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Solid-phase peptide synthesis and circular dichroism study of chiral β-peptoid homooligomers

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Summary. N-alkyl-β-alanine oligomers (β-peptoids) with α-chiral side chains [(*R*)- or (*S*)-1-(phenylethyl)amino groups] were synthesized and analyzed by CD spectroscopy. These chiral β-peptoid homomers exhibited chain-length-dependent and solvent-dependent ellipticity, strongly indicating the presence of a secondary structure in solution. The CD behaviour was only slightly temperature-dependent upon heating, as also previously observed for stable α-peptoid helices containing the same type of side chains. Thus, the data presented here comprise the first evidence for a chain length-dependent secondary folding of compounds with this novel peptidomimetic backbone design. In addition, applicability of a novel hyphenated technique, HPLC-SPE-NMR/MS, for analysis of crude SPPS reaction products was demonstrated.

Keywords: Peptidomimetics – Chiral β-peptoids – Solid-phase synthesis – Circular dichroism – Secondary folding

Abbreviations: β-Nrpe, (*R*)-*N*-(1-phenylethyl)-β-alanine; β-Nspe, (*S*)-*N*-(1-phenylethyl)-β-alanine; CD, circular dichroism; DMF, *N*,*N*-dimethylformamide; HRMS, high-resolution mass spectrometry; MRE, mean residue ellipticity; PyBOP®, benzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; RP-HPLC, reversed-phase high performance liquid chromatography; SPE, solid-phase extraction; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TFFH, fluoro-*N*,*N*,*N*,*N*-tetramethylformamidinium hexafluorophosphate

Introduction

Several types of biomimetic oligomers with the ability to adopt secondary folding, ''foldamers'' (Gellman, 1998), have recently been developed, and their 3D structures have been studied in detail (Hill et al., 2001). In particular, synthetic peptides (**1**) and analogues consisting of β-amino acids (i.e., β-peptides, **2**) have received considerable attention (Cheng et al., 2001; Seebach et al., 2004). The latter oligomers are interesting due to their high propensity to adopt secondary structures, combined with stability towards proteases that cleave natural peptides (**1**). Helix formation is in most β-peptides stabilized by hydrogen bonding, but oligomers constructed from rigid nipecotic acid or pyrrolidine-3-carboxylic acid residues that lack the ability to form backbone hydrogen bonds, have also been found to exhibit secondary structures (Abele et al., 1999). *N*-alkylated glycine oligomers (i.e., peptoids **3**) (Simon et al., 1992; Zuckermann et al., 1992), comprise yet another remarkable class of peptidomimetic foldamers. Due to their lack of backbone chirality, rigidity, and hydrogen bonding capability, the peptoids require α-chiral side chains for the induction of a stable polypropylene-like helical structure (Kirshenbaum et al., 1998; Lee et al., 2005). Nevertheless, a threaded loop conformation was also recently reported (Huang et al., 2006).

New potential foldamer designs are of pertinent interest in order to expand the diversity of synthetic peptide-like constructs both for investigations of their biological activity and possibly in the context of materials science. The present article reports on members of a novel type of oligomers based on *N*-alkylated β-alanine residues, β-peptoids **4** (Hamper et al., 1998; Jia et al., 2002; Shuey et al., 2006). A molecular design containing chiral side chains similar to ours was reported while this manuscript was in preparation (Norgren et al., 2006).

Materials and methods

General

Starting materials were obtained from commercial suppliers and used without further purification. Rink amide resin (100–200 mesh, cross-linked

with 1% divinylbenzene) was obtained from IRIS Biotech (Marktredwitz, Germany). PyBOP was obtained from Novabiochem (Läufelingen, Switzerland). TFFH was from Advanced ChemTech (Louisville, KY, USA). (*S*)-1-phenylethylamine (>95% ee) and (*R*)-1-phenylethylamine (>95% ee) were purchased from Lancaster (Morecambe, England). DMF dried over molecular sieves (H_2O <0.01%) was from Fluka Chemie GmbH (Buchs, Switzerland), and dry dichloromethane was distilled from P_2O_5 and kept over 4 Å molecular sieves. Water for reversed-phase high-performance liquid chromatography (HPLC) was filtered through a 0.22 µm membrane filter (Millipore, Millipak40). Vacuum liquid chromatography was performed using Merck silica gel 60H, 5–40 µm (average size 15 µm). Solid-phase reactions were performed in Teflon filter vessels on a Scansys PLS 4 × 6 Organic Synthesizer equipped with a heating block. ^{1}H NMR spectra were recorded at 400.13 MHz, and ^{13}C NMR spectra were recorded at 100.61 MHz on a Bruker Avance 400 spectrometer using $CDCl_3$, D_2O or CD_3OD as solvents. Coupling constants (*J* values) are given in hertz (Hz). Multiplicities of ^{1}H NMR signals are reported as follows: s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; m, multiplet; br, broad signal. Preparative HPLC was performed using a Waters model 590 pump, a Waters Lambda-Max model 481 spectrophotometric detector, and a 25 × 2.12 cm Phenomenex Luna 5 C18(2) column. The chromatograph operated isocratically at a flow-rate of 14 ml/min, using water/acetonitrile/TFA as the mobile phase. High-resolution mass spectra (HRMS) for exact mass determination were recorded with a Bruker APEX Qe Fourier transform mass spectrometer (FTMS) equipped with a 9.4 tesla superconducting cryomagnet and an external electrospray ion source (Apollo II source). The spectra were externally calibrated with arginine cluster and measured in positive ion mode. The samples were dissolved in methanol, further diluted in 50% methanol containing 0.2% formic acid, and introduced into the electrospray ion source using a syringe pump with a flow of 2 µl/min.

