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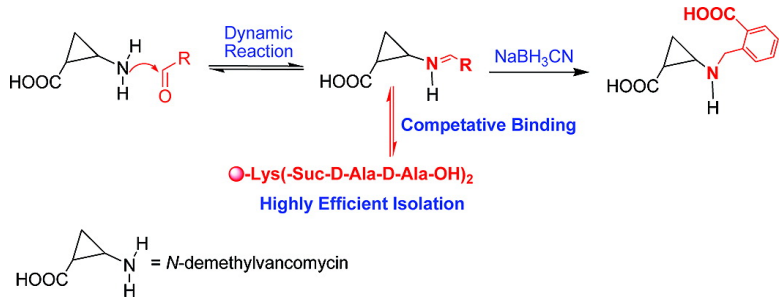

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Dynamic Selection of Novel Vancomycin N-Terminal Derivatives by Resin-bound Reversed D-Ala-D-Ala

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The most attractive advantage of dynamic combinatorial chemistry (DCC) is that it can screen the compound library as soon as compounds are synthesized. However, it is very difficult to analyze a dynamic combinatorial library with free probes using the state-of-the art analysis technologies. We report herein a method that uses a resin-immobilizing reversed peptide probe to screen vancomycin derivatives and provides a solution to this problem.

Introduction

N-demethylvancomycin (NDMVan), an analogue of vancomycin (Van), differs from Van at its N-terminus, where the *N*-methyl-leucine is replaced by leucine. This mimics the binding of vancomycin to the terminal H-D-Ala-D-Ala-OH (DADA) fragment of the growing peptidoglycan biosynthetic precursor through an intricate network of five hydrogen bonds.[1] The leucin residue forms a hydrophobic cavity to increase the hydrogen interaction. Therefore, the structure and mechanism effects of altering the leucin residue may interfere with the molecule's antibacterial activity. By allowing the free amino of leucin to form Schiff bases with aldehydes, a dynamic combinatorial chemistry (DCC) is developed in this study.

DCC makes use of reversible bond-forming reactions to create thermodynamically controlled dynamic combinatorial libraries (DCLs), in which the molecules interesting to the targeting receptor can be identified through competitive and physical binding selection.[2] Over the past decade, DCC has emerged as a technique for the in situ generation and screening of compounds with a broad range of desirable properties. DCLs are frequently analyzed by LC-MS. However, this requires a different spectrum of mass over electron (*m*/*e*) for each library member, as well as the ability to separate individual library members on a chromatography column; the difficulty of this task increases as the library size increases. Taking advantage of immobilized molecules having a simple spatial isolation that allows high-throughput combinatorial chemistry, we have designed, for the first time, a new method of Van DCC by using an immobilizing reversed dipeptide of DADA as a probe to amplify and simultaneously select the Van derivatives.

Results and Discussion

The literature indicates that two carboxyl oxygens of the DADA C-terminal form three hydrogen bonds with Van, which is a major contribution to Van's activity. Therefore, immobilization of DADA must leave its carboxyl group free and attach to the resin at its N-terminus, which is the reverse of normal peptide assembly. Our group has concluded that PEGA NH_2 resin is more compatible with Van analysis by HR/MAS NMR than other resins,[3] because it does not disturb the intermolecular interactions over a range of molecular sizes from 1500 to 2000 Dalton. The assembly of DADA and dynamic selection of vancomycin analogues are finally achieved by PEGA resin.

The strategy we pursued for the bead-bound reversed DADA synthesis is to use amino acid *t*-butyl esters as building blocks and is based on a peptide reversed assembly chemistry. Favorable features of this approach are that amino acid *t*-butyl esters are stable, and the deprotection process of *t*-butyl is mild. The synthesis and analysis of both monomer and dimer of DADA through a lysine branch were systematically investigated. Dimerization of dipeptide substrate aimed at mimicking the natural bindings between vancomycin and the substrate.[4] Releasable and immobilized DADA was finally synthesized to analyze the yield and purity because there are no universal methods to detect the synthesis efficacy of the peptide by a reversed strategy.

In the releasable form, the HMBA linker was first introduced to the PEGA NH_2 resin. This linker is sensitive to alkaline conditions with an ester bond between resin and linker, but it is stable under acidic conditions, that is, for *t*-butyl group removal. To the dimer of DADA, the introduction of two carboxyl groups into the resin was accomplished simultaneously with subsequent esterification of Fmoc-Lys(Boc)-COOH of HMBA resin and acylation by succinic anhydride, after removal of the Fmoc and Boc protecting

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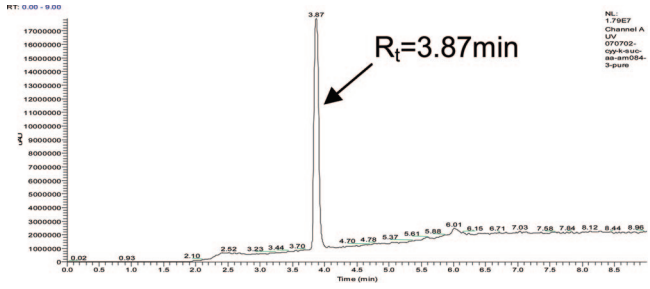


Figure 1. HPLC profile of L-Lys(-Suc-DADA)$_2$.

groups by 20% piperidine in DMF and 95% TFA in DCM, respectively. To determine the polypeptide by LC-MS analysis, an *o*-nitrobenzene amide was assembled onto the peptide at the last step. Treatment of the bead-bond product with 1.0 M NaOH/dioxane (v/v = 1:3) yields the anticipated compound in almost 100% purity as analyzed by the LC-MS system (Figure 1). The immobilized dimer of DADA was then directly assembled onto the PEGA resin without the HMBA linker (Scheme 1). Analysis of the final product by HR/MAS NMR revealed that the pure anticipated compound was obtained (Figure 2).

A complex was then investigated under incubation conditions with PBS (pH 6.0) for 24 h at 25 °C in an equal molar ratio of binding NDMVan and probe components. The resin was then washed twice with PBS, redistilled water three times, and finally, lyophilized before analysis. The interactions were assigned by HR/MAS NMR analysis directly (Figure 3). Clearly, NDMVan was attached to the resin, and the immobilized dimer of DADA strongly interacted with NDMVan, in that the key protons of the complexes were arrowed by the comparable assignments with the NMR of free complexes of H-L-Lys-DADA and NDMVan.

In particular, the large high-frequency shifts of NH of NDMVan upon complexation are fully in agreement with the free form of the observations by Williams et al. and the larger high-frequency shift of NH is correlated with stronger hydrogen bond between Van derivatives and ligand.[5] The lack of significant differences indicate that the interactions in the immobilized complex did mimic the natural properties between Van and DADA interaction, which implies that the resin-bound dipeptide probe is efficiently able to "fish" the Van derivatives.

