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Conformational Studies of Peptides Containing *cis*-3-Hydroxy-D-proline

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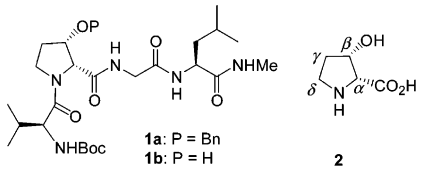
Abstract: Conformational analysis of peptides containing *cis*-3-hydroxy-D-proline (D-*cis*-3-Hyp) by NMR studies revealed that the 3-hydroxyl group in this amino acid plays a significant role in the overall three-dimensional structures of the peptides. When the D-*cis*-3-Hyp had its 3-hydroxyl group protected as the benzyl (Bn) ether, the peptide displayed a β-hairpin structure in both $CDCl_3$ and DMSO-d_6. Even after the removal of the Bn group, the resulting deprotected compound retained the same structure as in the protected version in $CDCl_3$. However, in polar solvent DMSO-d_6, the C-terminal strand of the hydroxyl-deprotected peptide flipped to the side of the hydroxyl group, breaking the hairpin to form a pseudo β-turn-like nine-membered ring structure involving an intramolecular hydrogen bond between LeuNH → HypC3-OH.

Proline plays a critical determinant role in protein folding and refolding, because it induces a reversal in backbone conformation resulting in the nucleation of turns as well as in the breaking of helices in proteins.[1] Whereas L-proline as the second residue ($i + 1$) is known to nucleate type I/II β-turns in peptides involving an intramolecular hydrogen bond between the C═O of the first (i) and NH of the fourth ($i + 3$) residue, its D-isomer favors antiparallel β-sheet formation via type I′/II′ β-turns.[2] It is well established that the type I′ and II′ β-turn conformations adopted by D-Pro-Xxx segments, with a φ value of ca. +60° for the pyrrolidine ring, promote nucleation of β-hairpin structures in proteins, stronger in the former.[3] While induction of proline causes a reversal in the backbone conformation in peptides, polyproline-based peptides exhibit helical structures as often seen, for example, in collagenous peptides[4] that have repeating units of Gly-X-Y composed predominantly of proline (X) and hydroxyproline (Hyp, Y) residues. The roles of 4-Hyp[5] and 3-Hyp[6] residues on the conformational stability of the collagen triple helix have been extensively investigated. However, no information is available on the effect of 3-Hyp residues on the structures of short linear peptides. This prompted us to undertake detailed structural analysis of peptide **1** containing *cis*-3-hydroxy-D-proline **2** (D-*cis*-3-Hyp) by multidimensional NMR techniques. The choice of the sequence was guided by the fact that similar sequences with proline in the middle, flanked on both sides with apolar residues, are known to adopt well-defined β-turn structures.[7]



Our studies revealed that whereas compound **1a** containing the *O*-Bn-protected D-*cis*-3-Hyp unit adopted a β-hairpin structure in both nonpolar ($CDCl_3$) and polar (DMSO-d_6) solvents, the free *cis*-3-hydroxyl group on the proline ring in peptide **1b** favored a pseudo β-turn-like nine-membered ring structure involving an intramolecular hydrogen bond between the 3-hydroxyl and the Leu amide located at the $i + 2$ position on the backbone in polar solvent DMSO-d_6. The structure of **1b** in nonpolar $CDCl_3$ was, however, found to be similar to that of **1a**. This is in conformity with our earlier observations that the free hydroxyl groups on sugar rings prevent furanoid sugar amino acid based short linear peptides from adopting regular β-turn structures, as these hydroxyl groups themselves act as hydrogen bond acceptors.[8] Similar secondary structures were also observed by us in the reverse turn peptidomimetics based on C_2-symmetric 2,5-dideoxy-2,5-anhydro-[9] and 2,5-dideoxy-2,5-imino-sugar diacids.[10] Recent works of Overhand and coworkers on the X-ray structure of furanoid sugar amino acid containing gramicidin S analogue showed the existence of a similar intramolecularly hydrogen-bonded reverse turn structure in which the ring hydroxyl acted as an acceptor, providing further support to our earlier findings.[11] That the *cis*-3-hydroxy-D-proline-containing