(S)-N-Fmoc-N-(1-phenylethyl)-β-alanine (Fmoc-β-Nspe-OH, ***12****)*

tert-Butyl acrylate (8.0 ml, 62 mmol) and (*S*)-(1-phenylethyl)amine (4.0 ml, 31 mmol) were dissolved in DMSO (7 ml) and stirred in a sealed vessel under microwave irradiation at 160 °C for 30 min (using a Biotage Initiater M.W. reactor). The mixture was cooled to room temperature, diluted with *Et*OAc (300 ml), and washed with water (4 × 100 ml) and brine (100 ml). The organic layer was then dried (Na_2SO_4), filtered, concentrated, and lyophilized. The crude Michael adduct (14.3 g, 93%) was used without further purification as previously described (Olsen et al., 2007). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.34$–7.23 (m, 5H, Ar–H), 3.80 (q, $J = 6.6$ Hz, 1H, CHPh), 2.72 and 2.68 (m, $J_{AA'BB'} \approx 6.5$ Hz, 2H, CH_2N), 2.44 (m, $J_{AA'BB'} \approx 6.5$ Hz, 2H, CH_2CO), 2.35 (br, s, 1H, NH), 1.43 (br, s, 9H, $C[CH_3]_3$), 1.39 (d, $J = 6.6$ Hz, 3H, $CHCH_3$); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 172.1$, 144.8, 128.5 (2C), 127.1, 126.7 (2C), 80.6, 58.3, 43.1, 35.6, 28.1 (3C), 24.1; ESI-MS: $C_{15}H_{23}NO_2$ requires $[M + H]^+$ at m/z 250.2, found, 250.2.

The Michael adduct (1.62 g, 6.55 mmol) was dissolved in *Me*OH (15 ml), 1 *M* $NaOH_{aq}$ was added (13 ml, 13 mmol, approximately 2 equiv), and the mixture was stirred on an oil bath at 60 °C for 2 h. *Me*OH was evaporated, water (100 ml) was added, and the aqueous phase was washed with *Et*OAc (2 × 75 ml). Then the aqueous layer was neutralized (pH ≈ 6) with 4 M HCl_{aq}, residual organic solvents were evaporated in vacuo, and the free acid was lyophilized. ^{1}H NMR (400 MHz, D_2O): $\delta = 7.55$ (m, 5H, Ar–H), 4.48 (q, $J = 6.9$ Hz, 1H, C*H*Ph), 3.18 and 3.07 (2m, $J_{AA'BB'} \approx 6.8$ Hz, 2H, CH_2N), 2.57 (t, $J_{AA'BB'} \approx 6.8$ Hz, 2H, CH_2CO), 1.73 (d, $J = 6.9$ Hz, 3H, $CHCH_3$); ^{13}C NMR (100 MHz, D_2O): $\delta = 180.1$, 138.4, 132.2, 131.9 (2C), 130.1 (2C), 60.8, 44.9, 34.7, 20.8; ESI-MS: $C_{11}H_{14}NO_2$ requires $[M + Na]^+$ at m/z 216.1, found, 216.2. Finally, this residue and Na_2CO_3 (1.38 g, 13 mmol) were suspended in water-1,4-dioxane 1:1 (40 ml) and stirred on an ice bath. Fmoc-Cl (2.52 g, 9.74 mmol) in 1,4-dioxane (10 ml) was added dropwise, and the mixture was stirred for 2 h at room temperature. 1,4-Dioxane was evaporated in vacuo, water (100 ml) was added, and the solution was extracted with Et_2O (3 × 75 ml). The organic layer was concentrated, and the residue was purified by vacuum liquid chromatography (5 × 5 cm column; elution with hexane, hexane–*Et*OAc 12:1, hexane–*Et*OAc–HOAc 100:10:1, hexane–*Et*OAc–HOAc 70:10:0.7, and hexane–*Et*OAc–HOAc 50:10:0.5) gave **12** as a colorless foam (1.54 g, 57% for two steps). TLC: R_f 0.18 (hexane–*Et*OAc–HOAc 3:1:0.03). ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 7.70$ (d, $J = 7.4$ Hz, 2H, Fmoc Ar–H), 7.54 (d, $J = 7.4$ Hz, 2H, Fmoc Ar–H), 7.37–6.95 (m, 9H, Fmoc Ar–H, Ph), 5.41–5.13 (m, 1H, C*H*Ph), 4.62 (br, s, 2H, Fmoc CH_2), 4.27 (br, s, 1H, Fmoc CH), 3.27–2.96 (br, m, 2H, CH_2NH), 2.41–1.83 (br, m, 2H, CH_2CO), 1.41 (d, $J = 6.5$ Hz, 3H, $CHCH_3$); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 176.7$, 156.4, 144.1 (2C), 141.6 (2C), 140.6, 128.7 (2C), 127.9 (2C), 127.8 (2C), 127.3 (4C), 124.9, 120.2 (2C), 67.1, 53.9, 47.7, 38.4, 33.9, 16.9; ESI-MS: $C_{26}H_{25}NO_4$ requires $[M + Na]^+$ at m/z 438.2, found, 438.3; HRMS: $C_{26}H_{25}NO_4$ requires $[M + H]^+$ at m/z 438.16758, found, 438.16755; $\Delta M = 0.07$ ppm.

