Macromolecular Self-Assembly of Diketopiperazine Tetrapeptides

Raymond J. Bergeron,* Otto Phanstiel IV, Guo Wei Yao, Sam Milstein,† and William R. Weimar

Contribution from the Department of Medicinal Chemistry, J. Hillis Miller Health Center, University of Florida, Gainesville, Florida 32610, and Emisphere Technologies, Inc., 15 Skyline Drive, Hawthorne, New York 10532

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Abstract: Basic solutions of tetrapeptides derived from L-aspartic acid diketopiperazines are shown to form microcapsules when acidified to pH 2.4. An initial structure–activity study clearly demonstrates that a very delicate balance exists between the tetrapeptides' structure and their ability to self-assemble. Scanning electron micrographs confirm that microcapsules and not solid microspheres are formed.

Introduction

There has recently been a renewed interest in molecular self-assembly[1,2] from both a mechanistic[3] and applications perspective.[4] While many amphiphiles and bolaamphiphiles have been shown to form hydrophobically-driven spherical micelles and vesicles, because of their fluid character, they lose their shape upon drying. However, a number of groups have demonstrated that, upon introduction of a secondary amide functionality into these systems, stable tubular vesicles are formed. In fact, these rods can be isolated in a dry state. For example, diacetylenic aldonamides prepared from aldonic acid γ-lactones of a number of monosaccharides were shown to form stable supramolecular assemblies as microtubules.[5,6] This assembly process was also observed with unsymmetric linear bolaamphiphiles in which the N^6 nitrogen of lysine was acylated with 12-aminododecanoic acid.[7] While these molecules are fascinating with regard to their tubule-forming properties, their size and degrees of rotational freedom make it difficult to identify those structural parameters responsible for self assembly. The identification of smaller molecules with fewer degrees of conformational freedom which indulge in supramolecular assembly would certainly be of value in mechanistic evaluations of such assembly processes. Furthermore, although it is true that many of the self-assembly investigations have focused on microtubule formation, studies of microcapsule formation are of equal importance from both applied and theoretical standpoints. The forces which drive macromolecular assembly to a microcapsular geometry are likely to be the same as those which control tubule formation.

In the late 1950s, Fox et al. demonstrated that poly(amino acids) generated from thermal melts form microspheres or "protocells". When basic solutions of these poly(amino acids) were acidified, self assembly occurred.[8–13] However, the precise structures of the thermoproteins were unknown as were the structural features responsible for their remarkable self-assembly. In 1974, Gyore and Ecet demonstrated that along with linear peptides diketopiperazine ring systems were generated during the thermal polymerization of amino acids.[14] These six-membered ring systems were presumably generated by intramolecular cyclization of the dimer prior to further chain growth or directly from a linear peptide.[15,16] Since diketopiperazines of one form or another may well have been constituents of the original Fox poly(amino acid) mixture, we elected to explore the diketopiperazine tetrapeptides as potential models for self-assembly. Furthermore, the diketopiperazine framework offered an opportunity to study the self-assembly properties of molecules with reduced degrees of freedom.

When trifunctional amino acids such as L-Glu and L-Asp cyclize (to form diketopiperazines), they generate a bis(carboxylic acid) platform which can be further condensed with other amino acids. These unique systems, because of the *cis* geometry imparted by the chiral components of the diketopiperazine ring,[17] provide an opportunity to systematically alter the structure of the terminal amino acids while holding the orientation between them fixed relative to noncyclic analogues.[18,19]

Results and Discussion

Synthesis. Two diketopiperazine platforms were utilized in this study, one from the self-condensation of L-aspartic acid and one from the self-condensation of L-glutamic acid. Each of these cyclic amides has two free carboxylic acid groups available for coupling of additional amino acids. Although there are a number of synthetic approaches to the diketopiperazine framework,[15,18,20,21] the *in situ* generation of an "activated" amino acid ester was employed.

† Emisphere Technologies, Inc.

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(1) Ghadiri, M. R.; Granja, J. R.; Milligan, R. A.; McRee, D. E.; Khazanovich, N. *Nature* **1993**, *366*, 324–327.
(2) Borman, S. *Chem. Eng. News* **1993**, *71* (May 3), 45–46.
(3) Perlstein, J. *J. Am. Chem. Soc.* **1994**, *116*, 455–470.
(4) Brumlik, C. J.; Martin, C. R. *J. Am. Chem. Soc.* **1991**, *113*, 3174–3175.
(5) Fuhrhop, J. H.; Blumtritt, P.; Lehmann, C.; Luger, C. *J. Am. Chem. Soc.* **1991**, *113*, 7437–7439.
(6) Frankel, D. A.; O'Brien, D. F. *J. Am. Chem. Soc.* **1991**, *113*, 7436–7437.
(7) Fuhrhop, J. H.; Spiroski, D.; Boettcher, C. *J. Am. Chem. Soc.* **1993**, *115*, 1600–1601.
(8) Fox, S. W.; Harada, K.; Vegotsky, A. *Experientia* **1959**, *15*, 81–84.
(9) Fox, S. W. *Q. J. of the Fla. Acad. Sci.* **1968**, *31*, 1–15.
(10) Fox, S. W. *Nature* **1965**, *205*, 328–340.
(11) Fox, S. W. *Sci. Technol.* **1968**, No. 74, 51–61.
(12) Fox, S. W.; Nakashima, T. *Biochim. Biophys. Acta* **1967**, *140*, 155–167.
(13) Steiner, S.; Rosen, R. U.S. Patent 4,925,673, 1990.
(14) Gyore, J.; Ecet M. *Therm. Anal., Proc. Int. Conf., 4th* **1974**, *2*, 387–394.
(15) Reddy, A. V.; Ravindranath, B. *Int. J. Peptide Protein Res.* **1992**, *40*, 472–476.
(16) Mazurov, A. A.; Andronati, S. A.; Korotenko, T. I.; Gorbatyuk, V. A.; Shapiro, Y. E. *Int. J. Peptide Protein Res.* **1993**, *42*, 14–19.
(17) Lannom, H. K.; Dill, K.; Danarie, M.; Lacombe, J. M.; Pavia, A. A. *Int. J. Peptide Protein Res.* **1986**, *28*, 67–78.
(18) Fusaoka, Y.; Ozeki, E.; Kimura, S.; Imanishi, Y. *Int. J. Peptide Protein Res.* **1989**, *34*, 104–110.
(19) Ogura, H.; Furuhata, K.; Furuhata, K. *Chem. Pharm. Bull.* **1975**, *23*, 2474–2477.
(20) Lee, B. H.; Gerfen, G. J.; Miller, M. J. *J. Org. Chem.* **1984**, *49*, 2418–2423.
(21) Buyle, R. *Helv. Chim. Acta* **1966**, *49* (No. 162), 1425–1429.

