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Synthesis of Structurally Diverse Bis-peptide Oligomers

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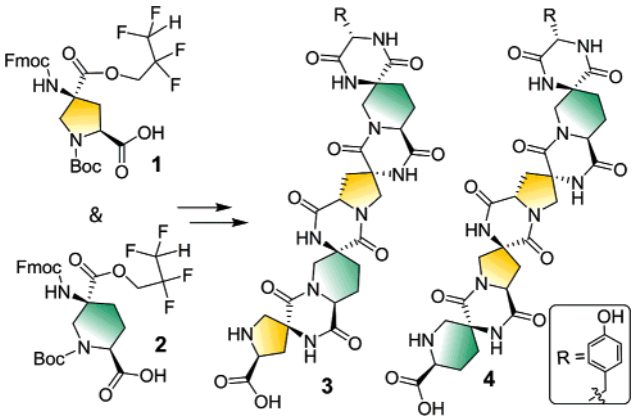

We have developed second-generation monomers **1** and **2** and improved conditions for rapidly and simultaneously closing multiple diketopiperazines on solid support. These new conditions involve either the microwave heating of a suspension of solid-supported amino-tetrafluoropropyl esters in acetic acid/triethylamine catalyst solution or continuous flow of catalyst solution through the resin, heated in a flow cell apparatus. We demonstrate that the new monomers **1** and **2** can be combined with the new conditions easily to synthesize previously inaccessible hetero and homo spiro ladder oligomers **3** and **4** and others.

The diketopiperazine (DKP) motif is found throughout chemistry. Many natural products containing 2,5-piperazinedione rings have been isolated, displaying a wide variety of biological functions.[1] Medicinal chemistry also makes considerable use of the diketopiperazine scaffold,[2] and DKPs have been found to be a useful scaffold for molecular recognition.[3] Our laboratory has developed an approach to the synthesis of nanometer-scale spiro ladder oligomers with controlled three-dimensional structures.[4−6] Our long-term goal is to develop these molecules for biomimetic and nanotechnology applications. Our oligomers are composed of cyclic monomers (Figure 1) joined by diketopiperazine rings (e.g., compound **3**). We assemble these oligomers in two phases: assembly followed by rigidification.

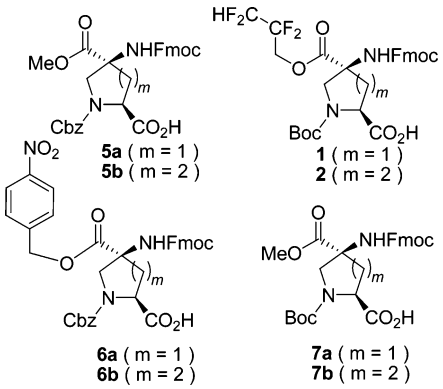

FIGURE 1. Protected bis-amino acid monomers used to optimize DKP formation on solid support. We name the monomers with $m = 1$ the "*pro4*" monomers and those with $m = 2$ the "*pip5*" monomers.

In the assembly phase, we assemble stereochemically pure, cyclic bis-amino acid monomers on solid support using the techniques of solid-phase peptide synthesis to form flexible oligomers. In the rigidification phase, we promote the simultaneous formation of a diketopiperazine between each adjacent

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pair of monomers in the oligomer to form a rigid spiro ladder oligomer. To form a spiro ladder oligomer containing N monomers, we must simultaneously close (N-1) diketopiperazines. To achieve this with useful yields, it is very important that the diketopiperazine formation reaction be fast and exhibit no side reactions.

Diketopiperazine formation has been the subject of several reviews.[7,8] A particularly facile route and the one that we initially used is an intramolecular aminolysis reaction between an amine and an ester catalyzed by 20% piperidine in dimethylformamide (DMF).[4] We found these conditions to be quite effective for *pro4* monomers, but they are not ideal for several reasons. Occasionally, precipitate would form in the reaction mixture and the yield of fully closed product would be low. We also discovered that for oligomers containing *hin(2S4R7R9R)*[5] or *pip5(2S5S)*[6] the DKP formation between monomers under 20% piperidine in DMF at room temperature was very slow or undetectable. The rate of DKP formation is known to be sensitive to the stereochemistry and nature of the substituents on the forming DKP ring.[9] We cannot use high temperatures or long reaction times to drive the aminolysis reaction toward completion because DKPs epimerize under basic conditions.[10]