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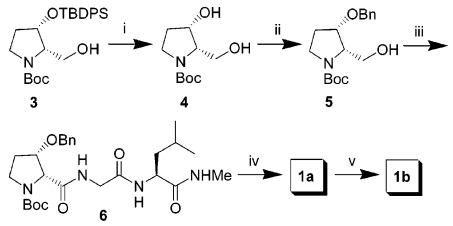
SCHEME 1[a]



[a] Reagents and conditions: (i) TBAF, THF, 0 °C to rt, 4 h, 95%; (ii) (a) TrCl, Et_3N, DMAP (cat.), CH_2Cl_2, 0 °C to rt, 12 h; (b) NaH, BnBr, TBAI (cat.), THF, 0 °C to rt, 8 h; (c) HCO_2H, Et_2O, 0 °C, 10 min, 73% (from **4**); (iii) (a) PDC, DMF, 0 °C to rt, 24 h; (b) HOBt, EDCI, CH_2Cl_2−DMF, 0 °C, 15 min and then TFA·H_2N-Gly-Leu-NHMe, DIPEA, 0 °C to rt, 12 h, 74% (from **5**); (iv) (a) TFA−CH_2Cl_2 (1:3), 0 °C to rt, 1 h; (b) Boc-Val-OH, HOBt, EDCI, CH_2Cl_2, 0 °C, 15 min and then the TFA-salt from step (iv) (a), DIPEA, 0 °C to rt, 12 h, 85% (from **6**); (v) $Pd(OH)_2$−C (20%) (cat.), H_2, MeOH, rt, 2 h, quantitative.

peptides exhibit hydroxyl-assisted turn structures, in polar solvents, similar to those found in their furanoid congeners may provide an insight into the role of the hydroxyl groups on the secondary structures of hydroxyproline containing peptides. This paper describes the synthesis and detailed conformational studies of the 3-hydroxy-D-proline containing peptides **1a** and **b**.

Synthesis of Peptides 1a and 1b. Scheme 1 outlines the synthesis of the core building block followed by the synthesis of the peptides **1a** and **1b**. Our synthesis started with the known intermediate **3**, which was prepared from D-mannitol according to the reported procedure.[12] Although oxidation of the primary hydroxyl group of **3** provided the silyl-protected D-*cis*-3-Hyp, the presence of the bulky silyl group in the molecule made the peptide couplings with D-*cis*-3-Hyp(ΣSi) very sluggish and prompted us to use benzyl protection in its place. Deprotection of the silyl group of **3** furnished the diol **4** in 95% yield. The secondary hydroxyl of diol **4** was next protected as *O*-Bn following a three-step protocol, protection of the primary hydroxyl as trityl ether, protection of the 3-OH as benzyl ether, and finally deprotection of the trityl group, to give **5** in 73% overall yield from **4**. Oxidation of the primary hydroxyl group of **5** with pyridinium dichromate[13] (PDC) in DMF furnished the requisite acid, which was used directly after aqueous workup in the peptide-coupling step.

The peptide coupling was carried out following standard solution-phase peptide synthesis methods[14] using 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDCI) and 1-hydroxybenzotriazole (HOBt) as coupling agents and dry DMF and/or CH_2Cl_2 as solvents. In the racemization free fragment condensation strategy that was followed, the Boc-D-*cis*-3-Hyp(Bn)-OH prepared from **5** was first coupled with the dipeptide H-Gly-Leu-NHMe as efficiently as with any normal amino acid using the reagents mentioned above to give the tripeptide Boc-D-*cis*-3-Hyp(Bn)-Gly-Leu-NHMe **6** in 74% yield from **5**. After removal of the Boc-protection of **6** using trifluoroacetic acid (TFA) in CH_2Cl_2, the resulting tripeptide, D-*cis*-3-Hyp(Bn)-Gly-Leu-NHMe, was reacted with Boc-Val-OH to give **1a** in 85% yield. Hydrogenation of **1a** using $Pd(OH)_2$−C in MeOH furnished **1b** in quantitative yield. The final products **1a** and **1b** were purified by silica gel column chromatography and fully characterized by spectroscopic methods before using them in the conformational studies.

