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Microwave-Assisted Cyclization of Unprotected Dipeptides in Water to 2,5-Piperazinediones and Self-Assembly Study of Products and Reagents

Marina Kurbasic[a]
Sabrina Semeraro[a]
Ana M. Garcia[a]
Slavko Kralj[a,b]
Evelina Parisi[a]
Caterina Deganutti[a]
Rita De Zorzi[a]
Silvia Marchesan*[a]

[a] Chemical & Pharmaceutical Sciences Dept., University of Trieste, Via L. Giorgieri 1, 34127 Trieste, Italy
[b] Materials Synthesis Department, Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia
smarchesan@units.it

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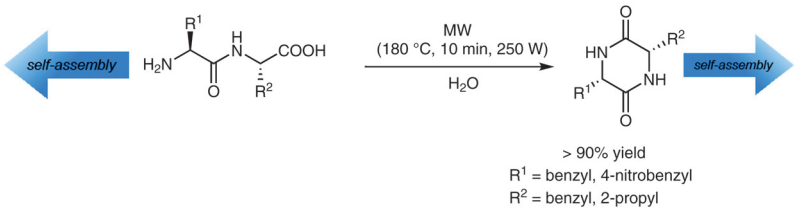

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Abstract Dipeptides and their cyclized 2,5-piperazinedione (or diketopiperazine, DKP) derivatives are attractive building blocks for supramolecular hydrogels. The Phe-Phe, (*p*-nitro)-Phe-Phe, and Phe-Val dipeptides and their corresponding DKPs are studied for self-assembly in water. The DKPs were obtained in high yields by microwave-assisted cyclization of the dipeptides in water, demonstrating that use of their methyl ester derivatives as reported in the literature is not necessary for successful cyclization. Single-crystal XRD structures are reported for two DKPs as well as stable hydrogels at neutral pH.

Key words dipeptide, diketopiperazine, self-assembly, hydrogels, amyloid, microwaves, 2,5-piperazinedione

Since the early report on *Science* in 2003 by Reches and Gazit,[1] diphenylalanine (Phe-Phe) has become a very popular self-assembling compound on its own,[2] in mixtures,[3] or as a motif in longer peptides and their derivatives.[4] A number of approaches have been successfully used to modulate the supramolecular behavior of related compounds, including use of D-amino acids in D,L-heterochiral sequences,[5] N-terminus protection with fluorenylmethyloxycarbonyl (Fmoc) protecting group[6] or other aromatic moieties,[7] as well as phenylalanine substitution with electron-donating or electron-withdrawing groups to stabilize dipolar interactions between neighboring aromatic rings.[8] In particular, *p*-nitro substitution of the Fmoc-Phe benzene ring proved to be the most effective amongst the substitutions tested, and resulted in the most rapid self-assembly.[8] Phe-Phe was also reported to self-assemble into metastable hydrogels, although it required use of an organic solvent to assist with its initial dissolution at the concentration required to gel an aqueous buffer.[9] In light of these findings, we reasoned that the compound (*p*-nitro)-Phe-Phe could be an interesting candidate to achieve a stable hydrogel without the use of organic solvents. Indeed, short peptides composed of 2–4 amino acids are highly attractive as hydrogelators thanks to their inherent biocompatibility, low-cost and ease of preparation also on a large scale, and benign fate in the environment. The applications are numerous and diverse, and range from the delivery of agents for plant growth stimulation,[10] imaging,[11] and drugs,[12] to supramolecular catalysts,[13] and organocatalysts,[14] to cite a few recent examples. However, Phe-Phe is notorious for its poor water solubility and it typically requires organic solvents for its dissolution, thus limiting the scope of this compound.

