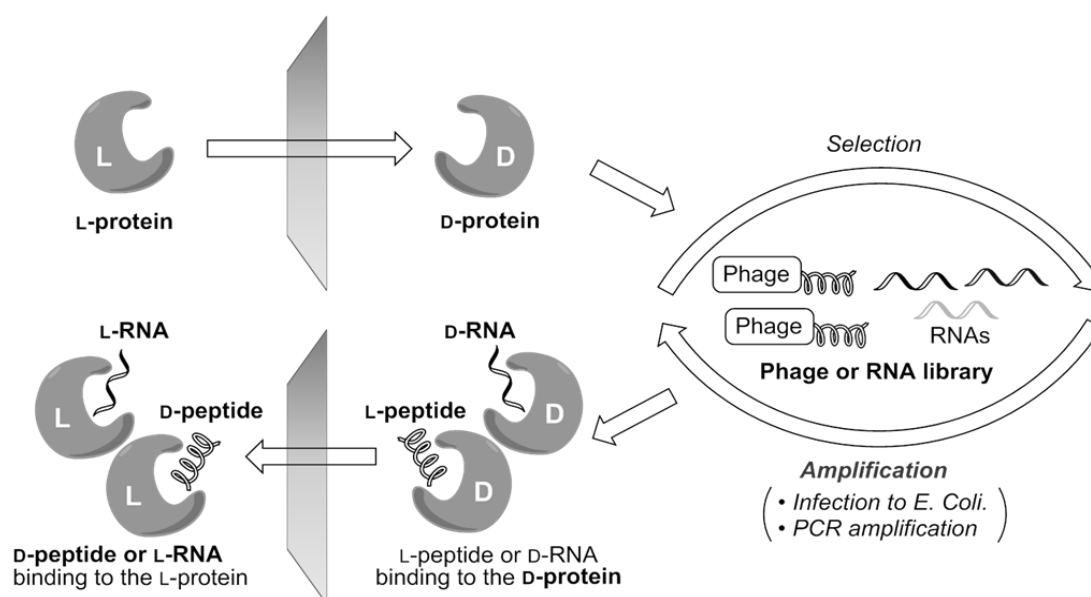


Figure 4. Illustration showing the generation of D-peptides and/or L-RNAs against L-proteins. Initially, a D-protein is synthesized. Naturally occurring phage or RNA molecules from a library that bind to the D-protein are then identified. Subsequently, amplification, cloning and sequencing of bound phages or RNAs are performed. Identified sequences are finally synthesized using D-amino acids or L-ribonucleotides.

screening an RNA library using target D-proteins, and some of them are now in clinical trials.²² In the mirror-image screening process, the hit peptides and RNAs are easily characterized by gene amplification and by phage polymerases, respectively. Small quantities of synthetic D-proteins are sufficient for screening a large number of peptides and RNAs with high diversity. However, for screening of natural products, these amplification techniques cannot be used. Preparation of significantly larger quantities of synthetic D-proteins is required for screening chiral natural products and their derivatives. To overcome these limitations, an effective detection and identification process of small-molecule hits *via* a high-throughput screening (HTS) process should be designed.

Chemical array screening is one of the effective approaches for the identification of small-molecule ligand–protein interactions.²³ This approach could explore protein–protein interaction (PPI) inhibitors against previously recognized binding pockets as well as search for potential binding molecules that interact with alternative or unexpected pockets present in the target proteins. In 2003, Kanoh and Osada *et al.* developed photo-crosslinked chemical array (Figure 5).²⁴ On the photo-crosslinked chemical array (76 × 25 mm), more than 3,000 small molecules are immobilized at a variety of positions to a carbene-based photo-cross linker without specific functional groups on the immobilized molecules. Fluorescence-labeled proteins are applied to the array and the hit compounds are detected by fluorescent signals using a microarray scanner. By using this technology, there have been some examples that have identified

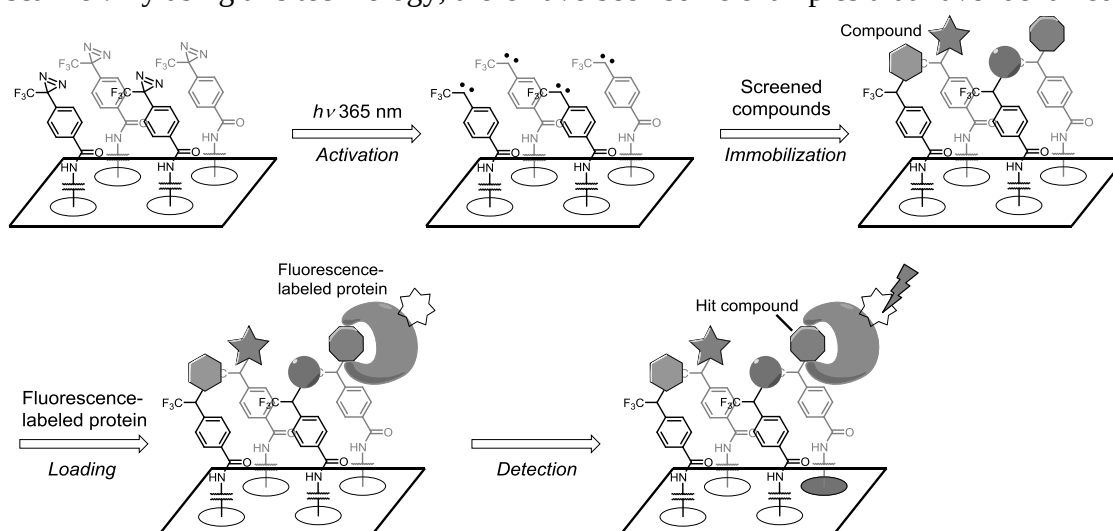


Figure 5. Schematic representation of the photo-crosslinked chemical array technology. Carbene produced by 365 nm ultraviolet light radiation immobilizes compounds in a functional-group-independent manner. The immobilized compounds are screened with fluorescence-labeled proteins, and the hit compounds are detected by fluorescent signals using microarray scanner.

bioactive compounds.²⁵ Because the compounds were spotted on microarray in a small volume and screened in HTS manner using minimal quantity of the synthetic fluorescent protein(s), the author expected screening process by using chemical array technology would be effective to identify novel hits from a virtual mirror-image library of natural products. Thus, the author decided to synthesize some domains of oncoproteins for the screening to identify novel anticancer agents.

In this thesis, the author describes development of screening process from a virtual mirror-image library of chiral natural products by using chemically synthesized mirror-image proteins.

In Chapter 1, Section 1, the author describes the chemical synthesis of L-MDM2 and L-MDMX proteins and development of screening process using chemical array and fluorescence polarization (FP) assay to identify novel peptidic inhibitors from in-house compound library.

In Chapter 1, Section 2, the author describes identification of a novel inhibitor against MDM2–p53 interaction from mirror-image library of natural products using D-MDM2 protein.

In Chapter 2, the author describes the chemical synthesis of both enantiomers of Grb2 SH2 domains and development of screening systems using chemical array and ELISA-based competitive assay.

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Chapter 1. Development of a Mirror-image Screening Process by Using Synthetic Proteins

Section 1. Affinity-based Screening of MDM2/MDMX-p53 Interaction Inhibitors by Chemical Array: Identification of Novel Peptidic Inhibitors

Summary

MDM2 and MDMX are oncoproteins that negatively regulate the activity and stability of the tumor suppressor protein p53. The inhibitors of protein-protein interactions (PPIs) of MDM2-p53 and MDMX-p53 represent potential anticancer agents. In this section, a novel approach for identifying MDM2-p53 and MDMX-p53 PPIs inhibitor candidates by affinity-based screening using a chemical array has been established. A number of compounds from an in-house compound library, which were immobilized onto a chemical array, were screened for interaction with fluorescence-labeled MDM2 and MDMX proteins. The subsequent fluorescence polarization (FP) assay identified peptidic compounds that inhibited MDM2-p53 and MDMX-p53 interactions.

MDM2 and MDMX are negative regulators of the tumor suppressor protein p53, and therefore confer tumor development and survival.¹ MDM2 and MDMX bind to the N-terminal transactivation domain of p53 with high affinity to inhibit functions associated with regulating responsive gene expression.² MDM2 has E3 ubiquitin ligase activity and thus also regulates the stability of p53 by proteasomal degradation, thereby conferring effective down-regulation of cellular p53 protein levels.³ MDMX has no ubiquitin ligase activity, yet forms a MDMX-MDM2 heterodimer via interaction between their C-terminal RING finger domains. This heterodimer stimulates MDM2-mediated ubiquitination of the p53 and subsequent degradation.⁴ As such, high expression of MDM2 and MDMX results in a reduction in the activity and expression level of the p53 in cancer cells, and PPI inhibitors against MDM2-p53 and/or MDMX-p53 interactions are expected as anticancer agents.

Inhibitory compounds that block the interaction between MDM2-p53 and MDMX-p53 have been identified using various types of screening technologies (Figure 1).⁵ For example, MDM2-p53 interaction inhibitors, nutlin-1, -2, and -3, were identified by surface plasmon resonance (SPR) analysis.⁶ SJ-172550 is an MDMX-p53 interaction inhibitor, which was identified by an FP assay.⁷ p53-like peptide, PMI and pDI (also named P4), were identified as MDM2 and MDMX inhibitors by phage

display.⁸ These facile technologies are based on the evaluation of inhibitory effects by compounds against p53 binding to MDM2 and MDMX. Among the inhibitors, some chiral compounds have different activities between their enantiomers. For example, nutlin-3a potently binds to MDM2 (IC_{50} : 0.09 μ M), while nutlin-3b (enantiomer of nutlin-3a) shows significantly less activity (IC_{50} : 13.6 μ M). Therefore, the author expected MDM2 and MDMX would have strong enantiomeric recognitions to small-molecules.

The author designed screening process using affinity-based chemical array screening and subsequent competitive FP assay to identify MDM2–p53 and MDMX–p53 interaction inhibitors efficiently. In this section, to investigate the feasibility of synthetic MDM2 and MDMX proteins for identification of their inhibitors, chemical synthesis of L-MDM2 and L-MDMX and application of the screening systems were performed.

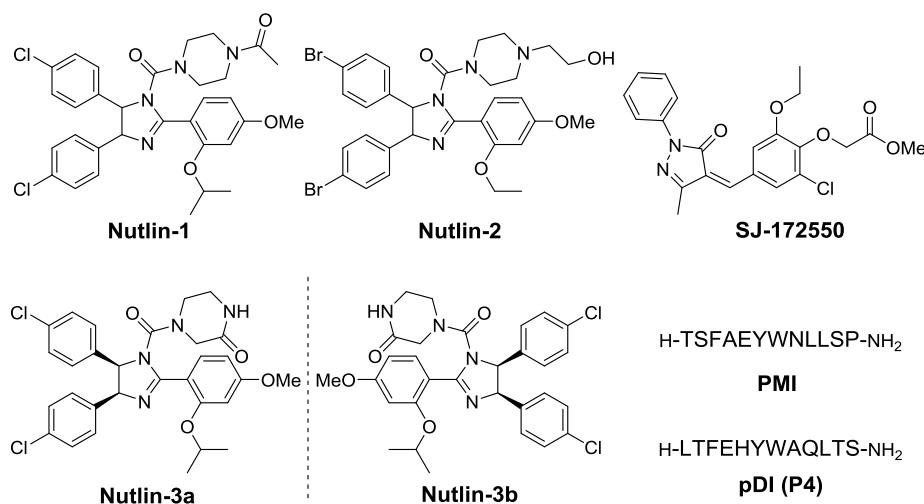
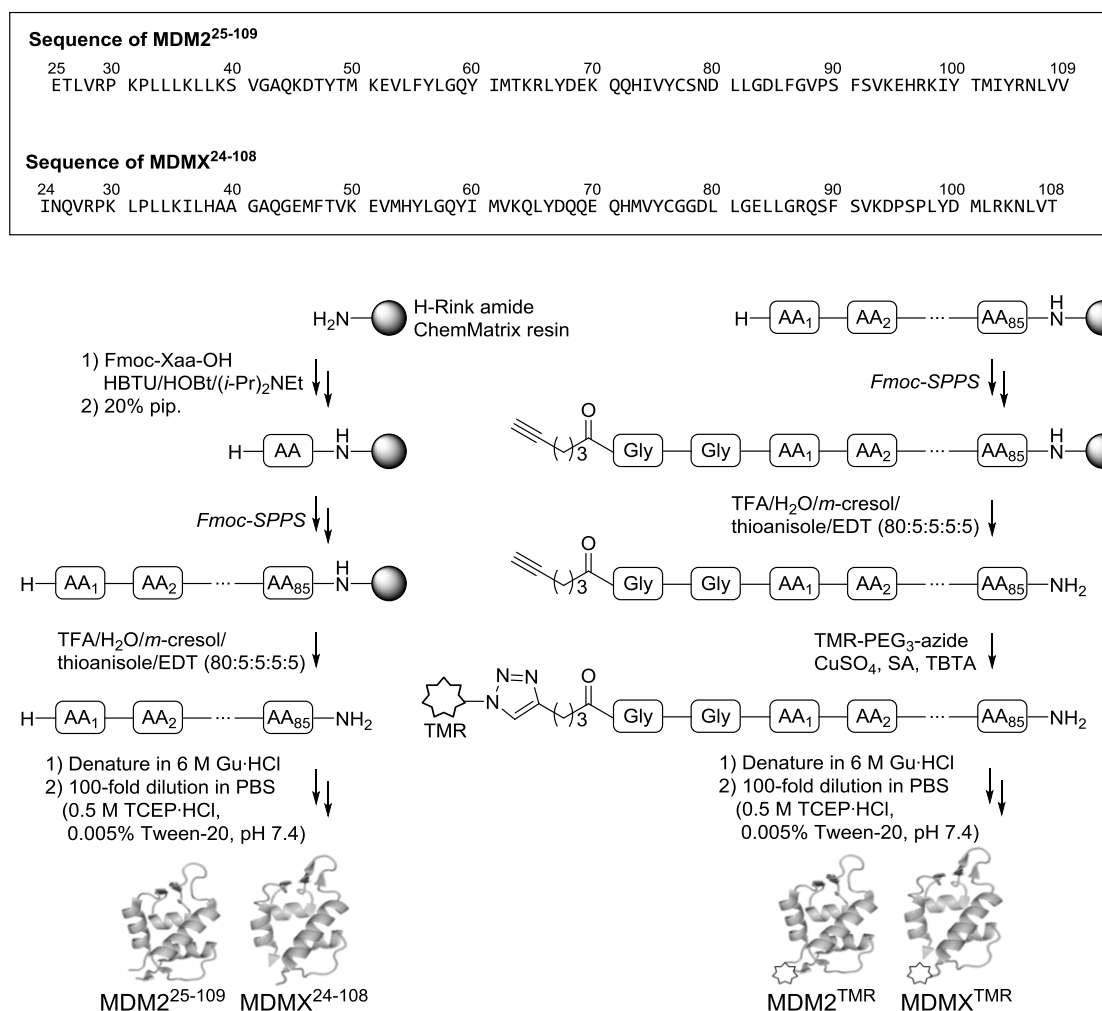


Figure 1. Structures of MDM2–p53 and/or MDMX–p53 interaction inhibitors.

Initially, the author investigated synthesis of MDM2 and MDMX and their fluorescent derivatives, which were used for chemical array screening. There have been a report on the synthesis of MDM2 and MDMX proteins by combining Boc-based solid-phase peptide synthesis (SPPS) with native chemical ligation.⁸ Although this approach would be applicable to mid-sized protein synthesis, a more facile stepwise Fmoc-based SPPS approach was undertaken in this study (Scheme 1).

The p53-binding domain of MDM2 and MDMX (designated MDM2^{25–109} and MDMX^{24–108}, respectively) were synthesized by standard Fmoc-based SPPS on an H-Rink Amide ChemMatrix resin using HBTU/HOBt/(*i*-Pr)₂NEt activation. After chain assembly, deprotection of the side chain protecting groups and cleavage from the resin

with the cocktail [TFA/thioanisole/*m*-cresol/EDT/H₂O (80:5:5:5:5)], followed by RP-HPLC purification afforded the expected peptides. For the preparation of fluorescent MDM2 and MDMX proteins bearing a single tetramethylrhodamine moiety (designated MDM2^{TMR} and MDMX^{TMR}, respectively), 5-hexynoic acid was conjugated via a diglycine linker on the resin. After purification of MDM2²⁵⁻¹⁰⁹ and MDMX²⁴⁻¹⁰⁸ with an N-terminal alkyne tag, treatment with TMR-(PEG)₃-azide in the presence of CuSO₄, sodium ascorbate (SA) and tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) provided fluorescent MDM2^{TMR} and MDMX^{TMR}, respectively. The synthetic MDM2 and MDMX proteins and their fluorescent derivatives, MDM2^{TMR} and MDMX^{TMR}, were denatured in 6 M guanidine hydrochloride (Gu·HCl) solution and refolded by 100-fold dilution of the solution to PBS buffer (pH 7.4) containing 0.5 mM tris(2-carboxyethyl)phosphine (TCEP) and 0.005% Tween-20.



Scheme 1. Synthesis of MDM2 and MDMX proteins.

To validate the structures of the synthetic proteins, the author evaluated circular

dichroism (CD) spectra of MDM2^{25–109} and MDMX^{24–108} (Figure 2). Negative bands at 208 nm and 222 nm indicated the presence of α -helix secondary structure elements, which is characteristic for MDM2 and MDMX, and supported by previous crystal structure and CD analyses.⁹

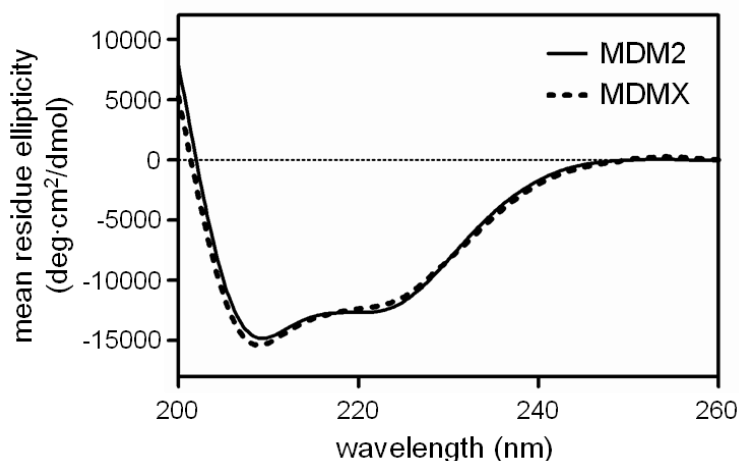


Figure 2. CD spectra of MDM2^{25–109} and MDMX^{24–108}. CD spectra of MDM2^{25–109} and MDMX^{24–108} were measured at room temperature in PBS containing 0.1 mM TCEP (pH 7.4).

To further verify the biological activity of the synthetic proteins, binding affinities of MDM2 and MDMX towards the p53 peptide sequence were evaluated by SPR analysis. Varying concentrations of MDM2 and MDMX proteins were analyzed by a NeutrAvidin-coated NLC sensor chip (BioRad) on which the biotinylated wild-type p53 peptide (biotinyl-aminocaproyl-GSGSSQETFSDLWKLLPEN-NH₂) was immobilized (Figure 3). MDM2^{25–109} and MDMX^{24–108} bound the p53 peptide with K_D values of 0.803 and 2.22 μ M, respectively, which supported the correct folding of these proteins.⁷ The binding affinities (K_D values) of MDM2^{TMR} and MDMX^{TMR} towards the p53 peptide were 0.616 and 2.14 μ M, respectively, indicating that N-terminal modification of synthetic MDM2 and MDMX had no effect on the folding of these proteins or the biological functions.¹⁰

The author next assessed the binding ability of MDM2^{TMR} and MDMX^{TMR} to the p53-like peptide (designated L-P4: H-LTFEHYWAQLTS-NH₂) immobilized on a microarray via carbene-mediated covalent bond formation. The enantiomer peptide (designated D-P4) was used as a negative control. MDM2^{TMR} and MDMX^{TMR} bound to L-p53 in a highly selective manner (Figure 4). Higher MDM2–p53 binding compared with the MDMX–p53 interaction was observed, which is consistent with the more potent binding affinity of MDM2 to the p53 peptide in the SPR analysis.

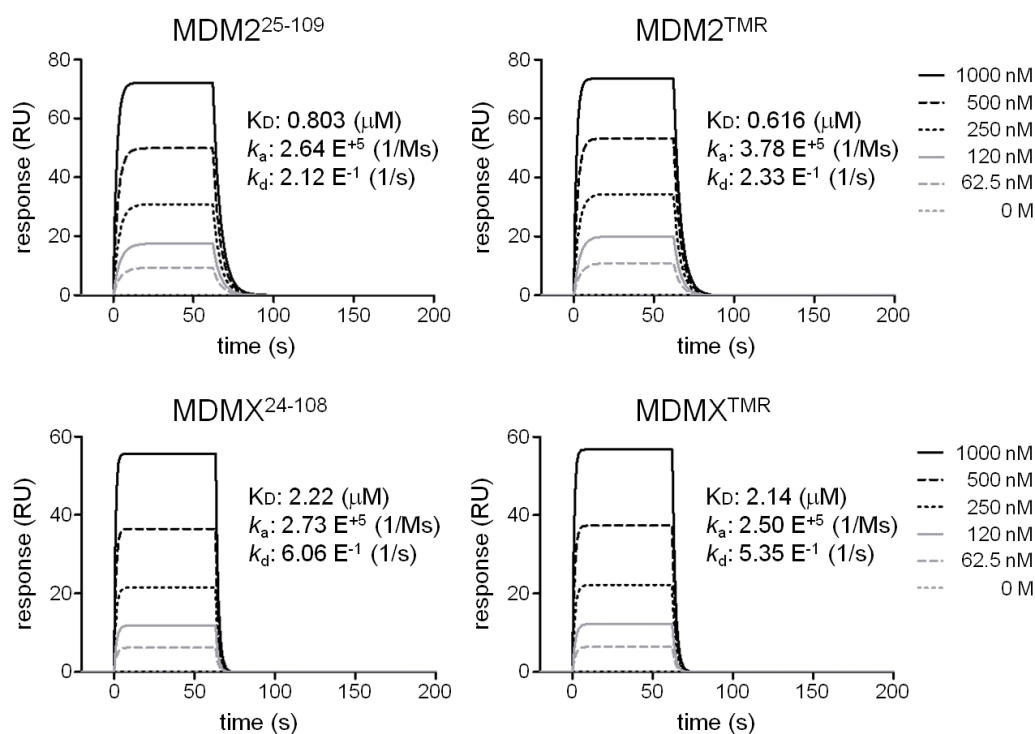


Figure 3. SPR analysis of MDM2²⁵⁻¹⁰⁹, MDM2^{TMR}, MDMX²⁴⁻¹⁰⁸ and MDMX^{TMR} towards p53 peptide. K_D values were determined by the calculation of k_a/k_d .

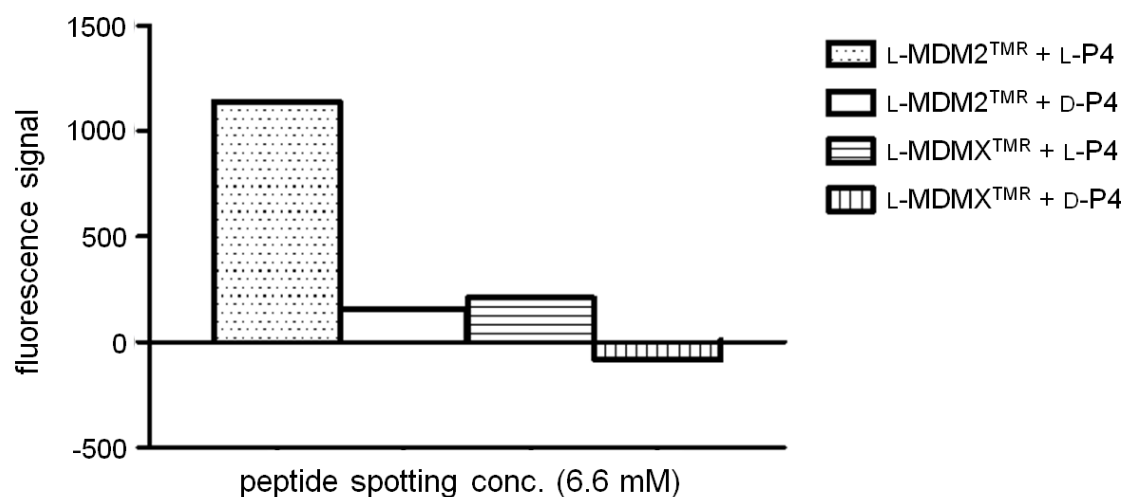


Figure 4. Chemical array analysis of MDM2^{TMR} and MDMX^{TMR} proteins using both enantiomers of p53-like peptide (P4). MDM2^{TMR} and MDMX^{TMR} binding (1 μM in 1% skim-milk-TBS-T) were assessed using a chemical array, on which L-P4 (H-LTFEHYWAQLTS-NH₂) or D-P4 was spotted at the concentration of 6.6 mM.

To identify the inhibitor candidates for MDM2-p53 and MDMX-p53 interactions, the author next carried out chemical array screening using the MDM2^{TMR} and MDMX^{TMR} proteins (Figure 5). Fifty-four hit compounds were identified from the

7,600 compounds of the in-house chemical library that were immobilized on the chemical array using 5 mM solutions. Of these, 6 compounds and 10 compounds showed selective binding to MDM2 and MDMX, respectively. The other 38 compounds exhibited binding to both MDM2 and MDMX.

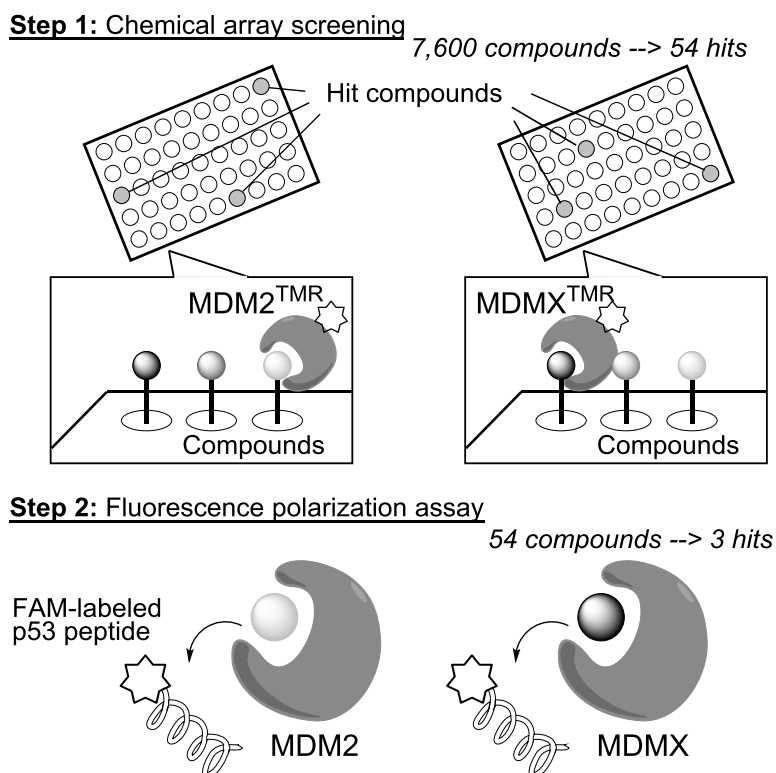


Figure 5. Two-step screening process using MDM2^{25–109} and MDMX^{24–108} proteins.

To select small molecules that bind to the p53 binding pocket(s) in MDM2 and MDMX, the inhibitory effects on MDM2–p53 and MDMX–p53 interactions were further evaluated for the 54 hit compounds by a FP assay using a fluorescein-labeled P4 peptide (FAM-LTFEHYWAQLTS-NH₂).¹¹ Three dual inhibitors against both interactions were obtained (Figure 6): KPYA52218 (**1**), KPYB00497 (**2**) and KPYB00556 (**3**) [IC₅₀(**1**) = 8.51 μM for MDM2, 32.4 μM for MDMX; IC₅₀(**2**) = 22.2 μM for MDM2, 42.1 μM for MDMX; IC₅₀(**3**) = 21.3 μM for MDM2, 52.5 μM for MDMX]. In the initial chemical array analysis, these compounds **1–3** were also identified to show dual binding to MDM2 and MDMX. Although MDM2 and MDMX share homologous sequences in their p53-binding domains, many of the known MDM2–p53 inhibitors do not effectively interact with MDMX–p53 probably because of the shallower and wider p53-binding pocket in MDMX.¹² Agents that disrupt MDM2–p53 interaction could inhibit tumor growth, but they are less effective in cancer cells that express high level of MDMX.¹³ These dual inhibitors represent promising

anticancer agent leads against cancer cells in which MDM2 and MDMX are overexpressed.¹⁴ To gain insight into the effect on cell proliferation, growth inhibition by compounds **1–3** against human lung carcinoma-derived A549 cells was assessed. Compounds **1** and **3** showed no effect on cell growth, whereas cell growth was inhibited by compound **2** ($EC_{50} = 13.7 \mu\text{M}$).

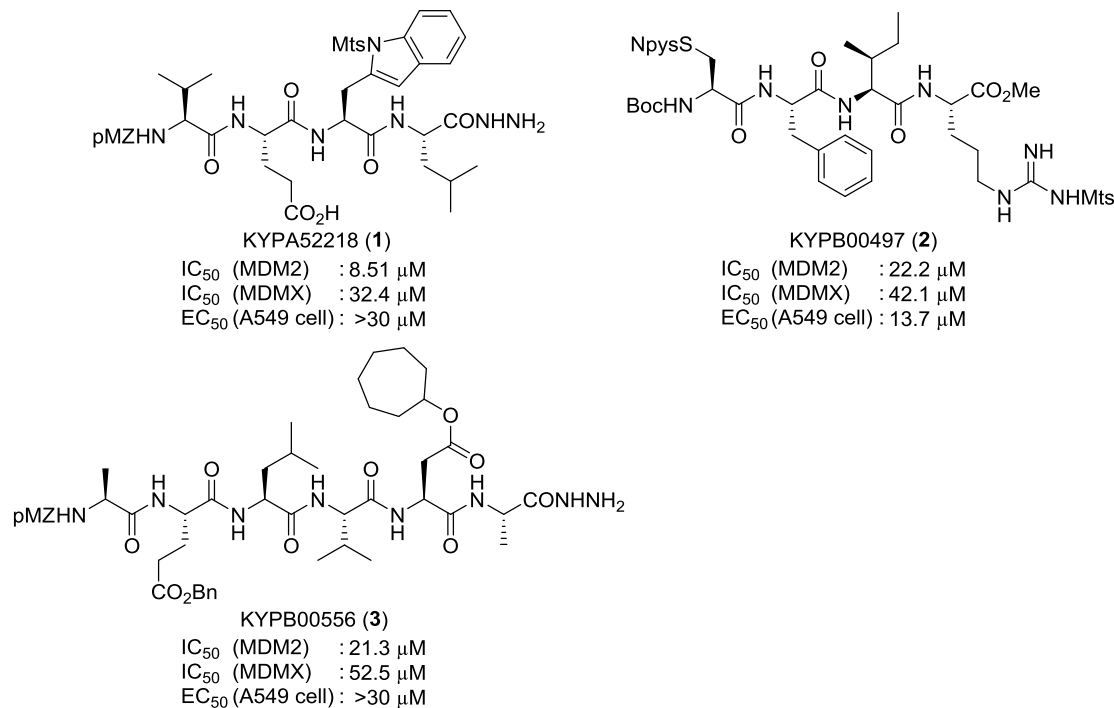


Figure 6. Structures and biological activities of the hit compounds

Of note, in this screening process, the biological data from FP assays did not necessarily correspond to the binding ability obtained in the initial microarray screening, possibly owing to structural modification of the molecules bound to the microarray and potential binding to other binding site(s) than the p53 binding pocket. However, the microarray technology successfully facilitated the selection process for the identification of potential MDM2/MDMX–p53 interaction inhibitors.