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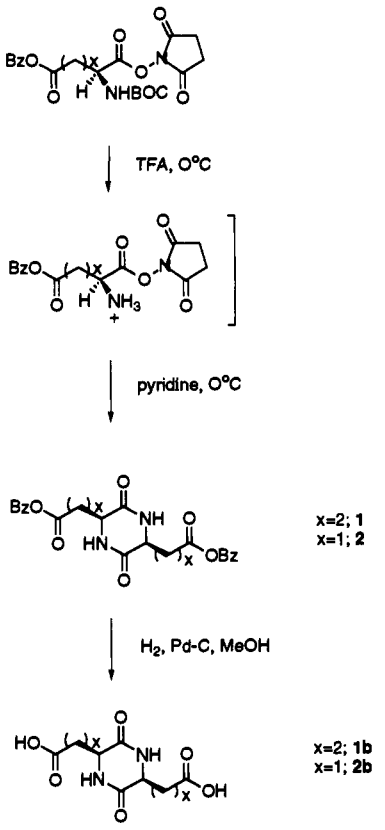
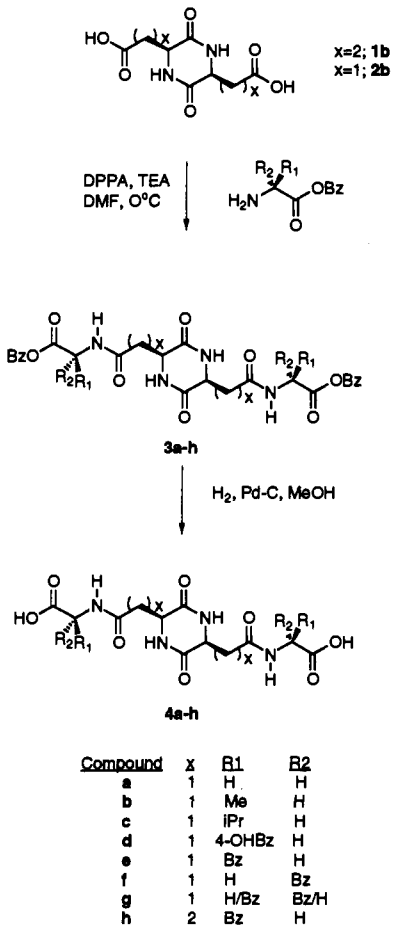
Scheme 1. Synthesis of Diketopiperazine Ring Systems



Scheme 2. Synthesis of Tetrapeptides



Compound	x	R1	R2
a	1	H	H
b	1	Me	H
c	1	iPr	H
d	1	4-OHBz	H
e	1	Bz	H
f	1	H	Bz
g	1	H/Bz	Bz/H
h	2	Bz	H

The glutamate systems were prepared from δ-benzyl-*N*α-BOC-L-glutamic acid *N*-hydroxy succinimide ester as shown in Scheme 1. Removal of the BOC group with TFA at 0 °C followed by neutralization with pyridine gave the diketopiperazine system **1** ($x = 2$) in 50% yield. Reduction of the terminal benzyl esters via hydrogenation over 10% Pd–C gave the diketopiperazine of L-Glu (**1b**) in quantitative yield. A similiar procedure was used to generate the diketopiperazine diester of L-Asp (**2**) (in 47% yield) and its corresponding free diacid **2b** (in 80% yield). The pendant carboxyls of **1b** and **2b** afforded sites of attachment for other appropriately protected amino acids. In order to model further chain growth of these difunctional monomers, a homologous series of amino acid benzyl esters including Gly, L-Ala, L-Val, L-Tyr, L-Phe, D-Phe, and D,L-Phe were attached to **2b** ($x = 1$) by the DPPA coupling method.[22] Subsequent hydrogenation gave the respective free tetrapeptides as illustrated in Scheme 2. Similarly, L-Phe benzyl ester was condensed with **1b** ($x = 2$) to give the diester **3h**, which was reduced to the tetrapeptide diacid **4h**. Using the same method, L-Phe-L-Phe-(diketo-L-Asp)-L-Phe-L-Phe (**4i**) was synthesized from its corresponding dibenzyl ester **3i** in 81% yield. The condensation reactions gave isolated yields ranging from 65 to 80%, and the ensuing debenzylation reactions were typically greater than 80%.

Microcapsule Formation. In order to identify which structural features of diketopiperazine tetrapeptides contribute to self-association, each diacid substrate (**1b**, **2b**, and **4a–i**) was tested for microcapsule formation as shown in Table 1. The compound of interest was dissolved in an aqueous Li_2CO_3 solution, and a light microscope was used to follow the changes which occurred on lowering the pH with a citric acid solution. Under these conditions, four different phenomena were observed: the peptides remained in solution, quickly crystallized, precipitated as an amorphous solid, or generated microcapsules. Compounds **1b**, **2b**, and **4a–d** all remained in solution upon reduction of pH, and recrystallization or precipitation was observed after 24 h on the microscope slide, i.e. only after appreciable evaporation had taken place. Compounds **4e–g** all formed microcapsules, while **4h** crystallized and **4i** spontaneously precipitated.

A comparison of the homologous series in Table 1 (compounds **4a–4e**) reveals the importance of the amino acid side chain. The peptides **4a–4d** all remained in solution during the assembly experiments. However, when the amino acid side chain is benzyl (**4e–g**), the peptides self-assemble into microcapsules. This was evidenced by an immediate formation of a homogeneous milk-white suspension, which upon microscopic examination revealed the presence of small spheres.

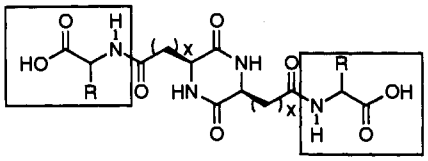
Recently it has been suggested by Ghadiri[1] in his studies with peptide nanotubes that the insolubility of the regenerated acids contributes to an ordered phase transition toward self-assembly. However, insolubility is not the only criterion for this assembly process, since the hexapeptide **4i** (Table 1) precipitated as an amorphous solid. Even more important is the aforementioned observation that replacement of a hydrogen on the phenylalanine aromatic ring with a hydroxyl results in a compound that does not form microcapsules. These results suggest a very delicate balance between the structure and macromolecular assembly of these tetrapeptides. It is clear that even small structural alterations within this group drastically alter the physiochemical properties of these tetrapeptides.

Chirality of the Attached Amino Acid. Fuhrhop and co-workers have observed a "chiral bilayer effect" in their studies of unsymmetric bolaamphiphiles, wherein separate L and D enantiomers (and not the racemate) were observed to self-assemble into molecular monolayers.[7,23–25] They speculated that electro-

(22) Shioiri, T.; Ninomiya, K.; Yamada, S. *J. Am. Chem. Soc.* **1972**, *94*, 6203–6205.