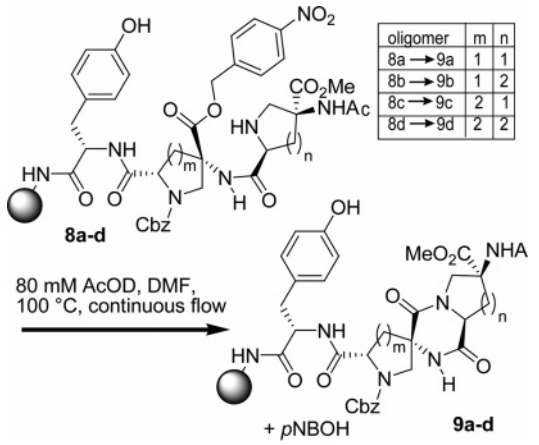
DKP formation is catalyzed by carboxylic acids[9] as well as base.[11] The mechanism and the rate-determining steps are different under acidic conditions than basic conditions.[9] We hypothesized that we could use carboxylic acids to catalyze DKP formation in difficult cases. We also hypothesized that using acid-catalyzed conditions would allow us to use higher temperature to accelerate the reaction without fear of epimerization. Thus, we have undertaken a search for improved conditions for closing diketopiperazines.

To explore different solvents, catalyst concentrations, and temperatures, we chose to close the diketopiperazines on solid support. Accordingly, we substituted the benzyloxycarbonyl (Cbz) protecting group of monomers **5a** and **5b** with the *tert*-butoxycarbonyl (Boc) protecting group to create **7a** and **7b** and substituted the methyl esters of monomers **5a** and **5b** with *para*-nitrobenzyl esters to give monomers **6a** and **6b.** We carried out oligomer syntheses on trifluoromethylsulfonic acid cleavable linkers (MBHA and hydroxyl methyl) and then measured the kinetics of DKP formation in a flow cell containing resin bound dimers **8a**−**d** (Scheme 1) by quantitating the amount of *para*-nitrobenzyl alcohol in the effluent as different catalyst solutions were flowed over the resin at varying temperatures.

We carried out a series of diketopiperazine closures examining how the nature of the monomers affects the rate of intramolecular aminolysis using acetic acid-*d* as a bifunctional catalyst (Table 1, Scheme 1). An 80 mM solution of acetic acid-*d* in DMF at 100 °C is a good catalyst for the formation of **9a**, **9b**, and **9c** but four times slower for the formation of **9d**. Deutero acetic acid was used because epimerization of **8a**−**d** would give rise to an increase in molecular weight of at least 1 Da. When the dimers were cleaved from the resin, no epimerization was observed using these conditions (data not shown).

To explore more optimal acetic acid-catalyzed DKP formation conditions, we investigated the effect of solvent and temperature

SCHEME 1. Solid Supported Diketopiperazine Formation Model Reactions[a]



[a] These bis-amino acid dimers are assembled from one of **6a** or **6b** followed by one of **7a** or **7b**.

TABLE 1. Half-Life of Diketopiperazine Formation for Different Dimers in 80 mM AcOD, DMF, 100 °C

sequence	half-life, $t_{1/2}$ min
pro4-pro4 (**8a** → **9a**)	6.5
pro4-pip5 (**8b** → **9b**)	12
pip5-pro4 (**8c** → **9c**)	16
pip5-pip5 (**8d** → **9d**)	78

TABLE 2. Effect of Solvent and Temperature on Diketopiperazine Closure in the Reaction 8b → 9b Using 80 mM AcOD as the Catalyst

	half-life, $t_{1/2}$ (min), for	
temp (°C)	DMF	*o*-xylene
40	126	n/a
70	31	115
100	12	18
130	<4	<2

on AcOD-catalyzed formation of **9b** (Scheme 1, Table 2). Increasing the temperature accelerates the rate of DKP formation in both solvents, and the temperature effect is stronger in *o*-xylenes. Dimethylformamide decomposes above 100 °C and is thus not a suitable solvent at high temperatures. These observations led us to choose *o*-xylene as the standard solvent for subsequent experiments. Gisin and Merrifield found that the maximum rate of acetic acid-catalyzed DKP formation occurred at an acetic acid (AcOH) concentration of 100 mM.[9]