In conclusion, the author has established screening protocols for binding molecules of MDM2 and MDMX by chemical array technology using synthetic fluorescent proteins. The preliminary screening of an in-house chemical library identified a number of peptidic binding molecules towards MDM2 and MDMX. The subsequent FP assay identified three dual inhibitors of MDM2–p53 and MDMX–p53 interactions. This new screening strategy for MDM2 and MDMX proteins using the affinity-based chemical array and subsequent FP assay should facilitate the identification process of MDM2–p53 and MDMX–p53 inhibitors.

Experimental Section

General Methods

Fmoc-protected amino acids were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan), Kokusan Chemical Co. Ltd. (Kanagawa, Japan) or Sigma-Aldrich JAPAN (Tokyo, Japan). Other chemicals were purchased from Nacalai Tesque Inc. (Kyoto, Japan). For analytical HPLC for MDM2 and MDMX proteins, Cosmosil 5C18-AR300 (4.6 × 250 mm, Nacalai Tesque Inc.) was employed with a linear gradient of CH₃CN containing 0.1% (v/v) TFA at a flow rate of 1 mL/min. Cosmosil 5C18-ARII column (4.6 × 250 mm, Nacalai Tesque Inc.) was employed for the analysis of the other peptides and peptidic compounds. ¹H NMR and ¹³C NMR spectra were recorded using a JEOL ECA-500 spectrometer. ¹H NMR spectra were tabulated as follows: chemical shift, multiplicity (br: broad, s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet), number of protons, and coupling constants.

Synthesis of MDM2²⁵⁻¹⁰⁹ and MDMX²⁴⁻¹⁰⁸

MDM2²⁵⁻¹⁰⁹ and MDMX²⁴⁻¹⁰⁸ were synthesized using automatic peptide synthesizer (PSSM-8, Shimadzu Corporation Ltd.) by Fmoc-based SPPS on H-Rink Amide ChemMatrix resin (0.4-0.6mmol/g). For the side-chain protection, *t*-Bu ester for Asp and Glu; 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg; *t*-Bu for Thr, Tyr and Ser; Boc for Lys and Trp; and Trt for Gln, Asn, His, and Cys were employed for side-chain protection. Fmoc-Phe-OH (5 eq) was coupled by using HBTU (5 eq), HOBt (5 eq) and (*i*-Pr)₂NEt (10 eq) in DMF-NMP, and the other Fmoc-protected amino acids were coupled by using HBTU (5 eq), HOBt (5 eq), (*i*-Pr)₂NEt (10 eq) in DMF. In each coupling of amino acid, coupling reaction was carried out for 45 min twice. Fmoc-protecting group was removed by treating the resin with 20% piperidine in DMF. After chain assembly, the resulting protected peptide resin was treated with TFA/thioanisole/*m*-cresol/EDT/H₂O (80:5:5:5:5) at room temperature for 2 h. After removal of the resin by filtration, the filtrate was poured into ice-cold dry Et₂O. The resulting powder was collected by centrifugation and then washed three times with ice-cold dry Et₂O. The crude product was purified by HPLC on a Cosmosil 5C18-AR300 preparative column (Nacalai Tesque, 20 × 250 mm) with a linear gradient of CH₃CN containing 0.1% TFA at a flow rate of 8 mL/min, affording the expected peptides as white powder. The polypeptides were characterized by ESI-MS and purity was calculated as >95% by HPLC.

MS (ESI) for MDM2²⁵⁻¹⁰⁹: Calcd for C₄₆₂H₇₃₉N₁₁₇O₁₂₃S₄: 10027.42; observed: [M+12H]¹²⁺ *m/z* = 836.80, [M+11H]¹¹⁺ *m/z* = 912.45, [M+10H]¹⁰⁺ *m/z* = 1003.91, [M+9H]⁹⁺ *m/z* = 1115.26, [M+8H]⁸⁺ *m/z* = 1254.33, [M+7H]⁷⁺ *m/z* = 1443.55, [M+6H]⁶⁺ *m/z* = 1672.32

MS (ESI) for MDMX²⁴⁻¹⁰⁸: Calcd for C₄₃₈H₇₀₁N₁₁₇O₁₂₁S₆: 9733.08; observed: [M+12H]¹²⁺ *m/z* = 811.68, [M+11H]¹¹⁺ *m/z* = 885.91, [M+10H]¹⁰⁺ *m/z* = 974.42, [M+9H]⁹⁺ *m/z* = 1082.62, [M+8H]⁸⁺ *m/z* = 1217.89, [M+7H]⁷⁺ *m/z* = 1391.30, [M+6H]⁶⁺ *m/z* = 1623.57

Synthesis of MDM2^{TMR} and MDMX^{TMR}

Protected MDM2²⁵⁻¹⁰⁹ and MDMX²⁴⁻¹⁰⁸ were synthesized according to the identical procedure for the synthesis of MDM2²⁵⁻¹⁰⁹ and MDMX²⁴⁻¹⁰⁸. After assembling the full length sequence, two glycine residues (5 eq) and 5-hexynoic acid (5 eq) were coupled with HBTU (5 eq), HOBt (5 eq) and (*i*-Pr)₂NEt (10 eq) in DMF. The resulting protected peptides were subjected to the deprotection, cleavage from resin, and HPLC purification. 200 mM CuSO₄ in H₂O (20 μL, 4 μmol) and 200 mM tris[(1-benzoyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) in DMSO (20 μL, 4 μmol) were added to H₂O-DMSO-*t*-BuOH (15:5:27, 470 μL) and the solution was stirred for 3 min. Then, a sodium ascorbate solution (200 mM, 40 μL, 8 μmol) was added to the mixture in several pulses. This reagent cocktail was added to the mixture of MDM2 or MDMX (200 nmol) and TMR-(PEG)₃-azide (1 μmol) in DMSO (100 μL). After 15 min, the reaction mixture was purified by RP-HPLC on a Cosmosil 5C18-AR300 preparative column (Nacalai Tesque, 20 × 250 mm) with a linear gradient of CH₃CN containing 0.1% TFA at a flow rate of 8 mL/min. This purification afforded the TMR-labeled polypeptide dimer with a disulfide bond via the Cys residues (Cys⁸² in MDM2; Cys⁸¹ in MDMX). The collected TMR-labeled polypeptide dimer was dissolved in 10 mM TCEP·HCl in 6 M Gu·HCl/H₂O solution at 37 °C for 5 h, followed by RP-HPLC purification on a Cosmosil 5C18-AR300 preparative column (Nacalai Tesque, 20 × 250 mm) with a linear gradient of CH₃CN containing 0.1% TFA at a flow rate of 8 mL/min. This purification procedure afforded the monomer polypeptide as a pink powder (80 nmol, 40%).

MS (ESI) for MDM2^{TMR}: Calcd for C₅₀₅H₇₈₉N₁₂₅O₁₃₃S₄: 10865.79; observed: [M+13H]¹³⁺ *m/z* = 836.55, [M+12H]¹²⁺ *m/z* = 906.36, [M+11H]¹¹⁺ *m/z* = 989.10, [M+10H]¹⁰⁺ *m/z* = 1087.60, [M+9H]⁹⁺ *m/z* = 1208.20, [M+8H]⁸⁺ *m/z* = 1359.00, [M+7H]⁷⁺ *m/z* = 1553.07 [M+6H]⁶⁺ *m/z* = 1812.23

MS (ESI) for MDMXTM: Calcd for C₄₈₁H₇₅₁N₁₂₅O₁₃₁S₆: 10571.44; observed: [M+14H]¹⁴⁺ *m/z* = 756.19, [M+13H]¹³⁺ *m/z* = 814.28, [M+12H]¹²⁺ *m/z* = 881.98, [M+11H]¹¹⁺ *m/z* = 962.15, [M+10H]¹⁰⁺ *m/z* = 1058.36, [M+9H]⁹⁺ *m/z* = 1175.58, [M+8H]⁸⁺ *m/z* = 1322.82, [M+7H]⁷⁺ *m/z* = 1511.24, [M+6H]⁶⁺ *m/z* = 1762.70

Folding of Synthetic MDM2 and MDMX Proteins

Lyophilized polypeptide (1 mg/mL) was dissolved in 6 M Gu·HCl containing 1 mM TCEP·HCl, followed by 100-fold dilution with PBS (pH 7.4) containing 0.5 mM TCEP·HCl and 0.005% Tween-20 at 4 °C. The solution was stored at 4 °C overnight, and the solution was concentrated using a MWCO 3000 centrifugal filtration membrane (Millipore, Amicon-Ultra 3kDa).

Synthesis of p53 Peptides and Their Derivatives

All p53 and p53-like peptides were synthesized by Fmoc-based SPPS on Rink-amide resin (0.66 mmol/g, 45.5 mg, 0.025 mmol). Fmoc-protected amino acids (3 eq) were coupled by using DIPCI (3 eq) and HOBt (3 eq) in DMF. The Fmoc-protecting group was removed by treating the resin with 20% piperidine in DMF. Coupling of biotin (0.125 mmol) was carried out with HBTU (5 eq), HOBt (5 eq) and (*i*-Pr)₂NEt (10 eq) in DMF. Coupling of 5-carboxyfluorescein (5 eq) was carried out with DIPCI (5 eq) and HOBt (10 eq) in DMF. The resulting protected peptide resin was treated with TFA/thioanisole/*m*-cresol/EDT/H₂O (80:5:5:5:5) at room temperature for 2 h. After removal of the resin by filtration, the filtrate was poured into ice-cold dry Et₂O. The resulting powder was collected by centrifugation and then washed three times with ice-cold dry Et₂O. The crude product was purified by HPLC on a Cosmosil 5C18-ARII preparative column (Nacalai Tesque, 20 × 250 mm). All peptides were characterized by ESI-MS or MALDI-TOF-MS and the purity was ascertained by HPLC as >95%.

Biotin-labeled p53 peptide: MS (MALDI-TOF) calcd for C₁₀₈H₁₆₆N₂₇O₃₅S [M+H]⁺ 2434.18; found 2434.23

L-P4: MS (MALDI-TOF) calcd for C₇₁H₁₀₀N₁₇O₁₉ [M+H]⁺ 1494.74; found 1494.61.

D-P4: MS (MALDI-TOF) calcd for C₇₁H₁₀₀N₁₇O₁₉ [M+H]⁺ 1494.74; found 1494.67.

Fluorescein-labeled P4 peptide: MS (ESI) calcd for C₉₂H₁₁₀N₁₇O₂₅ [M+H]⁺ 1852.79, found 1852.95

SPR Analysis

Binding kinetics analysis was carried out on a ProteOn XRP360 instrument using an N-terminal biotinylated wild-type p53 peptide immobilized on a NeutrAvidin-coated

NLC sensor chip (43 RUs) at 20 °C. PBS (pH 7.4) containing 0.005% Tween-20 was used as the running buffer.

Screening by the Chemical Array

The photoaffinity linker-coated (PALC) slides were prepared according to previous reports using amine-coated slides and the photoaffinity linker.¹⁵ A solution of compounds (5 mM in DMSO) from the in-house chemical library was immobilized onto the PALC glass slides with a chemical arrayer equipped with 24 stamping pins. The slides were exposed to UV irradiation of 4 J/cm² at 365 nm using a CL-1000L UV crosslinker (UVP, CA). The slides were washed successively with DMSO, DMF, acetonitrile, THF, dichloromethane, EtOH, and ultra-pure water (5 min, 3 times each), and dried. MDM2^{TMR} (1 μM in 1% skim-milk-TBS-T) and MDMX^{TMR} (1 μM in 1% skim-milk-TBS-T) were incubated with the glass slide for 1 h, and then washed with TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) (5 min, 3 times). The slides were dried and scanned at 532 nm on a GenePix scanner. The fluorescence signals were quantified with GenePixPro.

Fluorescence Polarization Assay

Fluorescence polarization assays were carried out in PBS containing 0.005% Tween-20 using a fluorescein-labeled P4 peptide (0.5 nM) and MDM2²⁵⁻¹⁰⁹ (10 nM) or MDMX²⁴⁻¹⁰⁸ (50 nM) in black 96-well non-binding surface assay plates (Corning). For the inhibitor assays, compounds were preincubated with the synthetic protein for 30 min. The fluorescein-labeled P4 peptide was then added and incubated for 30 min. The unlabeled L-P4 peptide was used as the positive control. FP signals were analyzed using an EnVision Xcite plate reader with a 480-nm excitation filter and a 535-nm emission filter.

Cell Growth Inhibition Assay

A549 cells were cultured in DMEM medium (Sigma) supplemented with 10% (v/v) FBS at 37 °C in a 5% CO₂-incubator. Cell based assays using A549 cells were performed in 96-well plates (BD Falcon). A549 cells were seeded at 500 cells/well in 50 μL of DMEM, and placed for 6 h. Chemical compounds in DMSO were diluted 250-fold with the culture medium in advance. Following the addition of the fresh culture medium (40 μL), the chemical diluents (30 μL) were also added to the cell cultures. The final volume of DMSO in the medium was equal to 0.1% (v/v). The cells under chemical treatment were incubated for a further 72 h. The wells in the plates were

washed twice with the cultured medium without phenol-red. After 1 h incubation with 100 μ L of the medium, the cell culture in each well was supplemented with the MTS reagent (20 μ L, Promega), followed by incubation for an additional 40 min. Absorbance at 490 nm of each well was measured using a Wallac 1420 ARVO SX multilabel counter (Perkin Elmer).

Characterization Data of Hit Compounds (1-3)

KPYA 52218 (**1**). ^1H NMR (500 MHz, DMSO- d_6) δ 0.77 (t, J = 7.7 Hz, 6H), 0.85 (d, J = 6.3 Hz, 3H), 0.90 (d, J = 6.9 Hz, 3H), 1.45 (m, 1H), 1.56 (m, 1H), 1.64 (m, 1H), 1.74 (m, 1H), 1.87 (m, 1H), 1.95 (m, 1H), 2.21 (dd, J = 5.7, 10.3 Hz, 2H), 2.25 (s, 3H), 2.40 (s, 6H), 2.91 (dd, J = 10.3, 14.9 Hz, 1H), 3.10 (d, J = 6.0, 12.0 Hz, 1H), 3.73 (s, 3H), 3.88 (dd, J = 6.9, 8.6 Hz, 1H), 4.32 (m, 1H), 4.38 (m, 1H), 4.64 (m, 1H), 4.92 (q, J = 12.4 Hz, 2H), 6.88 (d, J = 8.6 Hz, 2H), 7.08 (s, 2H), 7.17-7.28 (m, 5H), 7.67 (s, 1H), 7.77 (dd, J = 2.3, 6.3 Hz, 1H), 7.82 (d, J = 8.0 Hz, 1H), 8.17 (d, J = 8.0 Hz, 1H), 8.42 (d, J = 8.0 Hz, 1H), 10.85 (br, 1H); ^{13}C NMR (500 MHz, DMSO- d_6) δ 17.9, 19.1, 20.4, 21.4, 21.9, 22.8, 24.0, 26.8, 29.7, 30.1, 40.6, 49.6, 51.3, 52.1, 55.0, 60.1, 65.3, 111.6, 113.7, 115.5, 120.0, 122.5, 124.3, 125.0, 128.7, 129.6, 129.8, 132.3, 133.8, 139.5, 144.2, 156.2, 158.9, 170.9, 171.3, 174.0; MS (MALDI-TOF) calcd for $\text{C}_{45}\text{H}_{59}\text{N}_7\text{NaO}_{11}\text{S}$ $[\text{M}+\text{Na}]^+$ 928.39, found 928.37.

KPYB 00497 (**2**). ^1H NMR (500 MHz, DMSO- d_6) δ 0.78 (t, J = 7.4 Hz, 3H), 0.82 (d, J = 6.9 Hz, 3H), 1.04 (m, 1H), 1.2-1.4 (m, 3H), 1.38 (s, 9H), 1.56 (m, 1H), 1.66 (m, 2H), 2.21 (s, 3H), 2.57 (s, 6H), 2.79 (dd, 1H), 2.9-3.1 (m, 5H), 3.59 (s, 3H), 4.1-4.2 (m, 3H), 4.59 (m, 1H), 6.47 (br, 1H), 6.91 (s, 2H), 7.1-7.2 (m, 5H), 7.24 (d, J = 8.0 Hz, 1H), 7.60 (dd, J = 4.6, 8.0 Hz, 1H), 7.87 (d, J = 8.0 Hz, 1H), 7.97 (d, J = 8.0 Hz, 1H), 8.35 (d, J = 8.0 Hz, 1H), 8.64 (dd, J = 1.1, 8.6 Hz, 1H), 8.89 (dd, J = 1.1, 4.6 Hz, 1H); ^{13}C NMR (500 MHz, DMSO- d_6) δ 10.8, 15.0, 20.3, 22.4, 24.1, 27.9, 28.1, 36.9, 37.4, 40.3, 51.7, 51.8, 53.2, 54.1, 56.3, 78.5, 122.0, 126.2, 127.8, 129.3, 131.0, 134.5, 136.9, 137.2, 139.6, 142.6, 154.1, 155.0, 155.1, 156.3, 170.0, 170.1, 171.0, 172.1; MS (MALDI-TOF) calcd for $\text{C}_{44}\text{H}_{61}\text{N}_9\text{NaO}_{11}\text{S}_3$ $[\text{M}+\text{Na}]^+$ 1010.35, found 1010.52.

KPYB 00556 (**3**). ^1H NMR (500 MHz, DMSO- d_6) δ 0.7-0.9 (m, 12H), 1.17 (d, J = 6.9 Hz, 3H), 1.21 (d, J = 7.4 Hz, 3H), 1.3-1.6 (m, 13H), 1.80 (m, 3H), 1.92 (m, 2H), 2.38 (m, 2H), 2.52 (dd, J = 1.78, 10.9 Hz, 1H), 2.75 (dd, J = 4.9, 16.3 Hz, 1H), 3.74 (s, 3H), 4.03 (m, 1H), 4.13 (dd, J = 6.9, 8.6 Hz, 1H), 4.23 (m, 1H), 4.30 (m, 1H), 4.35 (m, 1H), 4.60 (m, 1H), 4.80 (m, 1H), 4.92 (dd, J = 11.7, 30.6 Hz, 2H), 5.08 (s, 2H), 6.90 (d, J =

8.0 Hz, 2H), 7.27 (d, $J = 8.6$ Hz, 2H), 7.3-7.4 (m, 5H), 7.41 (d, $J = 8.0$ Hz, 1H), 7.77 (d, $J = 8.0$ Hz, 1H), 7.92 (d, $J = 8.0$ Hz, 1H), 7.96-7.98 (m, 2H), 8.24 (d, $J = 8.0$ Hz, 1H), 10.07 (br, 1H); ^{13}C NMR (500 MHz, DMSO- d_6) δ 17.9, 18.0, 19.0, 21.5, 22.3, 23.1, 24.0, 27.2, 27.7, 30.0, 30.7, 33.1, 36.0, 40.3, 47.1, 49.2, 50.1, 51.0, 51.5, 55.1, 57.4, 65.2, 65.4, 74.7, 113.7, 127.8, 127.9, 128.4, 128.8, 129.7, 136.2, 155.8, 159.0, 169.3, 169.9, 170.7, 170.8, 171.4, 171.7, 172.2, 172.5; MS (MALDI-TOF) calcd for $\text{C}_{49}\text{H}_{72}\text{N}_8\text{NaO}_{13}$ $[\text{M}+\text{Na}]^+$ 1003.51, found 1003.61.

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Chapter 1. Development of a Mirror-image Screening Process by Using Synthetic Proteins

Section 2. Screening of a Virtual Mirror-image Library of Natural Products

Summary

The author established a facile access to an unexplored mirror-image library of chiral natural product derivatives using D-protein technology. In this process, two chemical syntheses of mirror-image substances including a target protein and hit compound(s) allow the lead discovery from a virtual mirror-image library without synthesis of numerous mirror-image compounds.

To screen the mirror-image library of chiral natural products and their derivatives for MDM2–p53 interaction inhibitors, the author performed the designed screening process using chemical array screening and subsequent FP assay with synthetic proteins:¹ (1) the D-protein of the target molecule is prepared by chemical protein synthesis; (2) using the synthetic D-protein, chiral natural products are screened to identify hit compound(s); (3) the mirror-image structure(s) of the hit compound(s) is synthesized; and (4) the biological activities for the native target molecule (L-protein) would be assessed (Figure 1).

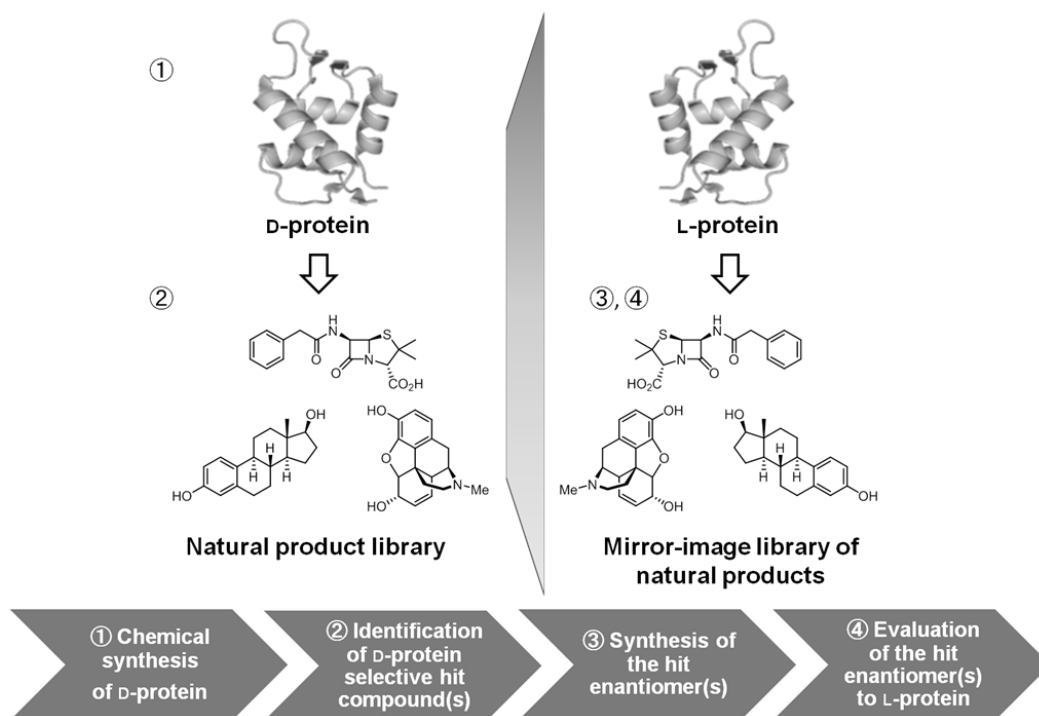


Figure 1. Concept of mirror-image screening of chiral natural products.

Initially, the author synthesized D-MDM2²⁵⁻¹⁰⁹ by standard Fmoc-based solid-phase peptide synthesis (SPPS) according to the identical protocol described in section 1 of chapter 1. The tetramethylrhodamine (TMR)-labeled protein (D-MDM2^{TMR}) was also prepared by conjugation of TMR-(PEG)₃-azide onto the N-terminal alkyne tag of D-MDM2²⁵⁻¹⁰⁹. The folded L-MDM2²⁵⁻¹⁰⁹ and D-MDM2²⁵⁻¹⁰⁹ had symmetric circular dichroism (CD) spectra, supporting the presence of mirror-image α -helices (Figure 2).

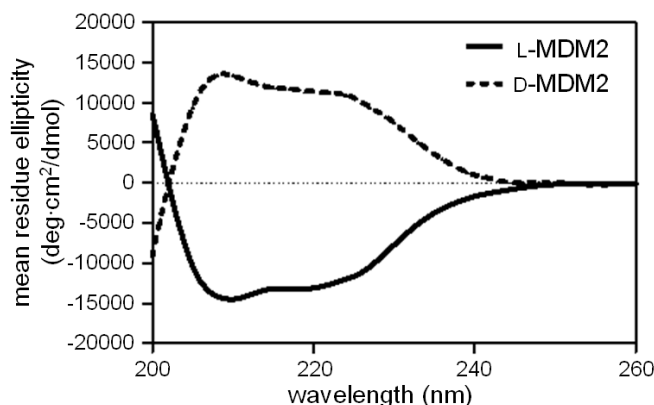
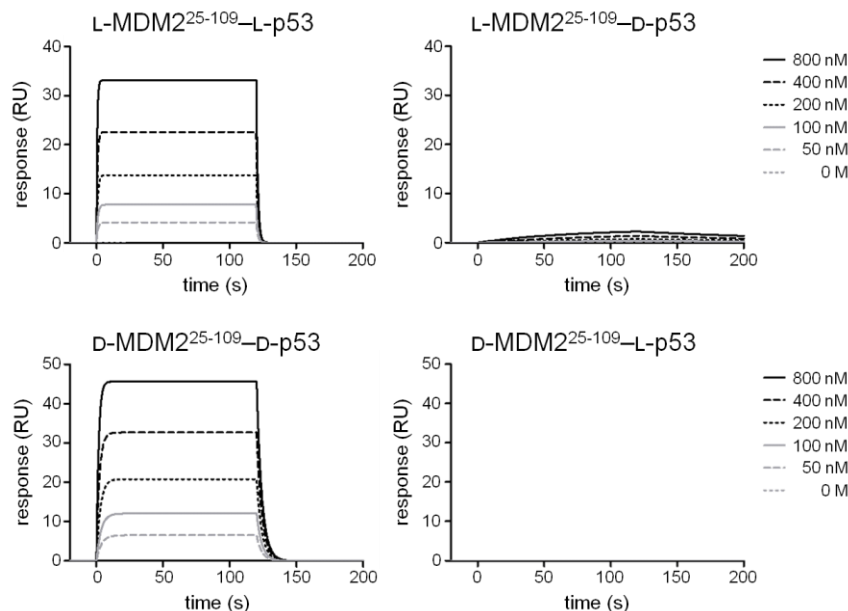


Figure 2. CD spectra of synthetic L-MDM2 and D-MDM2 proteins. Spectra of L-MDM2²⁵⁻¹⁰⁹ and D-MDM2²⁵⁻¹⁰⁹ were measured at 25 °C in PBS containing 0.5 mM tris(2-carboxyethyl)phosphine (TCEP) and 0.005% Tween-20 (pH 7.4).



Analyte/Ligand	k_a (1/Ms)	k_d (1/s)	K_D (mol/L)
L-MDM2 ²⁵⁻¹⁰⁹ /L-p53	$1.1 \pm 0.75 \text{ E}^+6$	$8.5 \pm 0.16 \text{ E}^-1$	$7.5 \pm 0.40 \text{ E}^-7$
D-MDM2 ²⁵⁻¹⁰⁹ /D-p53	$5.6 \pm 0.69 \text{ E}^+5$	$2.8 \pm 0.24 \text{ E}^-1$	$5.1 \pm 0.21 \text{ E}^-7$

Figure 3. SPR analysis of p53 binding to synthetic MDM2²⁵⁻¹⁰⁹. K_D values were determined by the calculation of k_a/k_d .

To verify the biological activity of the synthetic MDM2 proteins, the binding affinities toward biotinylated p53 peptides (biotinyl-aminocaproyl-GSGSSQETFSDL-WKLLPEN-NH₂) were evaluated by surface plasmon resonance (SPR) analysis (Figure 3). The high-affinity bindings of L-MDM2–L-p53 peptide and D-MDM2–D-p53 peptide were observed [K_D (L-MDM2–L-p53): $0.75 \pm 0.04 \mu\text{M}$; K_D (D-MDM2–D-p53): $0.51 \pm 0.02 \mu\text{M}$], while the binding activities of L-MDM2–D-p53 peptide and D-MDM2–L-p53 peptide were practically nil, suggesting high enantiomeric recognition of MDM2 toward p53.

The author next performed screening of natural products and their derivatives using both enantiomers of MDM2 protein. Chemical array screening of 22,293 compounds including natural products and their derivatives from RIKEN NPDepo was carried out using L-MDM2^{TMR} and D-MDM2^{TMR}. Among 43 initial hit compounds, four compounds showed inhibitory activity against L-MDM2–L-p53 or D-MDM2–D-p53 interactions in the competitive binding inhibition assay by fluorescence polarization (FP) (Figure 4). Of these, a chiral α -tocopherol derivative NP843 (**1a**) was identified to be a selective inhibitor against D-MDM2–D-p53 interaction (Figure 5).

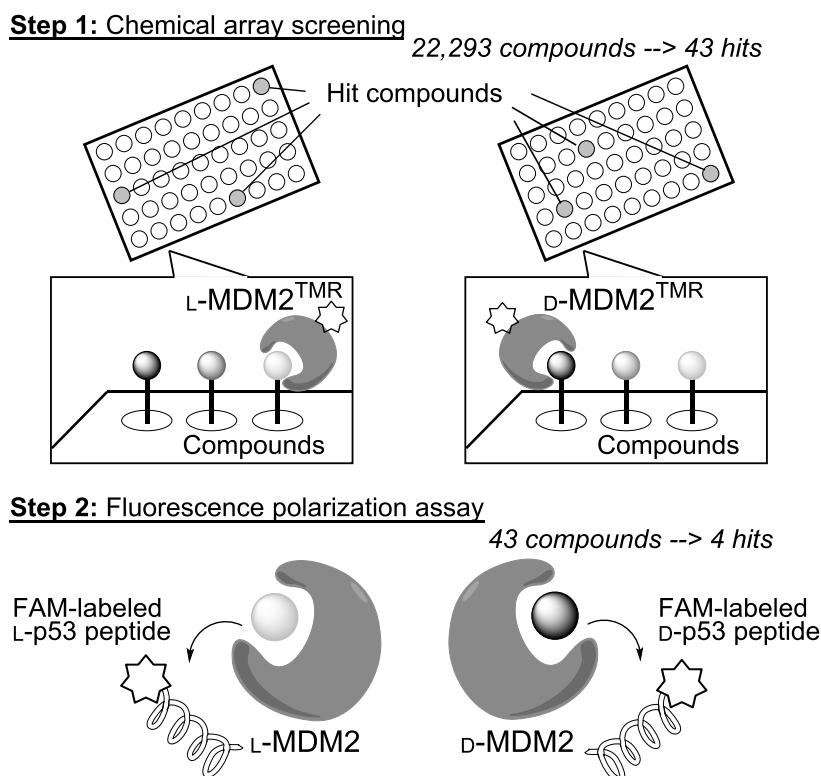


Figure 4. Two-step screening process using D-MDM2^{25–109}.

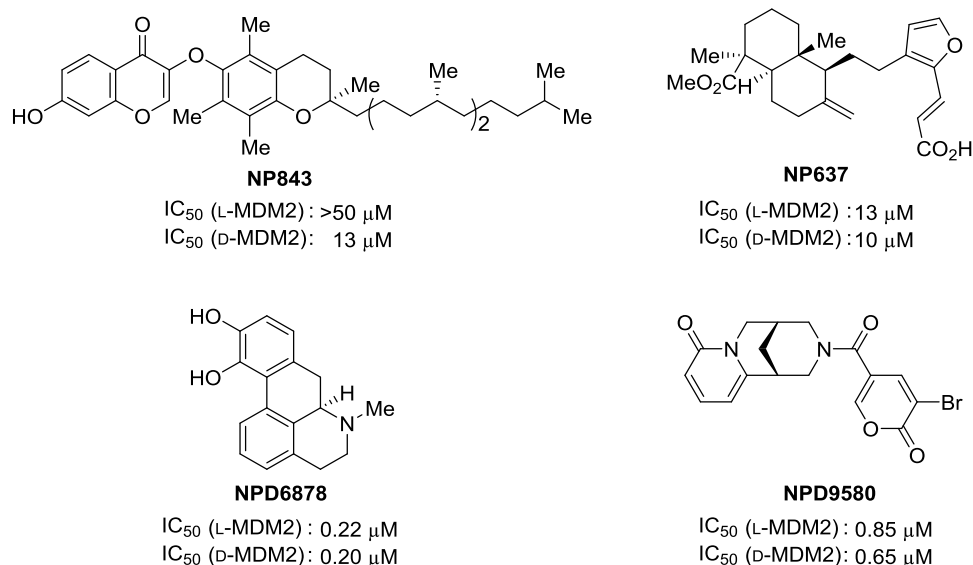


Figure 5. Structures and biological activities of MDM2–p53 inhibitors from mirror-image screening. IC_{50} values were measured by FP assay using 0.5 nM FAM-labeled P4 peptide and 10 nM MDM2 protein.