(23) Fuhrhop, J. H.; Schneider, P.; Rosenberg, J.; Boekema, E. *J. Am. Chem. Soc.* **1987**, *109*, 3387–3390.

Table 1. Microcapsule Formation Studies



4a-l

Compound	x	amino acid pendant	microcapsule formation?
1b	2	none	No
2b	1	none	No
4a	1	Gly	No
4b	1	L-Ala	No
4c	1	L-Val	No
4d	1	L-Tyr	No
4e	1	L-Phe	Yes
4f	1	D-Phe	Yes
4g	1	D,L-Phe	Yes
4h	2	L-Phe	No
4l	1	L-Phe-L-Phe	No

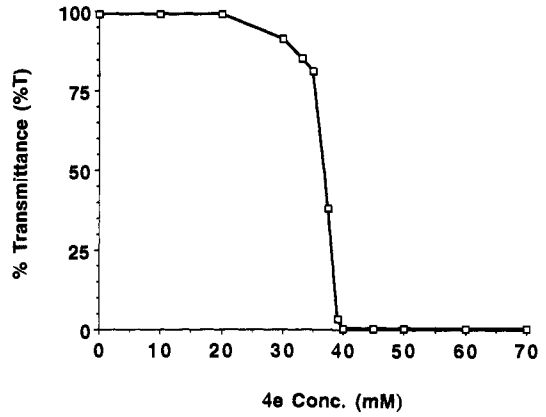


Figure 1. Percent of transmittance (at 400 nm) vs concentration of **4e** in 500 mM citric acid.

neutral fibers with high curvature are long-lived only if their surface is chiral. However, this chiral dependency was not observed in Fuhrhop's other study of microtubule supramolecular assembly and does not seem to apply to these spherical assemblies. Since each of the L- and D-Phe adducts (i.e., **4e** and **4f**, respectively) formed microcapsules, the chiral integrity of the pendant amino acid did not seem critical to microcapsule formation. This premise was tested by the attachment of racemic (D,L) Phe onto the framework of **2b** to give compound **4g**. As shown in Table 1, compound **4g** also self-assembled. The physical mixture of **4e**–**4f** also formed microcapsules. These observations suggest that the chirality of the attached amino acid is not a criterion for this phenomenon.

pH and Concentration Dependence. An investigation of the effects of diketopiperazine concentration and solution pH on the assembly process was conducted. The impact of these parameters on self-assembly was evaluated by following the change in solution turbidity while altering the pH at a fixed diketopiperazine (**4e**) concentration or by holding the pH constant and varying the concentration of peptide **4e**. The turbidity vs concentration curve (Figure 1) of **4e** in 500 mM citric acid clearly demonstrated a sharp transition from a clear solution (>95% T) to an opaque (0.2% T) suspension at concentrations of **4e** above 40 mM. A dense, homogeneous white suspension of microcapsules was observed. The influence of pH on turbidity was studied in solutions containing 50 mM **4e** in 500 mM lithium citrate buffers (Figure 2). The self-assembly of compound **4e** exhibited a very distinct

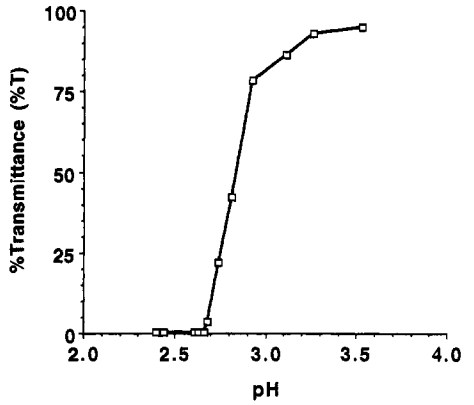


Figure 2. Percent of transmittance vs pH measurements with 50 mM **4e** and 500 mM lithium citrate.

dependence on the pH of the solution. The percent of transmittance (%T) was 0.2% at pH 2.70, while 100% at pH 3.3. The pK_a's for the terminal acid groups of **4e** were determined by titration to be 4.00 and 4.90, respectively. On the basis of these values for **4e**, the percentage of fully protonated diacid is >99% at pH 2.7 and 97.1% at pH 3.3. Therefore, the experimental data are consistent with a "protonation-induced" assembly process, wherein the visible manifestation of the assembly process (i.e., turbidity) is only apparent when the starting dianion is acidified to the fully protonated species **4e** in >97%. Viewed another way, the presence of one anionic species per 30 molecules of **4e** is sufficient to abort the supramolecular assembly. These results are in complete agreement with the aforementioned findings of Ghadiri, wherein the insolubilities of the regenerated acids were shown to play an important role in the assembly process.[1]

Hydrogen Bonding and Chain Length. Interestingly, the aspartate-based diketopiperazine tetrapeptides **4e–g** gave microcapsules whereas the corresponding glutamate diketopiperazine analogue (**4h**) did not. A comparison of **4e** (x = 1) and **4h** (x = 2) reveals the sensitivity of this system to chain length, wherein the incremental chain extension associated with the L-Glu derived diketopiperazine system resulted in crystals and not microcapsules. It is possible that the methylene chain length directly impacts the secondary structure of the molecule by influencing intramolecular hydrogen bonds. For example, the tetrapeptide **4e** can form a six-membered intramolecular H-bond between the diketopiperazine ring NH and the amide carbonyl of the pendant which may help orient the hydrophobic R group for proper macromolecular packing. This interaction is present as a seven-membered H-bond in **4h**, which may significantly alter the conformation necessary for macromolecular association.

This interplay between chain length, hydrogen bonding, concentration, pH, and secondary structure will be explored further in future NMR conformation studies.

Scanning Electron Micrographs. While there are numerous examples of microencapsulation with gelatin[26] and even with poly(amino acids),[13,27,28] this is the first time compounds of low molecular weight with clearly defined primary structure (**4e–g**) were observed to form microcapsules without additional additives. In order to prove that these entities were hollow and *not* solid microspheres or microparticles, compounds **4e,f** were observed under a scanning electron microscope (SEM). In Figure 3, the SEM photographs of **4e** clearly reveal the three-dimensional aspect of these microcapsules. Moreover, microscopy revealed a size distribution with diameters ranging from 0.2 to 10 μm. Since the

(24) Fuhrhop, J. H.; Krull, M.; Buldt, G. *Angew. Chem.* **1987**, *99*, 707–708.

(25) Fuhrhop, J. H.; Schneider, P.; Boekema, E.; Helfrich, W. *J. Am. Chem. Soc.* **1988**, *110*, 2861–2867.

(26) Deasy, P. B. *Microencapsulation and Related Drug Processes*; Drugs and the Pharmaceutical Sciences; Marcel Dekker Inc.: New York, 1984; Vol. 20, pp 61–95.