Because DMSO was used as the solvent for investigation of immobilized complexes by NMR and was used as an universal solvent for compound dissolution, we herein choose the mixture of DMSO and PBS (pH 6.00) as the reactant solvent, to ensure that both the reversible chemical bond formation and physical binding occurred simultaneously. Through competitive binding and amplification of Schiff base reactions and an appropriate reaction period, the compound that most strongly interacted with the immobilized DADA was ideally able to be amplified and attached to the resin, and efficiently separated from all other components. The selected compound was finally eluted from the resin with MeOH and quenched with $NaBH_3CN$. The eluent was analyzed using the LC-MS system to gain the final structure information.

In this study, the ratio of DMSO and PBS was investigated. The target of this experiment is to ensure the removal of both nonspecific and weaker bindings than NDMVan by organic DMSO interference. As the proportion of DMSO increased, the NDMVan remaining on the immobilized substrate decreased. When the DMSO in PBS reached 80% in volume, almost all the attached NDMVan was eluted from the resin (Figure 4). We finally used 90% DMSO in PBS as the solvent to ensure the absolute condition that only the most strongly binding vancomycin analogues were selected.

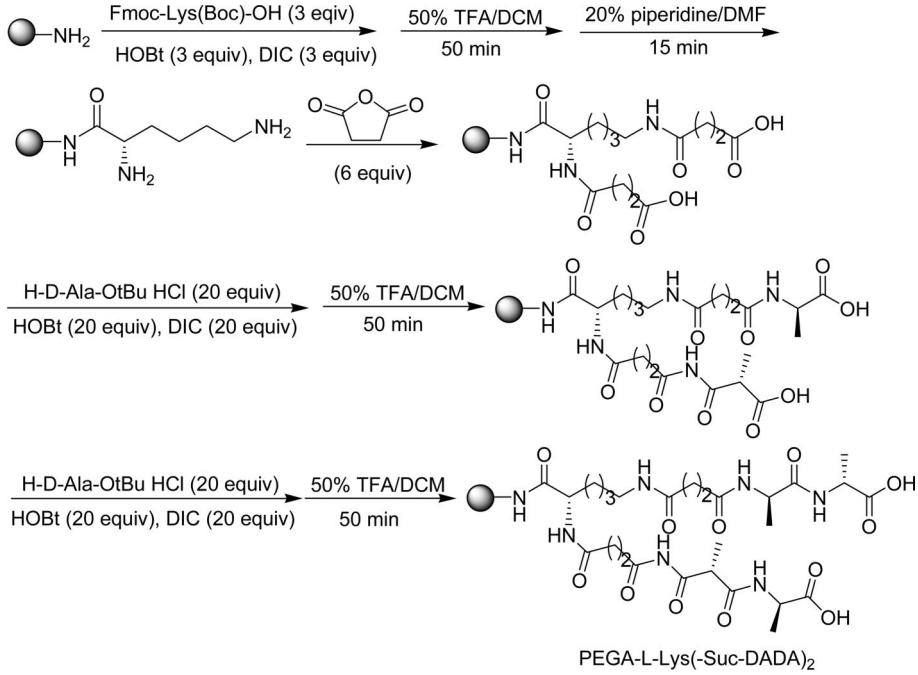
A total of 70 aldehydes were used in this study to verify this dynamic screening process. Ten of them were randomly grouped to ensure the clear analysis that is always a challenge for DCC. A dimer of immobilized substrate [PEGA-L-Lys(-suc-DADA)$_2$] in 90% DMSO/PBS (pH = 6.00) was incubated with NDMVan and seven individual groups of aldehydes at room temperature for 48 h. The resins were then collected through filtration and appropriate washing and were dried by lyophilization. The dried resins were placed in 0.1 mmol $NaBH_3CN$/methanol solution for 1 h and then analyzed directly by the LC-MS system. For comparison, the mother reactants of the corresponding aldehyde groups without the immobilized substrate were also quenched by $NaBH_3CN$ and analyzed. One of seven sublibraries (DCL-1) gave a new peak at a retention time of 6.98 min (Figures 5 and 6).

The sensitive and rapid analytical MS technique has characterized this novel NDMVan derivative (cyy202) with a proposed fragmentation pathway (Figure 7). The full-scan MS spectrum revealed that the amino group of leucine was selectively amino-alkylated by 2-carboxybenzaldehyde with $[M + H]^+$ (*m*/*z* 1570.1) and $[M + 2H]^{2+}$ (*m*/*z* 784.3). The main fragments 1425 (*m*/*z* 1425.8) and 1264 (*m*/*z* 1264.5), which were produced by losing vancosamine (143 Da) and disaccharide (305 Da), confirmed the anticipated structure. It should be noted that the cyy202 was undetected in the mother reactants of DCL-1 by sensitive LC-MS analysis, implying that the cyy202 was amplified for synthesis and completely selected by the immobilized probe.

We also examined the interactions of PEGA-L-Lys(-suc-DADA)$_2$ with cyy202 by HR/MAS NMR. As the analog of Van, the interaction between cyy202 and probe is similer to the Van. When the two kinds of complexes are compared, the typical chemical shift of the NH of cyy202 has a downfield shift to 11.73 ppm ($\Delta\delta$ 3.87 ppm) in comparison with 11.54 ($\Delta\delta$ 3.70 ppm) for the immobilized complex of NDMVan and DADA. Meanwhile, the methyl protons of the resin-bound probe were also shifted upfield from 1.18 to 0.42 ppm (Figure 8). These differences reflect that cyy202 was bound to DADA more strongly than NDMVan. The reason is that the substitution of 2-carboxybenzaldehyde on the amino of luecine has some disturbance on the conformation of hydrophobic cave that strengthen the interaction between cyy202 and probe.

The ability to form complexes with peptides has been shown to correlate with antibiotic power under a variety of circumstances.[6] The UV absorption spectrum was used to measure hydrogen bonding ability in this study. The binding properties of NDMVan and cyy202 with Ac_2-L-Lys-DADA were then pursued by UV absorption. With antibiotics alone as the reference, the Ac_2-L-Lys-DADA, dissolved in the same

Scheme 1. ISPS route of PEGA-L-Lys(-Suc-DADA)$_2$



solution, was titrated into the cell in the sample beam. The result indicates that the binding constant of cyy202 (1.69×10^6 L mol^{-1}) is ~2-fold to NDMVan (0.86×10^6 L mol^{-1}) (Figure 9).

Conclusion

In summary, with resin-bound reversed DADA as the probe, a vancomycin derivative (cyy202) was amplified and selected in a dynamic combinatorial library because of its stronger binding to DADA. This method provides a new solution to the difficulty in analyzing a dynamic chemical library through immobilized probes or receptors.

Experimental Section

Synthesis and Analysis of Probe. General. All amino acids used for peptides synthesis were purchased from GL Biochem (Shanghai, China) Ltd. All resins, the *o*-nitrobenzene amide used in the synthesis of peptides, and all aldehydes used in the DCLs were obtained from Sigma Aldrich corporation, Acros Ltd.

