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New Concepts

Short, Solubilized Polyalanines Are Conformational Chameleons: Exceptionally Helical If N- and C-Capped with Helix Stabilizers, Weakly to Moderately Helical If Capped with Rigid Spacers†

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ABSTRACT: Isolating spacers introduced between solubilizing lysine regions and a polyalanine core permit rigorous characterization of context-free alanine helices. The preferred building blocks for isolating spacers are amino acids with rigid, extended conformations such as proline, isonipecotic acid, and *tert*-leucine. Replacing isolating spacers by conventional N- and C-caps dramatically increases the helicity of dodecaalanine. Solubilized, isolated polyalanines provide optimal tools for testing polypeptide helicity algorithms, central to resolution of the protein folding problem.

A full understanding of the protein folding problem in chemical terms awaits clarification of the detailed structure-dependent energetics of formation of polypeptide sheets, turns, loops, and helices. Helicity correlations have received much recent attention, in part because unique insights into the stabilities of early protein folding intermediates (*1*) and native protein structures (*2*) would accrue from improved correlations of peptide helicity with amino acid composition and sequence. Although helix-stabilizing effects of particular interacting amino acid pairs have been documented (*3*), more fundamental helicity issues remain unresolved and controversial. The helical propensity of alanine, the simplest of the natural helix-forming amino acids, has been assigned a large range of values, primarily because there is no agreement on appropriate contexts in which it can be measured (*4*−*6*). Low water solubility and a high tendency toward aggregation render simple polyalanines unsuitable as contexts. Alanine-rich peptides solubilized by side chain-charged amino acids either spaced within the core region or sited at the ends (*7*, *8*) have been studied in their stead, but the high helicities of these peptides have been variously attributed to alanine itself or to interactions of the alanine regions with the charged solubilizers. The ideal alanine context is both solubilized and maximally insulated from helicity effects of neighboring amino acids. We now demonstrate such contexts.

Our strategy is embodied in the generic structure of Figure 1. Its novel feature is the pair of isolating spacers (IS)[1] that flank the polyalanine core and shield it from linked solubilizers (SO). This work breaks important ground; comparisons of helicities of these constructs with analogues lacking IS regions should settle the dispute about the helix-stabilizing role of charged caps. These new constructs are also ideal hosts for guest mutation tests of a rigorous helicity algorithm.

We first explored solubilization through optimization of the SO region of the Figure 1 general structure. Solubilization and suppression of aggregation have previously been reported

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[1] Abbreviations: IS, isolating spacer; SO, solubilizer; Fmoc, 9-fluorenylmethoxycarbonyl; PAL, NovaBiochem peptide amide linker; PEG, poly(ethylene glycol); DBU, diazabicycloundecene; DMF, dimethylformamide; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl-

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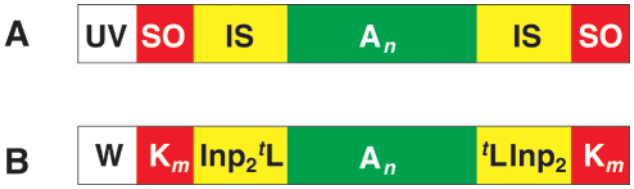

FIGURE 1: Schematics depicting water-solubilized, isolated, spaced polyalanine. (A) General form: features a UV reporter (white) for concentration determination, two SO regions (red), and two spacing regions (yellow) that isolate the core polyalanine of interest (green) from the effects of the UV reporter and the polar solubilizing regions. (B) Final form as developed in the text: the UV reporter (white) is W, and SO regions (red) are short (length = 3 or 4) polylysines. Rigid spacing regions (yellow) flanking the polyalanine core (green) are N-terminal $Inp_2{}^tL$ and C-terminal $^tLInp_2$ (see Figure 2). With SO = K_4, polyalanine is both soluble and unaggregated up to at least 28 residues.

for block copolymers in which polyalanine oligopeptides served as the core, flanked by charged polylysine or polyglutamate termini (*9*). We have adopted this proven strategy for shorter peptides, selecting for the SO functions of Figure 1 polylysine N- and C-terminal sequences of length sufficient to ensure solubiliity and lack of aggregation as judged by AUC (*10*).