(27) Anderson, J. M.; Gibbons, D. F.; Martin, R. L.; Hiltner, A.; Woods, R. *Biomed. Mater. Symp.* **1974**, No. 5, 197–207.

(28) Li, C.; Yang, D.; Kuang, L.; Wallace, S. *Int. J. Pharm.* **1993**, *94*, 143–152.

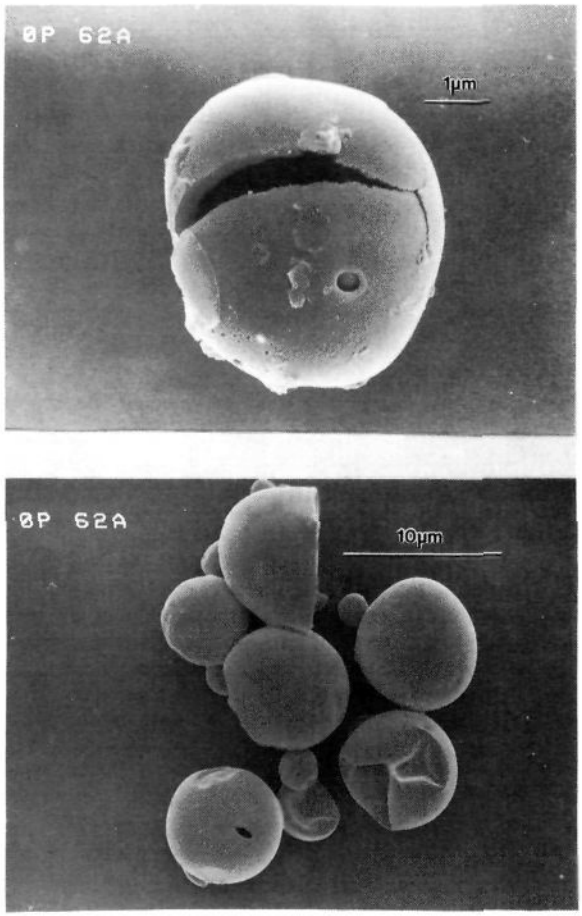


Figure 3. SEM pictures of L-Phe-(diketo-L-Asp)-L-Phe (**4e**): top photo, 15 400×; bottom photo, 3960×.

SEM technique requires a high vacuum for the electron beam, many of the microcapsules were imploded, further reflecting their hollow nature. Similar results were obtained with **4f**. To traverse the shell thicknesses observed in Figure 3 (170–250 nm) would require approximately 75–100 molecules oriented end to end (using an estimate of the molecular length of **4e** as 2.2 nm). However, an alternative and more likely situation would have the molecules *stacked* onto each other involving a greater number of assembled tetrapeptides to traverse the capsule shell.

Conclusions

The principal directive of this study was to identify a series of low molecular weight peptides which could be utilized to investigate the phenomenon of macromolecular self-assembly. The aspartate-based diketopiperazines in fact qualify as such peptides. Phenylalanine analogues **4e–g** dissolved in water formed microcapsules when the pH was lowered from 7.7 to 2.4. The choice of systems weighed heavily on earlier observations that solutions derived from amino acid thermal melts were shown to form microspheres and the fact that diketopiperazines were significant components of these complicated mixtures. Because the solution conformation of such small molecules can be assigned with some confidence, the stage is now set for an exploration of why some of the tetrapeptides self-assemble while others do not. We have initiated an NMR study of the respective solution conformations of **4d,e** and **4h**.

Experimental Section

General Procedure. All reagents were purchased from Sigma Chemical Co and were used without further purification. Silica gel (40 mm) obtained from J. T. Baker was used for flash column chromatography. NMR spectra were recorded on a Varian EM-390, VXR-300, or QE-300 instrument and were run with chemical shifts given in parts per million downfield from an internal tetramethylsilane or sodium 3-(trimethylsilyl)-propionate standard. Mass spectra were carried out on a Kratos MS 80RFA or a Finnigan 4516 MS instrument. All optical rotations were run at 589 nm (the Na D-line) at 22 °C on a Perkin-Elmer 241 polarimeter, with c expressed as grams of compound per 100 mL. Elemental analyses were performed by Atlantic Microlabs, Norcross, GA. Melting points were uncorrected. Light microscopy was performed on a camera-mounted ZEISS light microscope. SEM pictures were obtained on a Hitachi 4000 scanning electron microscope.

Diketopiperazine of L-Glutamic Acid Dibenzyl Ester (1). $N\alpha$-BOC-γ-benzyl-L-glutamic acid (6.0 g, 17.8 mmol) and N-hydroxysuccinimide (2.25 g, 19.6 mmol) were dissolved in anhydrous THF (150 mL). The solution was cooled to 0 °C, and dicyclohexylcarbodiimide (DCC, 4.04 g, 19.6 mmol) dissolved in 40 mL of dry THF was added dropwise over 30 min. The ice bath was removed and the solution allowed to warm to room temperature and stirred overnight. The reaction was monitored by TLC (20% EtOH/$CHCl_3$). The solution was filtered and the filtrate concentrated. Dry EtOAc was added, swirled, and filtered and the filtrate concentrated to give the crude N-hydroxysuccinimide (NHS) ester of $N\alpha$-BOC-γ-benzyl-L-glutamic acid as a viscous semisolid (8.7 g). Trifluoroacetic acid (TFA, 1.3 mL) was added dropwise to a portion of this NHS ester (0.50 g, 1.02 mmol) at 0 °C. The reaction was warmed to room temperature and stirred for 30 min. The volatiles were removed under reduced pressure, and the crude TFA salt was dissolved in 3 mL of dry DMF. This DMF solution was added dropwise to pyridine (120 mL) at 0 °C. The solution was allowed to slowly warm to room temperature and stirred overnight. The volatiles were removed under reduced pressure, and the crude yellow solid (0.85 g) was recrystallized from EtOAc to give pure **1** (0.11 g, 50%). mp = 275–277 °C. ^{1}H NMR (DMSO-d_6): δ 8.26 (s, 2H, NH), 7.46 (s, 10H, aromatic), 5.16 (s, 4H, CH_2), 3.98 (t, 2H, CH), 2.58 (m, 4H, CH_2), 2.06 (m, 4H, CH_2). Anal. Calcd for $C_{24}H_{26}N_2O_6$: C, 65.74, H, 5.98, N, 6.39. Found: C, 65.73, H, 6.03, N, 6.35. Mass spectrum: theory 438.18, found 439 (M + 1). Optical rotation: $[\alpha]_D$ –23.4° (c = 1, dioxane).