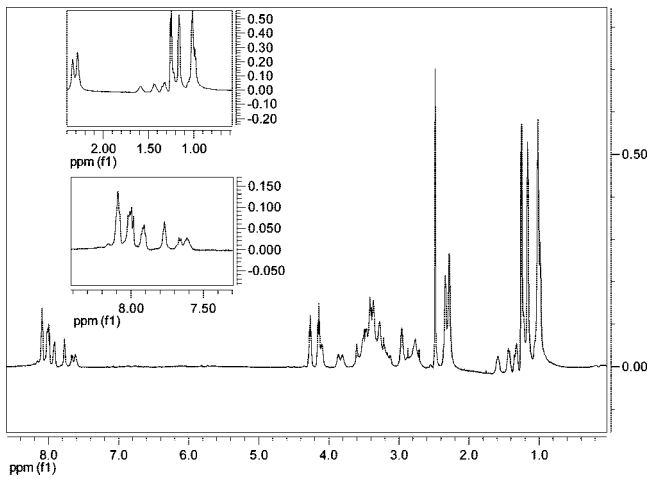


Figure 2. ^{1}H HR/MAS NMR spectrum of PEGA-L-Lys(-Suc-DADA)$_2$ swollen in DMSO-d_6 at 298 K with presaturation at 3.55 ppm.

All NMR spectra were recorded on a Varian Unity INOVA-500 MHz and SYS-600 MHz spectrometer equipped with a 4 mm ^{1}H-observe Nano NMR probe. The spin rate was ~2K Hz for all samples at 25 °C. The HR/MAS NMR spectrum of complexes were formed by addition of PEGA-L-Lys(-Suc-DADA)$_2$ (0.002 mmol) to a solution of cyy202 or NDMVan (0.002mmol) in 40 μL of DMSO-d_6 at 298 K with a presaturation at 3.55 ppm; the power was −16, and the delay was 1.5 s.

Mass spectra were acquired on an Finnigan LCQ-Advantage (ESI) LC-MS system, and HRMS specture were acquired on Agilent LC-MSD/TOF (ESI) system with Kromasil column (4.6 × 50 mm C_{18}, 300 Å, 5 μ) and ionization in positive ion mode.

Synthesis and Analysis of Suc-DADA. To PEGA amine resin (150 mg, 0.06 mmol, 0.4 mmol/g) was added a solution of HMBA (27 mg, 0.18 mmol, 3 equiv to resin), HOBt (24 mg, 0.18 mmol, 3 equiv to resin), and DIC (28 μL, 0.18 mmol, 3 equiv to resin) in DMF (4 mL). After the mixture was shaken at room temperature for 3 h, the ninhydrine text indicated that the coupling reaction was complete. Then, succinic anhydrous (18 mg, 3 equiv, 0.18 mmol), DMAP (4 mg, 0.5 equiv, 0.03 mmol), and DMF (4 mL) were added to resin. After 24 h, the resin was washed (DMF, MeOH, CH_2Cl_2, 3 × 5 mL) and filtered under vacuum. A mixture of H-D-Ala-OtBu·HCl (109 mg, 10 equiv, 0.6 mmol), DIPEA (101 μL, 10 equiv, 0.6 mmol), HOBt (81 mg, 10 equiv, 0.6 mmol), and DIC (94 μL, 10 equiv, 0.6 mmol) in DMF (4 mL) was added to the resin and was shaken for 24 h. The resin was washed by DMF, MeOH, and CH_2Cl_2 in turn. Then, the *t*-butyl group was removed by treatment with 50% TFA/DCM (v/v) (50 min, 4 mL). The second D-Ala was coupled to the resin by the same coupling and

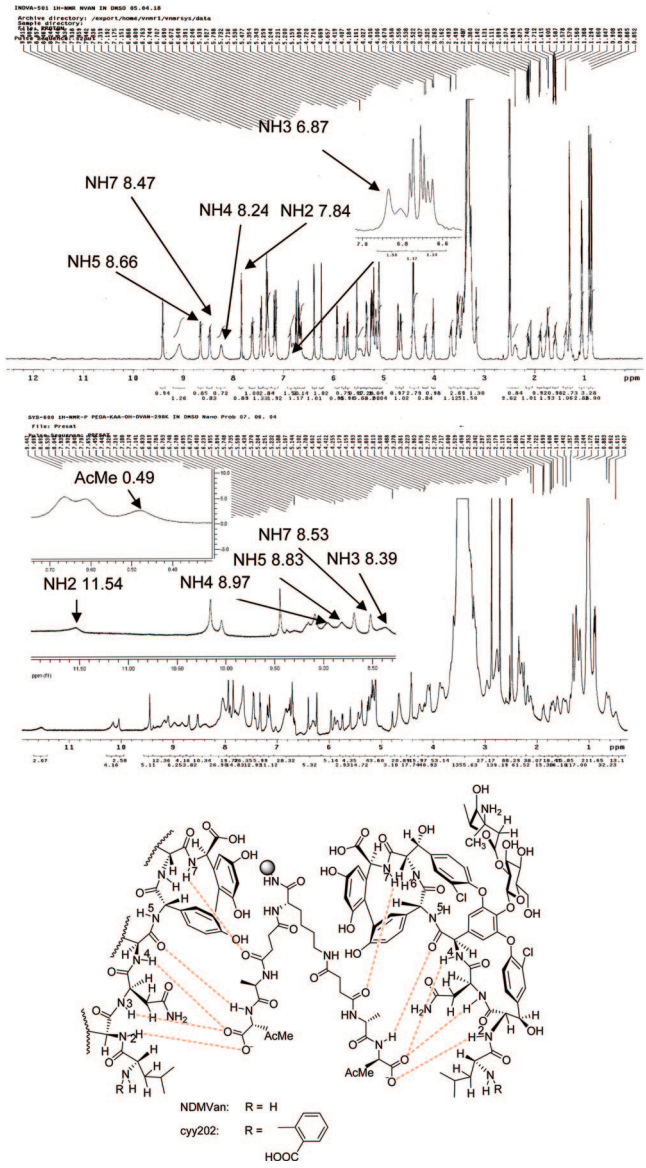


Figure 3. (top) ^{1}H HR/MAS NMR of NDMVan. (bottom) ^{1}H HR/MAS NMR down- and upfield spectrum of NDMVan attached by PEGA-L-Lys(-Suc-DADA)$_2$ in DMSO-d_6 at 298 K.