A successful isolating spacer (IS) must leave the helicity of the alanine core invariant when small structural changes are made in the IS or K_m regions. In accord with convention (*11*, *12*) we use the circular dichroism molar ellipticity at 222 nm, molar $[\theta]_{222}$, as a measure of alanine core helicity, under the testable assumption that contributions to $[\theta]_{222}$ from the IS, K_m, and W regions are modest as expected. For a fixed A_n we evaluate a candidate for IS by probing the sensitivity of its molar $[\theta]_{222}$ to small changes in IS structure and in the length of K_m.

MATERIALS AND METHODS

Peptides were prepared on a 0.025 mmol scale by automated continuous-flow solid-phase synthesis on a PE Biosystems Pioneer peptide synthesizer using standard Fmoc chemistry on cross-linked PAL and PEG functionalized polystyrene at a loading of 0.17 mequiv/g. Fmoc removal was accomplished using a 1:1:48 (v/v/v) mixture of DBU/piperidine/DMF and was monitored by UV absorbance of the cleaved Fmoc chromophore. Coupling conditions involved a 3-fold excess each of Fmoc-amino acid and HATU and excess DIEA in DMF. Peptides were cleaved with an 82.5% TFA, 5% phenol, 5% water, 5% thioanisole, 2.5% 1,2-dithioethane cocktail for 2 h and precipitated by dropwise addition to cold, dry diethyl ether. The precipitate was washed three times with diethyl ether, dried under vacuum, dissolved in 18.2 MΩ water, and purified by HPLC. HPLC was performed using Waters DeltaPak C_{18} 300 Å or YMC ODS-AQ 200 Å reverse-phase packing and a mobile phase of 0.05% TFA in water/0.042% TFA in acetonitrile and monitored at 214 and 280 nm. The criteria for purity were symmetrical peak shape and baseline resolution by analytical HPLC. Purified peptides were lyophilized twice from 18.2 MΩ purified water and stored in polypropylene conical tubes at or below 0 °C. Electrospray MS taken on a Waters Micromass ZMD 4000 yielded *m*/*z* ratios within 0.5 mass unit of the expected values. UV spectra between 250 and 300 nm all showed the same characteristic peak shape of the W chromophore with a maximum at 279 nm.

CD experiments were performed on an Aviv 62DS spectrometer equipped with a thermoelectric temperature controller and an Osram XBO 450 W high-pressure xenon lamp. Background scans (taken immediately prior to use) for each CD cell (Hellma 1.0 or 10.0 mm strain-free Suprasil) and all data were collected as an average of five scans at 1.0 nm bandwidth and 0.5 nm step size. Concentrations were determined by the UV absorption of W with $\epsilon_{280} = 5.56 \times 10^3\ M^{-1}\ cm^{-1}$ (*13*) on a double-beam Varian Cary 100 Bio UV−visible spectrometer. Except as noted, ellipticities are expressed as molar, rather than per residue, quantities due to uncertainty in defining the limiting helical lengths of most peptides.

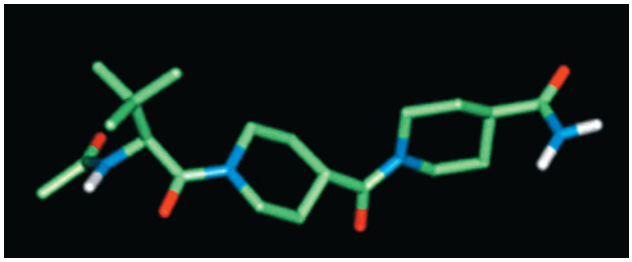

FIGURE 2: Three-dimensional representation of the IS element $^tLInp_2$. The bulky *tert*-butyl group biases the geometry of ^{t}L toward the extended conformation. The rigid γ-amino acid linkage of Inp requires a fully extended conformation. This picture was generated with Quanta 98 from Molecular Simulations Inc.