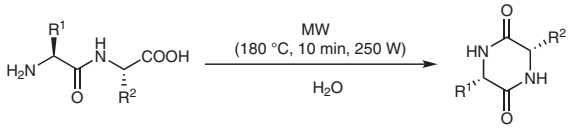
Dipeptides have been reported to cyclize to 2,5-piperazinediones (or diketopiperazines, DKPs), at a range of pH values, and upon heating.[15] DKPs are found in numerous natural products, as well as in processed food and beverages, and occur also as secondary metabolites in microorganisms and fungi. DKPs are very interesting for medicinal chemistry, thanks to their ability to mimic a peptidic turn that is a common feature of many bioactive compounds, and indeed they have been reported to have anticancer, antibiotic, antiviral properties, to cite a few.[15–17] They are also attractive as chiral catalysts in organic chemistry.[15] Recently, they have raised interest even in the area of the origins of life, since they have been reported to form spontaneously from proline-containing dipeptides under alkaline conditions and in simulated prebiotic conditions.[18] In the area of supramolecular chemistry, DKPs have been long known for their ability to pack into high-order structures, both as

crystals and in the (organo)gel phase.[19] From a synthetic point of view, the most recent dipeptide cyclization strategies are microwave-assisted and have been reported in water starting from dipeptides protected at the C-terminus as ester derivatives, and either N-protected[20] or N-deprotected.[21] Use of protected dipeptides as reagents is sensible in light of the fact that their synthesis requires use of protecting groups. Key advantages relative to the more traditional thermal heating are 1) short reaction times, 2) absence of epimerization, and 3) generally high yields, although amino acids with steric demand near the peptide backbone, such as Phe and Val, have been reported to be less reactive and result in lower yields.[20,21] In light of the prominent role of Phe in self-assembling peptide sequences and their derivatives, similar water-based and facile protocols with quantitative yields also for Phe-bearing dipeptides would be highly attractive. An additional advantageous feature would be the possibility to directly use unprotected dipeptides as reagents, since they are widely available as products from enzymatic reactions, or in extracts from natural sources.

This work reports the microwave-assisted synthesis in water of DKPs from unprotected dipeptides, namely (*p*-nitro)-Phe-Phe, Phe-Phe, and Phe-Val, all in excellent yields with a reaction time as short as 10 minutes. Products were characterized by ^{1}H NMR, ^{13}C NMR, ESI-MS, HRMS, melting point, FT-IR, circular dichroism, polarimetry, and single-crystal XRD [see sections 1, 5, and 7 of Supporting Information (SI)]. In addition, we assessed the supramolecular behavior of the DKPs and parent dipeptides, and report on self-assembling systems in phosphate buffer at neutral pH, characterized by circular dichroism, Thioflavin T fluorescence as amyloid stain, transmission electron microscopy (TEM), and rheometry (see sections 2–6 of SI).

(*p*-Nitro)-Phe-Phe was synthesized by Fmoc-based solid-phase peptide synthesis, and purified by reversed phase HPLC, while Phe-Phe and Phe-Val are commercially available. Each dipeptide was dissolved in water and underwent microwave-assisted cyclization as detailed in Table 1. Each compound was successfully converted into the corresponding 2,5-diketopiperazine in nearly quantitative yield within 10 minutes, as confirmed by ^{1}H NMR, ^{13}C NMR, ESI-MS, HRMS, FT-IR, circular dichroism, polarimetry, and single-crystal XRD. In particular, the latter showed that the presence of the nitro group did not affect the conformation of cyclo(Phe-Phe) (Figure 1). In both DKP1 and DKP2, the typical turn-mimetic structure was seen,[22] with DKP in planar conformation, as expected for benzyl derivatives, which feature the aromatic substituent folded on top.[15] The CH-π interaction reported for DKP2[23] between the two side chains maintained the same distance in DKP1 and DKP2, regardless of the nitro-substitution, thus revealing absence of electronic effects on molecular conformation due to the presence of the nitro group (Figure 1). In terms of molecular packing, both DKP1 and DKP2 featured the typical extended network of hydrogen bonds involving DKP rings (Figure 2).[24]