TABLE 1. ^{1}H NMR Chemical Shifts (δ in ppm) and Coupling Constants (J in Hz) of 1a ($CDCl_3$, 500 MHz, 30 °C)[a]

protons	Val	3-Hyp	Gly	Leu
NH	5.48 (d) (J = 6.3)		6.90 (dd) (J = 5.0, 7.6)	7.39 (d) (J = 8.5)
CαH	3.96 (dd) (J = 6.3, 8.2)	4.57 (d) (J = 5.9)	4.26 (dd) (J = 7.6, 17.3)	4.40 (ddd) (J = 5.2, 8.5, 9.8)
CαH′			3.53 (dd) (J = 5.0, 17.3)	
CβH	2.04 (m)	4.42 (m)		1.73 (m)
CβH′				1.68 (m)
CγH	1.03 (d) (J = 6.6)	2.22 (m)		1.64 (m)
CγH′	0.97 (d) (J = 6.7)	1.99 (m)		
CδH		4.06 (ddd) (J = 6.5, 9.5, 10.4)		0.91 (d) (J = 6.1)
CδH′		3.71 (ddd) (J = 3.9, 7.6, 10.4)		0.89 (d) (J = 6.1)

[a] Others: 6.66 (q, J = 4.8 Hz, N*H*Me), 4.61−4.60 (m, 2 H, OC*H*$_2$Ph), 2.70 (d, J = 4.8 Hz, NH*Me*), 1.37 (s, 9 H, Boc), 7.25−7.35 (m, 5 H, aromatic).

Conformational Analysis. NMR Studies. NMR studies of **1a**,**b** were carried out both in $CDCl_3$ and DMSO-d_6. The spectra were well resolved and most of the spectral parameters could be obtained easily and are reported in the Tables 1−4. While the assignments were carried out with the help of total correlation spectroscopy (TOCSY),[15] nuclear Overhauser effect spectroscopy (NOESY)/rotating frame nuclear Overhauser effect spectroscopy (ROESY)[16] experiments provided the information on the proximity of protons, the details of which are provided in the supplementary information. Variable-temperature studies were carried out to measure the temperature coefficients of the amide proton chemical shifts ($\Delta\delta/\Delta T$), which provided information about their involvements in intramolecular hydrogen bonds. The spectra were recorded between 30 and 70 °C (at 30, 40, 50, 60, and 70 °C) in DMSO-d_6, and the temperature coefficients $\Delta\delta/\Delta T$ were determined from the slopes of the linear regression lines obtained from the chemical shift vs temperature plots shown in the supplementary information.[17] The cross-peak intensities in the ROESY spectra were used for obtaining the restraints in the simulated molecular dynamics (MD) calculations.[18]

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TABLE 2. ^{1}H NMR Chemical Shifts (δ in ppm), Coupling Constants (J in Hz), and Temperature Coefficients ($\Delta\delta/\Delta T$ in ppb/K) of 1a (DMSO-d_6, 500 MHz, 30 °C)[a]

protons	Val	3-Hyp	Gly	Leu
NH	6.83 (d) (J = 8.5)		8.20 (dd) (J = 5.8, 6.3)	7.63 (d) (J = 8.4)
CαH	4.04 (dd) (J = 7.3, 8.5)	4.50 (d) (J = 6.7)	3.77 (dd) (J = 6.3, 17.0)	4.23 (ddd) (J = 4.8, 8.4, 10.1)
CαH′			3.61(dd) (J = 5.8, 17.0)	
CβH	1.96 (m)	4.26 (m)		1.53 (ddd) (J = 4.8, 10.1, 13.7)
CβH′				1.42 (ddd) (J = 4.8, 9.1, 13.7)
CγH	0.83 (d) (J = 6.5)	2.11 (m)		1.59 (m)
CγH′	0.83 (d) (J = 6.5)	2.02 (m)		
CδH		3.69 (dt) (J = 7.1, 10.2)		0.85 (d) (J = 6.5)
CδH′		3.54 (m)		0.81 (d) (J = 6.5)
$\Delta\delta/\Delta T$	−8.4		−4.3	−2.3

[a] Others: 7.63 (q, J = 4.4 Hz, N*H*Me), 4.66−4.59 (m, 2 H, OC*H*$_2$Ph), 2.53 (d, J = 4.4 Hz, NH*Me*), 1.37 (s, 9 H, Boc), 7.25−7.35 (m, 5 H, aromatic).