RESULTS AND DISCUSSION

Candidates for IS might embody flexibility or rigidity. Among the natural amino acids, glycine and proline are the obvious choices, since they respectively occupy the most and least ϕ, ψ conformational spaces (*14*, *15*). Short polyglycine sequences have been frequently incorporated into designer protein sequences to create flexible linkers between regions of defined secondary structure. The rigidity of polyproline conformations is well documented (*16*). Unnatural amino acids offer a wider range of conformational options. Inp = 4-carboxypiperidine, a simple achiral proline analogue, maximizes rigidity and linear extension while the side chain bulk of ^{t}L = *tert*-leucine strongly favors extended peptide conformations relative to helices (*15*). Significantly, CD analysis shows that although it is compatible with helical structure, ^{t}L is a helix breaker when incorporated within core peptide regions (*17*).

Our CD studies were first carried out with peptides $WK_mISA_{12}ISK_mNH_2$ containing IS regions composed of G_j, $2 \leq j \leq 7$, as well as P_3, Inp_3, $^tL_3$, and a combination of Inp_2 and ^{t}L (Figure 2). An alanine core length of 12 was chosen since Lifson−Roig modeling suggests that a helix of this length should be maximally sensitive to capping stabilization. Figure 3A shows CD spectra of two peptides that use G_j and G_k as IS isolating spacers. The spectra have

uronium hexafluorophosphate; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; MS, mass spectroscopy; *m*/*z*, mass-to-charge ratio; UV, ultraviolet; CD, circular dichroism; ϵ_{280}, molar extinction coefficient at 280 nm; Inp, isonipecotic acid (4-carboxypiperidine); ^{t}L, *tert*-leucine; AUC, analytical ultracentrifugation; MW, molecular weight; $[\theta]$, molar ellipticity; β, β-aminoalanine.

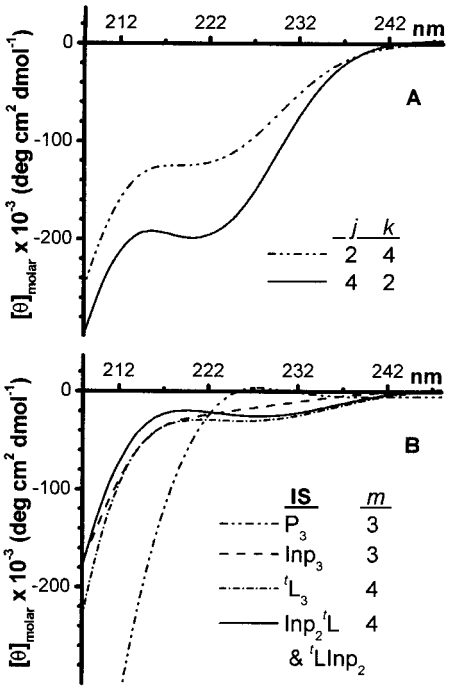

FIGURE 3: Tests of flexible and rigid IS candidates. The helicity as measured by molar $[\theta]_{222}$ of an effectively isolated, spaced peptide is insensitive to structural alterations in the IS region. (A) Molar ellipticity ($[\theta]$) vs wavelength (λ) for $WK_3G_jA_{12}G_kK_3$ (j, k = 2, 4 or 4, 2) in pure water at 2 °C and 140−200 μM. The structural exchange of G_2 and G_4 significantly enhances the partially helical character of the peptide. This large variation of $[\theta]_{222}$ due to a simple permutation in the flexible IS region excludes glycine spacers as IS candidates. (B) Molar $[\theta]$ vs λ for $WK_mISA_{12}ISK_m$ (IS containing P, Inp, ^{t}L; m = 3, 4) in pure water at 2 °C and 10−20 μM. Consistently nonhelical CD spectra are observed for peptides with structurally diverse rigid spacers.

a partially helical form but exhibit significantly different $[\theta]_{222}$, and thus these $G_{j,k}$ must be rejected as IS choices. In further studies the length of G_j was varied through $j = 3-7$, but convergence with increasing length to a constant $[\theta]_{222}$ was not seen, and peptide aggregation was detected for j = 7. Possible explanations for these failures are a length-dependent C-terminal helix-stabilizing capacity of G_2 (*18*) or stabilizing interactions between Lys ϵ-NH_3^+ cations and the C-terminal helix dipole that are tolerated by the exceptionally flexible (*15*) glycine IS regions.