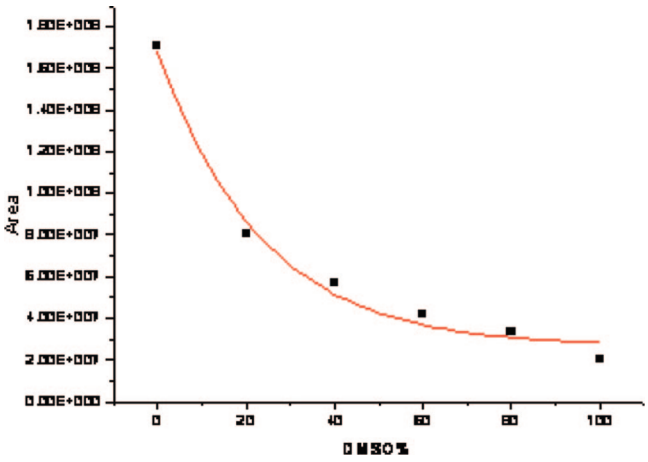


Figure 4. Eluation of the fished NDMVan with DMSO in PBS (pH 6.00).

deprotection conditions. Then *o*-nitrobenzene amide (am084, 61 mg, 5 equiv, 0.3 mmol), HOBt (41 mg, 5 equiv, 0.3 mmol), DIC (47 μL, 5 equiv, 0.3 mmol), and DIPEA (51

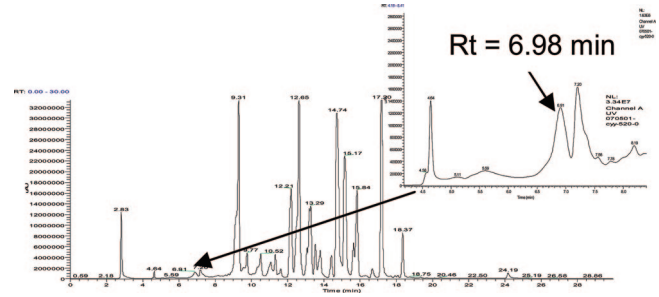


Figure 5. HPLC profile of the reductive amination of eluted solution from probe in DCL-1.

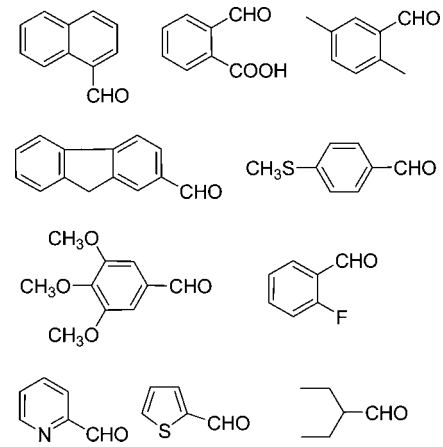


Figure 6. Aldehydes that have been involved in the solid-phase DCL-1.

μL, 5 equiv, 0.3 mmol) were added into DMF (4 mL) to react with the resin for 24 h. The dried peptide resin was added a mixture of 1 M NaOH/dioxane (1:3, v/v, 4 mL). The mixture was agitated at room temperature for 15 min. After filtration, the filtrates were neutralized and evaporated under reduced pressure. Finally, the residue was purified on an ODS-C_{18} reversed-phase column with MeCN/H_2O. The fraction of 10−20% (MeCN in H_2O) was freeze-dried on a lyophilizer to give the pure peptide as a white powder (5.5 g, 22.5%). ^{1}H NMR (600 MHz, DMSO-d_6, 25 °C): δ 1.14 (d, J = 7.2 Hz, 3H; CH_3), 1.17 (d, J = 7.2 Hz, 3H; CH_3), 2.31 (m, 2H; CH_2), 2.34 (m, 2H; CH_2), 2.84 (t, J = 7.2 Hz, 2H; CH_2), 3.31 (m, 2H; CH_2), 4.15 (m, 4H; CH_2), 4.15 (m, 4H; CH_2), 7.48 (d, J = 8.4 Hz, 2H; aromatic CH_2), 7.82 (t, J = 6.6 Hz, 1H; NH), 7.87 (d, J = 7.8 Hz, 1H; NH), 8.12 (d, J = 9 Hz, 2H; aromatic CH_2), 8.19 (d, J = 6.6 Hz, 1H; NH). ^{13}C NMR (600 MHz, DMSO-d_6, 25 °C): δ 171.98 (CO), 171.98 (CO), 171.98 (CO), 171.98 (CO), 147.90 (C), 146.04 (C), 130.12 (CH_2), 123.28 (CH_2), 48.66 (CH_2), 48.29 (CH_2), 39.09 (CH_2), 39.09 (CH_2), 39.09 (CH_2), 34.70 (CH_2), 18.00 (CH_3), 17.65 (CH_3). HRMS *m*/*z* calcd for $[2M + H]^+$: 817.3290; found 817.3355.

Synthesis and Analysis of L-Lys(-Suc-DADA)$_2$. To PEGA amine resin (210 mg, 0.084 mmol, 0.4 mmol/g) was added a solution of HMBA (38 mg, 0.25 mmol, 3 equiv to resin), HOBt (34 mg, 0.25 mmol, 3 equiv to resin), and DIC (40 μL, 0.25 mmol, 3 equiv to resin) in DMF (4 mL). After shaking at room temperature for 3 h, the ninhydrine text indicated the coupling reaction completed. A mixture of Fmoc-L-Lys(Boc)-OH (118 mg, 3 equiv, 0.25 mmol), HOBt (34 mg, 3 equiv, 0.25 mmol), DIC (40 μL, 3 equiv, 0.25 mmol), and

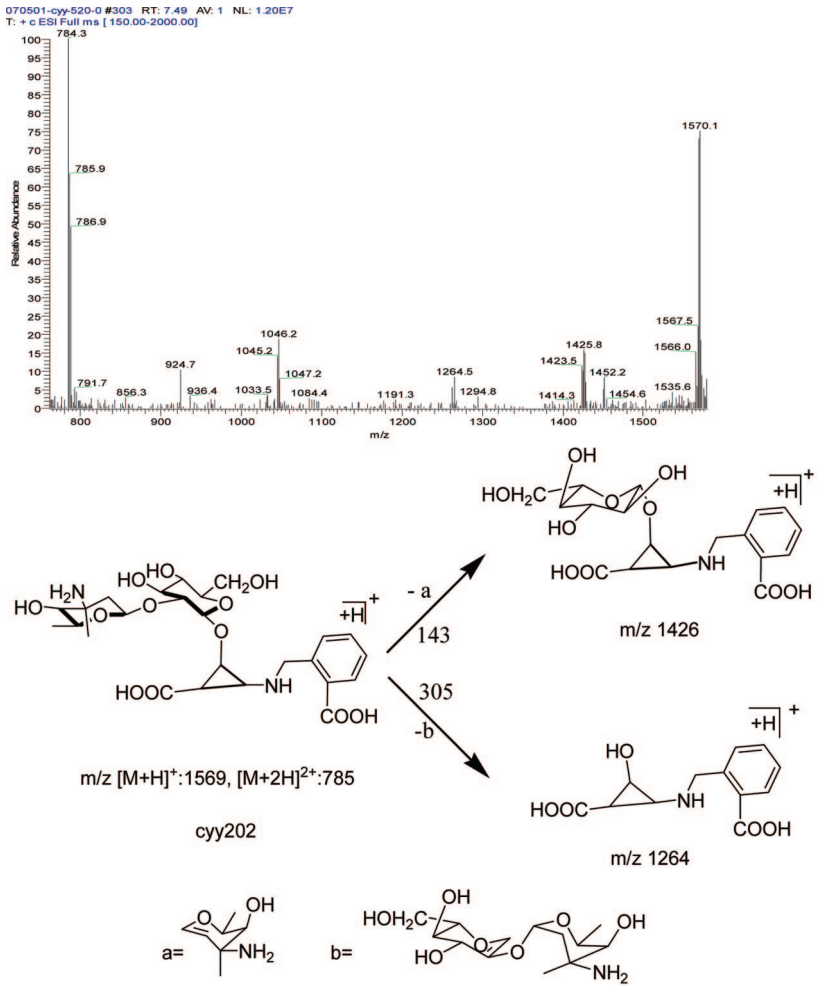


Figure 7. MS fragmentation of cyy202 in DCL-1.