Table 1 Microwave-Assisted Dipeptide Cyclization in Water to DKP



Dipeptide	DKP	R^1	R^2	Yield (%)
(*p*-nitro)-Phe-Phe	**1**	4-nitrobenzyl	benzyl	quantitative
Phe-Phe	**2**	benzyl	benzyl	95
Phe-Val	**3**	benzyl	2-propyl	90

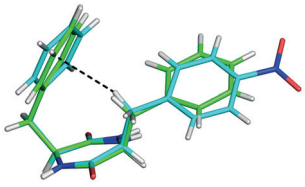


Figure 1 Single-crystal XRD structures of DKP1 (cyan) and DKP2 (green)

Having achieved a water-based synthesis of the desired DKPs, we sought to compare the self-assembly behavior among the three parent dipeptides and their corresponding DKPs. To avoid use of organic solvents, each dipeptide was first dissolved in a phosphate solution at pH 12, followed by addition of an equal amount of a phosphate buffer at pH 6 to achieve a final pH of ~7.3, following a standard procedure.[25] To our delight, both (*p*-nitro)-Phe-Phe and Phe-Phe could be dissolved with this approach without the need for organic solvents at the final concentration of 15 mM that was sufficient to obtain a self-supporting hydrogel (see Figure S25 of Section 2 in SI). Oscillatory rheology confirmed rapid gel formation for both compounds (Figure S35 in SI). TEM micrographs (see section 3 of SI) revealed elongated nanostructures compatible with nanotubes as previously reported for Phe-Phe (Figures S26 and S27 in SI).[1] On the contrary, oscillatory rheology (Figure S35 in SI) confirmed that Phe-Val remained soluble as seen by naked eye at concentrations as high as 100 mM (see Figure S25-C in SI). This is not too surprising, considering that Phe-Val is notably more hydrophilic than the other two dipeptides, as apparent from reverse-phase HPLC retention times, which are an experimental indication of hydrophobicity. They corresponded to 8.4 and 8.1 minutes for (*p*-nitro)-Phe-Phe and Phe-Phe, respectively, and to just 3.5 minutes for Phe-Val. A difference between the supramolecular behavior of (*p*-nitro)-Phe-Phe and Phe-Phe was that the latter displayed syneresis over time, in agreement with previous reports on metastable gels, which were formed upon dissolution of

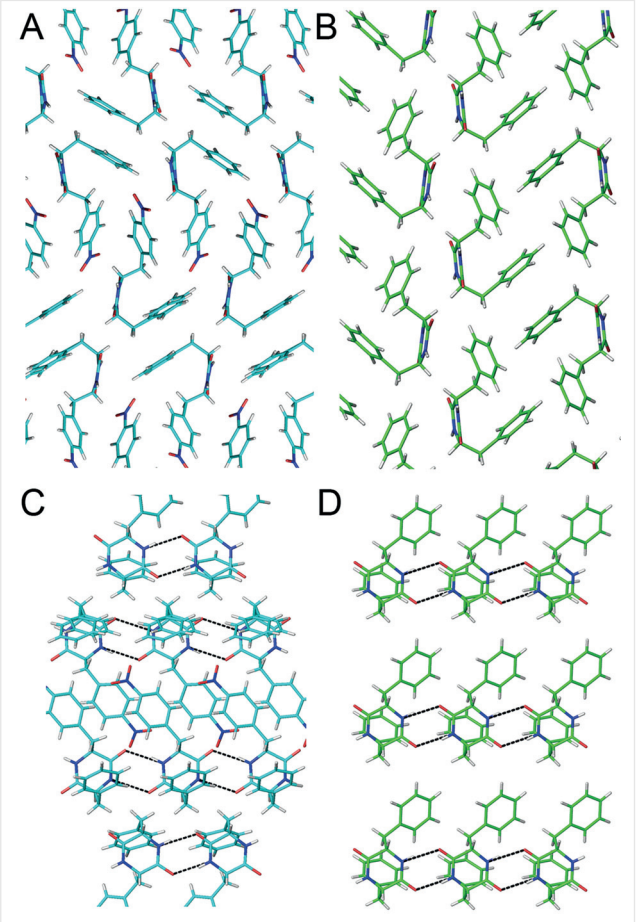


Figure 2 Molecular packing of DKP1 (A) and DKP2 (B), and hydrogen bonding network for DKP1 (C) and DKP2 (D)