Diketopiperazine of L-Glutamic Acid (1b). The dibenzyl ester **1** (0.90 g, 2.05 mmol, 4.1 mequiv) was dissolved in a mixture of EtOAc/MeOH (6:1, 470 mL) and 10% Pd–C (0.20 g) added. The black suspension was degassed three times and hydrogen gas introduced. The reaction was monitored by TLC (30% EtOH/$CHCl_3$). The catalyst was filtered off and washed five times with boiling MeOH and EtOAc (to dissolve some of the diacid product which had precipitated). The filtrate was concentrated to give **1b** as a white solid (0.53 g, 100%). mp = 234–236 °C. ^{1}H NMR (DMF-d_7): δ 4.00 (t, 2H, CH), 2.49 (m, 4H, CH_2), 2.10 (m, 4H, CH_2). Anal. Calcd for $C_{10}H_{14}N_2O_6$: C, 46.51; H, 5.46; N, 10.85. Found: C, 46.72; H, 5.50; N, 10.82. High-resolution mass spectrum: theory 259.0930 (M + H), found 259.033 (M + H). Optical rotation: $[\alpha]_D$ –52° (c = 1, DMSO).

Diketopiperazine of L-Aspartic Acid Dibenzyl Ester (2). Compound **2** was prepared using the same procedure as described for **1**, except using β-benzyl-$N\alpha$-BOC-L-aspartic acid (24.0 g, 74.2 mmol), NHS (9.40 g, 81.7 mmol), and DCC (16.85 g, 81.7 mmol) in dry THF to give 37.13 g of the crude NHS ester. This NHS ester (37.13 g) was reacted with TFA (85 mL) at 0 °C to give the crude TFA salt. The salt was neutralized in dry DMF (100 mL) and pyridine (3.5 L) at 0 °C and recrystallized from EtOAc after workup to give **2** as a white solid (7.13 g, 47%). mp = 157 °C. ^{1}H NMR ($CDCl_3$): δ 7.31 (s, 10H, aromatic), 6.72 (s, 2H, NH), 5.12 (s, 4H, CH_2), 4.35 (m, 2H, CH), 3.00 (m, 4H, CH_2). Anal. Calcd for $C_{22}H_{22}N_2O_6$: C, 64.38; H, 5.40; N, 6.83. Found: C, 64.27; H, 5.39; N, 6.79. High-resolution mass spectrum: theory 410.1478, found 410.1503. Optical rotation: $[\alpha]_D$ –69.5° (c = 1, $CHCl_3$).

Diketopiperazine of L-Aspartic Acid (2b). The dibenzyl ester **2** (6.15 g, 15 mmol, 30 mequiv) was dissolved in MeOH (250 mL) and Pd–C (0.90 g) added. The black suspension was degassed three times and hydrogen gas introduced. The reaction was monitored by TLC (30% EtOH/$CHCl_3$). The catalyst was filtered off and washed five times with boiling MeOH (to dissolve some of the diacid product which had precipitated). The filtrate was concentrated to give a white solid which was rinsed with MeOH and dried to give **2b** as a white solid (2.78 g, 80%). mp = 254–255 °C. ^{1}H NMR ($CDCl_3$–DMSO-d_6, 1:1 by volume): δ 7.80 (s, 2H, NH), 4.20 (t, 2H, CH), 2.82 (d, 4H, CH_2). Anal. Calcd for $C_8H_{10}N_2O_6$: C, 41.75; H, 4.38; N, 12.17. Found: C, 41.72; H, 4.39; N, 12.09. Optical rotation: $[\alpha]_D$ –37° (c = 1, DMSO).

Bn-Gly-(diketo-L-Asp)-Gly-Bn (3a). Compound **3a** was prepared from glycine benzyl ester p-toluenesulfonate salt using the same procedure as

described below for **3b**. Recrystallization from $CHCl_3/CH_3OH$ (1:1) gave pure **3a** as tiny white crystals (73%). mp = 228–230 °C. ^1H NMR (DMSO-d_6): δ 8.50 (m, 2H), 7.80 (s, 2H), 7.38 (m, 10H), 5.18 (s, 4H), 4.22 (m, 2H), 3.96 (m, 4H), 2.72 (m, 4H). ^{13}C NMR (DMSO-d_6): δ 170.1, 169.8, 167.1, 135.8, 128.4, 128.0, 127.9, 65.9, 51.1, 40.7, 37.2. Anal. Calcd for $C_{26}H_{28}N_4O_8$: C, 59.53; H, 5.38; N, 10.68. Found: C, 59.43; H, 5.37; N, 10.69. Optical rotation: $[\alpha]_D$ –34° (*c* = 1, DMSO).

Bn-L-Ala-(diketo-L-Asp)-L-Ala-Bn (3b). The *cis*-diketopiperazine of L-aspartic acid (**2b**) (0.69 g, 3 mmol) and L-alanine benzyl ester hydrochloride (1.36 g, 6.3 mmol) were dissolved in 10 mL of dry DMF, and diphenylphosphoryl azide (DPPA, 1.73 g, 6.3 mmol) was added dropwise at 0 °C over 5 min. After the mixture was stirred for 10 min, triethylamine (TEA, 1.38 g, 12.6 mmol) was added over 5 min. The reaction mixture was stirred at 0 °C for a further 30 min and allowed to warm to room temperature overnight under a nitrogen atmosphere. Removal of the volatiles under reduced pressure gave an oily residue which was dissolved in 30 mL of methylene chloride and washed once with 20 mL of H_2O, 1 N HCl, saturated $NaHCO_3$, and H_2O. The organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated to give a pale yellow syrup which was recrystallized from CH_3OH to provide **3b** as a white powder (0.9 g, 72%). mp = 218–219 °C. ^1H NMR (DMSO-d_6): δ 8.50 (d, *J* = 6.9 Hz, 2H), 7.81 (s, 2H), 7.36 (m, 10H), 5.12 (s, 4H), 4.42–4.35 (m, 2H), 4.19 (m, 2H), 2.63 (m, 4H), 1.30 (d, *J* = 7.3 Hz, 6H). ^{13}C NMR (DMSO-d_6): δ 172.4, 169.5, 167.0, 136.0, 128.4, 127.7, 65.8, 51.1, 47.7, 37.1, 16.9. Anal. Calcd for $C_{28}H_{32}N_4O_8$: C, 60.86; H, 5.84; N, 10.14. Found: C, 60.76; H, 5.87; N, 10.08. Optical rotation: $[\alpha]_D$ –67° (*c* = 1, DMSO).