CD spectra (Figure 3B) of A_{12} peptides flanked by the rigid IS elements P_3, Inp_3, $^tL_3$, or a combination of Inp_2 and ^{t}L lack evidence of significant helicity at 222 nm and resemble spectra of unordered peptides (*11*). A more sensitive test of these isolating spacers was carried out with the longer alanine sequence $WK_4ISA_{19}ISK_4NH_2$, which exhibits detectable CD helicity. We observed molar $[\theta]_{222}$ values of -5.17×10^5 for IS = Inp_3, -5.05×10^5 for IS = P_3, and -5.16×10^5 for the IS N, C pair $Inp_2{}^tL$ and $^tLInp_2$. These values are identical within limits of measurement, a key first step in validation of the isolating spacer principle. For further work we picked the general sequence $WK_4Inp_2{}^tLA_n{}^tLInp_2K_4NH_2$ which embodies the IS functions used in this last example (see Figure 2). These IS choices combine the normal peptide backbone of the core-flanking ^{t}L residues with the maximum extension of the Inp_2 sequence. Further tests explored dependence of $[\theta]_{222}$ on the W site using the fixed sequence $K_mInp_2{}^tLA_n{}^tLInp_2K_m$ but with W residues positioned at the N-terminus, at the C-terminus, and at both termini. The respective $[\theta]_{222}$ values were -5.16×10^5, -5.06×10^5, and -5.16×10^5. There are thus no detectable effects on $[\theta]_{222}$ from changes in either the isolating spacer or UV reporter functions.

If solubilizing K_m regions are effectively isolated from the alanine core, CD spectra should be accurately approximated as a sum of independent contributions from core and caps. Figure 4A shows the wavelength dependences of $[\theta]$ values for three models, $WK_mInp_2{}^tLA_4{}^tLInp_2K_mNH_2$ (m = 3, 4, 7), for which the A_4 region is expected to contribute insignificantly at 222 nm. Ellipticities negligible at high λ and pronounced at low λ are consistent with CD spectra (*19*) expected for unordered polylysines with a short polyalanine segment. Figure 4B shows the corresponding wavelength dependences of molar $[\theta]$ values of three peptides, $WK_mInp_2{}^tLA_{19}{}^tLInp_2K_mNH_2$ (m = 3, 4, 7). These CD spectra differ for λ < 212 nm but not in the helical region as expected for peptides with noninteracting caps and identical core polyalanines. The difference spectra are shown in Figure 4C and are coincident within experimental error, validating the hypothesis of CD independence for core and cap. The residue length that contributes to the difference spectra must thus be the alanine core length, and the $[\theta]$ values can be expressed as per residue molar ellipticities. To establish their monomeric character, two $WK_mInp_2{}^tLA_n{}^tLInp_2K_mNH_2$ conjugates, with m = 4 and n = 19 and 28, were subjected to AUC. The data clearly indicate that the peptides are monomeric in 0.1 M aqueous NaCl and show the correct molecular weight with no detectable evidence of aggregation (see Supporting Information).

A fundamental issue addressed in this communication is the degree to which the helicity of a short polyalanine is influenced by changes in its N- and C-caps. Although strong helix capping interactions are well documented (*7*, *8*), capping effects for polyalanines have been recently argued to be minor (*4*). The data of Figure 5[2] for the first time allow a comparison between the nonhelical CD spectrum of an isolated A_{12} peptide and the helical spectra of analogues that bear a variety of interactive caps (*20*, *21*). Clearly, for short polyalanines N- and C-capping can generate exceptionally large helix-stabilizing effects.

This initial study has focused on polyalanines, but a single example suggests that the isolating solubilizing principle can be generalized. The highly lipophilic 19-residue core sequence $WK_4Inp_2{}^tLAIA_3L_2A_2IAIA_2L_2AIA{}^tLInp_2K_4NH_2$ is highly helical in water. AUC analysis in dilute solution in