DMAP (5 mg, 0.5 equiv, 0.042 mmol) in DMF (4 mL) was reacted with the resin for 24 h. The resin was washed (DMF, MeOH, CH_2Cl_2, 3 × 5 mL), and the Boc group was removed by treatment with 50% TFA/DCM (v/v, 50 min, 4 mL); then the Fmoc group was removed with 20% piperidine/DMF (v/v, 2 × 15 min, 4 mL). After the mixture was washed (DMF, MeOH, CH_2Cl_2, 3 × 5 mL), the succinic anhydrous (50 mg, 3 equiv, 0.50 mmol), DMAP (11 mg, 0.5 equiv, 0.08 mmol), and DMF (4 mL) were added to the resin. After 24 h, the resin was washed (DMF, MeOH, CH_2Cl_2, 3 × 5 mL) and filtered under vacuum. A mixture of H-D-Ala-OtBu·HCl (305 mg, 10 equiv, 1.68 mmol), DIPEA (283 μL, 10 equiv, 1.68 mmol), HOBt (227 mg, 10 equiv, 1.68 mmol), and DIC (263 μL, 10 equiv, 1.68 mmol) in DMF (4 mL) was added to the resin, and the mixture was shaken for 24 h. The resin was washed by DMF, MeOH, and CH_2Cl_2 in turn. Then, the *t*-butyl group was removed by treatment with 50% TFA/DCM (v/v) (50 min, 4 mL). The second D-Ala was coupled to the resin by the same coupling and deprotection conditions. Then *o*-nitrobenzene amide (am084, 169 mg, 5 equiv, 0.84 mmol), HOBt (114 mg, 5 equiv, 0.84 mmol), DIC (132 μL, 5 equiv, 0.84 mmol), and DIPEA (142 μL, 5 equiv, 0.84 mmol) was dissolved in DMF (4 mL) to react with the resin for 24 h. The dried peptide resin was added a mixture of 1 M NaOH/dioxane (1:3, v/v, 4 mL). The mixture was agitated at room temperature for 15 min. After filtration, the filtrates were neutralized and evaporated under reduced pressure. Finally, the residue was purified on an ODS-C_{18} reversed-phase column with MeCN/H_2O. The fraction of 10−20% (MeCN in H_2O) was freeze-dried on a lyophilizer to give the pure peptide as a white powder (18.2 g, 23.4%). ^{1}H NMR (600 MHz, DMSO-d_6, 25 °C): δ 1.14 1.18 (m, 2H; CH_2), 1.20 (m, 2H; CH_2), 1.29 (m, 2H; CH_2), 1.46 (m, 1H; CH), 1.59 (m, 1H; CH), 2.26 (m, 2H; CH_2), 2.35 (m, 2H; CH_2), 2.37 (m, 2H; CH_2), 2.43 (m, 2H; CH_2), 2.84 (m, 4H; CH_2), 2.84 (m, 4H; CH_2), 2.86 (m, 1H; CH), 2.97 (m, 1H; CH), 3.26 (2H, t × d, J_1 = 13.8 Hz, J_2 = 6.0 Hz; CH_2), 3.32 (t × d, J_1 = 13.8 Hz, J_2 = 6.0 Hz, 2H; CH_2), 3.94 (m, 1H; CH), 4.09 (q, J = 7.8 Hz, 2H; CH_2), 4.12 (q, J = 7.8 Hz, 2H; CH_2), 7.47 (d × d, J_1 = 8.4 Hz, J_2 = 1.8 Hz, 4H; aromatic CH), 7.67 (m, 2H; CH_2), 7.75 (d, J = 6.0 Hz, 1H; CH), 7.92 (m, 1H; NH), 8.07 (m, 2H; CH_2), 8.10 (d × d, J_1 = 8.4 Hz, J_2 = 1.8 Hz, 4H; aromatic CH), 8.44 (m, 2H; CH_2). ^{13}C NMR (600 MHz, DMSO-d_6, 25 °C): δ 172.96 (CO), 172.84 (CO), 172.17 (CO), 172.17 (CO), 172.17 (CO), 172.17 (CO), 171.58 (CO), 171.12 (CO), 147.82 (C), 147.82 (C), 146.03 (C), 146.03 (C), 130.06 (CH), 130.06 (CH), 123.26 (CH), 123.26 (CH), 49.30 (CH), 48.43 (CH), 48.43 (CH), 48.43 (CH), 48.43 (CH), 48.43 (CH), 38.56 (CH_2), 38.56 (CH_2), 34.71 (CH_2), 34.65 (CH_2), 31.95 (CH), 31.02 (CH_2), 30.87 (CH_2), 30.74 (CH_2), 30.58 (CH_2), 28.92 (CH_2), 22.91 (CH_2), 17.52 (CH_3), 17.48 (CH_3), 17.31 (CH_3), 17.29 (CH_3). HRMS *m*/*z* calcd for $[M + H]^+$: 927.4134; found 927.4200.

Synthesis and Analysis of PEGA-L-Lys(-Suc-DADA)$_2$. To PEGA amine resin (500 mg, 0.2 mmol, 0.4 mmol/g) was added a solution of Fmoc-L-Lys(Boc)-OH (281 mg, 3 equiv,

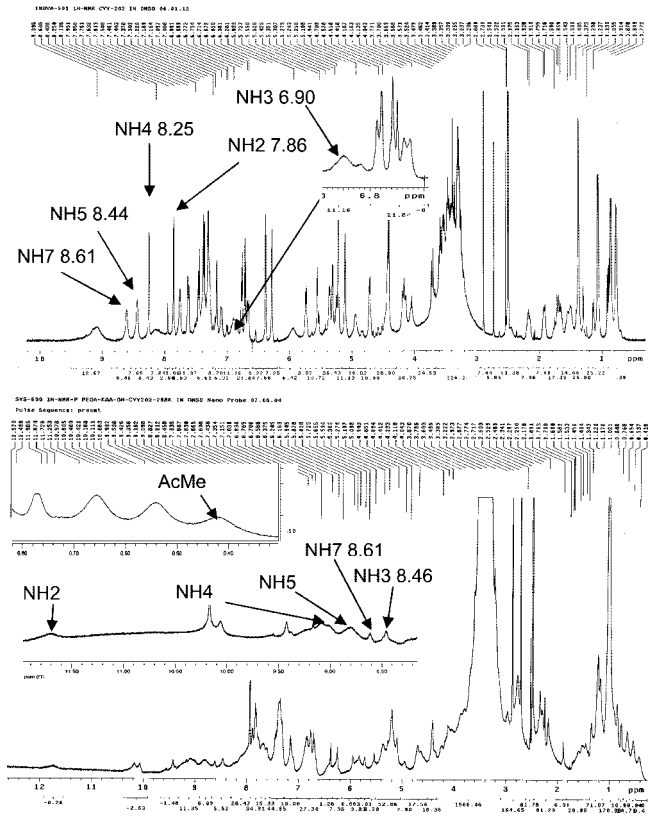


Figure 8. (top) ^{1}H HR/MAS NMR of cyy202. (bottom) ^{1}H HR/MAS NMR down- and upfield spectrum of cyy202 attached by PEGA-L-Lys(-Suc-DADA)$_2$ in DMSO-d_6 at 298 K.