Phe-Phe in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) and subsequent dilution with an aqueous buffer at pH 8 with the assistance of ultrasonication.[9] We were pleased to note that, instead, the unreported (*p*-nitro)-Phe-Phe hydrogel (see Figure S25-A in SI) was stable for 7 days, thus supporting the reported role of 4-nitro substitution in stabilizing the dipolar interactions between aromatic rings, hence the aromatic-interaction driven self-assembly in water.[6] Interestingly, we noted that after 7 days of self-assembly, 20% of (*p*-nitro)-Phe-Phe had spontaneously converted into DKP1, while Phe-Phe (and Phe-Val) had not, as revealed from ^{1}H NMR spectra of the freeze-dried hydrogels (Figures S42 and S43 in SI).

The corresponding DKPs were also thus assessed for their supramolecular behavior. Unfortunately these compounds are more hydrophobic than their parent dipeptides, hence they could only be dissolved at high concentration in 1,1,1,3,3,3-hexafluoroisopropanol (20% v/v of the final volume). Indeed their higher hydrophobicity relative to their parent dipeptides could be assessed experimentally by their reversed-phase HPLC retention times, which corresponded to 11.6, 11.2, and 10.1 minutes for DKP1-3, respectively (see Figures S10, S16, and S22 in SI). Dilution of the DKP solutions with phosphate buffer to a final pH of ~7.3 and a final concentration of 15 mM led to a self-supporting hydrogel only in the case of DKP1 (see Figure S25-D in SI), confirming once again a positive role for the 4-nitro substitution on Phe to promote self-assembly and hydrogelation. Self-assembly of DKP1 and DKP3 was unreported to date, while DKP2 was previously reported to self-assemble into fibers and organogels, and noted for its poor solubility in a number of solvents.[26] Oscillatory rheology revealed a gel nature for all DKPs 1–3, although gelation was rapid for DKP1–2, while a lag time of several minutes was observed for DKP3. TEM micrographs revealed the presence of elongated nanostructures compatible with fibrils in all cases, albeit DKP1 and DKP2 displayed evident bundling into heterogeneously-sized fibers (see Figures S28 and S29 in SI), while DKP3 formed homogenously-sized nanofibrils (see Figure S30 in SI). Thioflavin T fluorescence (see Figure S31 in SI), which is often used as a stain to detect amyloid structures,[27] indeed was significantly higher for DKP3 than for any other compound tested (i.e., DKP1–2 and the three parent dipeptides). These data agree with previous reports on increased fluorescence for the case of thin fibrils, as opposed to fibril bundles, due to an overall higher amyloid surface available for dye binding.[28]

In conclusion, we have reported the rapid and high-yield cyclization of unprotected dipeptides in water under microwave irradiation. This work confirmed a positive role played by *p*-nitro substitution on the benzene ring of phenylalanine for self-assembly both of the linear dipeptide Phe-Phe and for the corresponding DKP derivative, as previously reported for Fmoc-Phe by means of stabilization of dipole interactions.[8] Single-crystal XRD structures revealed absence of electronic effects due to the nitro group on DKP molecular conformation and intramolecular CH-π interaction. These data further support the hypothesis that the stabilizing role played by the nitro group in self-assembly is due to electronic effects in terms of intermolecular, as opposed to intramolecular, interactions.