The work of Capasso and Mazzarella suggests that triethylammonium acetate is at least 10 times faster at catalyzing DKP formation than acetic acid by itself.[12] In this study, it is likely that the triethylamine served to neutralize the acidic trifluoroacetate salt of the dipeptides that underwent diketopiperazine formation. In our system, we free-base our amines on solid support by washing the deprotected resin with triethylamine in DMF prior to diketopiperazine closure. Nonetheless, we examined the effect of adding triethylamine to the catalyst solution and found that DKP formation was accelerated by a factor of 2 to 3 in the formation of **9a** or **9d** (Scheme 1, Table 3) relative to when no triethylamine was added. The acid/base equilibrium between Et_3N:AcOH and Et_3NH^+:AcO^- is likely to lie on the

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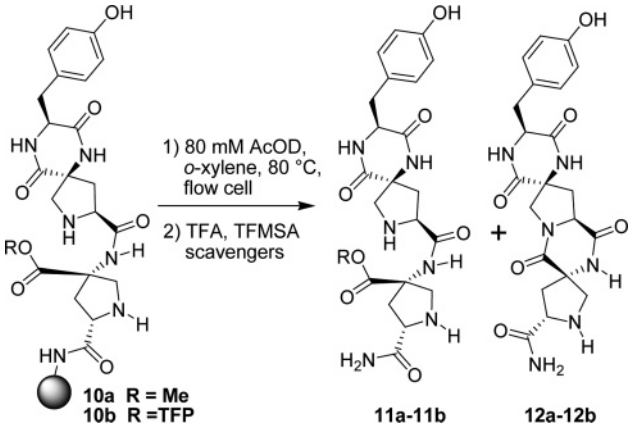
TABLE 3. Half-Life of Diketopiperazine Formation in the Reactions 8a → 9a and 8d → 9d: Exploring Different Concentrations of Acetic Acid and Triethylamine

conditions (solvent: *o*-xylene)	half-life $t_{1/2}$ (min) at 130 °C for **8d → 9d**	half-life $t_{1/2}$ (min) at 80 °C for **8a → 9a**
50 mM AcOD	9.5	18.8
80 mM AcOD	12.5	
100 mM AcOD	9.2	18
50 mM AcOD + 50 mM Et_3N	5	4.6
100 mM AcOD + 50 mM Et_3N	4.3	3.3
1 mM Et_3N	266	

SCHEME 2. Effect of pK_a of the Leaving Group on AcOH-Catalyzed Diketopiperazine Formation

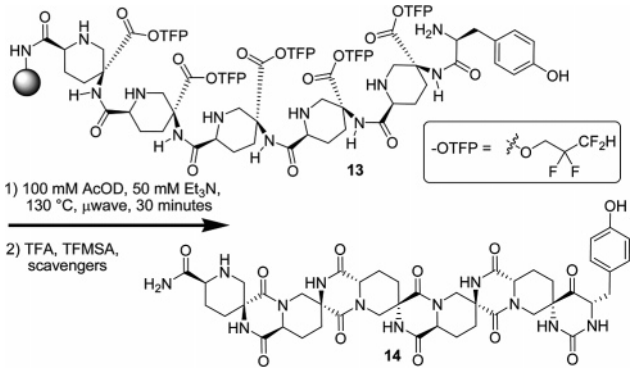


side of the neutral species in the apolar solvent *o*-xylene, and thus it should not reduce the concentration of acetic acid catalyst. Triethylamine could be increasing the rate of diketopiperazine formation by deprotonating any secondary ammonium ions on the resin in an acid/base pre-equilibrium.

After screening several esters, we developed the second-generation monomers **1** and **2** as a replacement for the methyl ester version of our first-generation monomers **7a** and **7b**. We explored trifluorethyl esters, trichloroethyl esters, and acetoxy esters and found that they were not suitable because they react with piperidine and DBU during our solid-phase oligomer assembly (data not shown). Ultimately, we found that 2,2,3,3-tetrafluoropropyl (TFP) esters are a good compromise of stability to piperidine and reactivity in diketopiperazine formation.