0.6 mmol), HOBt (81 mg, 3 equiv, 0.6 mmol), and DIC(94 μL, 3 equiv, 0.6 mmol) in DMF (4 mL). After the mixture was shaken at room temperature for 3 h, the ninhydrine test indicated that the coupling reaction was complete. The resin was washed (DMF, MeOH, CH_2Cl_2, 3 × 5 mL), and the Boc group was removed by treatment with 50% TFA/DCM (v/v, 50 min, 4 mL); the Fmoc group was removed with 20% piperidine/DMF (v/v, 2 × 15 min, 4 mL). A mixture of succinic anhydrous (120 mg, 3 equiv, 1.2 mmol) and DMF (4 mL) was reacted with the resin for another 2 h. The resin was washed (DMF, CH_2Cl_2, 3 × 5 mL) and filtered under vacuum. A mixture of H-D-Ala-OtBu·HCl (727 mg, 10 equiv, 4 mmol), DIPEA (674 μL, 10 equiv, 4 mmol), and HOBt (54 mg, 10 equiv, 0.4 mmol), DIC (620 μL, 10 equiv, 4 mmol) in DMF (4 mL) was added to the resin, and the mixture was shaken for 24 h. Then, the *t*-butyl group was removed by treatment with 50% TFA/DCM (v/v, 50 min, 4 mL). The second D-Ala was inversely coupled to the resin by the same coupling and deprotection conditions. Finally, the resin was lyophilized and directly detect by HR/MAS NMR. ^{1}H NMR (600 MHz, DMSO-d_6, 25 °C): δ 1.16 (m, 3H; CH_3), 1.16 (m, 3H; CH_3), 1.22 (m, 2H; CH_2), 1.26 (d, J = 6.6 Hz, 3H; CH_3), 1.26 (d, J = 6.6 Hz, 3H; CH_3), 1.33 (m, 2H; CH_2), 1.44 (m, 1H; CH), 1.59 (m, 1H; CH), 2.28 (m, 2H; CH_2), 2.28 (m, 2H; CH_2), 2.34 (m, 2H; CH_2), 2.34 (m, 2H; CH_2), 2.96 (m, 1H; CH_2), 4.10 (m, 1H; CH_2), 4.14 (q, J = 7.8 Hz, 2H; CH_2), 4.27 (q, J = 7.2 Hz, 2H; CH_2) 7.64 (m, 1H; NH), 7.77 (m, 1H; NH), 7.91 (m, 1H; NH), 8.00 (m, 2H; NH), 8.08 (m, 2H; NH).

Synthesis and Analysis of Ac$_2$-L-Lys-DADA. To Wang resin (2.0 g, 1.7 mmol, 0.85 mmol/g) was added a solution of Fmoc-D-Ala-OH (2.6 g, 5 equiv, 8.5 mmol), HOBt (1.2 g, 5 equiv, 8.5 mmol), DIC(1.3 mL, 5 equiv, 8.5 mmol), and DMAP (20 mg, 0.1 equiv, 0.17 mmol) in DMF (8 mL). After the mixture was shaken for 24 h at room temperature the solution of 15% Ac_2O/DCM (v/v) and DMAP (20 mg, 0.1 equiv, 0.17 mmoL) was added to the resin, and the mixture continue to react for another 30 min to end-cap unreacted hydroxyl groups on the Wang resin. After removal of the Fmoc group with 20% piperidine/DMF (v/v) (15 min, 2 × 8 mL), a mixture of another Fmoc-D-Ala-OH (1.6 g, 3 equiv, 5.1 mmol), HOBt (689 mg, 3 equiv, 5.1 mmol), and DIC (795 μL, 3 equiv, 5.1 mmol) in DMF (8 mL) was reacted with resin for 3 h. The ninhydrin test indicated that the coupling reaction was complete; the resin was washed (DMF, MeOH, CH_2Cl_2, 3 × 5 mL), and the Fmoc group was removed with 20% piperidine/DMF (v/v, 2 × 15 min, 4 mL). By the same method the (Ac)$_2$-L-Lys-OH (1.2 g, 3 equiv, 5.1 mmol) was coupled to the resin. The dried peptide resin was added a mixture of 50% TFA/DCM (10 mL), and the mixture was reacted at room temperature for 50 min. After filtration, the filtrates were evaporated under reduced pressure. Finally, the residue was purified on an ODS-C_{18} reversed-phase column with MeCN/H_2O. The fraction of 10% (MeCN in H_2O) was freeze-dried on a lyophilizer to give the pure peptide as a white powder (58.6 mg, 9.26%). ^{1}H NMR (600 MHz, DMSO-d_6, 25 °C): δ 1.18 (d, J = 6.9 Hz, 3H; CH_3), 1.23 (m, 2H; CH_2), 1.27 (d, J = 7.5 Hz, 3H; CH_3), 1.35 (m, 2H; CH_2), 1.50 (m, 2H; CH_2), 1.76 (s, 3H; CH_3), 1.82 (s, 3H; CH_3), 2.97 (d × t, J_1 = 12.6 Hz, J_2 = 6.6

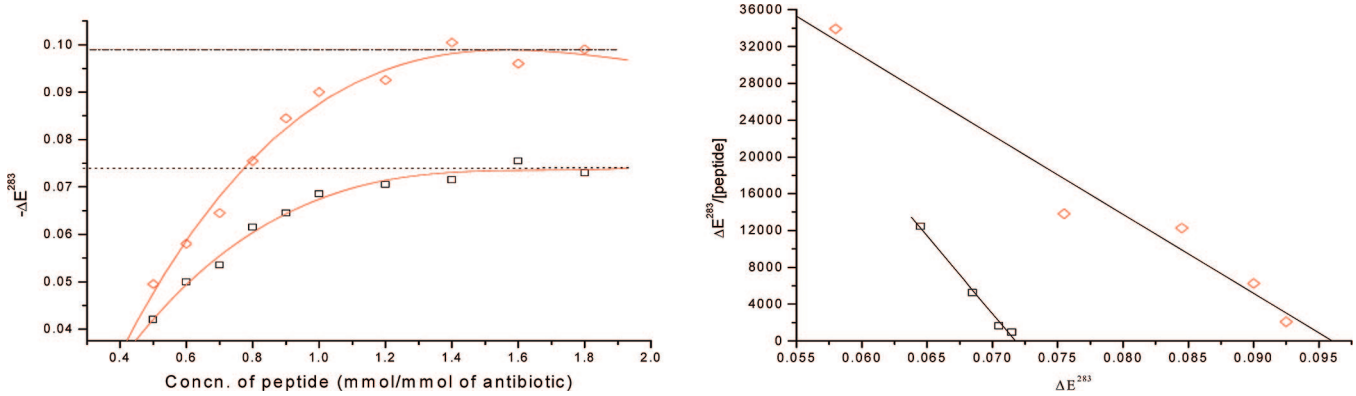


Figure 9. Absorption of cyy202 (□) and NDMVan (◇) during addition of peptide Ac$_2$-L-Lys-DADA (left). Scatchard plot of cyy202 and NDMVan (right).