The next step was to verify the bioactivity of the enantiomeric form of **1a** (*ent*-NP843, **1b**) toward L-MDM2–L-p53 interaction. Compound **1b** was prepared by chiral pool synthesis from three commercially available optically pure components (Scheme 1). Initially, (*R*)-Roche ester-derived tosylate **2** was reacted with Grignard reagent **3**, prepared from (*S*)-citronellyl bromide, in the presence of copper catalyst. The subsequent deprotection of trityl-group gave alcohol **4**, after treatment with 1N NaOH for hydrolysis of the formyl ester derived from **4**. C1 elongation of **4** was then performed via tosylation and cyanation followed by reductions to give an alcohol **6**, which was converted into phosphonium salt **8**. (*R*)-Trolox-derived aldehyde **9** was employed as a chiral chromane component for Wittig reaction. The subsequent two-step catalytic reduction including PtO_2 -mediated hydrogenation of olefins and subsequent Pd/C-mediated hydrogenolysis of the benzyl group provided the L- α -tocopherol (**10b**) in 84% yield. Alkylation of **10b** with bromide **11** followed by deprotection gave ketone **12b**. Construction of the left-part of the chromone substructure by treatment of **12b** with *N,N*-dimethylformamide dimethyl acetal (DMF-DMA) afforded **1b**.

L-MDM2–L-p53 interaction. Furthermore, the antiproliferative activity of *ent*-NP843 was investigated using cancer cells, SJSA-1 cells (MDM2 overexpressed)² and H1299 cells (no p53 expression).³ Unfortunately, no antiproliferative activities of *ent*-NP843 against SJSA-1 and H1299 cells were observed at 30 μ M (Table 1).

Table 1. Biological activities of NP843 and *ent*-NP843.

Compounds	IC ₅₀ (μ M) ^a		GI ₅₀ (μ M) ^b	
	L-MDM2	D-MDM2	SJSA-1 cells	H1299 cells
Nutlin-3a	0.32 $\pm$ 0.03	>30	5.2 $\pm$ 0.19	>30
NP843 (1a)	>30	6.5 $\pm$ 0.5	>30	>30
<i>ent</i> -NP843 (1b)	7.6 $\pm$ 1.9	>30	>30	>30

[a] IC₅₀ values for L/D-MDM2 were measured by FP assay using 1.0 nM fluorescein-labeled P4 peptide and 10 nM MDM2. [b] GI₅₀ values for SJSA-1 and H1299 cells were measured by MTS assay.

Table 2. Inhibitory Activities of NP843 and *ent*-NP843 by SPR Analysis and ELISA.

Compound	IC ₅₀ (μ M) ^a	
	SPR	ELISA
L-P4	0.011 $\pm$ 0.001	0.066 $\pm$ 0.007
D-P4	>1.0	>1.0
NP843 (1a)	>30	>30
<i>ent</i> -NP843 (1b)	3.1 $\pm$ 0.5	16.7 $\pm$ 1.1

[a] IC₅₀ values were evaluated in triplicate assays.

Further structure–activity study of compound **1b** and the derivatives revealed the structural requirements for inhibition of L-MDM2–L-p53 interaction (Table 3). The author designed two diastereomers of compound **1b**: epimer **1c** at the tetrasubstituted carbon on the chromane scaffold, and diastereomer **1d** with two epimeric methyl groups on the right-hand alkyl chain (mirror-image of **1c**) (Figure 6). Compound **1d**, having a tetrasubstituted carbon with (*S*)-configuration on the chromane skeleton (identical to that of compound **1b**) reproduced the bioactivity of **1b** against L-MDM2–L-p53 interaction [IC₅₀ (**1d**) = 6.9 $\pm$ 1.9 μ M for L-MDM2–L-p53], while compound **1c** with (*R*)-configuration (identical to that of compound **1a**) showed no inhibition. However, for D-MDM2–D-p53 inhibition, compound **1c** was potent [IC₅₀ (**1c**) = 7.8 $\pm$ 2.0 μ M for D-MDM2–D-p53]. These results indicate that the stereochemistry of the tetrasubstituted carbon on the chromane skeleton is dominant in the chiral recognition by L-MDM2

protein when compared with the stereocenters on the extended alkyl chain. To demonstrate the significance of the aliphatic chain length of **1b**, the author also designed derivatives **13a,b** and **14a,b** with shorter isoprene units (Figure 6). Derivatives **13a,b** and **14a,b** showed no inhibitory activity against L-MDM2–L-p53 interaction (nor against D-MDM2–D-p53 interaction). These results suggest a chain length of three isoprene units is indispensable for biological activity of compound **1b**.

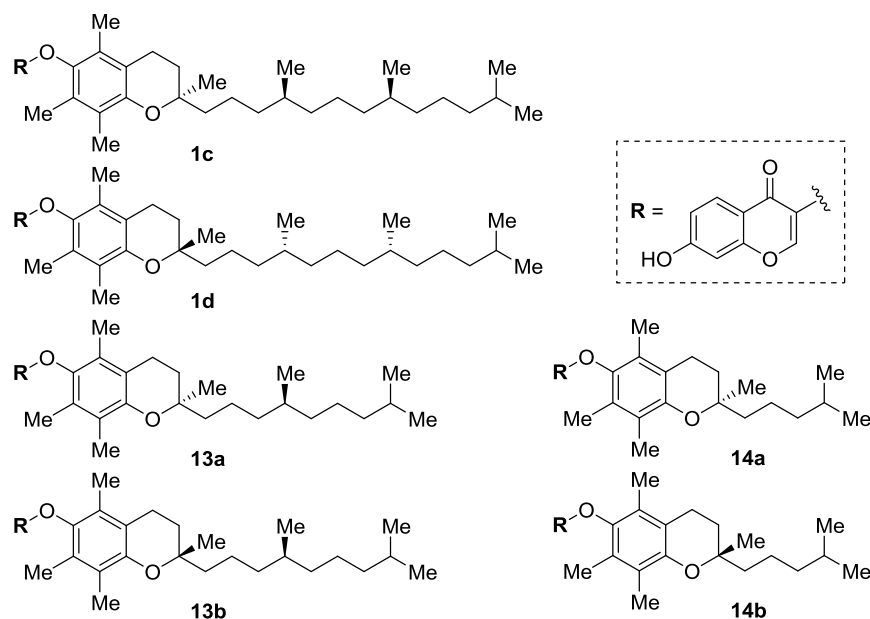


Figure 6. Structures of NP843 derivatives.

Table 3. Biological activity of NP843 derivatives.

Compounds	IC ₅₀ (μM) ^a	
	L-MDM2	D-MDM2
1c	>30	7.8 ± 2.0
1d	6.9 ± 1.9	>30
13a	>30	>30
13b	>30	>30
14a	>30	>30
14b	>30	>30

[a] IC₅₀ values for L/D-MDM2 were measured by FP assay using 1.0 nM fluorescein-labeled P4 peptide and 10 nM MDM2.

In this study, isolated natural products and derivatives, which were immobilized on microarray slides, were used as a screening resource. This approach would be further applicable to the mirror-image screening of mixture samples from natural resources

including crude plant extracts and fermentation broth extracts, which could provide unprecedented opportunity to explore diastereomeric interactions between the mirror-image structures of chiral ingredients and native proteins. Once the hit extract(s) is identified, structural determination of the active ingredient(s) and synthesis of its enantiomer(s) would be needed. In this way, novel hit compound(s) with “unnatural” chirality would be obtained with a high probability. It is the expectation that future analysis of screening data sets against a number of target molecules may give some insights into the value of mirror-image natural resources for drug discovery.

In this strategy, two chemical syntheses of mirror-image substances (target protein and initial hit compound), and chemical array technology using synthetic D-protein facilitated access to a variety of virtual mirror-image natural products. For further applications to other molecular targets, rapid and efficient preparations of mirror-image biomolecules and natural product hit(s) are essential because these cannot be generated *via* protein expressions or conventional cultivations. Recent progress in chemical protein synthesis has increased the availability of synthetic proteins, even with highly functional modification(s).⁴ Although this study demonstrates mirror-image screening for protein–protein interaction inhibitors, the targets could be extended to enzyme inhibitors and receptor ligands once the counterpart mirror-image biomolecule(s) are available.⁵ A number of recent advances in efficient synthetic strategies to access unique and complex frameworks in natural products should also contribute to the concise preparation of mirror-image structure(s) of natural product hit compounds. Thus, investigation of the bioactivities and functions of virtual mirror-image compounds is achievable only by using advanced synthetic organic chemistry technologies.

In summary, the author has established a novel screening process for a virtual mirror-image library of chiral natural products and derivatives. Chemically synthesized mirror-image MDM2 was applied for chemical array screening of a library of natural product derivatives, identifying novel tocopherol derivative NP843 (**1a**) as a D-MDM2–D-p53 interaction inhibitor. Chemical synthesis of the enantiomeric compound (**1b**) enabled the validation of the inhibitory activity of **1b** against L-MDM2–L-p53 interaction. The selective recognition by MDM2 was attributed to the stereochemistry of the tetrasubstituted carbon on the chromane skeleton of **1b**. The aliphatic side chain of three isoprene units was also needed for inhibition. In contrast to conventional screenings, this process could identify hit compounds from unavailable mirror-image chiral natural products, thus providing unprecedented lead compounds for

drug discovery. This is the first application of mirror-image protein technology to the screening of chiral small molecules including natural products.

Experimental Section

General Methods

^1H NMR and ^{13}C NMR spectra were recorded using a JEOL ECA-500 spectrometer. Chemical shifts are reported in δ (ppm) relative to Me_4Si (in CDCl_3). ^{13}C NMR spectra were referenced to the residual CHCl_3 (in CDCl_3). ^1H NMR spectra were tabulated as follows: chemical shift, multiplicity (br: broad, s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet), number of protons, and coupling constants. Exact mass (HRMS) spectra were recorded on a JMS-HX/HX 110A mass spectrometer or Shimadzu LC-ESI-IT-TOF-MS equipment. Melting points were measured by a hot stage melting point apparatus (uncorrected). For flash chromatography, Wakogel C-300E (Wako) was employed. For analytical HPLC for MDM2 proteins, Cosmosil 5C18-AR300 (4.6×250 mm, Nacalai Tesque Inc.) was employed with a linear gradient of CH_3CN containing 0.1% (v/v) TFA aq. at a flow rate of 1 mL/min. Cosmosil 5C18-ARII column (4.6×250 mm, Nacalai Tesque Inc.) was employed for the analysis of the other peptides. The purity of all compounds for biological evaluations was determined by combustion analysis (>95%).

Synthesis of MDM2²⁵⁻¹⁰⁹ and MDM2^{TMR} Proteins

L-MDM2²⁵⁻¹⁰⁹, L-MDM2^{TMR}, D-MDM2²⁵⁻¹⁰⁹ and D-MDM2^{TMR} proteins were synthesized by the identical protocol in Section 1.¹ Briefly, protected MDM2²⁵⁻¹⁰⁹ was synthesized by a standard protocol of Fmoc-based SPPS using automatic peptide synthesizer (PSSM-8, Shimadzu Corporation Ltd.) on H-Rink Amide ChemMatrix resin (0.4-0.6 mmol/g) using HBTU/HOBt/(*i*-Pr)₂NEt activation. The resulting protected peptide resin was treated with TFA/thioanisole/*m*-cresol/EDT/H₂O (80:5:5:5:5) at room temperature for 2 h. After removal of the resin by filtration, the filtrate was poured into ice-cold dry Et₂O. The resulting powder was collected by centrifugation and then washed with ice-cold dry Et₂O three times. The crude product was purified by HPLC to provide MDM2²⁵⁻¹⁰⁹ proteins as white powder (Figure 7).

For the synthesis of MDM2^{TMR} proteins, two glycines and 5-hexynoic acid were coupled with HBTU/HOBt/(*i*-Pr)₂NEt. The resulting protected peptides were treated with the deprotection cocktail as above to provide alkyne-conjugated proteins. Subsequently, treatment of the proteins with TMR-(PEG)₃-azide in the presence of Cu(I) provided TMR-labeled MDM2^{TMR} proteins as pink powder (Figure 7).

ESI-MS for L-MDM2²⁵⁻¹⁰⁹: Calcd for C₄₆₂H₇₃₉N₁₁₇O₁₂₃S₄: 10027.42; observed: [M+12H]¹²⁺ *m/z* = 836.74, [M+11H]¹¹⁺ *m/z* = 912.76, [M+10H]¹⁰⁺ *m/z* = 1003.86, [M+9H]⁹⁺ *m/z* = 1115.39, [M+8H]⁸⁺ *m/z* = 1254.76, [M+7H]⁷⁺ *m/z* = 1434.00.¹

ESI-MS for D-MDM2²⁵⁻¹⁰⁹: Calcd for C₄₆₂H₇₃₉N₁₁₇O₁₂₃S₄: 10027.42; observed: [M+12H]¹²⁺ *m/z* = 836.68, [M+11H]¹¹⁺ *m/z* = 912.77, [M+10H]¹⁰⁺ *m/z* = 1003.94, [M+9H]⁹⁺ *m/z* = 1115.46, [M+8H]⁸⁺ *m/z* = 1254.85, [M+7H]⁷⁺ *m/z* = 1433.42.

ESI-MS for L-MDM2^{TMR}: Calcd for C₅₀₅H₇₈₉N₁₂₅O₁₃₃S₄: 10865.79; observed: [M+13H]¹³⁺ *m/z* = 836.94, [M+12H]¹²⁺ *m/z* = 906.78, [M+11H]¹¹⁺ *m/z* = 989.03, [M+10H]¹⁰⁺ *m/z* = 1087.90, [M+9H]⁹⁺ *m/z* = 1208.38, [M+8H]⁸⁺ *m/z* = 1359.59.¹

ESI-MS for D-MDM2^{TMR}: Calcd for C₅₀₅H₇₈₉N₁₂₅O₁₃₃S₄: 10865.79; observed: [M+13H]¹³⁺ *m/z* = 836.65, [M+12H]¹²⁺ *m/z* = 906.65, [M+11H]¹¹⁺ *m/z* = 989.11, [M+10H]¹⁰⁺ *m/z* = 1088.17, [M+9H]⁹⁺ *m/z* = 1208.45, [M+8H]⁸⁺ *m/z* = 1359.80.

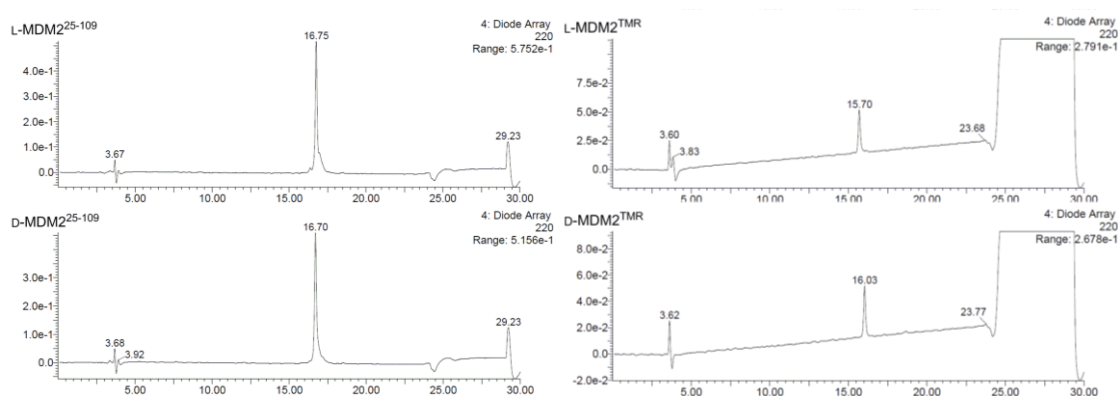


Figure 7. LC–MS chromatograms of purified synthetic MDM2²⁵⁻¹⁰⁹ and MDM2^{TMR} proteins. *HPLC conditions:* HPLC analysis was performed at 25 °C on a Cosmosil 5C18-AR300 preparative column (Nacalai Tesque, 4.5 × 250 mm) with a linear gradient of 30–50% CH₃CN containing 0.1% TFA at a flow rate of 1 mL/min over 20min.

Folding of Synthetic MDM2²⁵⁻¹⁰⁹ and MDM2^{TMR} Proteins

Folding of synthetic MDM2 proteins were carried out by the identical protocol in Section 1.¹ Lyophilized polypeptide was dissolved in 6 M guanidine hydrochloride (Gu·HCl) (1 mg/mL) followed by 100-fold dilution with PBS (pH 7.4) containing 0.5 mM TCEP·HCl and 0.005% Tween-20 at 4 °C. The solution was stored at 4 °C overnight, and the solution was concentrated using a MWCO 3000 centrifugal filtration membrane (Millipore, Amicon-Ultra 3 kDa) (3 times).

Synthesis of p53 Peptides and Their Derivatives

p53 peptides were synthesized by Fmoc-based SPPS on Rink-amide resin (0.66 mmol/g, 45.5 mg, 0.025 mmol) according to the identical protocol in the previous report.¹ Fmoc-protected amino acids (3 eq) were coupled by using DIPCI (3 eq) and HOBt (3 eq) in DMF. The Fmoc-protecting group was removed by treatment of the resin with 20% piperidine in DMF. Coupling of biotin (0.125 mmol) was carried out with HBTU (5 eq), HOBt (5 eq) and (*i*-Pr)₂NEt (10 eq) in DMF. Coupling of 5-carboxyfluorescein (5 eq) was carried out with DIPCI (5 eq) and HOBt (10 eq) in DMF. The resulting protected peptide resin was treated with TFA/thioanisole/*m*-cresol/EDT/H₂O (80:5:5:5:5) at room temperature for 2 h. After removal of the resin by filtration, the filtrate was poured into ice-cold dry Et₂O. The resulting powder was collected by centrifugation and then washed with ice-cold dry Et₂O three times. The crude product was purified by HPLC on a Cosmosil 5C18-ARII preparative column (Nacalai Tesque, 20 × 250 mm). All peptides were characterized by MALDI-TOF-MS.

Biotin-labeled L-p53 peptide (biotinyl-aminocaproyl-GSGSSQETFSDLWKLLP-EN-NH₂): MS (MALDI-TOF) calcd for C₁₀₈H₁₆₆N₂₇O₃₅S [M+H]⁺ 2434.19; found: 2434.13.

Biotin-labeled D-p53 peptide: MS (MALDI-TOF) calcd for C₁₀₈H₁₆₆N₂₇O₃₅S [M+H]⁺ 2434.19; found: 2434.12.

L-P4 (H-LTFEHYWAQLTS-NH₂): MS (MALDI-TOF) calcd for C₇₁H₁₀₀N₁₇O₁₉ [M+H]⁺ 1494.74; found: 1494.75.

D-P4: MS (MALDI-TOF) calcd for C₇₁H₁₀₀N₁₇O₁₉ [M+H]⁺ 1494.74; found: 1494.73.

5-Carboxyfluorescein-labeled L-P4 (5-FAM-LTFEHYWAQLTS-NH₂): MS (MALDI-TOF) calcd for C₉₂H₁₁₀N₁₇O₂₅ [M+H]⁺ 1852.79; found: 1852.80.

5-Carboxyfluorescein-labeled D-P4: MS (MALDI-TOF) calcd for C₉₂H₁₁₀N₁₇O₂₅ [M+H]⁺ 1852.79; found: 1852.76.

(2S,6R)-2,6,10-Trimethylundec-9-en-1-ol (4)

To a stirred mixture of Mg (1.00 g, 41.2 mmol) in THF (6.6 mL) was added dropwise (S)-citronellyl bromide (6.00 g, 27.4 mmol) in THF (16.8 mL) over 2 h using syringe pump at 60 °C to give Grignard reagent **3**. To a stirred solution of **2**⁶ (6.9 g, 14.2 mmol) in THF (31.0 mL) were added the reagent **3** and 0.1 M Li₂CuCl₄ in THF (27.0 mL, 2.70 mmol) at -40 °C. The resulting mixture was stirred at -40 °C overnight and the reaction was quenched with saturated NH₄Cl at 0 °C. The whole was extracted with Et₂O and the extract was washed with H₂O and brine, and dried over MgSO₄. The filtrate was concentrated under reduced pressure. The oily residue was dissolved in hexane and the solution was filtrated through a short pad of silica gel and the filtrate was concentrated

under reduced pressure. To a stirred solution of the residue in dry Et₂O (120 mL) was added HCO₂H (120 mL) dropwise at 0 °C under argon, and the stirring was continued for 1 h. After toluene (150 mL) was added, the solution was concentrated under reduced pressure. 1N NaOH in MeOH/H₂O (1:1, 100 mL) was added to the residue and the mixture was stirred for 10 min. The mixture was concentrated under reduced pressure. The residue was extracted with Et₂O and the extract was dried over MgSO₄. The filtrate was concentrated under reduced pressure, and the residue was purified by column chromatography to give the title compound **4** as a colorless oil (1.67 g, 55%). [α]_D²⁵ -7.5 (c 1.10, CHCl₃); IR (neat): 3319 (OH), 1035 (C-O); ¹H NMR (500 MHz, CDCl₃) δ 0.86 (d, *J* = 6.3 Hz, 3H), 0.93 (d, *J* = 6.3 Hz, 3H), 1.04-1.42 (m, 9H), 1.60 (s, 3H), 1.58-1.65 (m, 1H), 1.68 (s, 3H), 1.89-2.03 (m, 2H), 3.42 (dd, *J* = 10.3, 6.3 Hz, 1H), 3.51 (dd, *J* = 10.6, 6.0 Hz, 1H), 5.09-5.11 (m, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 16.6, 17.6, 19.6, 24.3, 25.5, 25.7, 32.4, 33.4, 35.8, 37.1, 37.2, 68.4, 125.0, 131.0. HRMS (FAB) calcd for C₁₄H₂₉O (MH⁺): 213.2213; found: 213.2217.

(2*R*,6*S*)-2,6,10-Trimethylundec-9-en-1-ol (*ent*-4)

According to the procedure described for the preparation of **4**, compound *ent*-2 (5.00 g, 10.3 mmol) was converted into *ent*-4 with (*R*)-citronellyl bromide (6.40 g, 29.2 mmol) as a colorless oil (996 mg, 46%). [α]_D²⁵ +7.4 (c 1.17, CHCl₃); IR (neat): 3357 (OH), 1034 (C-O); ¹H NMR (500 MHz, CDCl₃) δ 0.86 (d, *J* = 6.9 Hz, 3H), 0.92 (d, *J* = 6.3 Hz, 3H), 1.03-1.41 (m, 9H), 1.60 (s, 3H), 1.58-1.65 (m, 1H), 1.68 (s, 3H), 1.89-2.03 (m, 2H), 3.42 (dd, *J* = 10.3, 6.3 Hz, 1H), 3.51 (dd, *J* = 10.6, 6.3 Hz, 1H), 5.09-5.11 (m, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 16.6, 17.6, 19.6, 24.3, 25.5, 25.7, 32.4, 33.4, 35.8, 37.1, 37.2, 68.4, 125.0, 131.0. HRMS (FAB) calcd for C₁₄H₂₉O (MH⁺): 213.2213; found: 213.2223.

(3*S*,7*R*)-3,7,11-Trimethyldodec-10-enenitrile (5**)**

To a stirred solution of compound **4** (1.26 g, 5.93 mmol) in pyridine (4.3 mL) was added TsCl (1.40 g, 7.72 mmol) at 0 °C. After the mixture was stirred for 2 h, saturated aqueous solution of citric acid was added. The whole was extracted with Et₂O and the extract was washed with H₂O and brine, and dried over MgSO₄. The extract was concentrated under reduced pressure after filtration through a short pad of silica gel to give a crude sulfonate. To a stirred solution of the sulfonate in DMSO (13.0 mL) was added NaCN (0.581 g, 11.86 mmol) at room temperature. After the mixture was stirred overnight, saturated aqueous solution of NH₄Cl was added. The whole was extracted with CHCl₃ and the extract was washed with H₂O and brine, and dried over MgSO₄.

The filtrate was concentrated under reduced pressure, and the residue was purified by column chromatography to give the title cyanide **5** as a colorless oil (1.17 g, 89%). $[\alpha]_D^{25} +5.2$ (c 1.02, CHCl₃); IR (neat): 2247 (C≡N); ¹H NMR (500 MHz, CDCl₃) δ 0.87 (d, *J* = 6.3 Hz, 3H), 1.07 (d, *J* = 6.9 Hz, 3H), 1.07-1.17 (m, 2H), 1.23-1.43 (m, 7H), 1.60 (s, 3H), 1.69 (s, 3H), 1.81-1.89 (m, 1H), 1.89-2.03 (m, 2H), 2.23 (dd, *J* = 16.6, 6.9 Hz, 1H), 2.32 (dd, *J* = 16.6, 5.7 Hz, 1H), 5.09-5.11 (m, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 17.6, 19.5 (2C), 24.2, 24.4, 25.5, 25.7, 30.5, 32.3, 36.2, 36.8, 37.0, 119.0, 124.9, 131.1. HRMS (FAB) calcd for C₁₅H₂₈N (MH⁺): 222.2216; found: 222.2224.

(3*R*,7*S*)-3,7,11-Trimethyldodec-10-enenitrile (*ent*-5)

According to the procedure described for the preparation of **5**, compound *ent*-**4** (950 mg, 4.47 mmol) was converted into *ent*-**5** as a colorless oil (948 mg, 96%). $[\alpha]_D^{25} -5.3$ (c 1.08, CHCl₃); IR (neat): 2247 (C≡N); ¹H NMR (500 MHz, CDCl₃) δ 0.87 (d, *J* = 6.3 Hz, 3H), 1.07 (d, *J* = 6.9 Hz, 3H), 1.09-1.17 (m, 2H), 1.21-1.43 (m, 7H), 1.61 (s, 3H), 1.69 (s, 3H), 1.81-1.89 (m, 1H), 1.89-2.03 (m, 2H), 2.24 (dd, *J* = 16.6, 6.9 Hz, 1H), 2.32 (dd, *J* = 16.6, 5.7 Hz, 1H), 5.08-5.11 (m, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 17.6, 19.5 (2C), 24.2, 24.4, 25.5, 25.7, 30.5, 32.3, 36.2, 36.8, 37.0, 119.0, 124.9, 131.1. HRMS (FAB) calcd for C₁₅H₂₈N (MH⁺): 222.2216; found: 222.2224.

(3*S*,7*R*)-3,7,11-Trimethyldodec-10-en-1-ol (6**)⁶**

To a stirred solution of **5** (1.00 g, 4.52 mmol) in CH₂Cl₂ (5.0 mL) was added dropwise 1.0 M DIBAL-H in THF (4.97 mL, 4.97 mmol) at -78 °C under argon. After the mixture was stirred for 2 h, saturated aqueous solution of sodium potassium tartrate was added and the mixture was warmed to room temperature. The whole was extracted with Et₂O and the extract was dried over MgSO₄. The extract was concentrated under reduced pressure after filtration through a short pad of silica gel to give crude aldehyde. To a stirred solution of the aldehyde in CH₂Cl₂ (5.0 mL) was added dropwise DIBAL-H in THF (1.0 M, 4.97 mL, 4.97 mmol) at -78 °C under argon. After the mixture was stirred for 2 h, saturated aqueous solution of sodium potassium tartrate was added. The whole was extracted with Et₂O and the extract was dried over MgSO₄. The extract was concentrated under reduced pressure, and the residue was purified by column chromatography to give the title alcohol **6** as a colorless oil (0.848 g, 83%). $[\alpha]_D^{25} -2.3$ (c 0.90, CHCl₃); IR (neat): 3299 (OH), 1058 (C-O); ¹H NMR (500 MHz, CDCl₃) δ 0.86 (d, *J* = 6.3 Hz, 3H), 0.90 (d, *J* = 6.3 Hz, 3H), 1.07-1.41 (m, 11H), 1.54-1.64 (m, 1H), 1.61 (s, 3H), 1.69 (s, 3H), 1.89-2.03 (m, 2H), 3.64-3.73 (m, 2H), 5.09-5.11 (m, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 17.6, 19.6, 19.7, 24.3, 25.6, 25.7, 29.5, 32.4, 37.0, 37.2, 37.5,

40.0, 61.3, 125.0, 124.9, 131.0. HRMS (FAB) calcd for C₁₅H₃₁O (MH⁺): 227.2369; found: 227.2363.

(3R,7S)-3,7,11-Trimethyldodec-10-en-1-ol (*ent*-6)

According to the procedure described for the preparation of **6**, compound *ent*-5 (900 mg, 4.07 mmol) was converted into *ent*-6 as a colorless oil (606 mg, 66%). [α]_D²⁵ +2.0 (*c* 1.05, CHCl₃); IR (neat): 3299 (OH), 1055 (C-O); ¹H NMR (500 MHz, CDCl₃) δ 0.86 (d, *J* = 6.3 Hz, 3H), 0.90 (d, *J* = 6.3 Hz, 3H), 1.04-1.41 (m, 11H), 1.52-1.63 (m, 1H), 1.60 (s, 3H), 1.68 (s, 3H), 1.89-2.03 (m, 2H), 3.64-3.73 (m, 2H), 5.08-5.12 (m, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 17.6, 19.6, 19.7, 24.3, 25.6, 25.7, 29.5, 32.4, 37.0, 37.2, 37.5, 39.9, 61.3, 125.0, 124.9, 131.0. HRMS (FAB) calcd for C₁₅H₃₁O (MH⁺): 227.2369; found: 227.2363.

(6R,10S)-12-Bromo-2,6,10-trimethyldodec-2-ene (7)

To a stirred solution of compound **6** (800 mg, 3.53 mmol) in pyridine (2.70 mL) was added TsCl (876 mg, 4.60 mmol) at 0 °C. After the mixture was stirred for 2 h, saturated aqueous solution of citric acid was added. The whole was extracted with Et₂O and the extract was washed with H₂O and brine, and dried over MgSO₄. The extract was concentrated under reduced pressure after filtration through a short pad of silica gel to give a crude sulfonate. To a stirred solution of the sulfonate in acetone (5 mL) was added LiBr (1.80 g, 17.7 mmol). After the mixture was stirred for 1 h under reflux, the whole was concentrated under reduced pressure. To the residue was added H₂O and the whole was extracted with Et₂O and dried over MgSO₄. The filtrate was concentrated under reduced pressure, and the residue was purified by column chromatography to give the title bromide **7** as a colorless oil (923 mg, 90%). [α]_D²⁵ +5.8 (*c* 1.00, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.86 (d, *J* = 6.9 Hz, 3H), 0.89 (d, *J* = 6.9 Hz, 3H), 1.04-1.41 (m, 9H), 1.61 (s, 3H), 1.68 (s, 3H), 1.61-1.68 (m, 2H), 1.85-2.03 (m, 3H), 3.38-3.49 (m, 2H), 5.09-5.11 (m, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 17.6, 19.0, 19.6, 24.1, 25.6, 25.7, 31.6, 32.3, 32.4, 36.8, 37.1 (2C), 40.0, 125.0, 131.0. Anal. calcd. for C₁₅H₂₉Br: C, 62.28; H, 10.10. Found: C, 62.31; H, 10.32.