Bn-L-Val-(diketo-L-Asp)-L-Val-Bn (3c). L-Valine benzyl ester *p*-toluenesulfonate salt (0.80 g, 2.1 mmol) was condensed with **2b** (0.23 g, 1 mmol) using the same procedure as described for **3b**. The crude product was flash chromatographed on silica gel (EtOAc, R_f = 0.2) to furnish pure **3c** (0.42 g, 69%). mp = 161–162 °C. ^1H NMR ($CDCl_3$): δ 7.42 (s, 2H), 7.33 (s, 10H), 7.10 (d, *J* = 8.7, 2H), 5.21 (d, 2H), 5.10 (d, 2H), 4.61 (m, 2H), 4.30 (m, 2H), 3.44 (m, 4H), 2.14 (m, 2H), 0.85 (dd, 12H). ^{13}C NMR ($CDCl_3$): δ 171.9, 170.1, 167.0, 135.1, 128.5, 128.4, 128.2, 67.1, 57.0, 51.7, 38.0, 31.1, 18.9, 17.5. Anal. Calcd for $C_{32}H_{40}N_4O_8$: C, 63.14; H, 6.62; N, 9.20. Found: C, 63.13; H, 6.65; N, 9.11. Optical rotation: $[\alpha]_D$ –75° (*c* = 1, MeOH).

Bn-L-Tyr-(diketo-L-Asp)-L-Tyr-Bn (3d). Compound **3d** was prepared from reaction of **2b** (0.46 g, 2 mmol) and L-tyrosine benzyl ester *p*-toluenesulfonate salt (1.86 g, 4.2 mmol) using the same procedure as described for **3b**. The crude product was purified by flash chromatograhy on silica gel with $CHCl_3/CH_3OH$ (10:1, R_f = 0.4) to give **3d** (0.94 g, 65%). mp 106–108 °C. ^1H NMR (DMSO-d_6): δ 9.29 (s, 2H), 8.51 (d, *J* = 7.3 Hz, 2H), 7.77 (s, 2H), 7.34 (m, 10H), 6.97 (d, *J* = 8.4 Hz, 4H), 6.66 (d, *J* = 8.4 Hz, 4H), 5.04 (s, 4H), 4.44 (m, 2H), 4.17 (m, 2H), 2.78 (m, 8H). ^{13}C NMR (DMSO-d_6): δ 171.5, 169.8, 167.2, 156.1, 135.7, 130.1, 128.4, 128.0, 127.8, 126.8, 115.1, 65.9, 54.3, 51.1, 37.1, 36.2. Anal. Calcd for $C_{40}H_{40}N_4O_{10}$: C, 65.21; H, 5.47; N, 7.60. Found: C, 64.82; H, 5.41; N, 7.49. Optical rotation: $[\alpha]_D$ –41° (*c* = 1, MeOH).

Bn-L-Phe-(diketo-L-Asp)-L-Phe-Bn (3e). Compound **3e** was prepared from the condensation of L-Phe benzyl ester *p*-toluenesulfonate salt (39.95 g, 93.4 mmol) and **2b** (10.0 g, 43.5 mmol) using the same procedure as described for **3b**. The crude product was precipitated from EtOAc, and flash chromatography (10% EtOH/$CHCl_3$) gave pure **3e** (23.2 g, 75%). mp = 148–149 °C. TLC (10% EtOH/$CHCl_3$): R_f = 0.45. ^1H NMR ($CDCl_3$): δ 7.30 (m, 18H), 7.01 (m, 4H), 6.87 (d, 2H), 5.12 (dd, 4H), 4.90 (dd, 2H), 4.21 (d, 2H), 3.00 (m, 6H), 2.62 (dd, 2H). Anal. Calcd for $C_{40}H_{40}N_4O_8$: C, 68.17; H, 5.72; N, 7.95. Found: C, 68.00; H, 5.70; N, 7.87. Optical rotation: $[\alpha]_D$ –36° (*c* = 0.25, $CHCl_3$).

Bn-D-Phe-(diketo-L-Asp)-D-Phe-Bn (3f). *N-t*-Boc-D-Phe benzyl ester (14.2 g, 40 mmol) was dissolved in 50 mL of trifluoroacetic acid at 0 °C and stirred for 30 min. Evaporation of the resulting mixture gave a yellow oil which was dissolved in 30 mL of diethyl ether. The amine salt was precipitated, filtered, and dried (13.67 g, 93%). mp = 110–111 °C. The above trifluoroacetate salt (13.2 g, 34 mmol) was condensed with **2b** (3.45 g, 15 mmol) by the method described for **3b**. Removal of the volatiles under reduced pressure gave a yellow oil which was precipitated from MeOH (30 mL) and chromatographed on silica gel (10% EtOH/$CHCl_3$, R_f = 0.4) to afford **3f** (8.4 g, 80%). mp = 172–173 °C. ^1H NMR ($CDCl_3$): δ 7.20 (m, 20H), 6.70 (d, *J* = 8.1 Hz, 2H), 5.20 (d, 2H), 5.12 (d, 2H), 4.90 (m, 2H), 4.30 (m, 2H), 3.07 (m, 4H), 2.77 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 171.6, 169.3, 166.5, 135.5, 134.8, 129.1, 128.6, 127.1, 67.5, 53.2, 51.7, 38.6, 37.8. Anal. Calcd for $C_{40}H_{40}N_4O_8$: C, 68.17; H, 5.72; N, 7.95; Found: C, 67.83; H, 5.79; N, 7.80. Optical rotation: $[\alpha]_D$ –63° (*c* = 0.94, 6% MeOH/$CHCl_3$).

Bn-D,L-Phe-(diketo-L-Asp)-D,L-Phe-Bn (3g). D,L-Phenylalanine benzyl ester hydrochloride salt (0.584 g, 2.1 mmol) was condensed with **2b** (0.23 g, 1 mmol) by the method described for **3b**. After workup, removal of the volatiles under reduced pressure gave a yellow oil which was chromatographed on silica gel (10% EtOH/$CHCl_3$, R_f = 0.4) to afford **3g** (0.4 g, 57%). mp = 152–154 °C. ^1H NMR (DMSO-d_6): δ 8.56 (d, *J* = 7.9 Hz, 2H), 7.77 (d, *J* = 9.4 Hz, 2H), 7.24 (m, 20H), 5.05 (m, 4H), 4.58 (m, 2H), 4.19 (m, 2H), 3.00 (m, 8H). ^{13}C NMR ($CDCl_3$): δ 171.3, 169.7, 167.0, 136.8, 135.6, 129.1, 128.3, 128.0, 127.9, 127.8, 126.6, 65.9, 53.7, 51.0, 38.6, 37.0. Anal. Calcd for $C_{40}H_{40}N_4O_8$: C, 68.17; H, 5.72; N, 7.95; Found: C, 68.17; H, 5.71; N, 7.95. Optical rotation: $[\alpha]_D$ –66° (*c* = 0.33, DMSO).