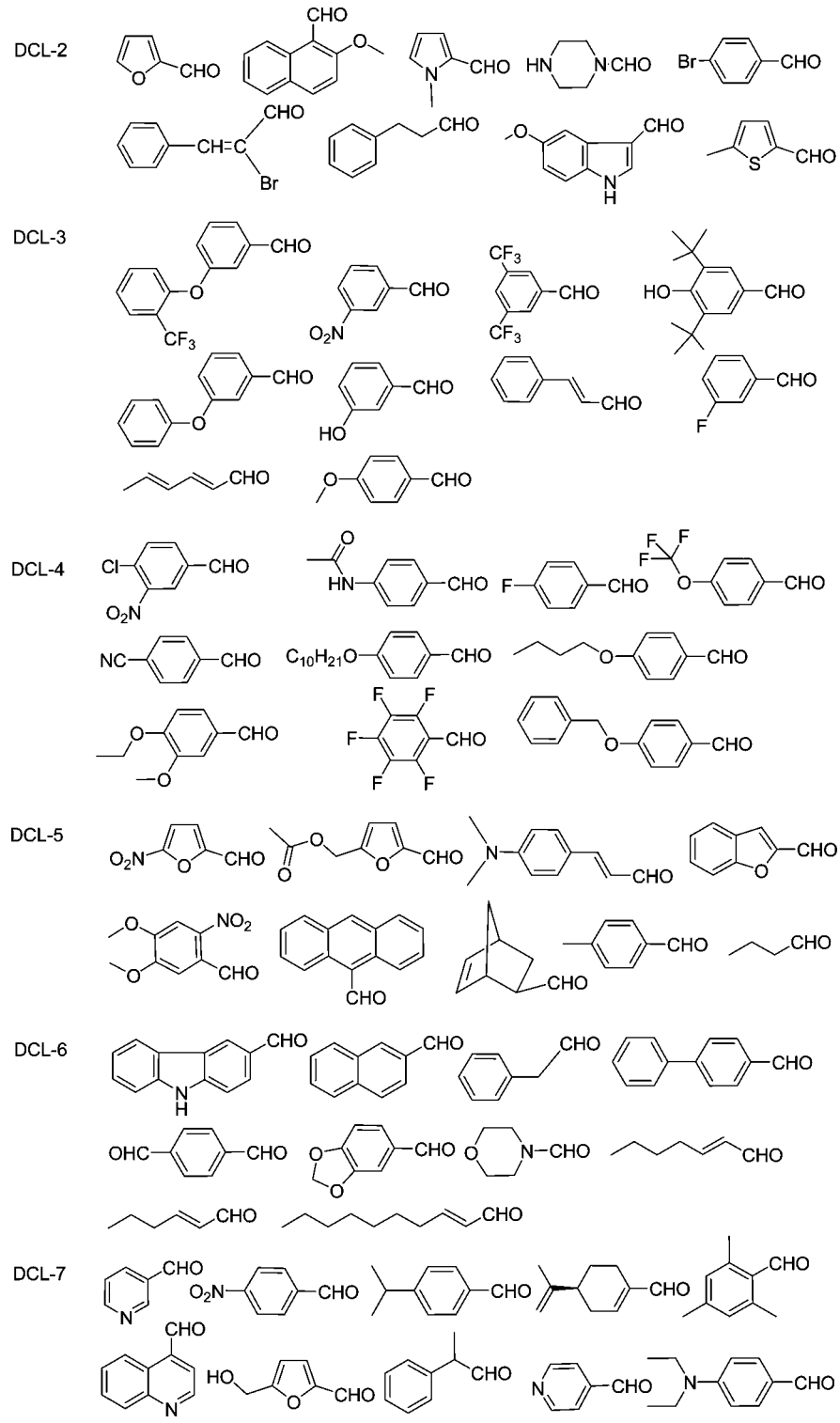


Figure 10. Aldehydes involved in the solid-phase dynamic combinatorial library, except DCL-1.

Hz, 2H; CH_2), 4.15 (m, 2H; CH_2), 4.28 (t, $J = 7.2$ Hz, 1H; CH), 7.76 (t, $J = 5.4$ Hz, 1H; NH), 7.99 (d, $J = 7.2$ Hz, 1H; NH), 8.05 (d, $J = 7.5$ Hz, 1H; NH), 8.13 (d, $J = 7.8$ Hz, 1H; NH), 12.47 (s, 1H; NH). ^{13}C NMR (600 MHz, DMSO-d_6, 25 °C): δ 173.98 (CO), 171.91 (CO), 171.45 (CO), 169.45 (CO), 168.94 (CO), 52.70 (CH), 52.46 (CH), 47.46 (CH), 38.37 (CH_2), 31.72 (CH_2), 28.87 (CH_2), 22.83 (CH_2), 22.61 (CH_3), 22.50 (CH_3), 18.12 (CH_3), 17.11 (CH_3). HRMS *m*/*z* calcd for $[M + H]^+$: 373.2009; found 373.2044.

Preparation and HPLC-MS Analysis of Resin-Bound Probe DCL. Selection of Solvent Used in the Resin-Bound Probe DCL. NDMVan (25 mg) and PEGA-L-Lys(-Suc-DADA)$_2$ (15 mg) were incubated in the mixture of DMSO/PBS (0% DMSO, 20% DMSO, 40% DMSO, 60% DMSO, 80% DMSO, and 100% DMSO) solutions for 24 h at room temperature. The resins were collected through a filtration and dried enough by lyophilizer. The dried resins were put into methanol (600 μL) for 1 h and then analyzed

the methanol solution directly by LC-MS (214 nm) according to the concentration gradation of DMSO from high to low.

Mass spectra were acquired on a Finnigan LCQ-Advantage (ESI) LC-MS system, using Kromasil column (4.6 × 50 mm C_{18}, 300 Å, 5 μ) and ionization in positive ion mode. Gradient of all HPLC analysis: 5% MeCN (0.1% TFA) to 100% MeCN (0.1% TFA) over 5 min. The injection volume was 20 μL, and flow was 1 mL/min.