(6S,10R)-12-Bromo-2,6,10-trimethyldodec-2-ene (*ent*-7)

According to the procedure described for the preparation of **7**, compound *ent*-6 (580 mg, 2.56 mmol) was converted into *ent*-7 as a colorless oil (669 mg, 90%). [α]_D²⁵ -5.8 (*c* 1.01, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.86 (d, *J* = 6.9 Hz, 3H), 0.89 (d, *J* = 6.9 Hz, 3H), 1.07-1.41 (m, 9H), 1.61 (s, 3H), 1.68 (s, 3H), 1.61-1.68 (m, 2H), 1.84-2.03 (m,

3H), 3.38-3.49 (m, 2H), 5.09-5.11 (m, 1H); ^{13}C NMR (125 MHz, CDCl_3) δ 17.6, 19.0, 19.6, 24.1, 25.6, 25.7, 31.6, 32.2, 32.4, 36.8, 37.1 (2C), 40.0, 125.0, 131.0. Anal. calcd. for $\text{C}_{15}\text{H}_{29}\text{Br}$: C, 62.28; H, 10.10. Found: C, 62.22; H, 10.08.

Triphenyl((3*S*,7*R*)-3,7,11-trimethyldodec-10-en-1-yl)phosphonium Bromide (8**)**

A mixture of the bromide **7** (757 mg, 2.61 mmol) and PPh_3 (750 mg, 2.87 mmol) was heated to 100 °C and the mixture was stirred for 15 h. After cooling, Et_2O was added to the mixture and the resulting white precipitate was washed with Et_2O to remove the excess PPh_3 . The residue was dried under vacuum to give the title phosphonium salt **8**, which was used without further purification.

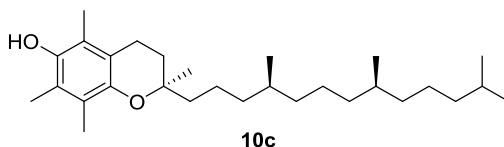
Triphenyl((3*S*,7*R*)-3,7,11-trimethyldodec-10-en-1-yl)phosphonium Bromide (*ent*-8**)**

According to the procedure described for the preparation of **8**, compound *ent*-**7** (600 mg, 2.07 mmol) was converted into *ent*-**8**.

(*S*)-2,5,7,8-Tetramethyl-2-((4*S*,8*S*)-4,8,12-trimethyltridecyl)chroman-6-ol (10b**)⁷**

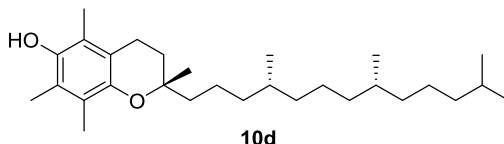
To a stirred solution of phosphonium salt **8** (ca. 2.61 mmol) in THF (15.0 mL) was added LHMDS (1.0 M, 2.30 mL, 2.30 mmol) in THF dropwise at -40 °C under argon. After the mixture was stirred for 30 min, the aldehyde **9**⁸ (675 mg, 2.08 mmol) in THF (5.0 mL) was added to the mixture. The stirring was continued for 30 min at the same temperature and for 1 h at 0 °C. After the reaction was quenched with saturated aqueous solution of NH_4Cl , the whole was extracted with Et_2O and the extract was washed with H_2O and brine, and dried over Na_2SO_4 . The filtrate was concentrated under reduced pressure. The oily residue was dissolved in hexane and the solution was filtrated through a short pad of silica gel to remove phosphine oxide and the filtrated was concentrated under reduced pressure. To a stirred solution of the crude alkene in TBME (9.5 mL) was added PtO_2 (37.8 mg, 0.167 mmol). The mixture was stirred under an atmosphere of H_2 at room temperature. After 30 min, the reaction mixture was filtrated through Celite and the filtrate was concentrated. To a stirred solution of the residue in MeOH (35.0 mL) was added 10% Pd/C (222 mg, 0.208 mmol). The mixture was stirred under an atmosphere of H_2 at room temperature. After 30 min, the reaction mixture was filtrated through Celite. The filtrate was concentrated under reduced pressure, and the residue was purified by column chromatography to give the title compound **10b** as a pale yellow oil (759 mg, 84%). $[\alpha]_{\text{D}}^{25}$ -0.7 (c 1.01, CHCl_3); ^1H NMR (500 MHz, CDCl_3) δ 0.83-0.87 (m, 12H), 1.02-1.53 (m, 21H), 1.23 (s, 3H), 1.73-1.84 (m, 2H), 2.11 (s, 6H), 2.16 (s, 3H), 2.60 (t, J = 6.9 Hz, 2H), 4.17 (s, 1H); ^{13}C NMR (125 MHz,

CDCl₃) δ 11.3, 11.8, 12.2, 19.6, 19.7, 20.7, 21.0, 22.6, 22.7, 23.8, 24.4, 24.8, 28.0, 31.5, 32.7, 32.9, 37.3, 37.4 (3C), 39.4, 39.8, 74.5, 117.3, 118.4, 121.0, 122.6, 144.5, 145.5. HRMS (ESI) calcd for C₂₉H₅₀O₂ (M⁺): 430.3811; found: 430.3806.



(R)-2,5,7,8-Tetramethyl-2-((4S,8S)-4,8,12-trimethyltridecyl)chroman-6-ol (10c)

According to the procedure described for the preparation of **10b**, compound *ent*-**9** (675 mg, 2.07 mmol) was converted into compound **10c** by the reaction with **8** (ca. 2.61 mmol) as a pale yellow oil (573 mg, 57%). [α]_D²⁵ +1.0 (c 1.10, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.83-0.87 (m, 12H), 1.03-1.58 (m, 21H), 1.23 (s, 3H), 1.73-1.84 (m, 2H), 2.11 (s, 6H), 2.16 (s, 3H), 2.60 (t, *J* = 6.9 Hz, 2H), 4.17 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.3, 11.8, 12.2, 19.7 (2C), 20.8, 21.0, 22.6, 22.7, 23.8, 24.4, 24.8, 28.0, 31.4, 32.7, 32.8, 37.3, 37.4 (2C), 37.5, 39.4, 39.8, 74.5, 117.3, 118.4, 121.0, 122.6, 144.5, 145.5. HRMS (ESI) calcd for C₂₉H₅₀O₂ (M⁺): 430.3811; found: 430.3803.



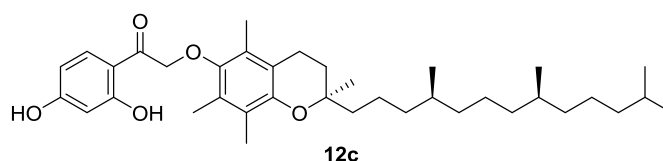
(S)-2,5,7,8-Tetramethyl-2-((4R,8R)-4,8,12-trimethyltridecyl)chroman-6-ol (10d)

According to the procedure described for the preparation of **10b**, compound **9** (540 mg, 1.66 mmol) was converted into compound **10d** by the reaction with *ent*-**8** (ca. 2.08 mmol) as a pale yellow oil (573 mg, 80%). [α]_D²⁵ -1.0 (c 1.00, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.83-0.87 (m, 12H), 1.03-1.58 (m, 21H), 1.23 (s, 3H), 1.73-1.84 (m, 2H), 2.11 (s, 6H), 2.16 (s, 3H), 2.60 (t, *J* = 6.9 Hz, 2H), 4.17 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.3, 11.8, 12.2, 19.7 (2C), 20.8, 21.0, 22.6, 22.7, 23.8, 24.4, 24.8, 28.0, 31.5, 32.7, 32.9, 37.3, 37.4 (2C), 37.5, 39.4, 39.8, 74.5, 117.3, 118.4, 121.0, 122.6, 144.5, 145.5. HRMS(ESI) calcd for C₂₉H₅₀O₂ (M⁺): 430.3811; found: 430.3801.

1-(2,4-Dihydroxyphenyl)-2-[(S)-2,5,7,8-tetramethyl-2-((4S,8S)-4,8,12-trimethyltridecyl)chroman-6-yl]oxy}ethan-1-one (12b)

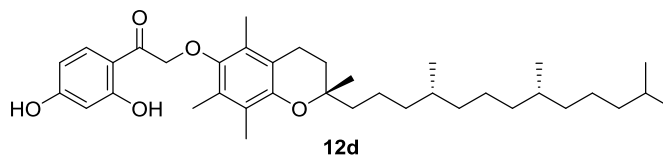
To a stirred solution of compound **10b** (600 mg, 1.39 mmol) in DMF (1.4 mL) were added K₂CO₃ (385 mg, 2.78 mmol) and compound **11** (745 mg, 1.81 mmol) at room

temperature. After the mixture was stirred overnight, the reaction was quenched with H₂O. The whole was extracted with Et₂O and the extract was washed with H₂O and brine, and dried over MgSO₄. The filtrate was concentrated under reduced pressure. To a stirred solution of the residue in EtOAc (15.0 mL) was added 10% Pd/C (149 mg, 0.140 mmol). The mixture was stirred under an atmosphere of H₂ at room temperature. After the reaction was completed, the reaction mixture was filtrated through Celite. Purification by column chromatography gave the title compound **12b** as pale yellow solid (583 mg, 72%): mp 87–88 °C; [α]_D²⁵ –4.0 (c 1.01, CHCl₃); IR (neat): 3315 (OH), 1627 (C=O); ¹H NMR (500 MHz, CDCl₃) δ 0.84–0.87 (m, 12H), 1.04–1.57 (m, 21H), 1.24 (s, 3H), 1.73–1.85 (m, 2H), 2.09 (s, 3H), 2.14 (s, 3H), 2.18 (s, 3H), 2.58 (t, *J* = 6.6 Hz, 2H), 4.92 (s, 2H), 5.80 (br s, 1H), 6.36 (dd, *J* = 8.6, 2.3 Hz, 1H), 6.42 (d, *J* = 2.3 Hz, 1H), 7.52 (d, *J* = 7.7 Hz, 1H), 12.40 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.8, 11.9, 12.8, 19.7 (2C), 20.6, 21.0, 22.6, 22.7, 23.8, 24.4, 24.8, 28.0, 31.2, 32.7, 32.8, 37.3, 37.4 (3C), 39.4, 40.0, 74.0, 75.0, 103.8, 108.0, 111.9, 117.8, 123.2, 125.7, 127.6, 130.9, 147.8, 148.3, 162.8, 165.3, 198.0. Anal. calcd. for C₃₇H₅₆O₅: C, 76.51; H, 9.72. Found: C, 76.41; H, 9.89.



1-(2,4-Dihydroxyphenyl)-2-[(*R*)-2,5,7,8-tetramethyl-2-((4*S*,8*S*)-4,8,12-trimethyltridecyl)chroman-6-yl]oxy}ethan-1-one (12c**)**

According to the procedure described for the preparation of **12b**, compound **10c** (100 mg, 0.232 mmol) was converted into compound **12c** as pale yellow solid (88.9 mg, 66%): mp 86–87 °C; [α]_D²⁵ +3.7 (c 0.55, CHCl₃); IR (neat): 3351 (OH), 1626 (C=O); ¹H NMR (500 MHz, CDCl₃) δ 0.84–0.87 (m, 12H), 1.04–1.61 (m, 21H), 1.24 (s, 3H), 1.73–1.85 (m, 2H), 2.08 (s, 3H), 2.14 (s, 3H), 2.18 (s, 3H), 2.58 (t, *J* = 6.9 Hz, 2H), 4.92 (s, 2H), 6.36 (dd, *J* = 8.9, 2.6 Hz, 1H), 6.42 (d, *J* = 2.9 Hz, 1H), 7.51 (d, *J* = 9.2 Hz, 1H), 12.40 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.8, 11.9, 12.8, 19.7 (2C), 20.6, 21.0, 22.6, 22.7, 23.8, 24.4, 24.8, 28.0, 31.1, 32.7, 32.8, 37.3, 37.4 (2C), 37.5, 39.4, 40.1, 74.0, 75.0, 103.8, 108.1, 111.9, 117.8, 123.2, 125.7, 127.6, 130.9, 147.8, 148.3, 162.9, 165.3, 198.0. Anal. Calcd. for C₃₇H₅₆O₅: C, 76.51; H, 9.72. Found: C, 76.35; H, 9.79.



1-(2,4-Dihydroxyphenyl)-2-[(S)-2,5,7,8-tetramethyl-2-((4R,8R)-4,8,12-trimethyltridecyl)chroman-6-yl]oxy}ethan-1-one (12d)

According to the procedure described for the preparation of **12b**, compound **10d** (500 mg, 1.16 mmol) was converted into compound **12d** as pale yellow solid (483 mg, 72%): mp 86–87 °C; $[\alpha]_D^{25}$ –3.9 (c 1.11, CHCl₃); IR (neat): 3350 (OH), 1626 (C=O); ¹H NMR (500 MHz, CDCl₃) δ 0.84–0.87 (m, 12H), 1.04–1.61 (m, 21H), 1.24 (s, 3H), 1.73–1.85 (m, 2H), 2.08 (s, 3H), 2.14 (s, 3H), 2.18 (s, 3H), 2.58 (t, *J* = 6.9 Hz, 2H), 4.92 (s, 2H), 6.36 (dd, *J* = 8.9, 2.6 Hz, 1H), 6.42 (d, *J* = 2.9 Hz, 1H), 7.51 (d, *J* = 9.2 Hz, 1H), 12.40 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.8, 11.9, 12.8, 19.7 (2C), 20.6, 21.0, 22.6, 22.7, 23.8, 24.4, 24.8, 28.0, 31.1, 32.7, 32.8, 37.3, 37.4 (2C), 37.5, 39.4, 40.1, 74.0, 75.0, 103.8, 108.1, 111.8, 117.8, 123.2, 125.7, 127.6, 130.9, 147.7, 148.3, 163.0, 165.3, 198.0. HRMS (ESI) calcd for C₃₇H₅₇O₅ (MH⁺): 581.4201; found: 581.4207.

7-Hydroxy-3-[(R)-2,5,7,8-tetramethyl-2-((4R,8R)-4,8,12-trimethyltridecyl)chroman-6-yl]oxy}-4H-chromen-4-one (1a, NP843)

mp 228–229 °C; $[\alpha]_D^{25}$ +5.0 (c 1.00, CHCl₃); IR (neat): 3163 (OH); ¹H NMR (500 MHz, CDCl₃) δ 0.84–0.87 (m, 12H), 1.03–1.59 (m, 21H), 1.25 (s, 3H), 1.75–1.85 (m, 2H), 2.02 (s, 3H), 2.06 (s, 3H), 2.09 (s, 3H), 2.58 (t, *J* = 6.9 Hz, 2H), 6.86 (d, *J* = 2.3 Hz, 1H), 7.07–7.10 (m, 2H), 8.22 (d, *J* = 9.2 Hz, 1H), 8.67 (br s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.8 (2C), 12.6, 19.6, 19.7, 20.6, 21.0, 22.6, 22.7, 23.7, 24.4, 24.8, 28.0, 31.2, 32.7, 32.8, 37.3, 37.4 (3C), 39.4, 40.0, 75.3, 102.7, 115.5, 117.0, 118.2, 123.7, 125.3, 127.1, 127.6, 140.0, 142.8, 143.4, 149.0, 157.8, 162.2, 172.6. Anal. calcd. for C₃₈H₅₄O₅: C, 77.25; H, 9.21. Found: C, 77.13; H, 9.40.

7-Hydroxy-3-[(S)-2,5,7,8-tetramethyl-2-((4S,8S)-4,8,12-trimethyltridecyl)chroman-6-yl]oxy}-4H-chromen-4-one (1b)

To a stirred solution of compound **12** (100 mg, 0.172 mmol) in THF (0.76 mL) was added DMF-DMA (27.7 μ L, 0.260 mmol) under argon, and the mixture was stirred under reflux for 4 h. Then, aqueous 1N HCl (1 mL) and MeOH (1 mL) were added and the mixture was stirred overnight. The whole was extracted with Et₂O and dried over Na₂SO₄. The filtrate was concentrated under reduced pressure, and the residue was purified by column chromatography to give the title compound **1b** as white solid (23.3

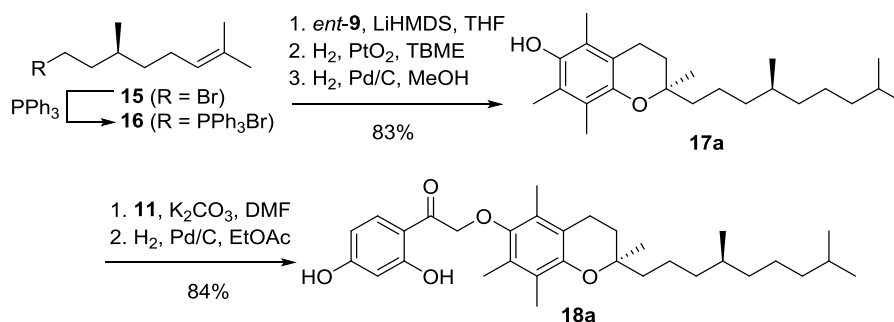
mg, 23%): mp 228–229 °C; $[\alpha]_D^{25} -5.3$ (c 1.00, CHCl₃); IR (neat): 3133 (OH); ¹H NMR (500 MHz, CDCl₃) δ 0.84–0.87 (m, 12H), 1.03–1.59 (m, 21H), 1.25 (s, 3H), 1.75–1.85 (m, 2H), 2.02 (s, 3H), 2.06 (s, 3H), 2.09 (s, 3H), 2.58 (t, $J = 6.9$ Hz, 2H), 6.86 (d, $J = 2.3$ Hz, 1H), 7.07–7.10 (m, 2H), 8.22 (d, $J = 9.2$ Hz, 1H), 8.55 (br s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.8 (2C), 12.6, 19.6, 19.7, 20.6, 21.0, 22.6, 22.7, 23.8, 24.4, 24.8, 28.0, 31.2, 32.7, 32.8, 37.3, 37.4 (3C), 39.4, 40.0, 75.2, 102.7, 115.5, 117.0, 118.2, 123.7, 125.3, 127.1, 127.6, 140.0, 142.8, 143.4, 149.0, 157.8, 162.1, 172.6. Anal. calcd. for C₃₈H₅₄O₅: C, 77.25; H, 9.21. Found: C, 77.22; H, 9.43.

7-Hydroxy-3-[(*R*)-2,5,7,8-tetramethyl-2-((4*S*,8*S*)-4,8,12-trimethyltridecyl)]chroman-6-yl]oxy}-4*H*-chromen-4-one (1c)

According to the procedure described for the preparation of **1b**, compound **12c** (50.0 mg, 0.0861 mmol) was converted into compound **1c** as white solid (12.2 mg, 24%): mp 229–231 °C; $[\alpha]_D^{25} +5.4$ (c 1.01, CHCl₃); IR (neat): 3125 (OH); ¹H NMR (500 MHz, CDCl₃) δ 0.84–0.87 (m, 12H), 1.03–1.64 (m, 21H), 1.25 (s, 3H), 1.75–1.85 (m, 2H), 2.02 (s, 3H), 2.06 (s, 3H), 2.09 (s, 3H), 2.58 (t, $J = 6.9$ Hz, 2H), 6.86 (d, $J = 2.3$ Hz, 1H), 7.07–7.10 (m, 2H), 8.22 (d, $J = 8.6$ Hz, 1H), 8.57 (br s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.8 (2C), 12.6, 19.7 (2C), 20.6, 21.0, 22.6, 22.7, 23.7, 24.4, 24.8, 28.0, 31.1, 32.7, 32.8, 37.3, 37.4 (3C), 39.4, 40.0, 75.3, 102.7, 115.5, 117.0, 118.3, 123.7, 125.3, 127.1, 127.6, 140.0, 142.8, 143.4, 149.1, 157.8, 162.1, 172.6. Anal. calcd. for C₃₈H₅₄O₅·0.15EtOAc: C, 76.75; H, 9.21. Found: C, 76.40; H, 9.21.

7-Hydroxy-3-[(*S*)-2,5,7,8-tetramethyl-2-((4*R*,8*R*)-4,8,12-trimethyltridecyl)]chroman-6-yl]oxy}-4*H*-chromen-4-one (1d)

According to the procedure described for the preparation of **1b**, compound **12d** (100 mg, 0.172 mmol) was converted into compound **1d** as white solid (23.5 mg, 23%): mp 229–231 °C; $[\alpha]_D^{25} -5.4$ (c 0.95, CHCl₃); IR (neat): 3126 (OH); ¹H NMR (500 MHz, CDCl₃) δ 0.84–0.87 (m, 12H), 1.03–1.64 (m, 21H), 1.25 (s, 3H), 1.75–1.85 (m, 2H), 2.02 (s, 3H), 2.06 (s, 3H), 2.09 (s, 3H), 2.58 (t, $J = 6.9$ Hz, 2H), 6.86 (d, $J = 2.3$ Hz, 1H), 7.07–7.10 (m, 2H), 8.22 (d, $J = 8.6$ Hz, 1H), 8.51 (br s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.8 (2C), 12.6, 19.7 (2C), 20.6, 21.0, 22.6, 22.7, 23.8, 24.4, 24.8, 28.0, 31.1, 32.7, 32.8, 37.3, 37.4 (3C), 39.4, 40.0, 75.3, 102.7, 115.4, 117.0, 118.3, 123.7, 125.3, 127.1, 127.6, 139.9, 142.8, 143.4, 149.1, 157.8, 162.1, 172.6. Anal. calcd. for C₃₈H₅₄O₅: C, 77.25; H, 9.21. Found: C, 77.36; H, 9.32.



(S)-(3,7-Dimethyloct-6-en-1-yl)triphenylphosphonium Bromide (**16**)

According to the procedure described for the preparation of **8**, (S)-citronellyl bromide **15** (3.99 g, 18.2 mmol) was converted into compound **16**, which was used without further purification.

(R)-2-((S)-4,8-Dimethylnonyl)-2,5,7,8-tetramethylchroman-6-ol (**17a**)

According to the procedure described for the preparation of **10b**, compound *ent*-**9** (800 mg, 2.47 mmol) was converted into compound **17a** using the phosphonium salt **16** (ca. 4.93 mmol) as a pale yellow oil (735 mg, 83%). [α]_D²⁵ +0.4 (c 1.00, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.83-0.88 (m, 9H), 1.05-1.53 (m, 14H), 1.23 (s, 3H), 1.73-1.84 (m, 2H), 2.11 (s, 6H), 2.16 (s, 3H), 2.60 (t, *J* = 6.9 Hz, 2H), 4.18 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.3, 11.8, 12.2, 19.7 (2C), 21.0, 22.6, 22.7, 23.8, 24.7, 28.0, 31.5, 32.7, 37.2, 37.5, 39.3, 39.8, 74.5, 117.3, 118.4, 121.0, 122.6, 144.5, 145.5. HRMS (ESI) calcd for C₂₄H₄₀O₂ (M⁺): 360.3028; found: 360.3026.

(S)-2-((S)-4,8-Dimethylnonyl)-2,5,7,8-tetramethylchroman-6-ol (**17b**)

According to the procedure described for the preparation of **10b**, compound **9** (800 mg, 2.47 mmol) was converted into compound **17b** using phosphonium salt **16** (ca. 4.93 mmol) as a pale yellow oil (730 mg, 82%). [α]_D²⁵ +1.5 (c 1.00, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.83-0.88 (m, 9H), 1.04-1.53 (m, 14H), 1.23 (s, 3H), 1.73-1.84 (m, 2H), 2.11 (s, 6H), 2.16 (s, 3H), 2.60 (t, *J* = 6.9 Hz, 2H), 4.18 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.3, 11.8, 12.2, 19.6, 20.7, 21.0, 22.6, 22.7, 23.8, 24.8, 28.0, 31.5, 32.7, 37.3, 37.5, 39.3, 39.7, 74.5, 117.3, 118.4, 121.0, 122.6, 144.5, 145.5. HRMS (ESI) calcd for C₂₄H₄₀O₂ (M⁺): 360.3028; found: 360.3035.

1-(2,4-Dihydroxyphenyl)-2-[(R)-2-((S)-4,8-dimethylnonyl)-2,5,7,8-tetramethylchroman-6-yl]oxy}ethan-1-one (**18a**)

According to the procedure described for the preparation of **12b**, compound **17a** (700 mg, 1.94 mmol) was converted into compound **18a** as a pale yellow solid (916 mg,

84%): mp 89–91 °C; $[\alpha]_D^{25} +3.8$ (c 0.70, CHCl₃); IR (neat): 3341 (OH), 1626 (C=O); ¹H NMR (500 MHz, CDCl₃) δ 0.84–0.87 (m, 9H), 1.06–1.61 (m, 14H), 1.24 (s, 3H), 1.73–1.85 (m, 2H), 2.09 (s, 3H), 2.14 (s, 3H), 2.18 (s, 3H), 2.57 (t, *J* = 6.6 Hz, 2H), 4.93 (s, 2H), 6.37 (dd, *J* = 8.9, 2.6 Hz, 1H), 6.42 (d, *J* = 2.9 Hz, 1H), 7.49 (d, *J* = 8.6 Hz, 1H), 12.37 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.8, 11.9, 12.8, 19.6, 20.6, 21.0, 22.6, 22.7, 23.8, 24.7, 28.0, 31.1, 32.7, 37.2, 37.5, 39.3, 40.0, 73.9, 75.0, 103.8, 108.2, 111.7, 117.8, 123.2, 125.7, 127.6, 130.8, 147.7, 148.3, 163.2, 165.3, 198.0. HRMS (ESI) calcd for C₃₂H₄₇O₅ (MH⁺): 511.3418; found: 511.3414.

1-(2,4-Dihydroxyphenyl)-2-[(S)-2-((S)-4,8-dimethylnonyl)-2,5,7,8-tetramethylchroman-6-yl]oxy}ethan-1-one (18b)

According to the procedure described for the preparation of **12b**, compound **17b** (700 mg, 1.94 mmol) was converted into compound **18b** as a pale yellow solid (766 mg, 79%): mp 89–91 °C; $[\alpha]_D^{25} -3.7$ (c 0.62, CHCl₃); IR (neat): 3363 (OH), 1626 (C=O); ¹H NMR (500 MHz, CDCl₃) δ 0.84–0.87 (m, 9H), 1.03–1.60 (m, 14H), 1.24 (s, 3H), 1.73–1.85 (m, 2H), 2.09 (s, 3H), 2.14 (s, 3H), 2.18 (s, 3H), 2.57 (t, *J* = 6.6 Hz, 2H), 4.93 (s, 2H), 6.26 (s, 1H), 6.37 (dd, *J* = 9.2, 2.3 Hz, 1H), 6.42 (d, *J* = 2.9 Hz, 1H), 7.50 (d, *J* = 9.2 Hz, 1H), 12.38 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.8, 11.9, 12.8, 19.6, 20.6, 21.0, 22.6, 22.7, 23.8, 24.7, 28.0, 31.2, 32.7, 37.3, 37.5, 39.3, 40.0, 73.9, 75.0, 103.8, 108.2, 111.7, 117.8, 123.2, 125.7, 127.6, 130.9, 147.7, 148.3, 163.1, 165.3, 198.0. HRMS (ESI) calcd for C₃₂H₄₇O₅ (MH⁺): 511.3418; found: 511.3419.

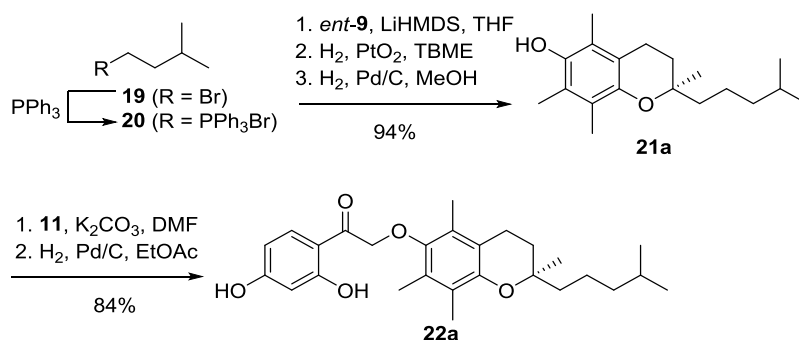
3-[(R)-2-((S)-4,8-Dimethylnonyl)-2,5,7,8-tetramethylchroman-6-yl]oxy}-7-hydroxy-4H-chromen-4-one (13a)

According to the procedure described for the preparation of **1b**, compound **18a** (100 mg, 0.196 mmol) was converted into compound **13a** as a white solid (20.5 mg, 20%): mp 239–241 °C; $[\alpha]_D^{25} +5.4$ (c 0.33, CHCl₃); IR (neat): 3116 (OH); ¹H NMR (500 MHz, CDCl₃) δ 0.84–0.87 (m, 9H), 1.06–1.61 (m, 14H), 1.25 (s, 3H), 1.75–1.85 (m, 2H), 2.02 (s, 3H), 2.05 (s, 3H), 2.09 (s, 3H), 2.58 (t, *J* = 6.7 Hz, 2H), 6.86 (d, *J* = 2.3 Hz, 1H), 7.07–7.10 (m, 2H), 8.22 (d, *J* = 8.7 Hz, 1H), 8.59 (br s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.8 (2C), 12.7, 19.6, 20.6, 21.0, 22.6, 22.7, 23.8, 24.7, 28.0, 31.1, 32.7, 37.2, 37.5, 39.3, 39.9, 75.3, 102.7, 115.5, 117.0, 118.3, 123.7, 125.3, 127.1, 127.6, 140.0, 142.8, 143.4, 149.0, 157.8, 162.1, 172.6. Anal. calcd. for C₃₃H₄₄O₅·0.25EtOAc: C, 75.24; H, 8.54. Found: C, 75.08; H, 8.60.

3-[(S)-2-((S)-4,8-Dimethylnonyl)-2,5,7,8-tetramethylchroman-6-yl]oxy}-7-hydroxy

-4*H*-chromen-4-one (13b)

According to the procedure described for the preparation of **1b**, compound **18b** (100 mg, 0.196 mmol) was converted into compound **13b** as a white solid (23.4 mg, 23%): mp 239–241 °C; $[\alpha]_D^{25}$ –5.6 (*c* 0.39, CHCl₃); IR (neat): 3131 (OH); ¹H NMR (500 MHz, CDCl₃) δ 0.86 (d, *J* = 6.3 Hz, 9H), 1.05–1.59 (m, 14H), 1.25 (s, 3H), 1.76–1.84 (m, 2H), 2.02 (s, 3H), 2.06 (s, 3H), 2.09 (s, 3H), 2.58 (t, *J* = 6.9 Hz, 2H), 6.85 (d, *J* = 1.7 Hz, 1H), 7.07 (dd, *J* = 9.2, 2.3 Hz, 1H), 7.09 (s, 1H), 8.22 (d, *J* = 8.6 Hz, 1H), 8.38 (br s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.8 (2C), 12.6, 19.6, 20.6, 21.0, 22.6, 22.7, 23.7, 24.7, 28.0, 31.2, 32.7, 37.3, 37.5, 39.3, 39.9, 75.3, 102.7, 115.7, 116.7, 118.3, 123.6, 125.3, 127.1, 127.4, 140.2, 142.8, 143.3, 149.0, 157.9, 162.5, 172.7. Anal. calcd. For C₃₃H₄₄O₅·0.1EtOAc: C, 75.76; H, 8.53. Found: C, 75.65; H, 8.58.



(Isopentyl)triphenylphosphonium Bromide (19)

According to the procedure described for the preparation of **8**, 1-bromo-3-methylbutane **19** (1.99 g, 13.2 mmol) was converted into compound **20**, which was used without further purification.