Bn-L-Phe-(diketo-L-Glu)-L-Phe-Bn (3h). Compound **3h** was prepared from the condensation of L-Phe benzyl ester *p*-toluenesulfonate salt (1.90 g, 4.46 mmol) and the diketopiperazine of L-Glu (**1b**) (0.50 g, 1.94 mmol) using the same procedure as described for **3b**. The crude product was recrystallized from EtOAc to give pure **3h** (1.10 g, 77%). mp = 118–119 °C. ^1H NMR (DMSO-d_6): δ 8.40 (d, 2H), 8.10 (s, 2H), 7.30 (m, 20H), 5.08 (m, 4H), 4.50 (m, 2H), 3.70 (t, 2H), 2.95 (m, 4H), 2.18 (m, 4H), 1.79 (m, 4H). High-resolution mass spectrum ($C_{42}H_{44}N_4O_8$): theory 733.3237 (M + H), found 733.3246 (M + H). Optical rotation: $[\alpha]_D$ –25° (*c* = 0.5, DMSO).

Bn-L-Phe-L-Phe-(diketo-L-Asp)-L-Phe-L-Phe-Bn (3i). *N-t*-Boc-L-Phe-L-Phe benzyl ester (1.20 g, 2.4 mmol) was dissolved in 10 mL of trifluoroacetic acid at 0 °C and stirred for 30 min. Evaporation of the volatiles under reduced pressure gave a white solid (1.35 g), which was combined with the diketopiperazine of L-Asp **2b** (0.25 g, 1.1 mmol, 2.2 mequiv) and dissolved in dry DMF. The solution was cooled to 0 °C and DPPA (0.66 g, 2.4 mmol) added, followed by TEA (1.08 g, 10.6 mmol). The solution was warmed to room temperature and stirred overnight. Removal of the volatiles under reduced pressure to give a white solid which was boiled in EtOAc and filtered to give pure **3i** (0.95 g, 87%). mp = 223–224 °C. ^1H NMR (DMSO-d_6): δ 8.45 (d, 2H), 8.15 (d, 2H), 7.15 (m, 30H), 5.00 (s, 4H), 4.47 (m, 4H), 4.10 (t, 2H), 3.00 (m, 8H), 2.65 (m, 4H). Anal. Calcd for $C_{58}H_{58}N_6O_{10}$: C, 69.72; H, 5.85; N, 8.41; Found: C, 69.83; H, 5.90; N, 8.36. Optical rotation: $[\alpha]_D$ –35.5° (*c* = 0.51, DMSO).

Gly-(diketo-L-Asp)-Gly (4a). Bn-Gly-(diketo-L-Asp)-Gly-Bn (**3a**) (0.71 g, 1.35 mmol) was hydrogenated using the same procedure as described below for **4b**. The crude product was purified by column chromatography (Sephadex LH-20, 15% EtOH/toluene) and recrystallized from methanol to afford pure **4a** (0.41 g, 89%). mp = 240–242 °C. ^1H NMR (DMSO-d_6): δ 8.37 (m, 2H), 7.78 (s, 2H), 4.21 (m, 2H), 3.78 (m, 4H), 2.62 (m, 4H). ^{13}C NMR (DMSO-d_6): δ 171.4, 170.2, 167.1, 51.3, 40.7, 37.5. Anal. Calcd for $C_{12}H_{16}N_4O_8$: C, 41.86; H, 4.69; N, 16.27. Found: C, 42.18; H, 4.84; N, 15.93. Mass spectrum: theory 344, found (M + 1) 345. Optical rotation: $[\alpha]_D$ –44° (*c* = 1, DMSO).

L-Ala-(diketo-L-Asp)-L-Ala (4b). A black suspension of Bn-Ala-(diketo-L-Asp)-Ala-Bn (**3b**) (0.83 g, 1.35 mmol) and 10% Pd–C (0.08 g, 10%) in 20 mL of THF was degassed and flushed with nitrogen three times, the flask was evacuated, and hydrogen gas was introduced. The hydrogenolysis was monitored by TLC (10% MeOH/$CHCl_3$, R_f = 0.33). The catalyst was filtered off and washed with hot DMF. Concentration of the filtrate and recrystallization of the crude solid from CH_3OH gave pure **4b** (0.5 g, 90%). mp = 234–235 °C. ^1H NMR (DMSO-d_6): δ 12.55 (br, 2H), 8.31 (d, *J* = 7.3 Hz, 2H), 7.78 (s, 2H), 4.20 (m, 4H), 2.66 (m, 4H), 1.26 (d, *J* = 7.3 Hz, 6H). ^{13}C NMR (DMSO-d_6): δ 174.2, 169.4, 167.0, 51.2, 47.5, 37.4, 17.3. Anal. Calcd for $C_{14}H_{20}N_4O_8$: C, 45.16; H, 5.41; N, 15.15. Found: C, 45.09; H, 5.45; N, 14.95. Optical rotation: $[\alpha]_D$ –65° (*c* = 1, DMSO).

L-Val-(diketo-L-Asp)-L-Val (4c). Compound **4c** was prepared by hydrogenation of its dibenzyl ester **3c** (0.4 g, 0.66 mmol) using the same procedure as described for **4b**, except that degassed MeOH was used as the solvent and during the workup. The crude solid was purified by column chromatography (Sephadex LH-20, 15% EtOH/toluene) to give pure **4c** as a white solid (0.25 g, 89%). mp = 217–218 °C. ^1H NMR (CD_3OD): δ 4.33 (m, 4H), 4.17 (s, 2H), 2.94 (m, 4H), 2.17 (m, 2H), 0.97 (dd, 12H). ^{13}C NMR (CD_3OD): δ 174.9, 172.2, 169.3, 59.1, 53.1, 39.2, 31.6, 19.6, 18.4; Anal. Calcd for $C_{18}H_{28}N_4O_8 \cdot 1H_2O$: C, 50.46; H, 6.59; N, 13.08. Found: C, 50.49; H, 6.65; N, 12.87. Optical rotation: $[\alpha]_D$ –54° (*c* = 1, MeOH).

L-Tyr-(diketo-L-Asp)-L-Tyr (4d). Compound **4d** was prepared by hydrogenation of its dibenzyl ester **3d** (0.65 g, 0.88 mmol) using the same procedure as described for **4c**. The reaction was monitored by TLC (11% EtOH/$CHCl_3$). Filtration of the catalyst and removal of the volatiles gave pure **4d** (0.48 g, 98%). mp = 154–156 °C. ^1H NMR (DMSO-d_6):

δ 9.30 (br s, 2H), 8.27 (d, J = 6.7 Hz, 2H), 7.72 (s, 2H), 7.01 (d, J = 7.1 Hz, 4H), 6.67 (d, J = 7.1 Hz, 4H), 4.35 (m, 2H), 4.17 (s, 2H), 2.70 (m, 8H). ^{13}C NMR (DMSO-d_6): δ 171.1, 169.7, 167.2, 156.0, 130.1, 127.5, 115.1, 54,1, 51.2, 37.4, 36.2. Anal. Calcd for $C_{26}H_{28}N_4O_{10}\cdot 1H_2O$: C, 54.35; H, 5.26; N, 9.75. Found: C, 54.42; H, 5.20; N, 9.63. Optical rotation: $[\alpha]_D$ −3.4° (c = 1, MeOH).