To examine the effect of the leaving group on the rate of DKP formation, we set out to measure the rate of the intramolecular aminolysis reaction in *pro4-pro4* dimers **10a** and **10b** under the same solvent and temperature conditions (Scheme 2). In **10a** a secondary amine attacks a methyl ester, while in **10b** it attacks a tetrafluoropropyl ester. Unlike *para*-nitrobenzyl alcohol, the release of methyl alcohol or tetrafluoropropyl alcohol cannot be observed and quantified by HPLC. Hence, a different method was used to monitor the progress of the reaction. We carried out the reaction by flowing catalyst solution over separate batches of resin-supported dimer (80 mM AcOD in *o*-xylene at 80 °C) for definite time intervals: 1 h for **10a** and 5 min for **10b**. After each reaction, we cleaved the oligomer from the resin and quantified the amount of closed product and open product. We found that the acceleration provided by substitution of the methyl ester by a tetrafluoropropyl ester is too large to be accurately quantified by this experiment. In the case of the methyl ester, it takes 6 h to obtain 70% formation

SCHEME 3. Test of Our Second-Generation Monomers and Diketopiperazine Formation Conditions Using a Pentamer of 2



of DKP; however, only 5 min are required for dimer containing TFP ester to obtain the same amount of DKP product. This experiment allowed us to quantify epimerization under these conditions. In the case of the methyl ester, a separate peak was observed for epimerized product. This epimer peak had a mass spectrum that indicated 50% deuterium incorporation, suggested by a large change in the ratios of M + 1, M + 2, and M + 3 peaks of the mass spectrum. This new peak represented only 3% epimerized product; thus, even after 6 h of heating at 80 °C in a flow cell in the presence of 80 mM AcOD/*o*-xylene, there is very little epimerization. We did not detect any epimerized product in the case of the TFP ester. These observations suggest that the acid-catalyzed DKP formation results in very little epimerization if the reaction time is kept short. By exchanging the methyl ester for more reactive esters, we can shorten the reaction time considerably.

To test our best catalysis conditions, we synthesized the monomer **2** and assembled a pentamer on a 4-methylbenzhydrylamine (MBHA) resin and on solid support removed the Boc protecting group on each monomer to form intermediate **13**. We suspended the resin bound **13** in 100 mM AcOD and 50 mM Et_3N and exposed it to microwaves at 130 °C for 30 min (Scheme 3). The resin cleavage product **14** was characterized by C_{18} reverse-phase HPLC with mass spectrometry (Figure 2). The major peak was the desired product **14**. Two small peaks representing intermediates that had failed to close one diketopiperazine were also observed, but there was no evidence of epimerization of **14**.

We then set out to synthesize a small library of structurally diverse bis-peptide oligomers containing *pip5(2S5S)* and *pro4-(2S4S)* monomers and test the efficacy of our newly optimized conditions for diketopiperazine formation. We designed four hetero sequences **15**−**18** and assembled them on hydroxymethyl polystyrene resin (Scheme 4). This was followed by on resin diketopiperazine formation in a flow cell apparatus catalyzed by 100 mM AcOH and 50 mM Et_3N in *o*-xylene at 130 °C. The resin cleavage products **3**, **4**, **19**, and **20** were purified by C_{18} reverse-phase preparative HPLC and characterized by high-resolution mass spectrometry. The analysis of the crude products before purification by C_{18} reverse-phase HPLC reveals that in all four cases diketopiperazine formation was very clean. Only trace amounts of any product with one diketopiperazine ring unclosed were detected (Figure 3).The oligomers **3**, **4**, **19**, and **20** gave rise to high-resolution mass spectra with m/z = 762.2927, 762.2844, 762.2880, and 762.2865, respectively (calculated 762.2847).

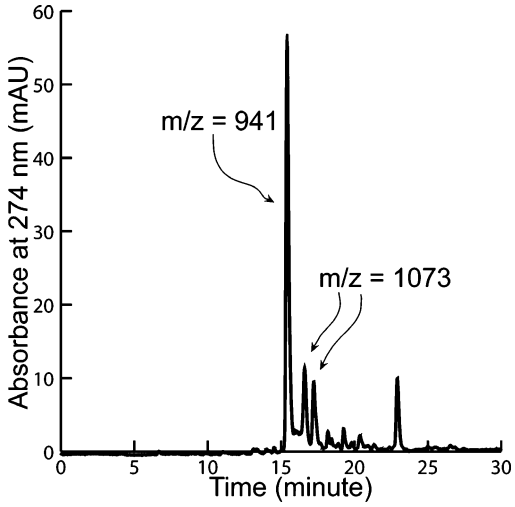


FIGURE 2. Unpurified reverse-phase C_{18} HPLC chromatograms of **14** (0.1% HCO_2H, 0−25% AcN over 30 min). The main peak is the desired product (m/z = 941).