(*R*)-2,5,7,8-Tetramethyl-2-(4-methylpentyl)chroman-6-ol (21a)

According to the procedure described for the preparation of **10b**, compound *ent*-**9** (800 mg, 2.47 mmol) was converted into compound **21a** using the phosphonium salt **20** (ca. 4.93 mmol) as a pale yellow oil (675 mg, 94%). $[\alpha]_D^{25}$ –0.9 (*c* 1.00, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.87 (d, *J* = 6.9 Hz, 6H), 1.14–1.19 (m, 2H), 1.22 (s, 3H), 1.37–1.58 (m, 5H), 1.73–1.84 (m, 2H), 2.11 (s, 6H), 2.16 (s, 3H), 2.60 (t, *J* = 6.9 Hz, 2H), 4.17 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.3, 11.8, 12.2, 20.7, 21.4, 22.6 (2C), 23.8, 27.9, 31.5, 39.4, 39.7, 74.5, 117.3, 118.4, 121.0, 122.6, 144.5, 145.5. HRMS (ESI) calcd for C₁₉H₃₀O₂ (M⁺): 290.2246; found: 290.2245.

(S)-2,5,7,8-Tetramethyl-2-(4-methylpentyl)chroman-6-ol (21b)

According to the procedure described for the preparation of **10b**, compound **9** (800 mg, 2.47 mmol) was converted into compound **21b** using compound **20** (ca. 4.93 mmol) as a pale yellow oil (621 mg, 86%). $[\alpha]_D^{25} +0.9$ (c 1.00, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 0.87 (d, *J* = 6.9 Hz, 6H), 1.14-1.19 (m, 2H), 1.22 (s, 3H), 1.37-1.58 (m, 5H), 1.73-1.84 (m, 2H), 2.11 (s, 6H), 2.16 (s, 3H), 2.60 (t, *J* = 6.9 Hz, 2H), 4.17 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.3, 11.8, 12.2, 20.7, 21.4, 22.6 (2C), 23.8, 27.9, 31.5, 39.4, 39.7, 74.5, 117.3, 118.4, 121.0, 122.6, 144.5, 145.5. HRMS (ESI) calcd for C₁₉H₃₀O₂ (M⁺): 290.2246; found: 290.2239.

(R)-1-(2,4-Dihydroxyphenyl)-2-([2,5,7,8-tetramethyl-2-(4-methylpentyl)chroman-6-yl]oxy)ethan-1-one (22a)

According to the procedure described for the preparation of **12b**, compound **21a** (600 mg, 2.07 mmol) was converted into compound **22a** as a pale yellow solid (765 mg, 84%); mp 86–87 °C; $[\alpha]_D^{25} +3.5$ (c 0.78, CHCl₃); IR (neat): 3351 (OH), 1626 (C=O); ¹H NMR (500 MHz, CDCl₃) δ 0.87 (d, *J* = 6.9 Hz, 6H), 1.18 (dd, *J* = 14.9, 6.9 Hz, 2H), 1.24 (s, 3H), 1.37-1.61 (m, 5H), 1.73-1.85 (m, 2H), 2.08 (s, 3H), 2.14 (s, 3H), 2.18 (s, 3H), 2.57 (t, *J* = 6.6 Hz, 2H), 4.93 (s, 2H), 6.36 (dd, *J* = 8.9, 2.3 Hz, 1H), 6.42 (d, *J* = 2.3 Hz, 1H), 6.52 (br s, 1H), 7.49 (d, *J* = 9.2 Hz, 1H), 12.37 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.8, 11.9, 12.8, 20.6, 21.3, 22.6 (2C), 23.8, 27.8, 31.1, 39.3, 39.9, 73.9, 75.0, 103.8, 108.1, 111.7, 117.8, 123.2, 125.7, 127.6, 130.8, 147.7, 148.3, 163.3, 165.3, 198.0. HRMS (ESI) calcd for C₂₇H₃₇O₅ (MH⁺): 441.2636; found: 441.2633.

(S)-1-(2,4-Dihydroxyphenyl)-2-([2,5,7,8-tetramethyl-2-(4-methylpentyl)chroman-6-yl]oxy)ethan-1-one (22b)

According to the procedure described for the preparation of **12b**, compound **21b** (600 mg, 2.07 mmol) was converted into compound **22b** as pale yellow solid (505 mg, 55%); mp 86–87 °C; $[\alpha]_D^{25} -3.9$ (c 1.11, CHCl₃); IR (neat): 3351 (OH), 1626 (C=O); ¹H NMR (500 MHz, CDCl₃) δ 0.87 (d, *J* = 6.9 Hz, 6H), 1.17 (dd, *J* = 14.9, 6.9 Hz, 2H), 1.24 (s, 3H), 1.37-1.61 (m, 5H), 1.73-1.85 (m, 2H), 2.09 (s, 3H), 2.14 (s, 3H), 2.18 (s, 3H), 2.57 (t, *J* = 6.6 Hz, 2H), 4.93 (s, 2H), 6.28 (br s, 1H), 6.37 (dd, *J* = 8.9, 2.3 Hz, 1H), 6.42 (d, *J* = 2.3 Hz, 1H), 7.50 (d, *J* = 9.2 Hz, 1H), 12.38 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 11.8, 11.9, 12.8, 20.6, 21.3, 22.6 (2C), 23.8, 27.8, 31.1, 39.3, 39.9, 73.9, 75.0, 103.8, 108.1, 111.7, 117.8, 123.2, 125.7, 127.6, 130.8, 147.7, 148.3, 163.1, 165.3, 198.0. Anal. calcd. for C₂₇H₃₆O₅: C, 73.61; H, 8.24. Found: C, 73.39; H, 8.49.

(R)-7-Hydroxy-3-[[2,5,7,8-tetramethyl-2-(4-methylpentyl)chroman-6-yl]oxy]-4H-chromen-4-one (14a)

According to the procedure described for the preparation of **1b**, compound **22a** (100 mg, 0.227 mmol) was converted into compound **14a** as white solid (23.0 mg, 23%): mp 255–258 °C; $[\alpha]_D^{25} +5.3$ (c 0.40, THF); IR (neat): 3265 (OH); ^1H NMR (500 MHz, THF- d_8) δ 2.69 (d, $J = 2.9$ Hz, 3H), 2.70 (d, $J = 2.9$ Hz, 3H), 3.01 (dd, $J = 14.9, 6.9$ Hz, 2H), 3.06 (s, 3H), 3.26–3.43 (m, 5H), 3.56–3.68 (m, 2H), 3.82 (s, 3H), 3.86 (s, 3H), 3.89 (s, 3H), 4.44 (t, $J = 6.9$ Hz, 2H), 8.47 (d, $J = 2.3$ Hz, 1H), 8.64 (dd, $J = 8.6, 2.3$ Hz, 1H), 8.84 (s, 1H), 9.87 (d, $J = 8.6$ Hz, 1H), 11.19 (s, 1H); ^{13}C NMR (125 MHz, THF- d_8) δ 10.9, 11.1, 11.8, 20.4, 21.4, 22.0 (2C), 23.2, 27.9, 31.1, 39.5, 39.9, 74.9, 101.9, 114.1, 117.5, 118.0, 123.2, 125.2, 126.9, 127.3, 139.2, 143.4, 143.8, 148.9, 157.6, 162.4, 170.0. Anal. calcd. for $\text{C}_{28}\text{H}_{34}\text{O}_5 \cdot 0.1\text{EtOAc}$: C, 74.25; H, 7.64. Found: C, 74.12; H, 7.61.

(S)-7-Hydroxy-3-[[2,5,7,8-tetramethyl-2-(4-methylpentyl)chroman-6-yl]oxy]-4H-chromen-4-one (14b)

According to the procedure described for the preparation of **1b**, compound **22b** (100 mg, 0.227 mmol) was converted into compound **14b** as white solid (22.7 mg, 22%): mp 255–258 °C; $[\alpha]_D^{25} -5.1$ (c 0.52, THF); IR (neat): 3147 (OH); ^1H NMR (500 MHz, THF- d_8) δ 2.69 (d, $J = 2.9$ Hz, 3H), 2.70 (d, $J = 2.9$ Hz, 3H), 3.01 (dd, $J = 14.9, 6.9$ Hz, 2H), 3.06 (s, 3H), 3.26–3.43 (m, 5H), 3.56–3.68 (m, 2H), 3.82 (s, 3H), 3.86 (s, 3H), 3.89 (s, 3H), 4.44 (t, $J = 6.9$ Hz, 2H), 8.47 (d, $J = 2.3$ Hz, 1H), 8.64 (dd, $J = 8.6, 2.3$ Hz, 1H), 8.84 (s, 1H), 9.87 (d, $J = 8.6$ Hz, 1H), 11.20 (s, 1H); ^{13}C NMR (125 MHz, THF- d_8) δ 10.9, 11.1, 11.8, 20.4, 21.4, 22.0 (2C), 23.2, 27.9, 31.1, 39.5, 39.9, 74.9, 101.9, 114.1, 117.5, 118.0, 123.2, 125.2, 126.9, 127.3, 139.2, 143.3, 143.8, 148.9, 157.6, 162.4, 170.0. Anal. calcd. for $\text{C}_{28}\text{H}_{34}\text{O}_5$: C, 74.64; H, 7.61. Found: C, 74.33; H, 7.74.

Screening by the Chemical Array

Photoaffinity linker-coated (PALC) slides were prepared according to previous reports using amine-coated slides and the photoaffinity proline linker.¹ A solution of compounds (2.5 mg/mL in DMSO) from the in-house chemical library (NPDepo, RIKEN) was immobilized onto the PALC glass slides with a chemical arrayer equipped with 24 stamping pins. The slides were exposed to UV irradiation of 4 J/cm² at 365 nm using a CL-1000L UV crosslinker (UVP, CA). The slides were washed successively with DMSO, DMF, acetonitrile, THF, dichloromethane, EtOH, and ultra-pure water (5 min, 3 times each), and dried. D- or L-MDM2^{TMR} (3 μM in 1% skim-milk-TBS-T) was incubated with the glass slide for 1 h, and then washed with TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) (5 min, 3 times). The slides were dried and

scanned at 532 nm on a GenePix scanner. The fluorescence signals were quantified with GenePixPro.

FP Assay

FP assays were carried out in PBS containing 2% DMSO and 0.005% Tween-20 using a fluorescein-labeled p53 (P4) peptide (0.5-1.0 nM) and MDM2²⁵⁻¹⁰⁹ (10 nM) in black 96-well non-binding surface assay plates (Corning).¹ The potential inhibitors and FAM-labeled P4 peptide in DMSO were diluted five-fold with PBS in advance. The protein (90 µL) was preincubated with the compound solution (5 µL) for 30 min. Then, the fluorescein-labeled P4 peptide (5 µL) was added and incubated for 30 min. The P4 peptide was used as the positive control. FP signals were analyzed using an EnVision Xcite plate reader (Perkin Elmer) with a 480-nm excitation filter and a 535-nm emission filter.

SPR Analysis

SPR Analyses of p53 binding to synthetic MDM2²⁵⁻¹⁰⁹ and MDM2^{TMR} were carried out using Biacore T200 SPR instrument. PBS (Nacalai Tesque, pH 7.4) containing 0.05 % Tween-20 was used as the running buffer at 25 °C. Biotinylated wild-type p53 peptides (biotinyl-aminocaproyl-GSGSSQETFSDLWKLLPEN-NH₂) were immobilized on a streptavidin (SA) sensor chip (L-p53: 45.1 RU, D-p53: 46.4 RU). All analytes were evaluated for 2 min as contact time, followed by 2 min dissociation at a flow rate of 30 µL/min.

For competitive inhibition assays, L-MDM2²⁵⁻¹⁰⁹ (30 nM) in the presense of varying concentration of inhibitors in PBS containing 0.05% Tween-20 and 1% DMSO were injected on SA sensor chip, where biotinylated wild-type L-p53 peptide was immobilized (127.3 RU).

Competitive Binding Inhibition Assay by a Standard ELISA

ELISA assays were carried out in HEPES buffer [20 mM HEPES (pH 7.4), 100 mM NaCl, 0.05% Tween-20, 0.1% BSA]. Precoated streptavidin 96-well plates (Nunc) were incubated with 300 µL/well of HEPES buffer containing 3% BSA for 1h. After three washes, biotinylated wild-type p53 peptide (100 nM) in HEPES buffer (100 µL/well) was added and incubated for 2 h. After three washes, 100 nM MDM2 (recombinant human MDM2, untagged, Sigma) in the presence of varying concentration of inhibitors in HEPES buffer containing 1% DMSO (100 µL/well) was added and incubated for 1 h. After three washes, 1:1000 dilution of anti-MDM2 rabbit IgG antibody (N-20, Santa

Cruz] in HEPES buffer (100 μ L/well) was added and incubated for 1 h. After three washes, 1:5000 dilution of HRP-conjugated anti-rabbit IgG antibody (Promega) in HEPES buffer (100 μ L/well) was added and incubated for 1 h. After three washes, TMB (3,3',5,5'-Tetramethylbenzidine) solution (WAKO, 100 μ L/well) was added and incubated for 1 h. Then, aqueous 2 N H_2SO_4 (10 μ L/well) was added. Absorbance at 450 nm was measured for each well using an EnVision Xcite plate reader. The IC_{50} values were calculated by using GraphPad Prizm (GraphPad software, San Diego, CA).

Cell Growth Inhibition Assay

SJSA-1 and H1299 cells were cultured in RPMI-1640 medium (high glucose) (WAKO) supplemented with 10% (v/v) FBS at 37 °C in a 5% CO_2 -incubator. Cell-based assays using SJSA-1 and H1299 cells were performed in 96-well plates (BD Falcon). Both cells were seeded at 500 cells/well in 50 μ L of DMEM, and placed for 6 h. Chemical compounds in DMSO were diluted 250-fold with the culture medium in advance. Following the addition of the fresh culture medium (40 μ L), the chemical diluents (30 μ L) were also added to the cell cultures. The final volume of DMSO in the medium was equal to 0.1% (v/v). The cells under chemical treatment were incubated for a further 72 h. The wells in the plates were washed with the cultured medium without phenol-red twice. After 1 h of incubation with 100 μ L of the medium, the cell culture in each well was supplemented with the MTS reagent (20 μ L, Promega), followed by incubation for an additional 40 min. Absorbance at 490 nm was measured for each well using an EnVision Xcite plate reader. The GI_{50} values were calculated by using GraphPad Prizm (GraphPad software, San Diego, CA).

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Chapter 2. Synthesis of Grb2 SH2 Domain Proteins for Mirror-image Screening Systems

Summary

Grb2 is an adaptor protein that mediates cellular signal transduction. Grb2 contains an SH2 domain that interacts with phosphotyrosine-containing sequences in EGFR and other signaling molecules, and it is a promising molecular target for anticancer agents. To identify novel inhibitors of the Grb2 SH2 domain from natural products and their mirror-image isomers, screening systems using both enantiomers of a synthetic Grb2 SH2 domain protein were established. A pair of synthetic procedures for the proteins were investigated: one employed a single native chemical ligation (NCL) of two segment peptides, and the other used the N-to-C-directed NCL of three segment peptides for easier preparation. Labeling at the N-terminus or the Ala¹¹⁵ residue of the Grb2 SH2 domain provided functional probes to detect binding to a phosphotyrosine-containing peptide. The resulting synthetic-protein-based probes were applied to bioassays including chemical array analysis and enzyme-linked immunosorbent assays (ELISAs).

Grb2 functions as an adaptor protein in signal transduction pathways that are triggered by activation of receptor tyrosine kinases (RTKs). The Grb2 SH2 domain recognizes the phosphotyrosine (pTyr)-containing sequence in epidermal growth factor receptor (EGFR)¹ and Shc adaptor proteins,² leading to activation of the Ras-mitogen-activated protein kinase (MAPK) pathway³ and the Gab1-mediated PI3K–Akt pathway.⁴ The Grb2 SH2 domain also interacts with fibroblast growth factor receptor (FGFR) substrate 2 (FRS2) to regulate the FGFR signaling pathway.⁵ Moreover, the Grb2 SH2 domain interacts with the non-RTK chimeric Bcr–Abl protein, which constitutively activates Abl tyrosine kinase activity to induce chronic myeloid leukemia.⁶ Because of these pivotal roles in cell growth and differentiation signaling pathways, the Grb2 SH2 domain is a promising molecular target for anticancer agents.⁷

To date, a variety of inhibitors against the Grb2 SH2 domain have been reported via structure-activity relationship studies (Figure 1).⁸ Peptides with a pYXNX motif, which were identified by affinity-based purification on agarose beads from a mixture of phosphorylated peptides, exhibited selective binding for the Grb2 SH2 domain.⁹ To discover more potent and selective peptides, rapid screening of an extensive library of pTyr peptides was performed.¹⁰ Phage display libraries were also employed to identify potent phosphorylated peptides¹¹ or cyclic peptides G1TE.¹² After revealing the bioactive β -turn conformation of potent peptide ligands in the binding pockets of Grb2 SH2 domain,¹³ a number of inhibitors with a macrocyclic scaffold were designed.¹⁴

These include nonphosphorus-containing analogues with resistance against phosphatase-mediated degradation and favorable cell permeability.¹⁵

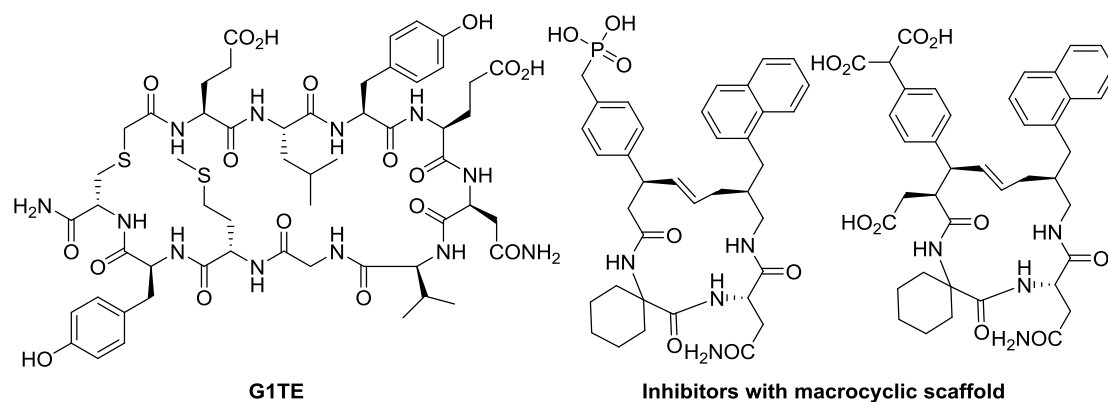


Figure 1. Structures of Grb2 SH2 domain inhibitors.

Despite a number of phosphopeptide-based inhibitors, there have been a limited number of reports on natural product-based Grb2 SH2 domain inhibitors (Figure 2). Asterriquinones were identified by screening extracts from *Aspergillus candidus* as Grb2 SH2 domain inhibitors that disrupt the Grb2–EGFR interaction.¹⁶ Sclerotiorinamine derivatives¹⁷ and actinomycin derivatives¹⁸ were reported to be Grb2 SH2 domain inhibitors that interfere with the Grb2–Shc interaction.

To validate the mirror-image screening strategy,¹⁹ the author chose the Grb2 SH2 domain and planned to apply both Grb2 SH2 domain enantiomers (L-Grb2 and D-Grb2) for the screening to identify the novel natural product-based inhibitors from both enantiomers of chiral natural products.

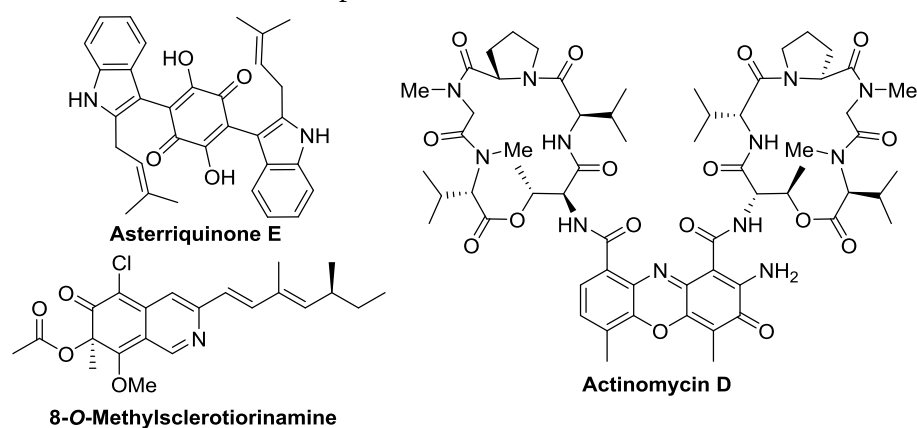


Figure 2. Structures of Grb2 SH2 domain inhibitors from natural products.

For synthesis of the Grb2 SH2 domain (Grb2^{53–154}), the author focused on two Gly-Ala sites (Gly⁸¹-Ala⁸² and Gly¹¹⁴-Ala¹¹⁵) as the ligation sites (Figure 3). Peptides

with a C-terminal Gly thioester are readily ligated with N-terminal Cys peptides²⁰ and chemical conversion of Cys to Ala is achievable by metal-free-desulfurization (MFD).²¹ Therefore, the author planned to employ a single or N-to-C-directed NCL strategy for synthesis of Grb2 SH2 domains. In a single NCL strategy, the N-terminal segment of Grb2⁵³⁻⁸¹ and the C-terminal segment of Grb2⁸²⁻¹⁵⁴ were ligated to produce [Cys⁸²]-Grb2⁵³⁻¹⁵⁴ (Figure 3A). This simple synthetic strategy required the solid-phase synthesis of the long C-terminal segment. In the alternative N-to-C-directed NCL strategy, three segments were successively ligated from the N-terminal Grb2⁵³⁻⁸¹ segment to the C-terminal Grb2¹¹⁵⁻¹⁵⁴ segment to provide [Cys⁸²/Cys¹¹⁵]-Grb2⁵³⁻¹⁵⁴ (Figure 3B). Although this strategy required an additional NCL process, three segments with ≤ 40 residues could be synthesized by solid-phase synthesis more easily.

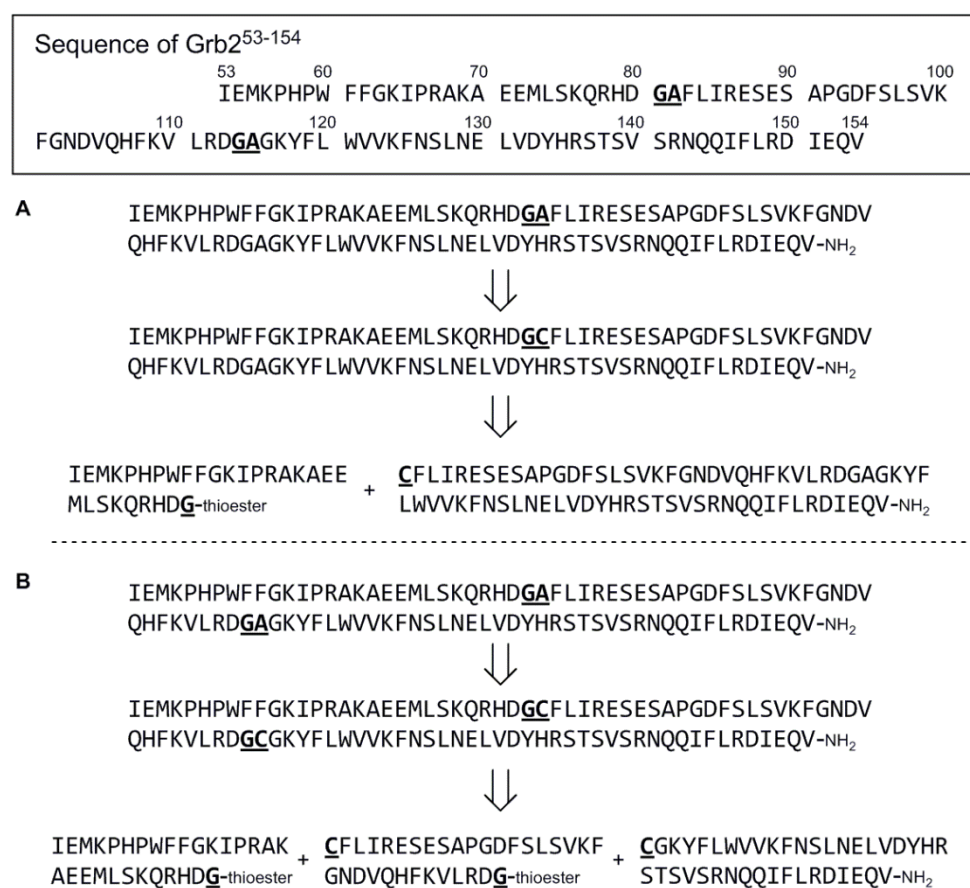


Figure 3. Sequence of the Grb2 SH2 domain. Ligation sites (Gly⁸¹-Ala⁸², Gly¹¹⁴-Ala¹¹⁵) are underlined and in bold type. (A) Retrosynthetic analysis of Grb2⁵³⁻¹⁵⁴ via a single NCL strategy. (B) Retrosynthetic analysis of Grb2⁵³⁻¹⁵⁴ via the N-to-C-directed NCL strategy.

Initially, the author synthesized a native sequence of the Grb2 SH2 domain (L-Grb2⁵³⁻¹⁵⁴) via NCL using two segments (Grb2⁵³⁻⁸¹ and Grb2⁸²⁻¹⁵⁴) (Figure 4). An

N-terminal segment for L-Grb2⁵³⁻⁸¹ (**L-1a**) was synthesized by standard Fmoc-based solid-phase peptide synthesis (SPPS) using HBTU/HOBt/(i-Pr)₂NEt activation on CLEAR amide resin with a first-generation Dawson linker.²² After chain assembly, the Dawson linker was converted to the benzimidazol-2-one form by treatment with 4-nitrophenyl chloroformate and (i-Pr)₂NEt. Final deprotection and cleavage from resin was achieved by treatment with 4-mercaptophenyl acetic acid (MPAA) and provided

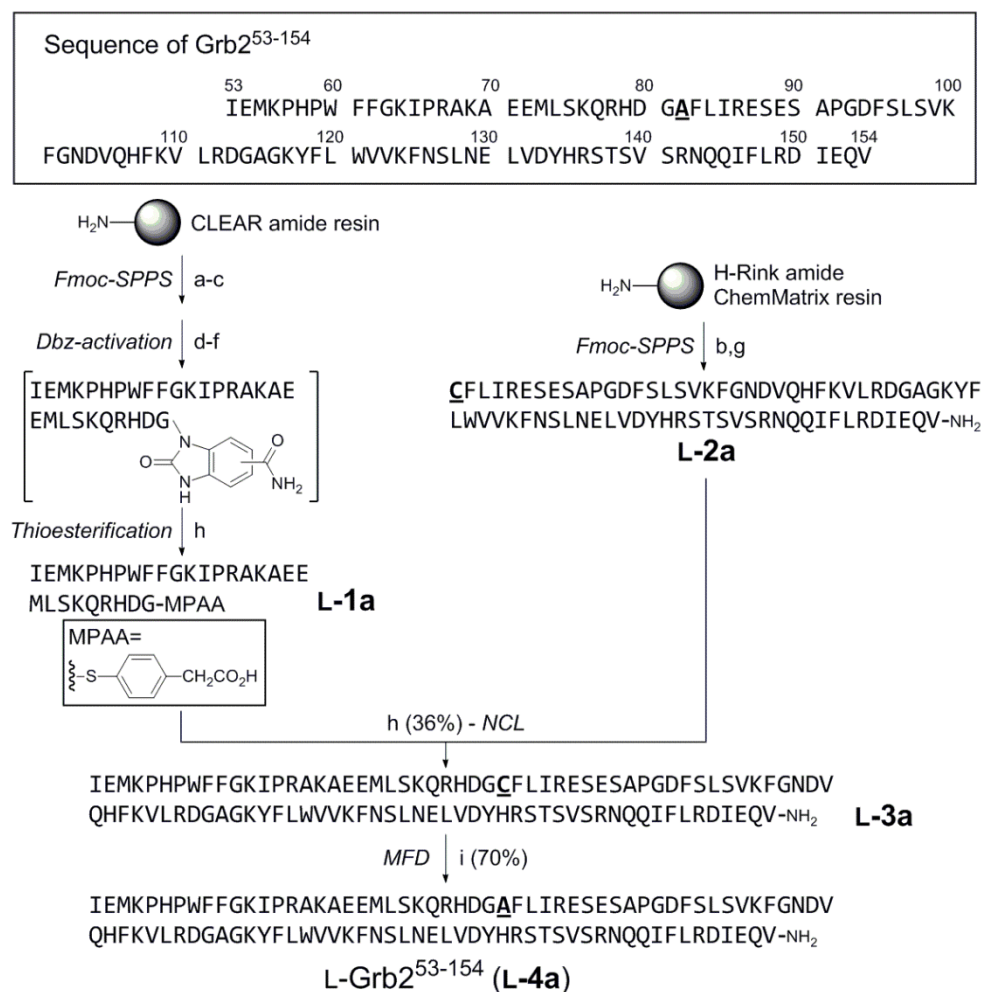


Figure 4. Synthesis of Grb2⁵³⁻¹⁵⁴. Sequence of Grb2⁵³⁻¹⁵⁴ and the synthetic scheme. Ligation sites (Cys⁸² and Ala⁸²) are underlined and in bold type. *Reagents and conditions:* (a) Fmoc-Dbz-OH, HBTU, HOBt, (i-Pr)₂NEt, DMF, then 20% piperidine/DMF; (b) Fmoc-Xaa-OH, HBTU, HOBt, (i-Pr)₂NEt, DMF, then 20% piperidine/DMF; (c) Boc-Ile-OH, HBTU, HOBt, (i-Pr)₂NEt, DMF; (d) 0.3 M 4-nitrophenyl chloroformate in DCM; (e) 0.5 M (i-Pr)₂NEt in DMF; (f) TFA/H₂O/*m*-cresol/thioanisole (80:10:5:5); (g) TFA/H₂O/*m*-cresol/thioanisole/EDT (80:5:5:5:5); (h) 6 M Gu·HCl, 200 mM MPAA, 20 mM TCEP·HCl in PBS (pH 7.0); (i) 6 M Gu·HCl, 100 mM Na₂HPO₄, 200 mM TCEP·HCl, 20 mM VA-044·2HCl, 40 mM MESNa (pH 6.5). Abbreviations: Dbz = 3,4-diaminobenzoic acid, Gu·HCl = guanidine hydrochloride, MES = 2-mercaptoethanesulfonic acid, TCEP = tris(2-carboxyethyl)-phosphine.

the expected thioester **L-1a**. The C-terminal segment L-Grb2⁸²⁻¹⁵⁴ (**L-2a**) was synthesized by standard Fmoc-based SPPS using HBTU/HOBt/(*i*-Pr)₂NEt activation on H-Rink Amide ChemMatrix amide resin. Ligation of **L-1a** and **L-2a** proceeded smoothly at room temperature to give [Cys⁸²]-L-Grb2⁵³⁻¹⁵⁴ (**L-3a**). The radical-mediated metal-free-desulfurization of **L-3a** with VA-044 produced native sequence L-Grb2⁵³⁻¹⁵⁴ (**L-4a**) at high purity (Figure 5). MESNa was used as a thiol additive instead of glutathione²³ because the author found the reaction completed faster.