Design and HPLC-MS Analysis of Resin-Bound Probe DCL. The aldehydes that involved in the whole DCL (except DCL-1) can be found in Figure 10

Mass spectra were acquired on an Finnigan LCQ-Advantage (ESI) LC-MS system, using Kromasil column (4.6 × 250 mm C_{18}, 300 Å, 5 μ) and ionization in positive ion mode. Gradient of all HPLC: 5% MeCN (0.1% TFA) to 100% MeCN (0.1% TFA) over 24 min. The injection volume was 20 μL; the flow was 1 mL/min, and the UV detector wavelength is 214 nm.

HR/MAS NMR experiment. All NMR spectra have been recorded on a Varian Unity INOVA-500 MHz and SYS-600 MHz spectrometer equipped with a 4-mm ^{1}H-observe nano NMR probe. The spin rate was ~2000 Hz for all samples at 25 °C. The HR/MAS NMR spectrum of complexes were formed by adding PEGA-L-Lys(-Suc-DADA)$_2$ (0.002 mmol) to a solution of cyy202 or NDMVan (0.002mmol) in 40 μL of DMSO-d_6 at 298 K with a presaturation at 3.55 ppm; the power was −16, and the delay was 1.5 s.

Bounding Constant Experiment. The combination of peptides with NDMVan derivatives were measured by observation of changes in the UV absorption spectrum. All the spectra were recorded on HeλIOSα of Thermo Spectronic Company. The solvent was 4% DMSO-PBS (pH 6.00), and all experiments were performed at 23 °C. The concentration of NDMVan derivatives were 0.17 mmol/L. The NDMVan derivatives were put into the reference cell, and the complex of NDMVan derivatives with ligand were put into the sample cell. The spectra recorded over the range of 260−320 nm, and the differential absorption reached greatest at 283 nm. The differential absorption of 283 nm was plotted against the concentration of added peptide. The resulting date was fined to the theoretical equation for 1:1 binding using least-squares methods. If the assumption is made that the plateau corresponds to 100% combination, when all the NDMVan derivatives are in the form of its complex with 1 molar proportion of peptide, and that lower values of the differential absorption are proportional to the concentration of the complex, the slope is bounding constant.

Synthesis of cyy202. The mixture of NDMVan (60 mg, 0.04 mmol), 2-carboxybenzaldehyde (18.2 mg, 0.12 mmol), and 10% DMSO/MeOH (v/v, 20 mL) was stirred at room temperature for 10 min. Then $NaBH_3CN$ (13.2 mg, 5 equiv, 0.2 mmol) was added into the mixture to react for another 2 h. After filtration, the white crude product was purified by an ODS-C_{18} reversed-phase column; 33.4 mg of cyy202 was obtained in a yield of 52.15%. ^{1}H NMR (600 MHz, DMSO-d_6, 25 °C): δ 7.86 (s, 1H; NH), 6.90 (s, 1H; NH), 8.25 (s, 1H; NH), 8.44 (s, 1H; NH), 8.61(s, 1H; NH), 7.86 (s, 1H; aromatic CH), 7.62 (d, J = 9.5 Hz, 1H; aromatic CH), 7.40 (m, 1H; aromatic CH), 7.30 (m, 3H; aromatic CH), 7.45 (d, J = 9.0 Hz, 1H; aromatic CH), 7.76 (d, J = 7.0 Hz, 1H; aromatic CH), 7.38 (d, J = 8.5 Hz, 1H; aromatic CH), 7.10 (s, 1H; aromatic CH), 6.76 (d, J = 9.0 Hz, 1H; aromatic CH), 7.16 (s, 1H; aromatic CH), 6.90 (s, 2H, $CONH_2$), 6.71 (d, J = 8.5 Hz, 1H; aromatic CH), 6.38 (s, 1H, aromatic CH), 5.73 (d, J = 7.5 Hz, 1H; aromatic CH), 6.66 (d, J = 11.0 Hz, 1H; NH), 6.28 (s, 1H; aromatic CH), 5.55 (s, 1H, aromatic CH), 5.31 (m, 1H; CH), 5.22 (s, 1H; aromatic CH), 5.11 (s, 1H; CH), 4.71 (d, J = 6.0 Hz, 1H; CH), 5.36 (d, J = 7.0 Hz, 1H, CH), 5.24 (m, 1H; NH), 5.11 (s, 1H; CH), 4.94 (s, 1H; CH), 4.43 (d, J = 6.0 Hz, 1H, CH), 4.16 (t, J = 12.0 Hz, 1H, CH), 3.59 (m, 1H; CH), 4.41 (m, 1H; CH), 3.72 (d, J = 10.5 Hz, 2H; CH_2), 3.54 (m, 1H; CH), 3.46 (m, 1H; CH), 2.45 (m, 1H, CH), 1.92 (d, J = 9.0 Hz, 1H; CH), 1.66 (m, 1H; CH), 1.37 (s, 3H; CH_3), 3.54 (m, 1H; CH), 3.30 (m, 1H; CH), 1.54 (s, 3H; CH_3), 1.06 (d, J = 6.0 Hz, 3H; CH_3), 0.77 (d, J = 4.5 Hz, 3H; CH_3), 0.85 (d, J = 4.5 Hz, 3H; CH_3). ^{13}C NMR (600 MHz, DMSO-d6, 25 °C): δ 173.1 (CO), 173.1 (CO), 172.3 (CO), 172.3 (CO), 172.3 (CO), 170.8 (CO), 170.8 (CO), 170.8 (CO), 168.6 (CO), 158.6 (C), 157.5 (C), 156.6 (C), 153.5 (C), 152.4 (C), 151.8 (C), 149.8 (C), 141.2 (C), 144.1 (C), 137.5 (C), 137.5 (CH), 135.5 (C), 133.4 (C), 131.4 (CH), 131.4 (CH_3), 131.4 (C), 130.2 (CH), 129.0 (CH), 129.0 (CH), 129.0 (CH), 129.0 (CH), 129.0 (CH), 128.5 (C), 127.8 (C), 127.8 (C), 126.7 (CH), 125.8 (CH), 124.9 (CH), 123.6 (C), 119.5 (C), 117.6 (CH), 109.0 (CH), 108.4 (CH), 106.4 (CH), 103.4 (CH), 102.2 (CH), 97.9 (CH), 78.8 (CH), 78.5 (CH), 78.5 (CH), 73.1 (CH), 72.6 (CH), 71.8 (CH), 64.7 (CH), 63.3 (CH), 60.3 (CH), 59.5 (CH), 56.4 (CH), 55.1 (CH), 43.0 (CH), 35.2 (CH_2), 25.6 (CH), 24.6 (CH_3), 24.1 (CH_3), 23.6 (CH_3), 18.5 (CH). HRMS m/z calcd for $[M + 2H]^{2+}$: 784.7257; found 784.7307.

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Supporting Information Available. The synthesis procedure and analysis of probe, the preparation and HPLC-MS analysis of resin-bound probe DCL, HR/MAS NMR, bonding constant experimental procedure, and full characterizations of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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