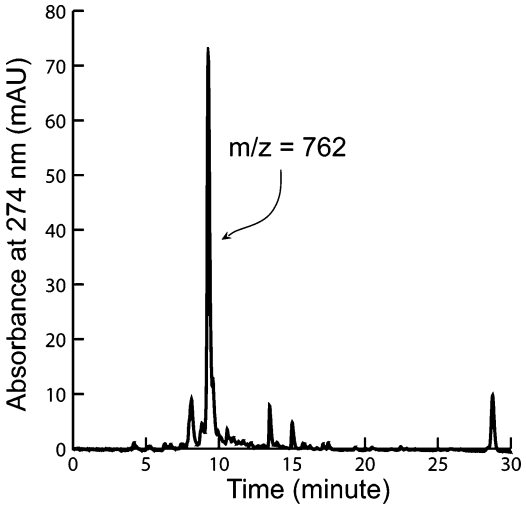


FIGURE 3. Reverse-phase C_{18} HPLC chromatograms of **20** (0.1% HCO_2H, 0−25% AcN over 30 min) before purification. The main peak is the desired product (m/z = 762).

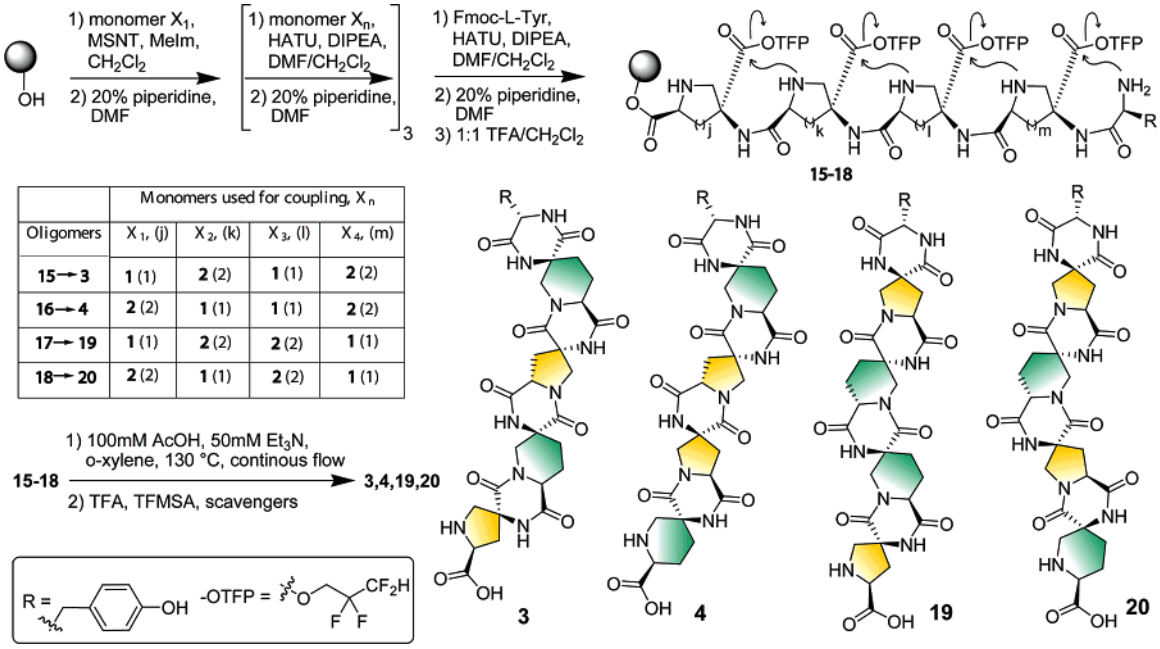
In conclusion, we have developed the second-generation monomers **1** and **2** and AcOD/Et_3N-catalyzed, microwave-assisted, and continuous flow conditions for simultaneously closing multiple diketopiperazines on solid support with insignificant amounts of epimerization. We are now using this new methodology to synthesize larger and more complex spiro ladder oligomers for biomimetic and nanotechnology applications.