HPLC-SPE-NMR/MS

Separations for the HPLC-SPE-NMR/MS experiments were performed on a 150 × 4.6 mm i.d., 3 µm particle size Luna C18(2) column at 40 °C using a system consisting of an quaternary solvent delivery pump equipped with a degasser, a column oven, an autosampler and a DAD UV-VIS detector (all Agilent 1100 series); 50 µl of a 10 mg/ml solution was injected and separation was achieved using a linear gradient of *Me*CN in water (0.1% HCOOH) from 5 to 95% over 30 min delivered at

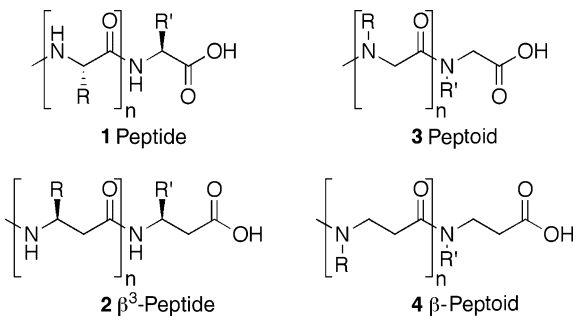
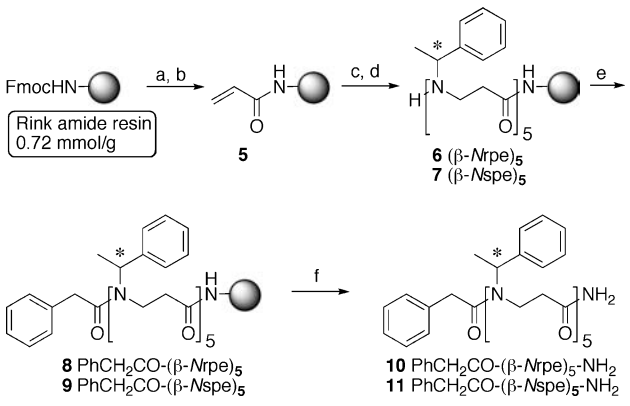


Fig. 1. Structures of peptides and peptidomimetics



Scheme 1. Solid-phase synthesis of β-peptoids **10** and **11**. Reagents and conditions: (a) 30% piperidine-DMF, 2 × 10 min; (b) acryloyl chloride (10 equiv), pyridine (15 equiv), CH_2Cl_2, 1 h; (c) >95% enantiopure (*R*)- or (*S*)-1-phenylethylamine (20 equiv, 2 M in DMSO), 50 °C, 16 h; (d) repetition of steps b and c four times; (e) $PhCH_2COOH$ (5 equiv), PyBOP (5 equiv), iPr_2EtN (10 equiv), DMF, 1.5 h; (f) TFA-CH_2Cl_2 95:5, 50 min