TABLE 3. ^{1}H NMR Chemical Shifts (δ in ppm) and Coupling Constants (J in Hz) of 1b ($CDCl_3$, 500 MHz, 30 °C)[a]

protons	Val	3-Hyp	Gly	Leu
NH	5.30 (d) (J = 6.4)		7.35 (dd) (J = 5.5, 6.9)	7.49 (d) (J = 8.7)
CαH	4.02 (dd) (J = 6.4, 7.6)	4.51 (d) (J = 6.0)	3.93 (dd) (J = 6.9, 17.2)	4.41 (ddd) (J = 5.0, 8.7, 9.3)
CαH			3.84 (dd) (J = 5.5, 17.2)	
CβH	2.01 (m)	4.72 (dt) (J = 4.8, 6.0)		1.67 (m)
CβH				1.56 (m)
CγH	1.03 (d) (J = 6.7)	2.15 (m)		1.60 (m)
CγH	0.97 (d) (J = 6.8)	2.10 (m)		
CδH		4.04 (m)		0.92 (d) (J = 6.1)
CδH		3.73 (ddd) (J = 4.2, 7.4, 10.2)		0.89 (d) (J = 5.9)

[a] Others: 6.66 (q, J = 4.8 Hz, N*H*Me), 2.76 (d, J = 4.8 Hz, NH*Me*), 1.40 (s, 9 H, Boc).

The ^3J coupling constant between the CαH and CβH protons of the pyrrolidine ring in **1a** (5.9 in $CDCl_3$, 6.7 in DMSO-d_6) and **1b** (6.0 in $CDCl_3$, 6.4 in DMSO-d_6) attributed to the dihedral angle of about 30° and the strong NOE correlations between CαH ↔ CβH, CαH ↔ Cδ(Pro-S)H, CβH ↔ Cδ(Pro-S)H are in agreement with a *cis* relationship between the CαH and CβH protons of the pyrrolidine ring.[19]

Conformational Analysis of 1a. Conformational studies of **1a** were carried out by using 1D and 2D NMR experiments in both nonpolar ($CDCl_3$) and polar solvents (DMSO-d_6). In $CDCl_3$ solution the downfield appearance

TABLE 4. ^{1}H NMR Chemical Shifts (δ in ppm), Coupling Constants (J in Hz) and Temperature Coefficients ($\Delta\delta/\Delta T$ in ppb/K) of 1b (DMSO-d_6, 500 MHz, 30 °C)[a]

protons	Val	3-Hyp	Gly	Leu
NH	6.82 (d) (J = 8.4)		8.11 (dd) (J = 5.5, 6.6)	7.73 (d) (J = 8.4)
CαH	4.03 (dd) (J = 7.5, 8.4)	4.26 (d J = 6.4)	3.73 (dd J = 6.6, 17.4)	4.19 (ddd J = 4.7, 8.4, 10.3)
CαH′			3.56 (dd J = 5.5, 17.4)	
CβH	1.95 (m)	4.42 (m)		1.48 (m)
CβH′				1.40 (m)
CγH	0.83 (d J = 6.4)	1.97 (m)		1.54 (m)
CγH′	0.83 (d J = 6.4)	1.90 (m)		
CδH		3.69 (m)		0.83 (d J = 6.6)
CδH′		3.53 (m)		0.79 (d J = 6.6)
$\Delta\delta/\Delta T$	−8.5		−4.2	−2.7

[a] Others: 7.57 (q, J = 4.7 Hz, N*H*Me), 5.81 (d, J = 4.0 Hz, OH), 2.55 (d, J = 4.7 Hz, NH*Me*), 1.38 (s, 9 H, Boc).