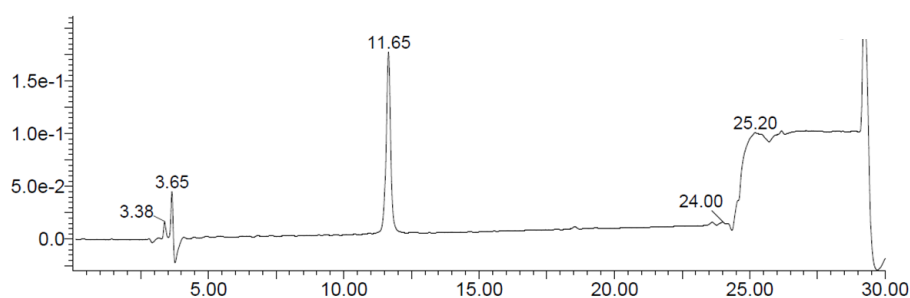


Figure 5. Analytical HPLC chromatogram of purified **L-4a**. HPLC analysis was performed at 25 °C with a linear gradient of 30–50% CH₃CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

For synthetic Grb2⁵³⁻¹⁵⁴ to be applied to chemical array screening, a tetramethylrhodamine (TMR)-labeled Grb2 SH2 domain protein was designed and synthesized (Figure 6). On the basis of the previous reports in which an N-terminal GST-fused Grb2 SH2 domain was expressed,²⁴ the author designed a TMR-labeled [Cys⁸²]-Grb2⁵³⁻¹⁵⁴ (L-Grb2^{TMR}) with modification at the N-terminus of the Grb2 SH2 domain. After solid-phase synthesis of the N-terminal Grb2⁵³⁻⁸¹ segment, the TMR moiety was introduced by on-resin modification at the N-terminus *via* a diglycine linker. Subsequent thioester formation using the identical protocol provided the TMR-labeled N-terminal segment **L-1b**. The ligation of **L-1b** and **L-2a** proceeded efficiently to afford the expected L-Grb2^{TMR} (**L-3b**) at high purity (Figure 7). Because the TMR moiety could be unstable under radical-mediated MFD conditions, L-Grb2^{TMR} (**L-3b**) was used for the biological studies without further conversion to the native sequence. The mirror-image protein (**D-3b**) was also synthesized using identical procedures.

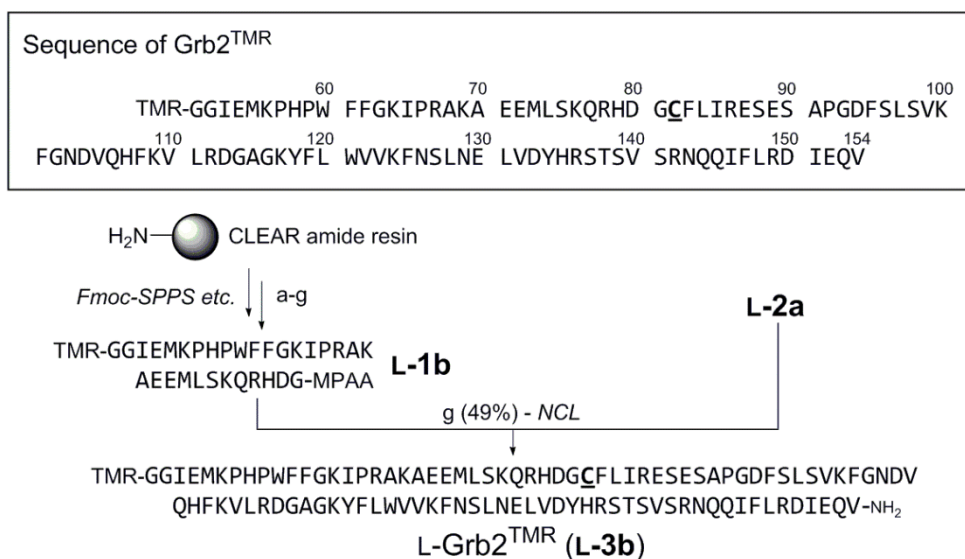


Figure 6. Synthesis of TMR-labeled [Cys⁸²]-Grb2^{53–154} (L-Grb2^{TMR}). (A) Sequence of L-Grb2^{TMR} and the synthetic scheme. Ligation sites (Cys⁸²) are underlined and in bold type. *Reagents and conditions:* (a) Fmoc-Dbz-OH, HBTU, HOBT, (*i*-Pr)₂NEt, DMF, then 20% piperidine/DMF; (b) Fmoc-Xaa-OH, HBTU, HOBT, (*i*-Pr)₂NEt, DMF, then 20% piperidine/DMF; (c) TMR, HOBT, DIPCl, DMF; (d) 0.3 M 4-nitrophenyl chloroformate in DCM; (e) 0.5 M (*i*-Pr)₂NEt in DMF; (f) TFA/H₂O/*m*-cresol/thioanisole (80:10:5:5); (g) 6 M Gu·HCl, 200 mM MPAA, 20 mM TCEP·HCl in PBS (pH 7.0).

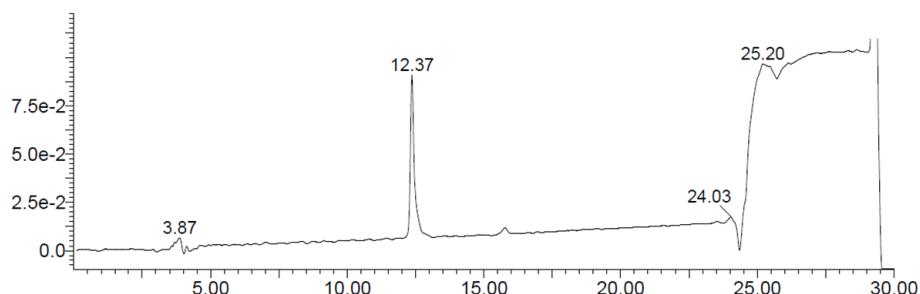


Figure 7. Analytical HPLC chromatogram of purified L-Grb2^{TMR} (L-3b). HPLC analysis was performed at 25 °C with a linear gradient of 30–50% CH₃CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

This two segment-based approach for Grb2^{53–154} needed to be improved for the large scale preparation of proteins that are required to screen a number of compounds, because the yields of the two segments were quite low (up to 2% yield from the starting resin). The low yields of N- and C-terminal segments were because of undesirable cleavage in the activation process of the Dawson linker and low efficiencies at the late stage of couplings, respectively. To overcome these problems, the author investigated an alternative synthetic strategy for Grb2^{53–154} via N-to-C-directed NCL using three segments (Grb2^{53–81}, Grb2^{82–114} and Grb2^{115–154}) (Figure 8).²⁵ To synthesize peptide

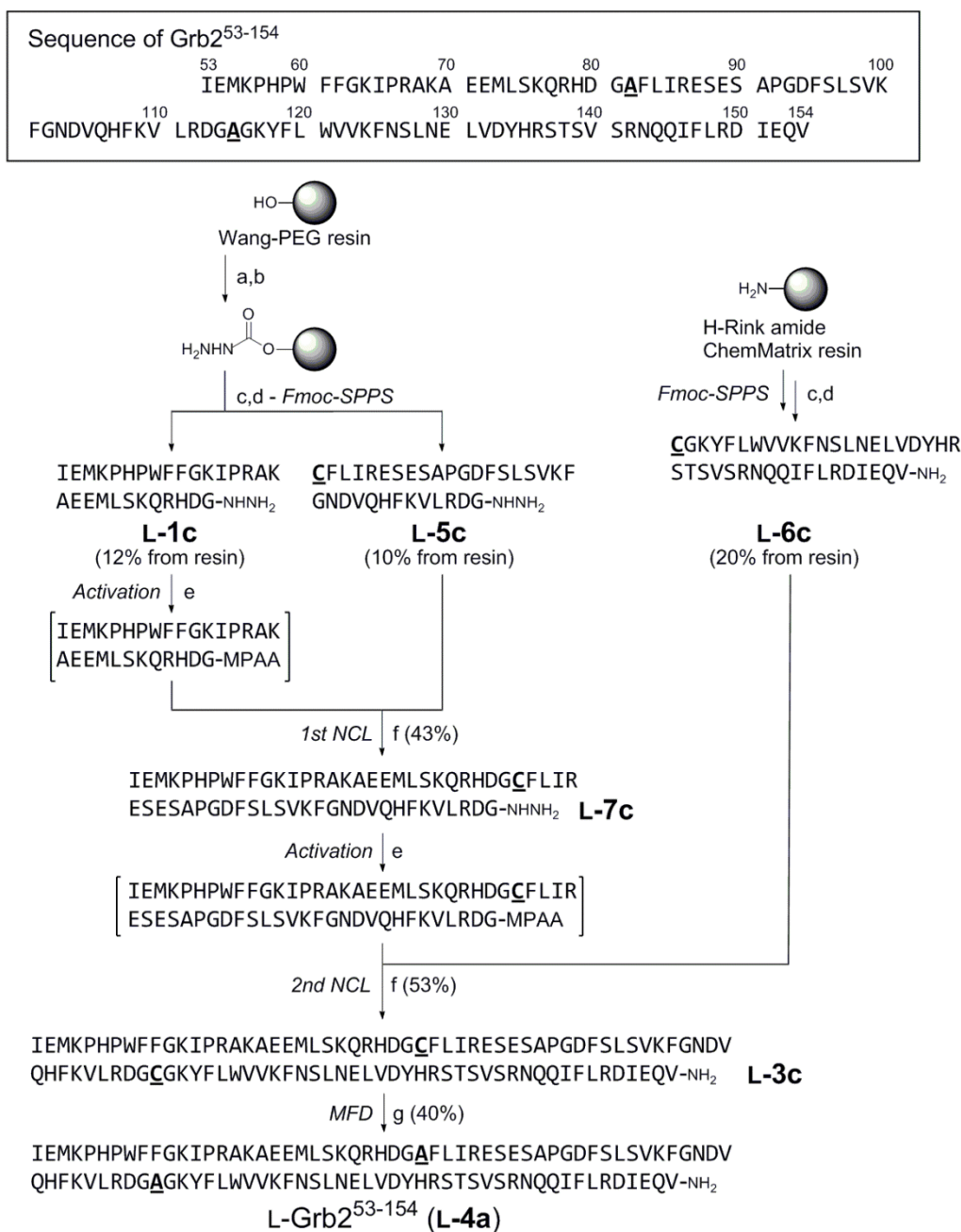


Figure 8. Synthesis of L-Grb2⁵³⁻¹⁵⁴ by three-segment hydrazide-based NCL. (A) Sequence of Grb2⁵³⁻¹⁵⁴ and the synthetic scheme by N-to-C-directed NCL using three segments. Ligation sites are underlined and in bold type. *Reagents and conditions:* (a) 4-nitrophenyl chloroformate, pyridine in DCM; (b) NH₂NH₂·H₂O in THF; (c) Fmoc-Xaa-OH, HBTU, HOBt, (*i*-Pr)₂NEt, DMF, then 20% piperidine/DMF; (d) TFA/H₂O/*m*-cresol/thioanisole/EDT (80:5:5:5); (e) NaNO₂, 6 M Gu·HCl, 100 mM Na₂HPO₄ (pH 3.0); (f) 6 M Gu·HCl, 150 mM Na₂HPO₄, 100 mM MPAA (pH 6.8-7.0); (g) 6 M Gu·HCl, 100 mM Na₂HPO₄, 200 mM TCEP·HCl, 20 mM VA-044·2HCl, 40 mM MESNa (pH 6.5).

hydrazides for the N-terminal and middle segments (Grb2⁵³⁻⁸¹ and Grb2⁸²⁻¹¹⁴), a hydrazine linker was attached to Wang-PEG resin by treatment of 4-nitrophenyl chloroformate and hydrazine. The peptide sequences were constructed by standard Fmoc-based SPPS using HBTU/HOBt/(i-Pr)₂N₂ activation. Cleavage from resin and final deprotection using a TFA cocktail followed by HPLC purification afforded the peptide hydrazides **L-1c** and **L-5c** at yields of more than 10%. The C-terminal segment **L-6c** was also obtained at a satisfactory yield by the standard protocol. Next, sequential N-to-C-directed NCL was investigated. The first NCL was conducted by NaNO₂-mediated activation of **L-1c** followed by the addition of **L-5c** in the presence of MPAA to provide the first NCL product **L-7c**. The second NCL was conducted by the same protocol using the hydrazide **L-7c** and the C-terminal segment **L-6c** to provide the full sequence **L-3c** with Cys⁸²/Cys¹¹⁵ substitutions. To obtain the native sequence, global desulfurization was performed to provide L-Grb2⁵³⁻¹⁵⁴ (**L-4a**) (Figure 9). Although solid-phase synthesis of three segments and two ligation processes were needed in this strategy, the overall yield of the target protein (0.91%) was improved compared with the previous synthesis using two segments (0.25%). The mirror-image protein, D-Grb2⁵³⁻¹⁵⁴ (**D-4a**), was also synthesized in the same manner.

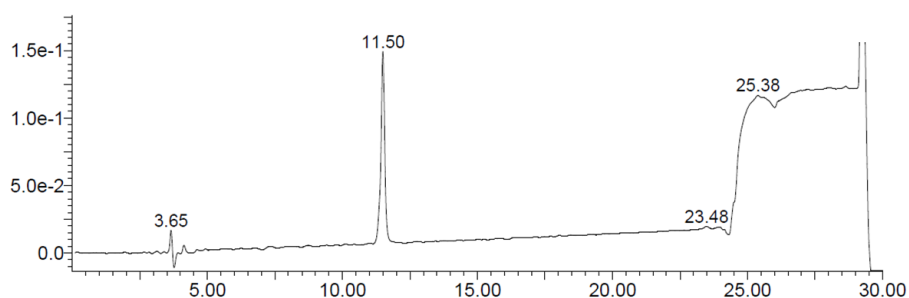


Figure 9. Analytical HPLC chromatogram of purified **L-4a**. HPLC analysis was performed at 25 °C with a linear gradient of 30–50% CH₃CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

Prior to assaying the biological activities of the synthetic Grb2 SH2 domain proteins, structural and functional analyses were performed. According to a procedure for refolding the Grb2 SH2 domain from *Escherichia. Coli* inclusion bodies,²⁶ the author investigated the folding conditions of chemically synthesized Grb2⁵³⁻¹⁵⁴. After the lyophilized synthetic proteins L-Grb2⁵³⁻¹⁵⁴ (**L-4a**) and D-Grb2⁵³⁻¹⁵⁴ (**D-4a**) were denatured in 6 M guanidine solution, the solutions were dialyzed in HEPES buffer (pH 7.4) to afford folded proteins. A similar L-Grb2⁵³⁻¹⁵⁴ spectrum was observed compared with reported spectra of other SH2 domains,²⁷ indicating that the synthetic Grb2 SH2 domain was correctly folded. Symmetrical circular dichroism (CD) spectra of

L-Grb2^{53–154} and D-Grb2^{53–154} supported the mirror-image structures of both Grb2 SH2 domain enantiomers (Figure 10).

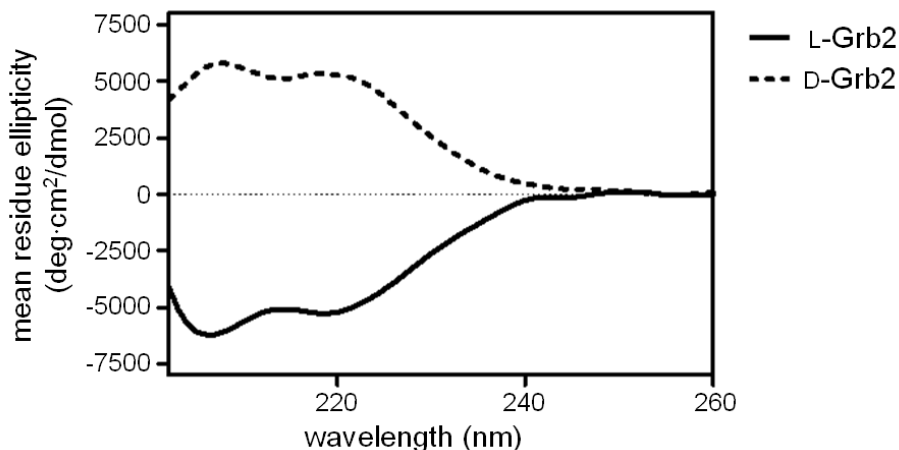


Figure 10. CD spectra of folded L-Grb2^{53–154} and D-Grb2^{53–154}

To validate the biological activities of the folded Grb2 SH2 domain proteins, their binding affinities toward both enantiomers of a pTyr-containing EGFR-derived peptide, biotin-EGFR^{1060–1076} (biotin-DTELPVPEpYINQSVPKR-NH₂), were evaluated by surface plasmon resonance (SPR) analysis. The selective binding of L-Grb2^{53–154} toward L-EGFR^{1060–1076} was observed with a K_D value of 329 nM, while no binding was observed toward D-EGFR^{1060–1076} (Figure 11). The selective binding of D-Grb2^{53–154} toward D-EGFR^{1060–1076} was observed with a K_D value of 447 nM, while no binding was observed toward L-EGFR^{1060–1076}, as well. The author also evaluated the binding activity of [Cys⁸²]-L-Grb2^{53–154}, which was obtained by folding **L-3a**. The binding affinity toward L-EGFR^{1060–1076} was identical (K_D: 327 nM), suggesting that the Ala82Cys mutation in the Grb2 SH2 domain had little effect on the binding toward the target pTyr-containing sequence, presumably because Ala⁸² is buried in the interior of the Grb2 SH2 domain.²⁸

The author next evaluated the binding activities of TMR-labeled Grb2 SH2 domains (L-Grb2^{TMR} and D-Grb2^{TMR}) toward pTyr-containing sequences by SPR analysis (Figure 12). L-Grb2^{TMR} and D-Grb2^{TMR} bound to L-EGFR^{1060–1076} and D-EGFR^{1060–1076} with K_D values of 180 and 242 nM, respectively. In contrast, no interaction was observed for L-Grb2^{TMR}-D-EGFR^{1060–1076} and D-Grb2^{TMR}-L-EGFR^{1060–1076}. These data suggested that TMR labeling at the N-terminus of the Grb2 SH2 domain did not interfere with binding or chiral recognition of pTyr-containing sequences.

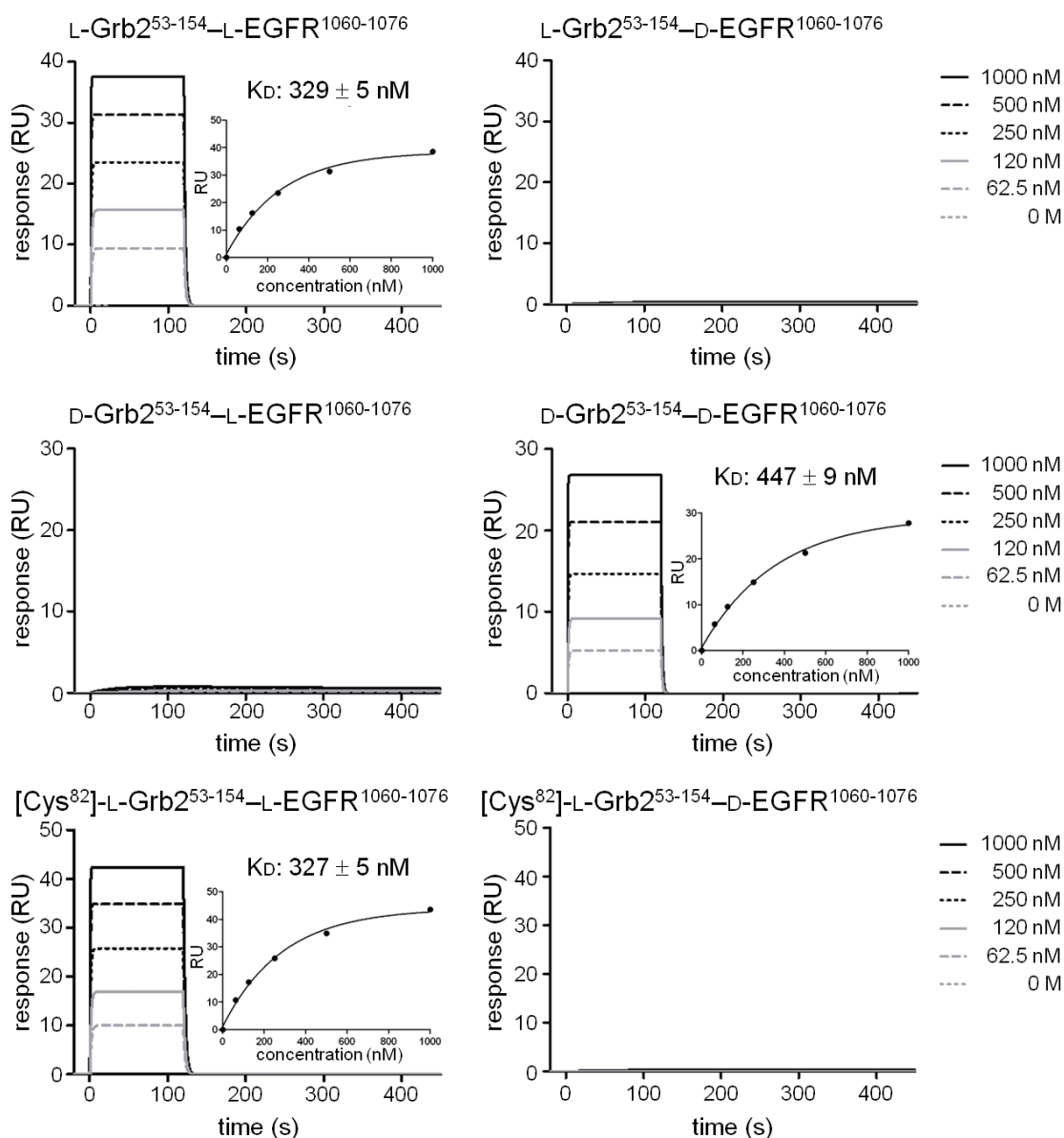


Figure 11. Representative data of SPR analysis of folded L-Grb2⁵³⁻¹⁵⁴ and [Cys⁸²]-L-Grb2⁵³⁻¹⁵⁴ for binding to N-terminally biotinylated L-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ and D-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ peptides. The steady-state bindings were evaluated from triplicate analyses.

The author also assessed the binding of TMR-labeled Grb2 SH2 proteins toward EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ peptides, which were immobilized on a chemical array *via* carbene-mediated covalent bond formation. Both L-Grb2^{TMR} and D-Grb2^{TMR} showed selective binding to L-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ and D-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ peptides, respectively, at different concentrations of spotted peptides (Figure 13). As such, these synthetic TMR-labeled Grb2 proteins, with good chiral recognition ability, could be employed for chemical array screenings.

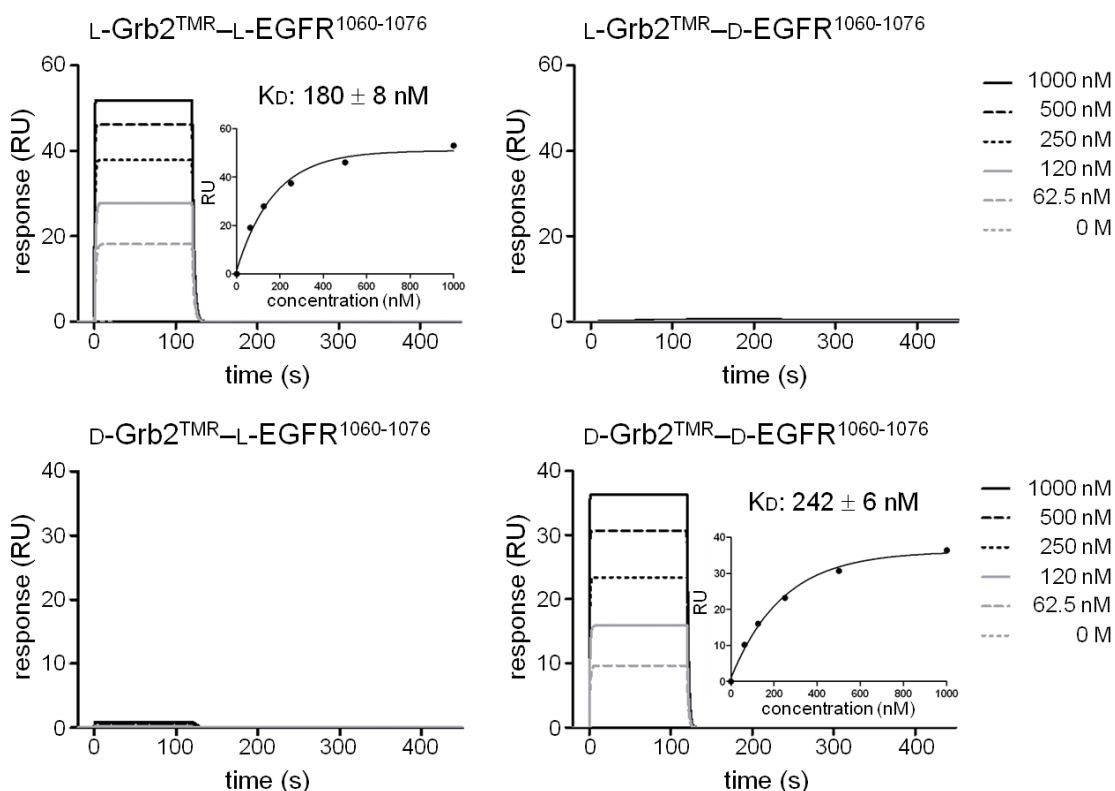


Figure 12. Representative data of SPR analysis of folded L-Grb2^{TMR} and D-Grb2^{TMR} toward L-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ and D-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ peptides. The steady-state binding affinities were evaluated from triplicate assays.

Using the both enantiomers of TMR-labeled Grb2 SH2 domains, chemical array screening for Grb2 SH2 domain was performed for 29,707 compounds from the Natural Products Depository (NPDepo in RIKEN) and our in-house library. Of these, 198 molecules and 503 molecules showed selective binding to L-Grb2^{TMR} and D-Grb2^{TMR}, respectively. The other 634 compounds exhibited binding to both L-Grb2^{TMR} and D-Grb2^{TMR}.

The chemically synthesized, functional Grb2 SH2 domain proteins should be applicable to chemical array technologies for the screening of a number of compounds. However, the hit compounds from chemical array analysis may bind to various pockets or to the surface of the SH2 domain. To identify compound(s) that binds to the pTyr-binding pocket of the Grb2 SH2 domain, thereby inhibiting the Grb2–EGFR interaction, the author next investigated synthetic Grb2⁵³⁻¹⁵⁴ in other bioassay systems. To date, a number of ELISA systems have been employed to test for inhibitory activity against interaction between the Grb2 SH2 domain and the pTyr peptide in EGFR. The majority of systems uses pTyr-containing peptides of EGFR or Shc immobilized on plates. The binding of the Grb2 SH2 domain is monitored by indirect detection using a

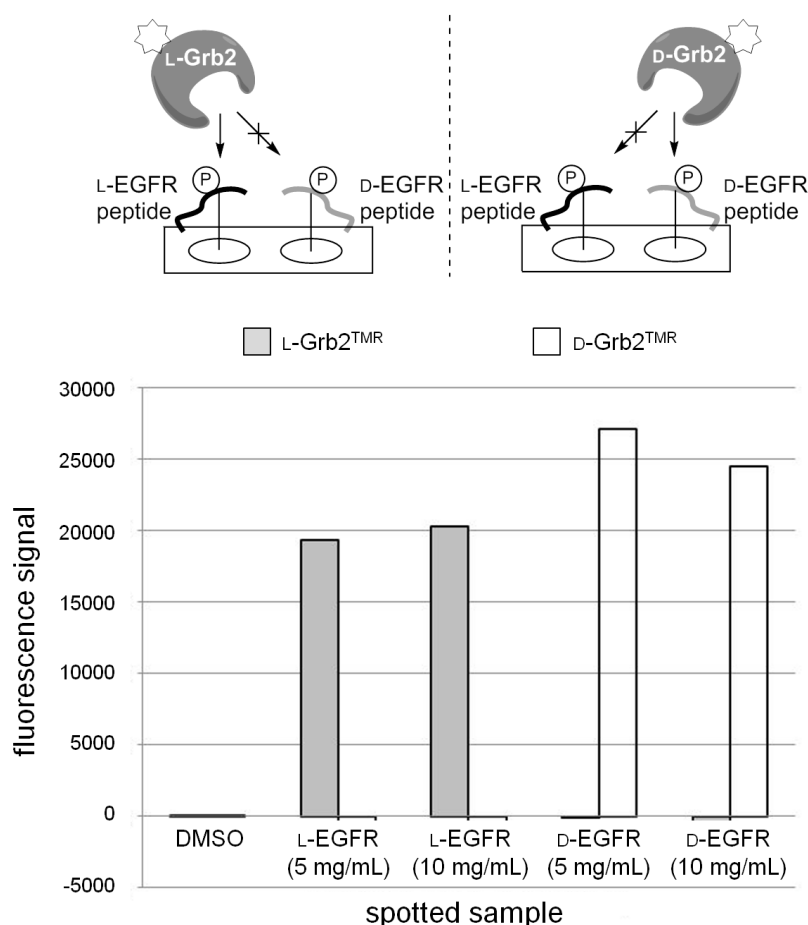


Figure 13. Binding activities of Grb2 SH2 domain proteins toward pTyr-containing peptides immobilized on a chemical array. The bindings of L-Grb2^{TMR} and D-Grb2^{TMR} were assessed using a chemical array, where L-EGFR^{1060–1076} or D-EGFR^{1060–1076} peptides were spotted at various concentrations.

GST-fused Grb2, an anti-GST antibody and an anti-IgG antibody conjugated with peroxidase.²⁹ These ELISA systems cannot be applied to mirror-image screening because commercially available antibodies cannot be used to detect mirror-image proteins and tag sequences consisting of D-amino acids. To establish a simple ELISA system for D-Grb2–D-EGFR interaction, native-form tags would be favorable for labeling D-Grb2 SH2 domain proteins and their counterpart pTyr-containing peptide (Figure 14). That is, D-Grb2 SH2 domain proteins with a D-biotin tag (native form) were designed for immobilization on a streptavidin (SA)-coated plate. The mirror-image D-pTyr-containing sequence, D-EGFR^{1060–1076} was labeled with a native 3×FLAG tag consisting of L-amino acids (sequence: DYKDHDGDYKDHDIDYKDDDDK). In this system, mirror-image protein–protein interaction between D-Grb2 and D-EGFR was assessed using a commercially available anti-FLAG antibody.

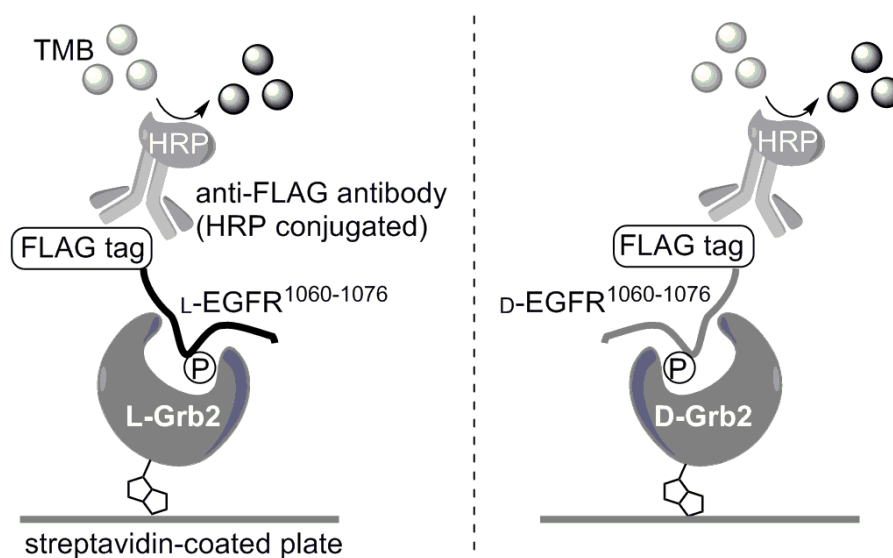


Figure 14. Schematic representation of the ELISA principle using synthetic proteins. Biotinylated L-Grb2 or D-Grb2 is immobilized on a streptavidin-coated plate. The binding of EGFR-derived pTyr peptide with a FLAG tag is detected by an HRP-conjugated anti-FLAG antibody and TMB substrate. Abbreviations: HRP = horseradish peroxidase, TMB = 3,3',5,5'-tetramethylbenzidine.