This work also investigated the self-assembly behavior in water of the three dipeptides and their corresponding DKPs, revealing that the 5 more hydrophobic compounds of the 6 tested formed supramolecular structures of elongated nanomorphology in aqueous buffer at neutral pH. In particular, only in the case of (*p*-nitro)-Phe-Phe and its DKP derivative, the compounds self-assembled into stable and self-supporting hydrogels at neutral pH, confirming a positive role played by the nitro group on hydrogelation. In the case of Phe-Val, dipeptide cyclization favored self-assembly by increasing hydrophobicity and allowed formation of homogenously sized nanofibrils for cyclo(Phe-Val).

Indeed, all DKPs were notably more hydrophobic than their parent linear dipeptides. On one hand, the increased hydrophobicity was an issue since all DKPs displayed poor

solubility, and it was not possible to avoid use of organic solvents to allow dissolution of these compounds into aqueous systems. On the other hand, cyclization of more hydrophilic dipeptides could allow to obtain self-assembling motifs with defined conformation from non-assembling sequences. Future application of this approach to other dipeptides could thus provide the means for the facile preparation of DKPs in water, and for the introduction of a variety of hydrophilic functional groups in geometrically-defined self-assembling motifs for potential biological applications.

2-Chlorotrytil resin, *O*-benzotriazole-*N*,*N*,*N*,*N*′-tetramethyluronium hexafluorophosphate (HBTU), HOAt, and Fmoc-protected L-phenylalanine were purchased from GL Biochem (Shanghai) Ltd. Fmoc-protected (*p*-nitro)-L-phenylalanine (CAS 95753-55-2), Phe-Phe (CAS 2577-40-4), and Phe-Val (CAS 3918-90-9) dipeptides were purchased from Sigma-Aldrich. All solvents were purchased of analytical grade from Merck. Piperidine, trifluoroacetic acid (TFA), *N*,*N*-diisopropylethylamine (DIPEA), and triisopropylsilane (TIPS) were from Acros. NaH_2PO_4 and Na_2HPO_4 were from BDH AnalaR. High purity Milli-Q H_2O (MQ H_2O) with a resistivity greater than 18 M Ω cm was obtained from an in-line Millipore RiOs/Origin system. Microwave-assisted reactions were carried out in a Discover SP MW reactor from CEM Corporation (Matthews, NC, USA). ^{1}H NMR spectra were recorded at 400 MHz and ^{13}C NMR spectra were recorded at 101 MHz on a Varian Inova Instrument with chemical shifts reported as ppm (in DMSO with TMS as internal standard). LC-MS analysis data were acquired on a Agilent 6120 LC-MS system with a C-18 analytical column (Luna 5 µm, 100Å, 150 × 2 mm). Electrospray HRMS data were obtained on a Bruker microTOF-Q. FT-IR spectra were recorded on a PerkinElmer Paragon 1000 FT-IR spectrophotometer, with solid compounds compressed into KBr pellets. Melting points were measured on a Gallen Kamp apparatus. Polarimetric measurements were carried out on compound solutions on a Jasco P-2000 Polarimeter. Circular dichroism spectra were acquired in 0.1 mm quartz cells in a Jasco J815 Spectropolarimeter, with 1s integrations, 1 accumulation, and a step size of 1 nm with a bandwidth of 1 nm. The fluorescence emission was analyzed using a Tecan Infinite M1000 pro. Rheological analyses were performed on a Malvern Kinexus-Pro oscillatory rheometer with 20 mm-wide parallel plate geometry. Transmission electron microscopy analyses were performed on JEM 2100 (Jeol, Japan) operated at 100 kV.

Peptide Synthesis

(*p*-Nitro)-Phe-Phe was not commercially available, and it was prepared from the corresponding amino acids by Fmoc-based solid-phase peptide synthesis, under standard conditions.[25]

2,5-Piperazinediones; General Procedure

Each dipeptide [10.7, 9.4, 8.1 mg for (*p*-nitro)Phe-Phe, Phe-Phe, and Phe-Val, respectively) was introduced in a 10 mL MW glass vial with of milliQ H_2O (2 mL) to reach a final concentration of 15 mM. The mixtures were dispersed with the assistance of ultrasonication in a water-bath for 5 min at r.t. The vials were sealed and placed in the MW reactor for 10 min at 180 °C and 250 W. The precipitate was vacuum filtered, washed with H_2O, and dried in vacuo to afford a white powder (DKP1, 10.2 mg, quantitative yield; DKP2, 8.6 mg, 95% yield; DKP3, 6.6 mg, 90% yield).