[2] For the calculation of per residue $[\theta]$ of Figure 5, the maximum helical lengths of the peptide have been set as follows: red, 12; black and green, 14; blue, 18. The red, green, and black curves give $[\theta]$ values for sequences of 12 conformationally independent alanine residues bearing N- and C-caps that add no independent residues to the sequence and therefore add no new conformations to the A_{12} manifold. The N-terminal cap Ac-Hel extends by two oriented proline-like residues the sequences of the preexisting A_{12} conformations that join it. Ac-Hel is thus counted as two residues in calculating per residue $[\theta]$; β-aminoalanine acts as a pure C-capping stabilizer (*21*), without lengthening preexisting conformations or generating new ones. Lys residues may terminate the chain with charge−dipole helix stabilization at the C-terminus or may join and extend the chain; the combined N and C K_3 caps of the peptide of the blue curve are counted as six residues. Cumulative interactions of Lys side chain charges are expected to destabilize even short polylysine helices; preliminary NOE evidence suggests that the N-terminal residues are nonhelical.

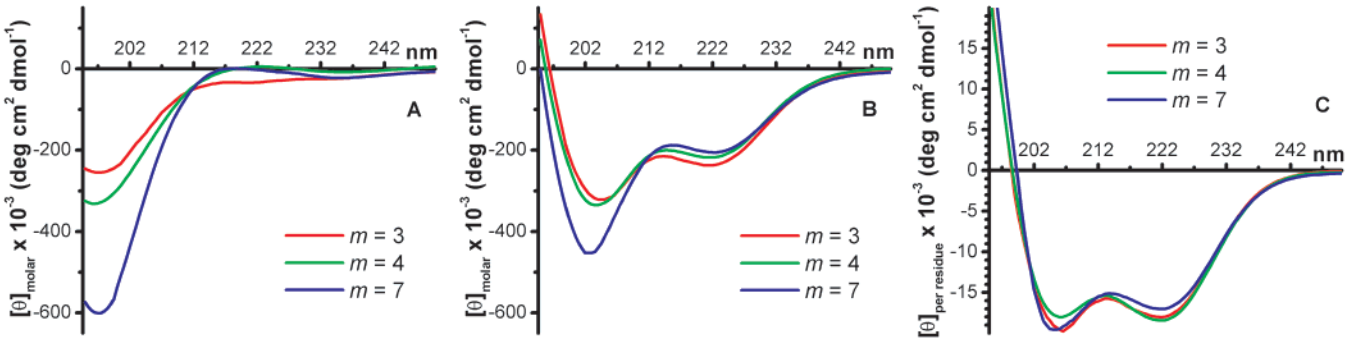

FIGURE 4: Test of the CD independence of core polyalanine regions and W, SO, and IS regions. (A) Molar $[\theta]$ vs λ for $WK_mInp_2{}^tLA_4{}^tLInp_2K_m$ ($m = 3, 4, 7$) in pure water at 25 °C and 10−20 μM. (B) Molar $[\theta]$ vs λ for $WK_mInp_2{}^tLA_{19}{}^tLInp_2K_m$ ($m = 3, 4, 7$) in pure water at 25 °C and 10−20 μM. (C) Per residue difference spectra of $WK_mInp_2{}^tLA_4{}^tLInp_2K_m$ and $WK_mInp_2{}^tLA_{19}{}^tLInp_2K_m$ ($m = 3, 4, 7$) in pure water at 25 °C and 10−20 μM. These corrected spectra correspond to a context-free CD spectrum of A_{19}, whose ellipticity is appropriately expressed in molar per residue units.