For this purpose, two biotinylated Grb2 SH2 domain proteins were designed and synthesized. On the basis of successful N-terminal TMR labeling of the Grb2 SH2 domain, biotin conjugation was attempted at the N-terminus *via* a diglycine linker (L-Grb2^{N-biotin}, **L-4d**). Additionally, the author chose the Ala¹¹⁵ residue, which is surface-located and away from the pTyr binding pocket, according to the crystal structure of the Grb2 SH2 domain (Figure 15), as an alternative position for biotin labeling.

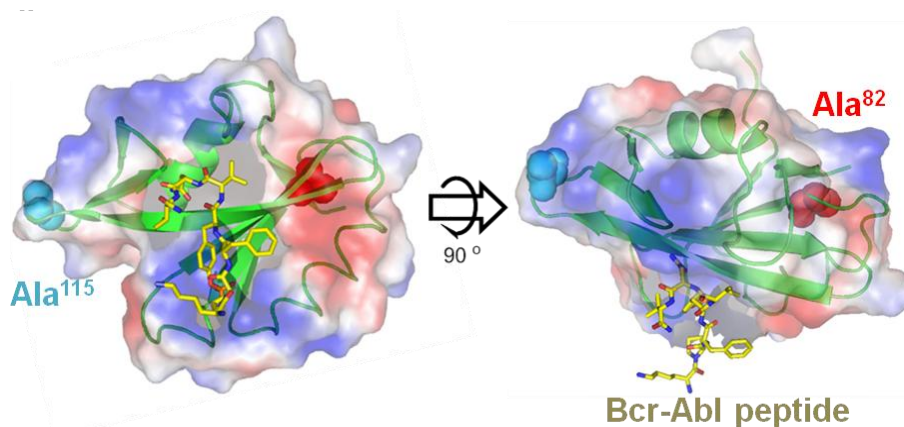


Figure 15. Structure of the Grb2 SH2 domain (PDB ID: 1TZE).²⁸ Ala⁸² (red) and Ala¹¹⁵ (cyan) are represented as spheres. The Bcr–Abl peptide bound to the Grb2 SH2 domain is represented as sticks.

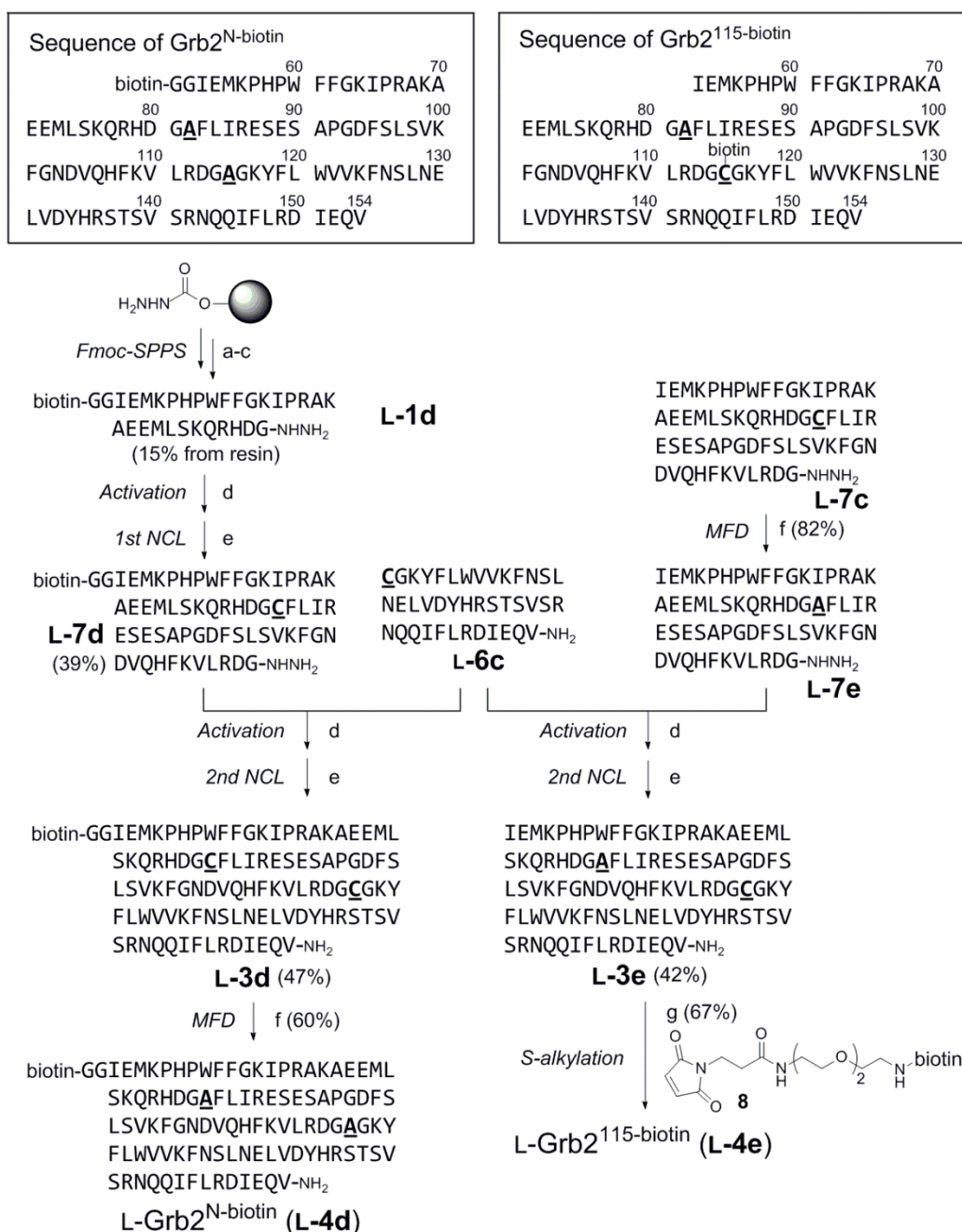


Figure 16. Design and synthesis of biotinylated Grb2 SH2 proteins. (A) Synthetic schemes for L-Grb2^N-biotin (**L-4d**) and L-Grb2¹¹⁵-biotin (**L-4e**). Amino acid sequences of Grb2^N-biotin and Grb2¹¹⁵-biotin. Ligation sites (Ala⁸², Ala¹¹⁵) are underlined and in bold type. *Reagents and conditions:* (a) Fmoc-Xaa-OH, HBTU, HOBt, (*i*-Pr)₂NEt, DMF, then 20% piperidine/DMF; (b) biotin, HBTU, HOBt, (*i*-Pr)₂NEt, DMF; (c) TFA/H₂O/*m*-cresol/thioanisole/EDT (80:5:5:5); (d) NaNO₂, 6 M Gu·HCl, 100 mM Na₂HPO₄ (pH 3.0); (e) 6 M Gu·HCl, 150 mM Na₂HPO₄, 100 mM MPAA (pH 6.8-7.0); (f) 6 M Gu·HCl, 100 mM Na₂HPO₄, 200 mM TCEP·HCl, 20 mM VA-044·2HCl, 40 mM MESNa (pH 6.5); (g) **8**, AcOH/DMSO/H₂O (1:2:1).

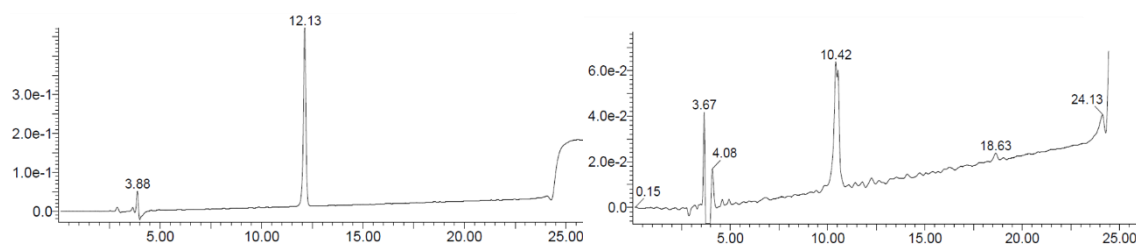


Figure 17. Analytical HPLC chromatograms of purified **L-4d** (left) and **L-4e** (right). HPLC analysis was performed at 25 °C with a linear gradient of 30–50% CH₃CN containing 0.1% TFA at a flow rate of 1 mL/min over 20 min.

L-Grb2^{N-biotin} (**L-4d**) was synthesized according to the procedure used for **L-4a** (Figure 16). Briefly, the first NCL using a biotinylated N-terminal segment **1d** provided a peptide hydrazide of [Cys⁸²]-L-Grb2^{53–114, N-biotin} (**L-7d**) at a yield of 39%. The second ligation using the C-terminal segment **L-6c** provided [Cys⁸², Cys¹¹⁵]-L-Grb2^{53–154, N-biotin} (**L-3d**) at a yield of 47%. The subsequent desulfurization of **L-3d** afforded the expected **L-4d** at high purity (Figure 17). The mirror-image protein (D-Grb2^{N-biotin}; **D-4d**) was also synthesized in the same manner using mirror-image materials except for native D-biotin.

The synthesis of L-Grb2^{115-biotin} (**L-4e**) with biotin labeling at the Ala¹¹⁵ position of the Grb2 SH2 domain began with desulfurization of Cys⁸² in **L-7c**, which was employed as an intermediate for the synthesis of native sequence **L-7a**, to give a peptide hydrazide of L-Grb2^{53–114} (**L-7e**). The subsequent NCL with **L-6c** provided [Cys¹¹⁵]-L-Grb2^{53–154} (**L-3e**) at a yield of 42%. The expected L-Grb2^{115-biotin} (**L-4e**) was synthesized by conjugation with biotin-PEG₂-maleimide **8** at Cys¹¹⁵ under acidic conditions (Figure 16). In this process, two stepwise NCLs and desulfurization of the intermediate facilitated the site-specific biotin labeling after all segments were assembled (Figure 17). This approach would be applicable to labeling proteins at the late stage of synthesis, facilitating the easy introduction of appropriate labeling group(s) for various screening processes.

The resulting synthetic biotinylated Grb2 SH2 domains (**L-4d**, **D-4d** and **L-4e**) were subjected to folding conditions to provide bioactive proteins (Figure 18). The binding affinities between biotinylated proteins and the counterpart pTyr peptides (EGFR^{1060–1076} peptides) were evaluated by SPR analysis, in which the biotinylated Grb2 SH2 domain proteins were immobilized on an SA sensor chip. L-EGFR^{1060–1076} peptide showed the desired binding toward L-Grb2^{N-biotin} (KD: 330 nM), while no binding of the mirror image D-EGFR^{1060–1076} peptide was observed, suggesting that N-terminal biotin labeling did not prevent chiral recognition at the binding pocket for the pTyr peptide. Also, D-EGFR^{1060–1076} peptide showed the desired binding toward D-Grb2^{N-biotin} (KD: 221 nM), while no binding of the mirror image L-EGFR^{1060–1076}

peptide was observed. The affinity between the L-Grb2^{115-biotin} and L-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ peptide was similar to that of L-Grb2^{N-biotin} (K_D: 364 nM). These data indicated that both the N-terminus and Ala¹¹⁵ in Grb2⁵³⁻¹⁵⁴ would be appropriate positions for biotin labeling without impairing the Grb2 SH2 domain–pTyr peptide and biotin–streptavidin interactions in the ELISA systems.

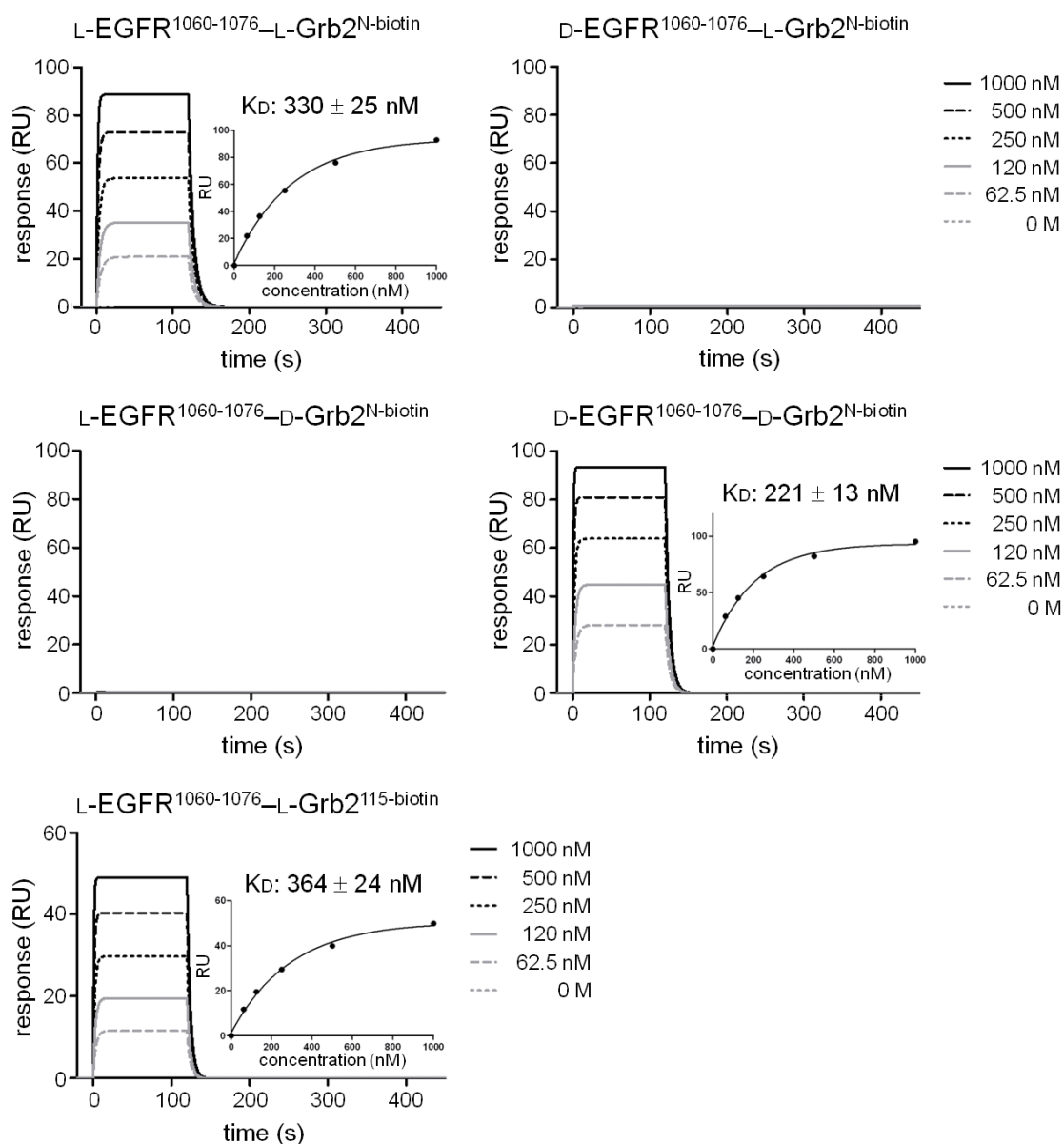


Figure 18. SPR analysis of biotinylated Grb2 SH2 domain proteins. Representative data of L-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ peptide binding to L-Grb2^{N-biotin} (A); D-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ binding to L-Grb2^{N-biotin} (B); and L-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ binding to L-Grb2^{115-biotin} (C). The steady-state bindings affinities were evaluated from triplicate assays.

Using the biotinylated Grb2 SH2 protein, ELISAs were established using

L-Grb2^{N-biotin} and D-Grb2^{N-biotin} to measure the inhibitory activity of potential Grb2 SH2 domain inhibitors. Initially, L-Grb2^{N-biotin} (1.8 ng/well) was immobilized on streptavidin-coated 96-well plates. Subsequently, the L-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ peptide, which possessed a FLAG tag at the N-terminus, was added (100 nM). The binding ratio of FLAG-labeled L-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ peptide to L-Grb2^{N-biotin} protein was detected using an

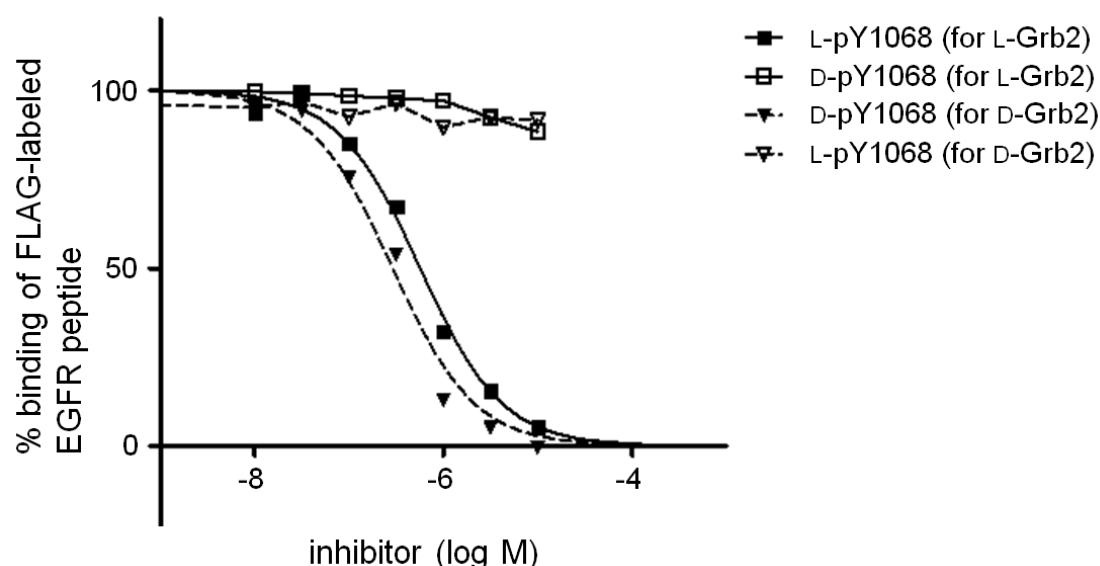


Figure 19. ELISA-based competition assays for Grb2 SH2 domain inhibitors. Representative dose-response curves of the assay using L-Grb2^{N-biotin} or D-Grb2^{N-biotin} are shown.

Table 1. Inhibitory activities of mirror-image pTyr-containing peptides (pY1068) against Grb2^{N-biotin}–EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ interactions in ELISAs.

compound	IC ₅₀ (μM) ^a	
	L-Grb2 SH2	D-Grb2 SH2
L-pY1068	0.60 ± 0.03	>10
D-pY1068	>10	0.39 ± 0.08

[a] The inhibitory activities were evaluated by ELISA-based competitive assays using L-Grb2^{N-biotin} or D-Grb2^{N-biotin}. IC₅₀ values were calculated from triplicate assays.

HRP-conjugated anti-FLAG antibody and TMB substrate. The mirror-image interaction between D-Grb2^{N-biotin} and D-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ was also monitored in the same manner. The bioactivity of a reported Grb2 SH2 domain inhibitor, pY1068, which is a pTyr-containing peptide of EGFR¹⁰⁶⁰⁻¹⁰⁷⁶, was evaluated for inhibitory activity against the L-Grb2^{N-biotin}–L-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ interaction (Figure 19 and Table 1). L-pY1068 potently inhibited the native interaction with an IC₅₀ value of 0.60 μM, while D-pY1068

did not. Similarly, D-pY1068 inhibited the mirror-image interaction between D-Grb2^{N-biotin}–D-EGFR^{1060–1076} with an IC₅₀ of 0.39 μM. Taken together, both enantiomers of chemically synthesized Grb2 SH2 domain proteins were successfully applied to the ELISA-based competition assays to identify inhibitors of Grb2–EGFR interaction.

In this study, the author investigated the chemical synthesis of Grb2 SH2 domain proteins and their application to the development of *in vitro* assay systems for Grb2 SH2 domain inhibitors. Initial attempt by single NCL at the Ala⁸² position using two segments produced synthetic Grb2 SH2 domain protein at moderate yield. When N-to-C-directed sequential NCL of three segments was employed, the desired Grb2 SH2 proteins were obtained with higher efficiency. To the best of the author's knowledge, this is the first report of the chemical synthesis of SH2 domain proteins. Based on these protocols, several labeled Grb2 SH2 proteins including Grb2^{TMR}, Grb2^{N-biotin} and Grb2^{115-biotin}, were synthesized. The synthetic proteins, which were subjected to folding under the appropriate conditions, exhibited the expected potent binding to phosphorylated EGFR peptide in chemical array analysis, SPR analysis, and ELISA. The L-Grb2 SH2 and D-Grb2 SH2 proteins selectively interacted with L-peptide and D-peptide of phosphorylated EGFR(1060–1076), respectively, suggesting that these proteins distinguish the native sequence and the mirror-image structure. In section 2 of chapter 1, chemical array screening and subsequent competitive fluorescence polarization assays using both enantiomers of synthetic L-MDM2 and D-MDM2 proteins identified novel inhibitors against MDM2–p53 interaction.¹ This mirror-image screening facilitated the identification process from a virtual library of mirror-image natural products. The *in vitro* assay systems using these chemically synthesized Grb2 SH2 domain proteins would be applicable to facile drug screening for the identification of novel Grb2 SH2 domain inhibitors from chiral substances (chiral natural products and L-peptides from phage display) and the virtual mirror-image compounds.

SH2 domains, which consist of a central antiparallel β-sheet flanked by two α-helices, are characteristic structural motifs common to a variety of critical intracellular signaling proteins. Because these mediate distinct signaling pathways,³⁰ selective inhibitors to impairing key intracellular SH2-mediated protein–protein interactions would be promising pharmaceutical agents.³¹ The developed synthetic protocols in this section are applicable to the preparation of a number of other SH2 domains, and their application in screening virtual mirror-image libraries will facilitate the identification of selective agents for these structurally conserved domains.

Experimental Section

General Methods

Fmoc-protected amino acids were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan) or Kokusan Chemical Co. Ltd. (Kanagawa, Japan). For analytical HPLC, Cosmosil 5C18-AR300 column (4.6 × 250 mm, Nacalai Tesque Inc.) was employed with a linear gradient of CH₃CN containing 0.1% (v/v) TFA at a flow rate of 1 mL/min. The products were detected by UV absorbance at 220 nm. For preparative HPLC, Cosmosil 5C18-AR300 column (20 × 250 mm, Nacalai Tesque Inc.) was employed with a linear gradient of CH₃CN containing 0.1% (v/v) TFA at a flow rate of 8 mL/min unless otherwise stated. All peptides were characterized by micromass ZQ LC-MS (Waters).

General Procedure for the Synthesis of Peptide Amides

Peptide chain elongation was performed on H-Rink Amide ChemMatrix resin (0.4–0.6 mmol/g) using an automatic peptide synthesizer (PSSM-8, Shimadzu) by Fmoc-based solid-phase peptide synthesis. For the side-chain protection, *t*-Bu ester for Asp and Glu; 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg; *t*-Bu for Thr, Tyr and Ser; Boc for Lys and Trp; and Trt for Gln, Asn, His, and Cys were employed for side-chain protection. Fmoc-protected amino acids (5 eq) were coupled by using HBTU (5 eq), HOBT (5 eq) and (*i*-Pr)₂NEt (10 eq) in DMF for 45 min twice. Fmoc-protecting group was removed by treatment with 20% piperidine in DMF for 5 min. The resulting protected peptide resin was treated with a TFA cocktail [TFA/H₂O/*m*-cresol/thioanisole/EDT (80:5:5:5:5)] for 2 h. After the removal of the resin by filtration, the filtrate was poured into ice-cold dry Et₂O. The resulting powder was collected by centrifugation and washed three times with ice-cold dry Et₂O. The crude product was purified by RP-HPLC to afford the expected peptide.

Peptide L-2a

By the standard procedure for peptide synthesis on H-Rink Amide ChemMatrix resin (160 mg, ca. 0.08 mmol), peptide **L-2a** was synthesized (8.43 mg, 1% yield). MS (ESI): Calcd for C₃₈₄H₅₈₈N₁₀₆O₁₁₀S₃: 8481.62; observed: [M+10H]¹⁰⁺ *m/z* = 849.04, [M+9H]⁹⁺ *m/z* = 944.03, [M+8H]⁸⁺ *m/z* = 1061.32, [M+7H]⁷⁺ *m/z* = 1213.18, [M+6H]⁶⁺ *m/z* = 1414.90.

Peptide D-2a

By the standard procedure for peptide synthesis on H-Rink Amide ChemMatrix resin (160 mg, ca. 0.08 mmol), peptide **D-2a** was synthesized (16.0 mg, 2% yield). MS (ESI): Calcd for $C_{384}H_{588}N_{106}O_{110}S_3$: 8481.62; observed: $[M+10H]^{10+}$ m/z = 849.18, $[M+9H]^{9+}$ m/z = 943.60, $[M+8H]^{8+}$ m/z = 1061.29, $[M+7H]^{7+}$ m/z = 1212.92, $[M+6H]^{6+}$ m/z = 1415.46.

Peptide L-6c

By the standard procedure for peptide synthesis, peptide **L-6c** was synthesized (19.5 mg, 20% yield) from H-Rink Amide ChemMatrix resin (40 mg, ca. 0.02 mmol). MS (ESI): Calcd for $C_{219}H_{337}N_{61}O_{61}S$: 4832.53; observed: $[M+6H]^{6+}$ m/z = 806.45, $[M+5H]^{5+}$ m/z = 967.69, $[M+4H]^{4+}$ m/z = 1209.21.

Peptide D-6c

By the standard procedure for peptide synthesis, peptide **D-6c** was synthesized (22.2 mg, 12% yield) from H-Rink Amide ChemMatrix resin (80.0 mg, ca. 0.04 mmol). MS (ESI): Calcd for $C_{219}H_{337}N_{61}O_{61}S$: 4832.53; observed: $[M+6H]^{6+}$ m/z = 806.50, $[M+5H]^{5+}$ m/z = 967.46, $[M+4H]^{4+}$ m/z = 1209.26.

Synthesis of Peptide MPAA Thioesters: Peptide L-1a

Fmoc-Dbz-OH (300 mg, 0.8 mmol) was loaded on CLEAR amide resin (0.32 mmol/g, 500 mg, 0.16 mmol) by HBTU (303 mg, 0.8 mmol), HOBt·H₂O (123 mg, 0.8 mmol) and (*i*-Pr)₂NEt (279 μ L, 1.6 mmol) in DMF for 2 h (three times). After the loading of the Dbz group on resin, the resins were split into eight portions (0.02 mmol each). The solid-phase peptide synthesis was performed using automatic peptide synthesizer by the standard protocol. For the coupling of an N-terminal Ile in peptide **1a**, Boc-Ile-OH (23.1 mg, 0.1 mmol) was employed with HBTU (37.9 mg, 0.1 mmol), HOBt·H₂O (15.3 mg, 0.1 mmol) and (*i*-Pr)₂NEt (34.8 μ L, 0.2 mmol) for 2 h using the 2 vessels (0.02 mmol $\times$ 2). After chain assembly, the resins were combined and treated with 0.3 M 4-nitrophenyl chloroformate in DCM (2.00 mL) for 2 h followed by 0.5 M (*i*-Pr)₂NEt in DMF (2.00 mL) for 1 h. Global deprotection and cleavage from resin was performed by treatment with a TFA cocktail [TFA/H₂O/*m*-cresol/thioanisole (80:10:5:5)] for 2 h. After removal of the resin by filtration, the filtrate was poured into ice-cold dry Et₂O. The resulting powder was collected by centrifugation and washed three times with ice-cold dry Et₂O. The resulting precipitate was dissolved in minimum amount of ligation buffer (6 M Gu·HCl, 200 mM MPAA, 20 mM TCEP·HCl in PBS, pH 7.0) for 30 min. Purification by RP-HPLC provided the peptide **1a** (2.96 mg, 2% yield). MS

(ESI): Calcd for $C_{165}H_{249}N_{45}O_{41}S_3$: 3615.26; observed: $[M+6H]^{6+}$ m/z = 603.25, $[M+5H]^{5+}$ m/z = 724.00, $[M+4H]^{4+}$ m/z = 904.79, $[M+3H]^{3+}$ m/z = 1205.95.

Peptide Thioester L-1b

By the identical procedure described for the synthesis of peptide **L-1a**, thioester **L-1b** was synthesized (2.85 mg, 1% yield) from Clear amide resin (156 mg, 0.05 mmol). TMR (64.6 mg, 0.15 mmol) was coupled by using DIPCI (23.2 μ L, 0.15 mmol), HOBT·H₂O (23.0 mg, 0.15 mmol) in DMF for 3 h. MS (ESI): Calcd for $C_{194}H_{275}N_{49}O_{47}S_3$: 4141.81; observed: $[M+7H]^{7+}$ m/z = 592.43, $[M+6H]^{6+}$ m/z = 691.32, $[M+5H]^{5+}$ m/z = 829.46, $[M+4H]^{4+}$ m/z = 1036.63, $[M+3H]^{3+}$ m/z = 1381.67.

Peptide Thioester D-1b

By the identical procedure described for the synthesis of peptide **L-1a** and **L-1b**, thioester **D-1b** was synthesized (1.95 mg, 1% yield) from Clear amide resin (125 mg, 0.04 mmol). MS (ESI): Calcd for $C_{194}H_{275}N_{49}O_{47}S_3$: 4141.81; observed: $[M+7H]^{7+}$ m/z = 593.22, $[M+6H]^{6+}$ m/z = 691.44, $[M+5H]^{5+}$ m/z = 829.48, $[M+4H]^{4+}$ m/z = 1036.78, $[M+3H]^{3+}$ m/z = 1381.94.

Preparation of Hydrazide Linker for the Solid-phase Synthesis of Peptide Hydrazides

4-Nitrophenyl chloroformate (1.01 g, 5.00 mmol) was added to the suspension of Wang-PEG resin (0.31 mmol/g, 1.60 g, 0.500 mmol) in dry pyridine (404 μ L, 5.00 mmol) and dry DCM (16 mL), and the suspension was agitated for 2 h. After filtration, the resin was treated with 1 M NH₂NH₂·H₂O in THF (16 mL) for 2 h.

Peptide Hydrazide L-1c

By the standard procedure for peptide synthesis, hydrazide **L-1c** was synthesized (20.7 mg, 12% yield) from Wang-PEG resin (161 mg, 0.05 mmol). MS (ESI): Calcd for $C_{157}H_{245}N_{47}O_{39}S_2$: 3479.10; observed: $[M+6H]^{6+}$ m/z = 580.70, $[M+5H]^{5+}$ m/z = 696.65, $[M+4H]^{4+}$ m/z = 870.72, $[M+3H]^{3+}$ m/z = 1160.61.

Peptide Hydrazide D-1c

By the standard procedure for peptide synthesis, hydrazide **D-1c** was synthesized (4.90 mg, 14% yield) from Wang-PEG resin (32.3 mg, 0.01 mmol). MS (ESI): Calcd for $C_{157}H_{245}N_{47}O_{39}S_2$: 3479.10; observed: $[M+6H]^{6+}$ m/z = 580.83, $[M+5H]^{5+}$ m/z = 696.88, $[M+4H]^{4+}$ m/z = 871.05, $[M+3H]^{3+}$ m/z = 1160.87.

Peptide Hydrazide L-1d

By the standard procedure for peptide synthesis, hydrazide **L-1d** was synthesized (29.2 mg, 15% yield) from Wang-PEG resin (161 mg, 0.05 mmol). Biotin (61.1 mg, 0.25 mmol) was coupled by using HBTU (94.8 mg, 0.25 mmol), HOBt·H₂O (38.3 mg, 0.25 mmol) and (*i*-Pr)₂NEt (89.1 μL, 0.5 mmol) in DMF for 2 h. MS (ESI): Calcd for C₁₇₁H₂₇₁N₅₁O₄₃S₃: 3819.50; observed: [M+6H]⁶⁺ *m/z* = 637.66, [M+5H]⁵⁺ *m/z* = 764.98, [M+4H]⁴⁺ *m/z* = 956.01, [M+3H]³⁺ *m/z* = 1274.14.