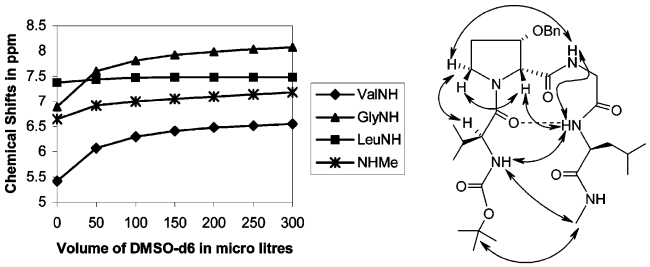

FIGURE 1. Schematic representation of the proposed structure of **1a** in $CDCl_3$ with some of the diagnostic long-range NOEs seen in its ROESY spectrum and the variation of the amide proton chemical shifts plotted against the volumes of DMSO-d_6 added to 600 μL of $CDCl_3$ solution of the peptide.

of LeuNH at 7.39 ppm suggested its participation in intramolecular H-bond, which was further confirmed by the small variation (0.11 ppm) in its chemical shift value when up to 33% v/v of DMSO-d_6 was added to the $CDCl_3$ solution in solvent titration studies (Figure 1). The NOE cross-peaks, as seen in Figure 1, between GlyNH ↔ LeuNH and LeuNH ↔ HypCαH, coupled with LeuNH H-bond supported the presence of a 10-membered β-turn structure around Hyp-Gly residues, which could nucleate either a type I′ or II′ β-turn. The cross-peak GlyNH ↔ HypCδH(pro-R) and a medium to weaker cross-peak between GlyNH ↔ HypCαH compared to that of LeuNH ↔ GlyCαH distinguish between the two and support the existence of a type I′ β-turn, which was further supported by the chemical shift difference between the GlyCα protons ($\Delta\delta$ = 0.70 ppm).[20] In addition, the cross-strand NOEs between LeuNH ↔ ValNH, ValNH ↔ NH*Me*, and Boc*Me* ↔ NH*Me* indicate the formation of a minimal hairpin.[21]

In DMSO-d_6 solution, the hydrogen bonding studies carried out with **1a** showed the small magnitude of the temperature coefficient for its LeuNH (−2.3 ppb/°K), indicating its participation in intramolecular H-bonding.

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FIGURE 2. Stereoview of the 20 superimposed energy-minimized structures of **1a** sampled during 100 cycles of the 600-ps constrained MD simulations following the simulated annealing protocol.

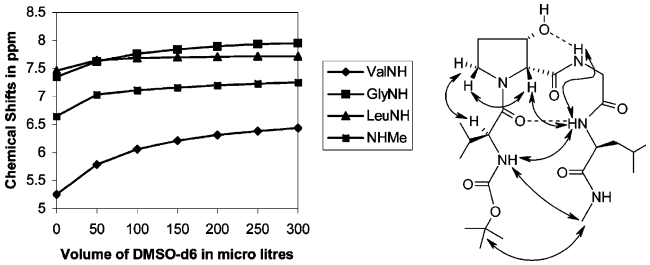

FIGURE 3. Schematic representation of the proposed structure of **1b** in $CDCl_3$ with some of the diagnostic long-range NOEs seen in its ROESY spectrum and the variation of the amide proton chemical shifts plotted against the volumes of DMSO-d_6 added to 600 μL of $CDCl_3$ solution of the peptide.

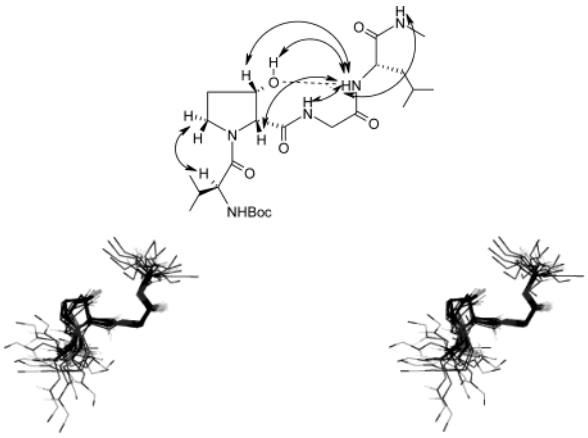

FIGURE 4. (Top) Schematic representation of the proposed nine-membered pseudo β-turn conformation of **1b** in DMSO-d_6 with some of the diagnostic long-range NOEs seen in its ROESY spectrum. (Bottom) Stereoview of the 20 superimposed energy-minimized structures of **1b** sampled during 100 cycles of the 600-ps constrained MD simulations following the simulated annealing protocol.