L-Phe-(diketo-L-Asp)-L-Phe (4e). Compound **4e** was prepared by hydrogenation of its dibenzyl ester **3e** (23.2 g, 32.9 mmol) using the same procedure as described for **4c**. The reaction was monitored by TLC (10% EtOH/$CHCl_3$). After workup, the crude solid was recrystallized in EtOAc to give pure **4e** as a white solid (15.83 g, 92%). mp 156–158 °C. ^{1}H NMR (CD_3OD): δ 7.25 (m, 10H), 4.65 (dt, 2H), 4.28 (t, 2H), 3.19 (dd, 2H), 2.98 (dd, 2H), 2.81 (m, 4H). ^{13}C NMR (CD_3OD) δ 174.7, 171.8, 169.1, 138.3, 130.3, 129.5, 127.8, 55.19, 53.0, 39.3, 38.4. Anal. Calcd for $C_{26}H_{28}N_4O_8$: C, 59.54; H, 5.38; N, 10.68. Found: C, 59.60; H, 5.49; N, 10.53. High-resolution mass spectrum: theory 525.1985 (M + H), found 525.2190 (M + H). Optical rotation: $[\alpha]_D$ −13° (c = 0.75, MeOH).

D-Phe-(diketo-L-Asp)-D-Phe (4f). Compound **4f** was prepared by hydrogenation of its dibenzyl ester **3f** (5.5 g, 7.8 mmol) using the same procedure as described for **4c**. The reaction was monitored by TLC (10% EtOH/$CHCl_3$). After workup, the crude solid was purified by column chromatography (Sephadex LH-20, 15% EtOH/toluene) to give pure **4f** as a white solid (4.0 g, 98%). mp = 195–197 °C. ^{1}H NMR (CD_3OD): δ 7.25 (m, 10H), 4.65 (dt, 2H), 4.21 (t, 2H), 3.21 (dd, 2H), 2.96 (dd, 2H), 2.75 (m, 4H). ^{13}C NMR (DMSO-d_6): δ 172.9, 169.7, 167.1, 137.4, 129.1, 128.2, 126.4, 53.6, 51.2, 37.2, 36.7. Anal. Calcd for $C_{26}H_{28}N_4O_8$: C, 59.54; H, 5.38; N, 10.68. Found: C, 59.45; H, 5.43; N, 10.58. High-resolution mass spectrum: theory 525.1985 (M + H), found 525.1972 (M + H). Optical rotation: $[\alpha]_D$ −48° (c = 1, DMSO).

D,L-Phe-(diketo-L-Asp)-D,L-Phe (4g). Compound **4g** was prepared by hydrogenation of its dibenzyl ester **3g** (0.23 g, 0.33 mmol) using the same procedure as described for **4c**. The reaction was monitored by TLC (10% EtOH/$CHCl_3$). After all the starting material had been consumed, the catalyst was filtered off and the filtrate concentrated to give pure **4g** as a white solid (0.16 g, 94%). mp = 138–140 °C. ^{1}H NMR (DMSO-d_6): δ 8.32 (s, 2H), 7.64 (s, 2H), 7.25 (m, 10H), 4.41 (m, 2H), 4.10 (m, 2H), 2.98 (m, 4H), 2.58 (m, 4H). ^{13}C NMR (DMSO-d_6): δ 173.0, 169.6, 167.1, 137.5, 129.2, 128.2, 126.5, 53.7, 51.1, 38.6, 37.4, 36.8. Anal. Calcd for $C_{26}H_{28}N_4O_8$: C, 59.54; H, 5.38; N, 10.68. Found: C, 59.40; H, 5.40; N, 10.62. Optical rotation: $[\alpha]_D$ −40° (c = 1, DMSO).

L-Phe-(diketo-L-Glu)-L-Phe (4h). Compound **4h** was prepared by hydrogenation of its dibenzyl ester **3h** (1.00 g, 1.36 mmol) using the same procedure as described for **4c** except in degassed EtOH. The reaction was monitored by TLC (10% EtOH/$CHCl_3$). After workup, the crude solid (0.75 g) was recrystallized in 30% EtOH/EtOAc to give pure **4h** as a white solid (0.31 g, 41%). mp = 135 °C (sublimes). ^{1}H NMR (10% CD_3OD/DMSO-d_6): δ 7.20 (m, 10H), 4.40 (dd, 2H), 3.68 (t, 2H), 3.05 (dd, 2H), 2.80 (dd, 2H), 2.17 (m, 4H), 1.79 (m, 4H). High-resolution mass spectrum ($C_{28}H_{32}N_4O_8$): theory 553.2298 (M + H), found 553.2219 (M + H). Optical rotation: $[\alpha]_D$ −35° (c = 0.51, DMSO).

L-Phe-L-Phe-(diketo-L-Asp)-L-Phe-L-Phe (4i). Compound **4i** was prepared by hydrogenation of its dibenzyl ester **3i** (0.92 g, 0.92 mmol) using the same procedure as described for **4b** in THF. After workup, the crude solid (0.9 g) was precipitated from boiling EtOAc to give pure **4i** (0.61 g, 81%). mp = 243–247 °C. ^{1}H NMR (DMSO-d_6): δ 8.25 (t, 4H), 7.20 (m, 20H), 4.47 (m, 2H), 4.37 (q, 2H), 4.15 (t, 2H), 3.00 (m, 6H), 2.67 (m, 4H), 2.31 (dd, 2H). Anal. Calcd for $C_{44}H_{46}N_6O_{10}$: C, 64.54; H, 5.66; N, 10.26. Found: C, 64.31; H, 5.75; N, 10.20. Optical rotation: $[\alpha]_D$ −41° (c = 1, DMSO).

Preparation of Microcapsules. The *bis* acid (0.1 mmol) is dissolved in 1.0 mL of aqueous Li_2CO_3 (0.1 M) to give a clear solution of the lithium salt in deionized water. A 50-μL sample of this 0.1 M solution is mixed with 50 μL of 0.86 M citric acid and shaken. An opaque white suspension was generated. Microscopic examination of the suspension revealed the presence of large numbers of tiny spheres which moved randomly throughout the field of inspection. A wide size distribution was observed qualitatively (ranging from 10 μm to submicron diameters).

Scanning Electron Microscopy (SEM). A typical procedure involved the generation of a white suspension by combining 50 μL of 0.86 M citric acid and 50 μL of a 0.1 M aqueous solution of the lithium salt of the peptide. The aqueous suspension was deposited on polylysine-coated glass coverslips and fixed with 2% OsO_4 for 4 h. The sample was washed with distilled water, air-dried, and sputter coated with gold. SEM photographs were then obtained.

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