LC-MS Analysis

Flow 0.3 mL/min. The gradient used consisted of MeCN/H_2O with 0.1% formic acid with the following program: t = 0–2 min, 25% MeCN; t = 17 min, 95% MeCN; t = 17–20 min, 95% MeCN.

Hydrogel Preparation

The peptides were dissolved in sodium phosphate buffer (0.1 M pH 11.8) at a concentration of 30 mM; subsequent addition of an equal volume of mildly acidic sodium phosphate buffer (0.1 M pH 5.8) resulted in a final concentration of 15 mM and a pH of 7.3 ± 0.2. DKPs were dissolved first in HFIP (20% v/v final volume) and then sodium phosphate buffer (0.1 M, pH 7.3) was added on top (80% v/v).

Circular Dichroism

To follow the self-assembly kinetics, the CD signal was monitored over a range of wavelengths from 185 to 280 nm at 25 °C (Peltier) every 5 min for 1 h. Samples were freshly prepared directly in the CD cell and the spectra immediately recorded. CD spectra were acquired for peptides at 15 mM (self-assembly conditions) and at 1 mM (conditions under minimum gelling concentration).

Thioflavin T Fluorescence

Gel precursor solutions (0.12 mL) were prepared as described above and immediately put on wells of Greiner 96 U Bottom Black P. After 4 h, 30 µL of Thioflavin T (22.2 µM in 20 mM glycine/NaOH pH 7.5, filtered with a 0.2 µm filter) solution was added in the wells. After 15 min, the fluorescence emission was analyzed, selecting an excitation wavelength of 446 nm and an emission wavelength of 490 nm, with a bandwidth of 20 nm. Each condition was repeated thrice in triplicate (n = 9). Average and standard deviations were calculated and plotted with Excel (see SI).

Oscillatory Rheometry

Each hydrogel was prepared in situ applying a gap of 1 mm. Time sweeps were recorded for 1 h at 1 Pa and 1 Hz, frequency sweeps were recorded at 1 Pa from 0.1 to 10 Hz and stress sweeps were recorded at 1 Hz from 1 Pa until the breaking point typical for every hydrogel, recognizable by the inversion of G′ and G″ values. Each analysis was repeated at least 2 times.

Transmission Electron Microscopy (TEM)

The small amount of supramolecular material was precisely deposited on a TEM grid (copper-grid-supported lacey carbon film), dried for 15 min at r.t., and contrasted by aq tungsten phosphate solution (pH 7.4). TEM micrographs were acquired on at least 25 different spots on TEM grid.

Peptide Crystallization

Crystals of DKP1 and DKP2 were grown using the sitting-drop vapor diffusion method. A small vial with a solution of (*p*-nitro)-Phe-Phe or DKP2 was introduced into a larger vial containing the reservoir solution. Thus, 800 µL of the dipeptide solution (1 mM in MeOH) or DKP2 (<1 mg/mL in MeCN) was deposited in a small vial and sealed with a reservoir containing either MeOH (3 mL, for DKP1) at 30% (v/v) in H_2O or MeCN (for DKP2) at 70% (v/v) in H_2O, to allow vapor diffusion until equilibration. Single crystals were grown for 3 weeks.