Experimental Section

Compound 8c. MBHA resin (23 mg, 14.6 μmol free amine, 0.62 mmol loading) was transferred to a 1-mL polypropylene solid-phase peptide synthesis (SPPS) reactor and allowed to swell for 30 min in DMF. In a 1.5-mL polypropylene microcentrifuge vial, Fmoc-L-Tyr(*t*-Bu)-OH (13.1 mg, 28.5 μmol) was dissolved in 20% CH_2Cl_2/DMF (143 μL). To this mixture was added DIPEA (9.9 μL, 57 μmol), followed by HATU (10.8 mg, 28.5 μmol). The resulting coupling mixture was mixed well and immediately added to the SPPS reactor carrying the resin. The resin and coupling mixture were agitated for 2 h. The coupling solution was drained, and the resin was washed two times each with DMF and CH_2Cl_2 alternately. Any residual free amine was acylated by treatment with 10% acetic anhydride/pyridine (250 μL) for 5 min. The resin was then washed five times with DMF and CH_2Cl_2 alternately, and the terminal Fmoc-protected amine was deblocked by treatment with 2% DBU/DMF for 5 min. UV−visible spectroscopic analysis of the piperidine fluorenyl adduct, formed by diluting the deprotection reaction solvent with 20% piperidine/DMF at 301 nm (ϵ = 7800 M^{-1} cm^{-1}), indicated quantitative coupling based on resin loading. The resin was washed five times with DMF and CH_2Cl_2 alternately.

Monomer **6b** (19.4 mg, 28.5 μmol) was coupled to the resin using HATU in a fashion similar to that described above. The coupling reaction was repeated one additional time followed by capping of any residual free amine and Fmoc deprotection using the procedure given above. The overall sequential procedure for

SCHEME 4. Assembly of Short Hetero Oligomers and on Resin Diketopiperazine Formation by Continous Flow Method Using Optimized Catalytic Conditions



	Monomers used for coupling, X_n			
Oligomers	X_1, (j)	X_2, (k)	X_3, (l)	X_4, (m)
15→3	**1** (1)	**2** (2)	**1** (1)	**2** (2)
16→4	**2** (2)	**1** (1)	**1** (1)	**2** (2)
17→19	**1** (1)	**2** (2)	**2** (2)	**1** (1)
18→20	**2** (2)	**1** (1)	**2** (2)	**1** (1)

coupling an amino acid moiety was repeated one additional time for monomer **5a**. The coupling yields were estimated as 90−95% on the basis of spectroscopic analysis described above.

After the coupling of the second monomer, the terminal amine was acylated by treatment with 10% acetic anhydride/pyridine (2 × 5 min). The resin was washed three times with DMF and CH_2Cl_2 alternately and then three times with MeOH and CH_2Cl_2 alternately. Boc group removal was affected by treatment of the resin with 1 mL of 1:1 TFA/DCM (2 × 15 min). The resin was washed with CH_2Cl_2 and MeOH many times and neutralized by washing a few times with 10% Et_3N/CH_2Cl_2. Finally, the resin was washed with CH_2Cl_2 and MeOH many times, followed by only CH_2Cl_2 and then dried under reduced pressure overnight. Other bis-amino acid sequences (**8a**, **8b**, and **8d**) were synthesized in the same manner.

Measurement of Kinetics of Diketopiperazine Formation. A tiny amount of resin (2 to 3 mg) carrying desired dimer (e.g., **8c**) was loaded in a homemade flow cell apparatus, and catalyst solution was made to flow through the resin at 100 μL/min at the desired temperature (details given in Supporting Information). The fractions were collected at 15-min intervals and analyzed by HPLC. Areas under the peaks were recorded for the reference and the *p*-nitrobenzyl alcohol and used for further calculations as shown in Supporting Information.

Compound 13. The homo oligomer **13** was synthesized on MBHA resin (10-mg resin, 0.62 mmol/g loading) following a procedure similar to the one described for **8c**. The only difference was that, instead of 2% DBU in DMF, 20% piperidine in DMF solution for 20 min was used to effect Fmoc deprotection. Accordingly, 5 units of monomer building block **2** were coupled sequentially to the resin, followed by Fmoc-L-Tyr(*t*-Bu)-OH. This was followed by removal of terminal Fmoc group to unmask the free amine and removal of Boc groups to reveal secondary amino groups. The resin was washed with CH_2Cl_2 and MeOH many times and neutralized by washing a few times with 10% Et_3N/CH_2Cl_2. Finally, the resin was washed with DCM and MeOH many times, followed by only CH_2Cl_2, and then dried under reduced pressure overnight to yield **13**.