The presence of NOEs, similar to those seen in $CDCl_3$, between GlyNH ↔ LeuNH, LeuNH ↔ ValNH, LeuNH ↔ HypCαH, ValNH ↔ NH*Me*, Boc*Me* ↔ NH*Me*, and the LeuNH hydrogen bond supported the formation of a minimal hairpin conformation as in $CDCl_3$.

The cross-peak intensities in the ROESY spectra, shown schematically in Figure 1, were used for obtaining the restraints in the simulated molecular dynamics (MD) calculations on **1a**. Molecular dynamics calculations were carried out using the Sybyl 6.8 program on a Silicon Graphics O2 workstation. The Tripos force field, with default parameters, was used throughout the simulations. The detailed protocol of the MD calculations is provided in Supporting Information. Figure 2 depicts the ensemble of the backbone superimposed turn structures of the 20 samples, collected during 600 ps simulated annealing protocol, which clearly shows structures with a type I′ β-turn around Hyp-Gly residues. The average pair-wise backbone RMSD for the structures is 0.34 ± 0.14 Å.

Conformational Analysis of 1b. Conformational study of **1b** was carried out in both nonpolar ($CDCl_3$) and polar (DMSO-d_6) solutions. The ^{1}H NMR spectrum in $CDCl_3$ was well resolved and the derived parameters are given in Table 3. The low field appearances of GlyNH (7.35 ppm) and LeuNH (7.49 ppm) suggested their involvements in intramolecular H-bonding. This was confirmed by the small variation in their chemical shifts (0.25 ppm for LeuNH and 0.60 ppm for GlyNH) values during solvent titration studies when 33% v/v of DMSO-d_6 was added to the $CDCl_3$ solution. The presence of NOE cross-peaks GlyNH ↔ LeuNH, LeuNH ↔ HypCαH, LeuNH ↔ ValNH, ValNH ↔ NH*Me*, and Boc*Me* ↔ NH*Me* and the association of LeuNH in H-bond are in agreement with a stable hairpin conformation. The cross-peak GlyNH ↔ HypCβH supported the formation of a six-membered hydrogen bond between GlyNH → Hyp*O*H. Such H-bonds are observed in threonine- and serine-containing peptides.[22]

Conformational signatures observed for **1b** in DMSO-d_6 were, however, very different from those seen in the $CDCl_3$ solution. The variable temperature experiments in DMSO-d_6 showed that only LeuNH had a small magnitude of the temperature coefficient of −2.7 ppb/°K indicating its participation in H-bonding. The NOE cross-peaks GlyNH ↔ LeuNH, LeuNH ↔ HypOH, LeuNH ↔ NHMe, LeuNH ↔ HypCβH, LeuNH ↔ HypCαH coupled with LeuNH H-bond hints a nine-membered pseudo β-turn-like structure involving Hyp-Gly-Leu residues.

The cross-peak intensities in the ROESY spectrum of **1b** in DMSO-d_6 (Figure 4) were used for obtaining the restraints in the MD calculations, which showed clearly the existence of a nine-membered pseudo β-turn-like structure as shown in Figure 4. The average pair-wise backbone RMSD is 1.22 ± 0.40 Å.

We believe that the hydroxyl group having a *cis* relationship with the adjacent Cα-carboxyl in D-*cis*-3-Hyp is playing an important role in the observed conformational switch on changing from nonpolar to polar solvent. In $CDCl_3$ solution, 3-Hyp-Gly dipeptide nucleates a type I′ β-turn by forming a regular 10-membered H-bond between LeuNH → ValC═O, which can further stabilize a minimal hairpin conformation. However, in polar solvent like DMSO-d_6, the hairpin conformation is unfolded, forcing the peptide chain veer toward the hydroxyl group. This results in the formation of a nine-membered H-bond between LeuNH → HypC3-*O*H, structures found earlier in furanoid sugar amino acid, 2,5-anhydo- and 2,5-imino-sugar diacid containing peptides.[8−10] We hope that the studies described here will help to understand the structures of other hydroxyproline containing peptides.

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Supporting Information Available: Experimental procedures, characterization data, and spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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