Single-Crystal X-ray Diffraction

Single crystals of DKP1and DKP2 were collected with a loop, cryoprotected by dipping the crystals in glycerol, and stored frozen in liquid

N_2. The crystals were mounted on the diffractometer at the synchrotron Elettra, Trieste (Italy), beamline XRD1, using the robot present at the facility. Temperature was kept at 100 K by a stream of N_2 on the crystals. Diffraction data were collected by the rotating crystal method using synchrotron radiation, wavelength 0.70 Å, rotation interval 1°/image, crystal-to-detector distance of 85 mm. A total of 360 images were collected for DKP1, and 360 for DKP2. Reflections were indexed and integrated using the XDS package,[29] space groups $P2_1$ for DKP1 and $P2_12_12$ for DKP2, were determined using POINTLESS[30] and the resulting data set were scaled using AIMLESS.[31] Phase information were obtained by direct methods using the software SHELXS.[32] Refinements cycles were conducted with SHELXL-14,[33] operating through the WinGX GUI,[34] by full-matrix least-squares methods on F2. Unit cell parameters and scaling statistics are reported in Table S1 (see SI). For DKP1, a phenyl group was refined in 2 statistical positions at partial occupancy, occupancy of each position was refined to give a total occupancy of 1. All the atoms except the H_2 atoms within the asymmetric unit have been refined with anisotropic thermal parameters. H_2 atoms were added at geometrically calculated positions and refined isotropically. Refinement statistics are reported in Table S1 (see SI).[35]

(4-NO_2)-Phe-Phe

White powder; yield: 55 mg (85%); mp 290 °C; $[\alpha]_D^{20}$ –25 (*c* 0.15, DMSO).

IR (KBr): 3306, 3091, 3038, 2935, 1714, 1669, 1599, 1557, 1523, 1437, 1397, 1352 cm^{-1}.

^1H NMR (400 MHz, CD_3OD): δ = 8.21–8.16 (m, 2 H, ArH), 7.60–7.45 (m, 2 H, ArH), 7.36–7.10 (m, 5 H, ArH), 4.71 (dd, *J* = 8.6, 5.3 Hz, 1 H, αCH), 4.15 (dd, *J* = 7.9, 5.8 Hz, 1 H, αCH), 3.35 (dd, *J* = 14.3, 5.8 Hz, 1 H, βCH), 3.27–3.14 (m, 2 H, βCH), 3.02 (dd, *J* = 14.1, 8.6 Hz, 1 H, βCH).

^{13}C NMR (101 MHz, CD_3OD): δ = 172.4, 167.7 (C=O), 147.6, 141.6 136.7, 130.5, 128.8, 128.1, 126.5, 123.5 (Ar), 53.9, 53.4 (αC), 39.2, 38,6 (βC).

MS ($C_{18}H_{19}N_3O_5$): *m*/*z* = 358.1 $[M + H]^+$; requires: 358.1.

HRMS: *m*/*z* $[M + H]^+$ calcd for $C_{18}H_{19}N_3O_5$: 358.1397 $[M + H]^+$; found: 358.1397.

(3*S*,6*S*)-6-Benzyl-3-(4-nitrobenzyl)-2,5-piperazinedione (DKP1)

White powder; yield: 10 mg (quant); mp 300 °C; $[\alpha]_D^{20}$ –20 (*c* 0.15, DMSO).

IR (KBr): 3306, 3095, 3038, 2935, 1713, 1669, 1599, 1510, 1438, 1397, 1392 cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6): δ = 8.15 (d, *J* = 1.0 Hz, 1 H, NH), 8.07–7.98 (m, 3 H, 1 H, NH, 2 H, ArH), 7.23–7.16 (m, 5 H, ArH), 7.14–7.04 (m, 2 H, ArH), 4.12 (dd, *J* = 5.8, 4.2 Hz, 1 H, αCH), 4.01 (dd, *J* = 5.8, 5.3 Hz, 1 H, αCH), 2.81 (m, *J* = 13.5, 4.3 Hz, 1 H, βCH), 2.69 (m, *J* = 13.4, 4.9 Hz, 1 H, βCH), 2.55–2.49 (m, 1 H, βCH), 2.20 (dd, *J* = 13.5, 6.4 Hz, 1 H, βCH).