Compound 14. A few beads of MBHA resin carrying homo oligomer sequence **13** were placed in a 10-mL microwave reaction vessel (10-mL capacity, glass) containing 5 mL of a solution of 100 mM AcOH and 50 mM Et_3N in *o*-xylene. The vessel was capped and placed in the microwave reactor (CEM Discover) and irradiated (300 W, maximum power, 130 °C, 5 min ramp) with continuous stirring. After 60 min, the tube was removed from the microwave reactor, and the resin was transferred to a 1-mL polypropylene SPPS reactor. The resin was washed with CH_2Cl_2 and MeOH many times, followed by only CH_2Cl_2, and then dried under reduced pressure overnight.

The reactor carrying the resin was kept in an ice bath, and 25 μL of thioanisole, 12.5 μL of 1,2-ethanedithiol (EDT), and 250 μL of TFA were added. The cleavage mixture was stirred for 5 min, and 25 μL of TFMSA was added. The stirring was continued for 1 h at 0 °C followed by 1 h at room temperature. The cleavage mixture was dripped into 50 mL of ether in an Eppendorf tube that caused desired product **14** to precipitate out. This precipitate was pelleted by centrifugation for 30 min at 4 °C at 3200 rpm. Ether was decanted, precipitate was dissolved in 200 μL of TFA, and product was precipitated a second time from ether. After centrifugation, ether was decanted and the pellet was dissolved in 200 μL of 1:1 acetonitrile/water. Quantities of 10 μL of this solution were injected into HPLC−MS for analysis. HPLC−MS: column, Waters XTerra MS C_{18} column 4.6 mm × 150 mm; mobile phase, MeCN (0.05% formic acid)/water (0.1% formic acid), 0−25% MeCN over 30 min; flow rate 0.80 mL/min; UV detection at 274 nm; t_R for **14**, 16.03 min; ES-LRMS *m*/*z* (ion), calcd 941.4 ($M + H^+$), obsd 941.2.

Compounds 15−18. Each of the hetero oligomers **15−18** was assembled on hydroxymethyl polystyrene resin (0.98 mmol/g loading) on a 20-mg scale. Monomer X_1 was attached to the hydroxymethylpolystyrene resin using the MSNT/MeIm method (Novabiochem 2004/05 catalogue, Method 2−12). The resin was allowed to swell in dry CH_2Cl_2 in a SPPS reactor for 10 min. In the case of oligomer **18**, 25 mg of **2** (40 μmol, 2 equiv) was dissolved in 300 μL of dry CH_2Cl_2. To this solution were added 2.3 μL of MeIm (28 μmol, 1.4 equiv) followed by 12 mg of MSNT (40 μmol). This coupling solution was added to the pre-swelled resin and gently agitated for 30 min. The coupling solution was drained, and the resin was washed many times with MeOH and CH_2Cl_2 alternately. Any unreacted alcohol groups were capped by treatment with 500 μL of 10% Ac_2O in pyridine for 5 min. Finally, the resin was drained and washed many times with MeOH and CH_2Cl_2, followed by only CH_2Cl_2. Other monomers, X_{2-4}, followed by Fmoc-L-Tyr(*t*-Bu)OH were coupled sequentially using HATU following a procedure similar to the one as described for oligomer **13**. Subsequently, Fmoc and Boc group were removed, and the resins were prepared for diketopiperazine closure using the previously described method for **13**.

Compounds 3, 4, 19, and 20. The resins carrying desired sequence (**15−18**) were transferred to the PTFE tubing of flow cell apparatus, and the catalytic solution (100 mM AcOH and 50 mM Et_3N in *o*-xylene) was pumped through the resin at 200 μL/min for 2 h at 130 °C. The resins were recovered and then cleaved using trifluoromethanesulfonic acid in the presence of scavengers as described for **14**. After a single precipitation in ether and centrifugation, the resulting pellets were dissolved in 10% acetonitrile in water and purified by preparative HPLC: mobile phase MeCN (0.05% formic acid)/water (0.1% formic acid), 0−25% MeCN over 30 min; flow rate 15 mL/min. The desired fractions were pooled and lyophilized giving compounds **3**, **4**, **19**, and **20**; HRESIQTOFMS calcd for $C_{35}H_{40}N_9O_{11}$ ($M + H^+$) 762.2847, found 762.2927 for **3**, found 762.2844 for **4**, found 762.2880 for **19**, found 762.2865 for **20**.

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Supporting Information Available: Experimental procedures, schematics of the flow cell apparatus, methodology for half-life estimation, and optimization of reaction condition for diketopiperazine formation, relevant NMR spectra, and other characterization data for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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