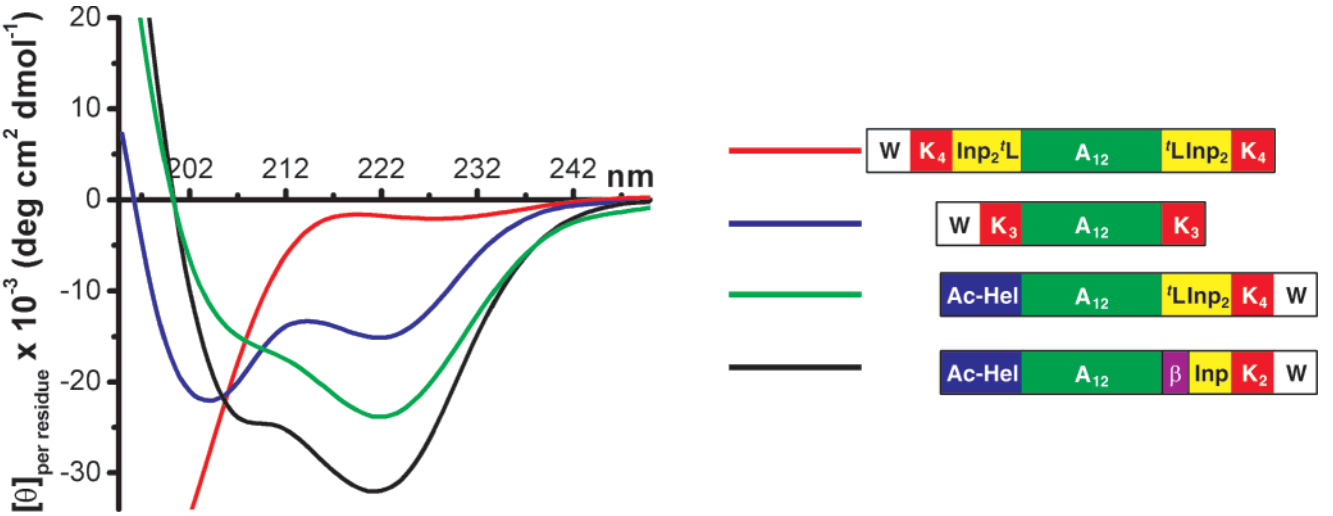

FIGURE 5: Caps greatly influence helicity. Per residue $[\theta]$ vs λ in pure water at 2 °C and 10−20 μM for N- and C-capped A_{12} peptides. The large change in $[\theta]_{222}$ seen (red curve to black curve) when the pair of isolating caps $Inp_2{}^tL$, ${}^tLInp_2$ is replaced by the pair Ac-Hel, β-aminoalanine defines the largest capping effect we have observed to date. The change in $[\theta]_{222}$ seen for the green curve reflects the substantial drop in helicity that results when β is replaced by ${}^tLInp_2$. The significant value of $[\theta]_{222}$ (blue curve) seen when both caps are replaced by K_3 sequences reflects the substantial helix-stabilizing effect of charged solubilizing residues directly linked to the A_{12} core. For this peptide, the overall length was used to define per residue $[\theta]$ even though helicity is expected to be weak or nonexistent in the N-terminal peptide cap. Since $[\theta]$ is calculated for a length including this cap, $[\theta]$ is likely to be underestimated.[2]

water alone shows it to be monomeric, although association with enhancement of helicity at high temperatures is observed at increased salt concentrations. This result suggests that the isolating solubilizing principle may permit characterization in water of the helicity of hitherto intractable hydrophobic peptides.

Polyalanines and alanine-rich peptides have previously been used to assign the helical propensity of alanine. Using NMR measurements applied to template-nucleated short alanine peptides Kemp and co-workers (*22*) have assigned a range $1.02 < w < 1.15$, consistent with earlier studies of the Scheraga group (*5*). Using ellipticity functions that relate fractional helicities to experimental values for $[\theta]_{222}$, the Baldwin and Stellwagen groups have assigned $1.6 < w < 1.8$ to alanine in $(A_4K)_n$ oligomers (*23*). These ellipticity functions have been recently shown to significantly overestimate fractional helicity, and we have provisionally proposed an alternative function (*24*). At 2 °C we find $1.1 < w < 1.3$ for short polyalanines typified by A_{12}; for longer polyalanines typified by A_{19} through A_{28}, $1.3 < w < 1.35$ (see Supporting Information). Definitive fitting of alanine w values awaits ongoing refinement of the ellipticity function, as well as assignment of the helix N- and C-capping parameters for the IS elements. The magnitude of capping effects observed in this study suggests that one can now generate a length range of structurally well-defined alanine-rich helices. Moreover, the helicities of core alanine regions should prove tunable over a wide range by changes in charge or polarity at a few sites within the capping regions (*25*). Such versatile structures are likely to find fertile practical applications in fields as diverse as medicinal chemistry, molecular recognition, supramolecular chemistry, and materials science.

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SUPPORTING INFORMATION AVAILABLE

AUC sedimentation equilibrium results, representative HPLC chromatograms, HPLC and MS data summaries, and calculations of w values. This material is available free of

charge via the Internet at http://pubs.acs.org.

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