^{13}C NMR (101 MHz, DMSO-d_6): δ = 166.7, 166.4 (C=O), 146.6, 145.5 136.58, 131.2, 130.6, 128.5, 126.8, 123.53 (Ar), 55.6, 55.2 (αC), 39.2, 38,6 (βC).

MS ($C_{18}H_{17}N_3O_4$): *m*/*z* = 358.1 $[M + H]^+$; requires: 357.1.

HRMS: *m*/*z* $[M + Na]^+$ calcd for $C_{18}H_{17}N_3O_4Na$: 362.1111 $[M + Na]^+$; found: 362.1111.

(3*S*,6*S*)-3,6-Dibenzyl-2,5-piperazinedione (DKP2)

White powder; yield: 9 mg (95%); mp 315 °C; $[\alpha]_D^{20}$ +52 (*c* 0.15, DMSO).

IR (KBr): 3318, 3205, 3162, 3088, 2968, 2927, 2876, 1675, 1662, 1497, 1460, 1339 cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6): δ = 7.90 (s, 2 H, NH), 7.28–7.16 (m, 6 H, ArH), 7.13–7.08 (m, 2 H, ArH), 3.96–3.93 (m, 2 H, αCH), 2.57–2.52 (m, 2 H, βCH), 2.23–2.18 (m, 2 H, βCH).

^{13}C NMR (101 MHz, DMSO-d_6): δ = 166.4 (C=O), 137.0, 130.1, 128.6, 126.9 (Ar), 55.8 (αC), overlapped with DMSO signal (βC).

MS ($C_{18}H_{18}N_2O_2$): *m*/*z* = 395.1 $[M + H]^+$; requires: 395.1.

HRMS: *m*/*z* $[M + Na]^+$ calcd for $C_{18}H_{18}N_2O_2Na$: 317.1260 $[M + Na]^+$; found: 362.1261.

(3*S*,6*S*)-3-Benzyl-6-(2-propyl)-2,5-piperazinedione (DKP3)

White powder; yield: 7 mg (90%); mp 270 °C; $[\alpha]_D^{20}$ –68 (*c* 0.15, DMSO).

IR (KBr): 3195, 3056, 2969, 2888, 1955, 1672 (br), 1613, 1456, 1346, 1315 cm^{-1}.

^1H NMR (400 MHz, DMSO-d_6): δ = 8.06 (s, 1 H, NH), 7.87 (s, 1 H, NH), 7.24–7.13 (m, 5 H, ArH), 4.18 (dd, *J* = 6.5, 4.9 Hz, 1 H, αCH), 3.50 (dd, *J* = 3.7, 1.9 Hz, 1 H, αCH), 3.13 (dd, *J* = 13.5, 5.0 Hz, 1 H, βCH), 2.85 (dd, *J* = 13.5, 5.0 Hz, 1 H, βCH), 1.67 (m, 1 H, βCH), 0.62 (d, *J* = 7.1 Hz, 3 H, γCH), 0.24 (d, *J* = 6.8 Hz, 3 H, γCH).

^{13}C NMR (101 MHz, DMSO-d_6): δ = 167.0, 166.8 (C=O), 136.8, 130.7, 128.5, 128.4, 126.9 (Ar), 59.6, 55.5 (αC), 38.2, 31.5 (βC), 18.7, 16.6 (γC).

MS ($C_{14}H_{18}N_2O_2$): *m*/*z* (%) = 247.1 $[M + H]^+$; requires: 247.1.

HRMS: *m*/*z* $[M + Na]^+$ calcd for $C_{14}H_{18}N_2O_2Na$: 269.1260 $[M + Na]^+$; found: 269.1260.

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Supporting Information

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(35) CCDC 1895316 (DKP1) and 1895262 (DKP2) contain the supplementary crystallographic data for this paper. The data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/getstructures.