Peptide Hydrazide D-1d

By the standard procedure for peptide synthesis, hydrazide **D-1d** was synthesized (10.9 mg, 14%) from Wang-PEG resin (64.5 mg, 0.02 mmol). MS (ESI): Calcd for C₁₇₁H₂₇₁N₅₁O₄₃S₃: 3819.50; observed: [M+6H]⁶⁺ *m/z* = 637.45, [M+5H]⁵⁺ *m/z* = 764.95, [M+4H]⁴⁺ *m/z* = 955.88, [M+3H]³⁺ *m/z* = 1274.09.

Peptide Hydrazide L-5c

By the standard procedure for peptide synthesis, hydrazide **L-5c** was synthesized (30.5 mg, 10% yield) from Wang-PEG resin (258 mg, 0.08 mmol). MS (ESI): Calcd for C₁₆₅H₂₅₅N₄₇O₄₉S: 3713.20; observed: [M+5H]⁵⁺ *m/z* = 743.93, [M+4H]⁴⁺ *m/z* = 929.33, [M+3H]³⁺ *m/z* = 1238.69.

Peptide Hydrazide D-5c

By the standard procedure for peptide synthesis, hydrazide **D-5c** was synthesized (10.5 mg, 7% yield) from Wang-PEG resin (129 mg, 0.04 mmol). MS (ESI): Calcd for C₁₆₅H₂₅₅N₄₇O₄₉S: 3713.20; observed: [M+4H]⁴⁺ *m/z* = 929.28, [M+3H]³⁺ *m/z* = 1238.64.

Native Chemical Ligation Using Peptide MPAA Thioester: Synthesis of Peptide L-3a

MPAA-thioester segment **L-1a** (1.40 mg, 0.387 μmol) and C-terminal segment **L-2a** (2.00 mg, 0.236 μmol) were dissolved in the ligation buffer (6 M Gu·HCl, 200 mM MPAA, 20 mM TCEP·HCl in PBS, pH 7.0; 470 μL), and the reaction was monitored by LC-MS. After reaction was completed, TCEP·HCl (13.5 mg, 47.0 μmol) was added to the reaction mixture, and the mixture was stirred for 30 min at room temperature. The crude products were purified by preparative HPLC to afford peptide **L-3a** (1.02 mg, 36% yield). MS (ESI): Calcd for C₅₄₁H₈₂₉N₁₅₁O₁₄₉S₃: 11928.67; observed: [M+15H]¹⁵⁺ *m/z* = 796.67, [M+14H]¹⁴⁺ *m/z* = 853.13, [M+13H]¹³⁺ *m/z* = 918.72, [M+12H]¹²⁺ *m/z* =

995.28, $[M+11H]^{11+}$ m/z = 1085.77, $[M+10H]^{10+}$ m/z = 1194.04, $[M+9H]^{9+}$ m/z = 1326.84.

Peptide L-3b

By the identical procedure described for the synthesis of peptide L-3a, peptide L-1b (1.50 mg, 0.362 μ mol) and peptide L-2a (1.40 mg, 0.165 μ mol) were converted into the peptide L-3b (1.00 mg, 49% yield). MS (ESI): Calcd for $C_{570}H_{855}N_{155}O_{155}S_3$: 12455.22; observed: $[M+15H]^{15+}$ m/z = 831.50, $[M+14H]^{14+}$ m/z = 891.31, $[M+13H]^{13+}$ m/z = 959.32, $[M+12H]^{12+}$ m/z = 1039.15, $[M+11H]^{11+}$ m/z = 1133.59, $[M+10H]^{10+}$ m/z = 1246.82, $[M+9H]^{9+}$ m/z = 1385.89.

Peptide D-3b

By the identical procedure described for the synthesis of peptide L-3a, peptide D-1b (1.95 mg, 0.471 μ mol) and peptide D-2a (1.84 mg, 0.217 μ mol) were converted into the peptide D-3b (1.40 mg, 52% yield). MS (ESI): Calcd for $C_{570}H_{855}N_{155}O_{155}S_3$: 12455.22; observed: $[M+15H]^{15+}$ m/z = 831.90, $[M+14H]^{14+}$ m/z = 890.86, $[M+13H]^{13+}$ m/z = 959.27, $[M+12H]^{12+}$ m/z = 1039.32, $[M+11H]^{11+}$ m/z = 1133.98, $[M+10H]^{10+}$ m/z = 1246.68.

Native Chemical Ligation Using Peptide-hydrazide: Synthesis of Peptide L-3c

$NaNO_2$ solution (200 mM, 8.40 μ L, 1.68 μ mol) was added to a solution of hydrazide L-7c (1.00 mg, 0.140 μ mol) in activation buffer (6 M Gu·HCl, 100 mM Na_2HPO_4 , pH 3.0; 139 μ L) at -20 °C.²⁴ After the mixture was stirred for 30 min, peptide L-6c (680 μ g, 1.41 μ mol) was added to the mixture in MPAA solution (6 M Gu·HCl, 200 mM MPAA, 200 mM Na_2HPO_4 , pH 7.0; 139 μ L). The mixture was warmed to room temperature and pH was adjusted to 6.8-7.0. After reaction was completed, TCEP·HCl (8.18 mg, 0.0285 mmol) was added to the reaction mixture to a final concentration of 100 mM. The crude products were purified by preparative HPLC to afford peptide L-3c (900 μ g, 53% yield). MS (ESI): Calcd for $C_{541}H_{829}N_{151}O_{149}S_4$: 11960.73; observed: $[M+15H]^{15+}$ m/z = 798.04, $[M+14H]^{14+}$ m/z = 855.34, $[M+13H]^{13+}$ m/z = 921.34, $[M+12H]^{12+}$ m/z = 997.92, $[M+11H]^{11+}$ m/z = 1088.70, $[M+10H]^{10+}$ m/z = 1197.22, $[M+9H]^{9+}$ m/z = 1330.61.

Peptide D-3c

By the identical procedure described for the synthesis of peptide L-3c, peptide D-7c (1.45 mg, 0.203 μ mol) and peptide D-6c (1.47 mg, 0.304 μ mol) were converted into the peptide D-3c (1.10 mg, 45% yield). MS (ESI): Calcd for $C_{541}H_{829}N_{151}O_{149}S_4$: 11960.73;

observed: $[M+15H]^{15+}$ $m/z = 798.55$, $[M+14H]^{14+}$ $m/z = 855.43$, $[M+13H]^{13+}$ $m/z = 921.37$, $[M+12H]^{12+}$ $m/z = 998.07$, $[M+11H]^{11+}$ $m/z = 1088.59$, $[M+10H]^{10+}$ $m/z = 1197.72$, $[M+9H]^{9+}$ $m/z = 1330.64$.

Peptide L-3d

By the identical procedure described for the synthesis of peptide **L-3c**, peptide **L-7d** (3.95 mg, 0.537 μ mol) and peptide **L-6c** (3.31 mg, 0.685 μ mol) were converted into the peptide **L-3d** (3.10 mg, 47% yield). MS (ESI): Calcd for $C_{555}H_{849}N_{155}O_{153}S_5$: 12301.13; observed: $[M+15H]^{15+}$ $m/z = 821.27$, $[M+14H]^{14+}$ $m/z = 879.82$, $[M+13H]^{13+}$ $m/z = 947.59$, $[M+12H]^{12+}$ $m/z = 1026.47$, $[M+11H]^{11+}$ $m/z = 1119.56$, $[M+10H]^{10+}$ $m/z = 1231.55$, $[M+9H]^{9+}$ $m/z = 1367.79$.

Peptide D-3d

By the identical procedure described for the synthesis of peptide **L-3d**, peptide **D-7d** (1.08 mg, 0.144 μ mol) and peptide **D-6c** (900 μ g, 0.187 μ mol) were converted into the peptide **D-3d** (850 μ g, 47% yield). MS (ESI): Calcd for $C_{555}H_{849}N_{155}O_{153}S_5$: 12301.13; observed: $[M+15H]^{15+}$ $m/z = 821.50$, $[M+14H]^{14+}$ $m/z = 879.54$, $[M+13H]^{13+}$ $m/z = 947.51$, $[M+12H]^{12+}$ $m/z = 1026.20$, $[M+11H]^{11+}$ $m/z = 1119.43$, $[M+10H]^{10+}$ $m/z = 1230.97$, $[M+9H]^{9+}$ $m/z = 1368.69$.

Peptide L-3e

By the identical procedure described for the synthesis of peptide **L-3c**, peptide **L-7e** (1.55 mg, 0.217 μ mol) and peptide **L-6c** (1.37 mg, 0.283 μ mol) were converted into the peptide **L-3e** (1.09 mg, 42% yield). MS (ESI): Calcd for $C_{541}H_{829}N_{151}O_{149}S_3$: 11928.67; observed: $[M+15H]^{15+}$ $m/z = 796.30$, $[M+14H]^{14+}$ $m/z = 853.26$, $[M+13H]^{13+}$ $m/z = 918.75$, $[M+12H]^{12+}$ $m/z = 995.29$, $[M+11H]^{11+}$ $m/z = 1085.66$, $[M+10H]^{10+}$ $m/z = 1194.14$, $[M+9H]^{9+}$ $m/z = 1326.83$.

Peptide L-7c

By the identical procedure described for the synthesis of peptide **L-3c**, peptide **L-1c** (5.96 mg, 1.75 μ mol) and peptide **L-5c** (5.00 mg, 1.35 μ mol) were converted into the peptide **L-7c** (4.20 mg, 43% yield). MS (ESI): Calcd for $C_{322}H_{496}N_{92}O_{88}S_3$: 7160.25; observed: $[M+11H]^{11+}$ $m/z = 652.07$, $[M+10H]^{10+}$ $m/z = 717.11$, $[M+9H]^{9+}$ $m/z = 796.59$, $[M+8H]^{8+}$ $m/z = 895.97$, $[M+7H]^{7+}$ $m/z = 1023.89$, $[M+6H]^{6+}$ $m/z = 1194.66$, $[M+5H]^{5+}$ $m/z = 1433.28$.

Peptide D-7c

By the identical procedure described for the synthesis of peptide **L-3c**, peptide **D-1c** (3.00 mg, 0.881 μmol) and peptide **D-5c** (2.50 mg, 0.677 μmol) were converted into the peptide **D-7c** (1.45 mg, 30% yield). MS (ESI): Calcd for $\text{C}_{322}\text{H}_{496}\text{N}_{92}\text{O}_{88}\text{S}_3$: 7160.25; observed: $[\text{M}+10\text{H}]^{10+}$ m/z = 717.19, $[\text{M}+9\text{H}]^{9+}$ m/z = 796.86, $[\text{M}+8\text{H}]^{8+}$ m/z = 896.19, $[\text{M}+7\text{H}]^{7+}$ m/z = 1024.15, $[\text{M}+6\text{H}]^{6+}$ m/z = 1194.25, $[\text{M}+5\text{H}]^{5+}$ m/z = 1433.24.

Peptide L-7d

By the identical procedure described for the synthesis of peptide **L-3c**, peptide **L-1d** (6.70 mg, 1.75 μmol) and peptide **L-5c** (5.00 mg, 1.35 μmol) were converted into the peptide **L-7d** (3.95 mg, 39% yield). MS (ESI): Calcd for $\text{C}_{336}\text{H}_{516}\text{N}_{96}\text{O}_{92}\text{S}_4$: 7500.64; observed: $[\text{M}+10\text{H}]^{10+}$ m/z = 751.15, $[\text{M}+9\text{H}]^{9+}$ m/z = 834.62, $[\text{M}+8\text{H}]^{8+}$ m/z = 938.69, $[\text{M}+7\text{H}]^{7+}$ m/z = 1072.76, $[\text{M}+6\text{H}]^{6+}$ m/z = 1251.64.

Peptide D-7d

By the identical procedure described for the synthesis of peptide **L-3c**, peptide **D-1d** (2.67 mg, 0.700 μmol) and peptide **D-5c** (2.00 mg, 0.538 μmol) were converted into the peptide **D-7d** (1.42 mg, 35% yield). MS (ESI): Calcd for $\text{C}_{336}\text{H}_{516}\text{N}_{96}\text{O}_{92}\text{S}_4$: 7500.64; observed: $[\text{M}+10\text{H}]^{10+}$ m/z = 750.72, $[\text{M}+9\text{H}]^{9+}$ m/z = 834.54, $[\text{M}+8\text{H}]^{8+}$ m/z = 938.66, $[\text{M}+7\text{H}]^{7+}$ m/z = 1072.71, $[\text{M}+6\text{H}]^{6+}$ m/z = 1251.02.

Desulfurization of Cysteine Residues: Synthesis of Peptide L-4a²³

VA-044 $\cdot$ 2HCl (2.20 mg, 6.80 μmol) was added to a solution of peptide **L-3a** (660 μg , 0.0553 μmol) in the desulfurization buffer (6 M Gu $\cdot$ HCl, 100 mM Na_2HPO_4 , 200 mM TCEP $\cdot$ HCl, 40 mM MESNa, pH 6.5; 340 μL). The mixture was incubated at 37 $^\circ\text{C}$ and the reaction was monitored by LC-MS. After the reaction was completed, the crude products were purified by preparative HPLC to afford peptide **L-4a** (460 μg , 70% yield). MS (ESI): Calcd for $\text{C}_{541}\text{H}_{829}\text{N}_{151}\text{O}_{149}\text{S}_2$: 11896.61; observed: $[\text{M}+14\text{H}]^{14+}$ m/z = 850.76, $[\text{M}+13\text{H}]^{13+}$ m/z = 916.25, $[\text{M}+12\text{H}]^{12+}$ m/z = 992.62, $[\text{M}+11\text{H}]^{11+}$ m/z = 1082.79, $[\text{M}+10\text{H}]^{10+}$ m/z = 1190.99, $[\text{M}+9\text{H}]^{9+}$ m/z = 1322.74.

Peptide L-4a from Peptide L-3c

By the identical procedure described for the synthesis of peptide **L-4a** from **L-3a**, peptide **L-3c** (600 μg , 0.0502 μmol) was converted into the peptide **L-4a** (240 μg , 40% yield). MS (ESI): Calcd for $\text{C}_{541}\text{H}_{829}\text{N}_{151}\text{O}_{149}\text{S}_2$: 11896.61; observed: $[\text{M}+15\text{H}]^{15+}$ m/z = 794.45, $[\text{M}+14\text{H}]^{14+}$ m/z = 851.03, $[\text{M}+13\text{H}]^{13+}$ m/z = 916.30, $[\text{M}+12\text{H}]^{12+}$ m/z = 992.72, $[\text{M}+11\text{H}]^{11+}$ m/z = 1082.89, $[\text{M}+10\text{H}]^{10+}$ m/z = 1191.42.

Peptide D-4a

By the identical procedure described for the synthesis of peptide L-4a, peptide D-3c (1.10 mg, 0.0920 μmol) was converted into the peptide D-4a (670 μg , 61% yield). MS (ESI): Calcd for $\text{C}_{541}\text{H}_{829}\text{N}_{151}\text{O}_{149}\text{S}_2$: 11896.61; observed: $[\text{M}+15\text{H}]^{15+}$ m/z = 794.58, $[\text{M}+14\text{H}]^{14+}$ m/z = 851.01, $[\text{M}+13\text{H}]^{13+}$ m/z = 916.29, $[\text{M}+12\text{H}]^{12+}$ m/z = 992.65, $[\text{M}+11\text{H}]^{11+}$ m/z = 1082.90, $[\text{M}+10\text{H}]^{10+}$ m/z = 1191.11, $[\text{M}+9\text{H}]^{9+}$ m/z = 1323.09.

Peptide L-4d

By the identical procedure described for the synthesis of peptide L-4a, peptide L-3d (3.10 mg, 0.252 μmol) was converted into the peptide L-4d (1.86 mg, 60% yield). MS (ESI): Calcd for $\text{C}_{555}\text{H}_{849}\text{N}_{155}\text{O}_{153}\text{S}_3$: 12237.01; observed: $[\text{M}+15\text{H}]^{15+}$ m/z = 816.97, $[\text{M}+14\text{H}]^{14+}$ m/z = 875.28, $[\text{M}+13\text{H}]^{13+}$ m/z = 942.47, $[\text{M}+12\text{H}]^{12+}$ m/z = 1020.91, $[\text{M}+11\text{H}]^{11+}$ m/z = 1113.61, $[\text{M}+10\text{H}]^{10+}$ m/z = 1225.03, $[\text{M}+9\text{H}]^{9+}$ m/z = 1361.02.

Peptide D-4d

By the identical procedure described for the synthesis of L-4a, peptide D-3d (850 μg , 0.0691 μmol) was converted into the peptide D-4d (490 μg , 57% yield). MS (ESI): Calcd for $\text{C}_{555}\text{H}_{849}\text{N}_{155}\text{O}_{153}\text{S}_3$: 12237.01; observed: $[\text{M}+14\text{H}]^{14+}$ m/z = 875.03, $[\text{M}+13\text{H}]^{13+}$ m/z = 942.37, $[\text{M}+12\text{H}]^{12+}$ m/z = 1020.72, $[\text{M}+11\text{H}]^{11+}$ m/z = 1113.45, $[\text{M}+10\text{H}]^{10+}$ m/z = 1224.75, $[\text{M}+9\text{H}]^{9+}$ m/z = 1360.98.

Peptide L-7e

By the identical procedure described for the synthesis of peptide L-4a, peptide L-7c (2.00 mg, 0.279 μmol) was converted into the peptide L-7e (1.66 mg, 82% yield). MS (ESI): Calcd for $\text{C}_{322}\text{H}_{496}\text{N}_{92}\text{O}_{88}\text{S}_2$: 7128.19; observed: $[\text{M}+10\text{H}]^{10+}$ m/z = 713.74, $[\text{M}+9\text{H}]^{9+}$ m/z = 793.14, $[\text{M}+8\text{H}]^{8+}$ m/z = 892.20, $[\text{M}+7\text{H}]^{7+}$ m/z = 1019.45, $[\text{M}+6\text{H}]^{6+}$ m/z = 1189.38, $[\text{M}+5\text{H}]^{5+}$ m/z = 1426.82.

Biotin Labeling on the Cys Residue: Synthesis of Peptide L-4e

The solution of maleimide-linked biotin **8** (132 μg , 0.251 μmol) in DMSO (15 μL) was added to the solution of peptide L-3e (300 μg , 0.0251 μmol) in AcOH (30 μL) and H_2O (15 μL), and the mixture was incubated at 37 $^\circ\text{C}$ overnight. The crude product was purified by preparative HPLC to afford peptide L-4e (210 μg , 67% yield). MS (ESI): Calcd for $\text{C}_{564}\text{H}_{864}\text{N}_{156}\text{O}_{156}\text{S}_4$: 12454.29; observed: $[\text{M}+14\text{H}]^{14+}$ m/z = 831.33, $[\text{M}+13\text{H}]^{13+}$ m/z = 890.68, $[\text{M}+12\text{H}]^{12+}$ m/z = 959.08, $[\text{M}+11\text{H}]^{11+}$ m/z = 1039.03, $[\text{M}+10\text{H}]^{10+}$ m/z = 1133.47, $[\text{M}+9\text{H}]^{9+}$ m/z = 1246.53.

L-pY1068 (EGFR¹⁰⁶⁰⁻¹⁰⁷⁶: H-DTELPVPEpYINQSVPKR-NH₂)

By the standard procedure for peptide synthesis, L-pY1068 was synthesized (6.27 mg, 15% yield) from NovaSyn®TGR resin (76.9 mg, 0.02 mmol). Fmoc-Tyr[PO(OBzl)OH]-OH (57.4 mg, 0.1 mmol) was coupled by using TBTU (32.1 mg, 0.1 mmol), HOBt·H₂O (15.3 mg, 0.1 mmol) and (*i*-Pr)₂NEt (34.8 μL, 0.2 mmol) in DMF for 3 h. MS (ESI): Calcd for C₉₂H₁₄₅N₂₄O₂₉P: 2082.29; observed: [M+3H]³⁺ *m/z* = 694.86, [M+2H]²⁺ *m/z* = 1042.25.

D-pY1068

By the identical procedure described for the synthesis of L-pY1068, D-pY1068 was synthesized (7.50 mg, 18% yield) from NovaSyn®TGR resin (76.9 mg, 0.02 mmol). MS (ESI): Calcd for C₉₂H₁₄₅N₂₄O₂₉P: 2082.29; observed: [M+3H]³⁺ *m/z* = 695.01, [M+2H]²⁺ *m/z* = 1042.12.

Biotin-labeled L-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ (biotin-DTELPVPEpYINQSVPKR-NH₂)

By the identical procedure described for the synthesis of L-pY1068, biotin-labeled L-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ was synthesized (5.6 mg, 24% yield) from NovaSyn®TGR resin (38.5 mg, 0.01 mmol). Biotin (12.2 mg, 0.05 mmol) was coupled by using HBTU (19.0 mg, 0.05 mmol) HOBt·H₂O (7.66 mg, 0.05 mmol) and (*i*-Pr)₂NEt (17.4 μL, 0.1 mmol) in DMF for 2 h. MS (ESI): Calcd for C₁₀₂H₁₅₉N₂₆O₃₁PS: 2308.58; observed: [M+3H]³⁺ *m/z* = 770.49, [M+2H]²⁺ *m/z* = 1155.36.

Biotin-labeled D-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶

By the identical procedure described for the synthesis of biotin-labeled L-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶, biotin-labeled D-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ was synthesized (14.0 mg, 30% yield) from NovaSyn®TGR resin (76.9 mg, 0.02 mmol). MS (ESI): Calcd for C₁₀₂H₁₅₉N₂₆O₃₁PS: 2308.58; observed: [M+3H]³⁺ *m/z* = 770.50, [M+2H]²⁺ *m/z* = 1155.35.

FLAG-labeled L-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ (H-DYKDHDGDYKDHDIDYKDDDDK-DTELPV-PEpYINQSVPKR-NH₂)

By the identical procedure described for the synthesis of L-pY1068, FLAG-labeled L-EGFR¹⁰⁶⁰⁻¹⁰⁷⁶ was synthesized (3.00 mg, 3% yield) from H-Rink Amide ChemMatrix resin (40 mg, ca. 0.02 mmol). MS (ESI): Calcd for C₂₀₇H₃₀₃N₅₄O₇₆P: 4794.98; observed: [M+6H]⁶⁺ *m/z* = 800.26, [M+5H]⁵⁺ *m/z* = 960.20, [M+4H]⁴⁺ *m/z* = 1199.89.

FLAG-labeled D-EGFR^{1060–1076}

By the identical procedure described for the synthesis of FLAG-labeled L-EGFR^{1060–1076}, FLAG-labeled D-EGFR^{1060–1076} was synthesized (34.8 mg, 9% yield) from H-Rink Amide ChemMatrix resin (160 mg, ca. 0.08 mmol). MS (ESI): Calcd for C₂₀₇H₃₀₃N₅₄O₇₆P: 4794.98; observed: [M+6H]⁶⁺ m/z = 800.25, [M+5H]⁵⁺ m/z = 960.14, [M+4H]⁴⁺ m/z = 1199.79.

Folding of Grb2 SH2 Domain Proteins

Lyophilized peptide was dissolved in denaturing buffer (6 M Gu·HCl, 20 mM HEPES, 100 mM NaCl, pH 8.5) at the concentration of 0.5 mg/mL. The solution was dialyzed three times against a 200-fold volume of a folding buffer (20 mM HEPES, 100 mM NaCl, 0.5 mM TCEP·HCl, pH 7.4) at 4 °C for 2 h using MWCO 3000 membrane.

Evaluation of Binding Activity on a Chemical Array

Photoaffinity linker-coated (PALC) slides were prepared according to previous reports using amine-coated slides and the photoaffinity PEG linker.¹⁹ A solution of compounds in DMSO was spotted onto the PALC glass slides with a chemical arrayer equipped with 24 stamping pins or a MultiSPRinter spotter (Toyobo) equipped with single stamping pin. The slides were exposed to UV irradiation of 4 J/cm² at 365 nm using a CL-1000L UV crosslinker (UVP, CA) for immobilization. The slides were washed successively with DMSO, DMF, acetonitrile, THF, dichloromethane, EtOH, and ultrapure water (5 min, three times each), and dried. L-Grb2^{TMR} or D-Grb2^{TMR} (3 μM in 1% skimmed-milk-TBS-T) was incubated with the glass slide for 1 h, and then washed with TBS-T [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween-20] (5 min, three times). The slides were dried and scanned at 532 nm on a GenePix scanner. The fluorescence signals were quantified with GenePixPro.

CD Spectra of L-Grb2^{53–154} and D-Grb2^{53–154}

CD spectra of L-Grb2^{53–154} and D-Grb2^{53–154} were recorded in 5 mM HEPES buffer containing 0.1 mM TCEP·HCl, pH 7.4 on a JASCO J-720 circular dichroism spectrometer at 20 °C.

SPR Analysis

SPR analyses of EGFR^{1060–1076} peptide binding to synthetic Grb2 and its derivatives were carried out using a Biacore T200 SPR instrument. 20 mM HEPES buffer containing 100 mM NaCl and 0.05% Tween-20 (pH 7.4) was used as the running buffer

at 25 °C. For evaluation of unlabeled and TMR-labeled Grb2 SH2 domain proteins, biotinylated EGFR^{1060–1076} peptide was immobilized on an SA sensor chip (L-EGFR^{1060–1076} peptide: 26.1 RU, D-EGFR^{1060–1076} peptide: 26.0 RU). For the evaluation of biotin-labeled Grb2 SH2 proteins, biotinylated Grb2 was immobilized on an SA sensor chip (L-Grb2^{N-biotin}: 1306.8 RU, D-Grb2^{N-biotin}: 1279.5 RU, L-Grb2^{115-biotin}: 1312.5 RU). All analytes were evaluated for 2 min of contact time, followed by 2 min dissociation at a flow rate of 30 µL/min. KD values were calculated by steady-state bindings from triplicate assays.

Competitive Binding Inhibition Assay by ELISA

ELISAs were performed in 20 mM HEPES buffer (pH 7.4) containing 100 mM NaCl, 0.05% Tween-20, 0.1% BSA. Precoated streptavidin 96-well plates (Nunc) were incubated with HEPES buffer containing 3% BSA (300 µL/well) for 1 h. After three washes, Grb2^{N-biotin} (1.5 nM) in HEPES buffer (100 µL/well) was added and incubated for 2 h. After three washes, 100 nM FLAG-labeled EGFR^{1060–1076} peptide in the presence of varying concentration of inhibitors in HEPES buffer containing 1% DMSO (100 µL/well) was added and incubated for 1 h. After three washes, a 1:10,000 dilution of HRP-conjugated anti-FLAG antibody (Sigma) in HEPES buffer (100 µL/well) was added and incubated for 1 h. After three washes, TMB solution (Wako, 100 µL/well) was added and incubated for 1 h. Next, aqueous 2N H₂SO₄ (10 µL/well) was added. Absorbance at 450 nm was measured for each well using an EnVision Xcite plate reader. The IC₅₀ values were calculated using GraphPad Prizm (GraphPad software, San Diego, CA).

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Chapter 3. Conclusions

1. The author has established screening protocols for binding compounds of MDM2 and MDMX by chemical array technology using synthetic fluorescent proteins. The preliminary screening of an in-house chemical library identified a number of peptidic binding compounds towards MDM2 and MDMX. The subsequent FP assay identified three dual inhibitors of MDM2–p53 and MDMX–p53 interactions.
2. The author has established a novel screening process for MDM2–p53 inhibitors from a virtual mirror-image library of chiral natural products and derivatives. Chemically synthesized mirror-image MDM2 was applied for chemical array screening of a library of natural product derivatives, identifying novel tocopherol derivative NP843 as a D-MDM2–D-p53 interaction inhibitor. Chemical synthesis of the enantiomeric compound enabled the validation of the inhibitory activity of *ent*-NP843 against the L-MDM2–L-p53 interaction. The selective recognition by MDM2 was attributed to the stereochemistry of the tetrasubstituted carbon on the chromane skeleton of *ent*-NP843. The aliphatic side chain of three isoprene units was also needed for the inhibitory activity.
3. The author investigated the chemical synthesis of Grb2 SH2 domain proteins and application to the development of in vitro assay systems for Grb2 SH2 domain inhibitors. Several labeled Grb2 SH2 proteins including Grb2^{TMR}, Grb2^{N-biotin} and Grb2^{115-biotin} were synthesized. Screening systems using both enantiomers of these proteins to some bioassays including SPR analysis, chemical array and ELISA, were developed.

In summary, the author developed a screening process from a virtual mirror-image library of natural products using synthetic D-protein technology. In contrast to conventional screening approaches, this process could identify hit compounds from unavailable mirror-image chiral natural products, thus providing unprecedented lead compounds for drug discovery.

Abbreviations

Abl:	abelson murine leukemia viral oncogene homologue 1	MES:	2-mercaptoethanesulfonic acid
Bcr:	breakpoint cluster region protein	MFD:	metal-free-desulfurization
CD:	circular dichroism	MPAA:	4-mercaptophenylacetic acid
DIPCI:	<i>N,N'</i> -diisopropylcarbodiimide	NCL:	native chemical ligation
EDT:	1,2-ethanedithiol	PALC:	photoaffinity linker-coated
ELISA:	enzyme-linked immunosorbent assay	PEG:	polyethylene glycol
FAM:	carboxyfluorescein	PI3K:	phosphoinositide 3-kinase
FP:	fluorescence polarization	PPI:	protein–protein interaction
GST:	glutathione S-transferase	RAS:	rat sarcoma
Gab1:	Grb2-associated binder 1	SA:	streptavidin
Grb2:	growth factor receptor bound protein 2	SH2:	src homology 2
Gu:	guanidine	SH3:	src homology 3
HBTU:	<i>O</i> -(1 <i>H</i> -benzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium hexafluorophosphate	SPPS:	solid-phase peptide synthesis
HOBt:	1-hydroxy-1 <i>H</i> -benzotriazole	SPR:	surface plasmon resonance
HRP:	horseradish peroxidase	TBTU:	<i>N,N,N',N'</i> -tetramethyl- <i>O</i> -(benzotriazol-1-yl)uronium tetrafluoroborate
HTS:	high-throughput screening	TCEP:	tris(2-carboxyethyl)phosphine
MDM2:	mouse double minute 2 homologue	TMB:	3,3',5,5'-tetramethylbenzidine
MDMX:	mouse double minute X homologue	TMR:	tetramethylrhodamine

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List of Publications

This study was published in the following papers.

Chapter 1.

- Section 1. Affinity-based Screening of MDM2/MDMX-p53 Interaction Inhibitors by Chemical Array: Identification of Novel Peptidic Inhibitors

Taro Noguchi, Shinya Oishi, Kaori Honda, Yasumitsu Kondoh, Tamio Saito, Tatsuhiko Kubo, Masato Kaneda, Hiroaki Ohno, Hiroyuki Osada, and Nobutaka Fujii

Bioorg. Med. Chem. Lett. **2013**, 23, 3802–3805.

- Section 2. Screening of a Virtual Mirror-image Library of Natural Products

Taro Noguchi, Shinya Oishi, Kaori Honda, Yasumitsu Kondoh, Tamio Saito, Hiroaki Ohno, Hiroyuki Osada, and Nobutaka Fujii

Chem. Commun. **2016**, 52, 7653–7656.

Chapter 2.

- Synthesis of Grb2 SH2 Domain Proteins for Mirror-image Screening Systems

Taro Noguchi, Hiroyuki Ishiba, Kaori Honda, Yasumitsu Kondoh, Hiroaki Ohno, Nobutaka Fujii, and Shinya Oishi

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