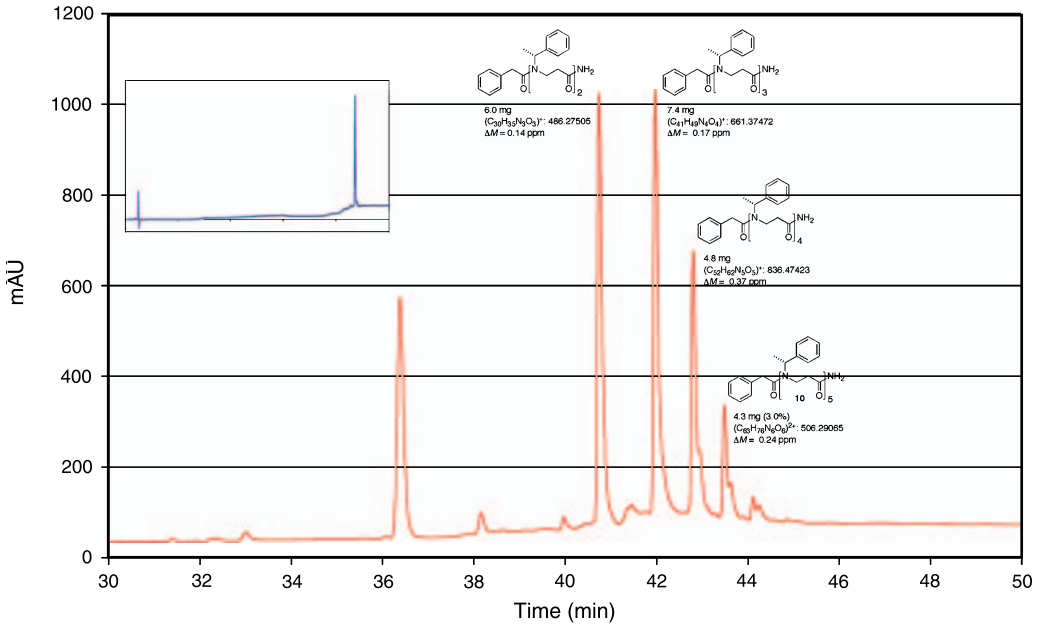


Fig. 2. Crude RP-HPLC trace with structures, isolated yields, and HRMS data for the compounds. The inset shows the RP-HPLC trace for compound **10** after purification

0.8 ml/min. Part of the flow (5%) was led to an Esquire LC ion-trap mass spectrometer operating in positive ion mode. The residual 95% of flow was diluted with a post-column make-up flow of water (2.0 ml/min) before peak trapping using a Prospekt II SPE unit (Spark Holland). The SPE device was coupled to a Bruker Avance 600 MHz spectrometer equipped with a 30 µl inverse $^{1}H\{^{13}C\}$ flow probe. HySphere GP (polydivinylbenzene polymer resin) SPE cartridges (10 × 2 mm i.d., Spark Holland) were used for trappings based on UV absorption of the eluate at 254 nm. Compounds **13** and **14** were trapped 8 times, the SPE cartridges were dried for 30 min each with a flow of N_2 gas, and the analytes were eluted and transferred with acetonitrile-d_3 (275 µl in total) into the NMR flow probe for data acquisition. 1D ^{1}H NMR spectra were recorded using a 1D NOESY pulse sequence with double solvent suppression.

Solid-phase peptide synthesis

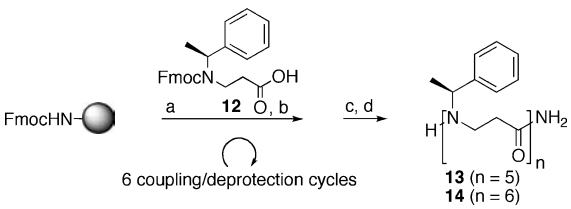
Fmoc-protected Rink amide resin (100 mg, 72 µmol; Rink, 1987) was treated with 20% piperidine-DMF (2 × 10 min), and washed with DMF, *Me*OH, and CH_2Cl_2 (3 × 5 ml, 5 min each). The resin was then agitated with Fmoc-β-*N*spe-OH (5 equiv), TFFH (5 equiv), and *i*Pr_2*Et*N (10 equiv) in dry DMF (1 ml) under N_2 for 1.5 h, and washed with DMF, *Me*OH, and CH_2Cl_2 (3 × 5 ml, 5 min each). This two-step procedure was performed six times, and coupling efficiencies were checked by the Kaiser test (week brown when positive). Residual free amino groups were capped with Ac_2O (0.5 ml) and pyridine (0.5 ml) in CH_2Cl_2 (3 ml), the resin was washed, the terminal Fmoc group was removed with 30% piperidine-DMF (2 × 15 min, 50 °C), and the usual washing procedure was applied again. The crude product was cleaved from the support with TFA–CH_2Cl_2 (95:5, 3 ml, 2 h), and an HPLC-SPE-NMR/MS analysis was performed as above (data not shown). The oligomers were purified to homogeneity by preparative RP-HPLC. Compound **13**, 7 mg (11% based on the initial loading given by the commercial supplier). Analytical $HPLC_{254\,nm}$: >95% (t_R = 18.0 min); HRMS (m/z): $[M]^{2+}$ calcd for $C_{55}H_{70}N_6O_5$, 447.26984; found, 447.26978; ΔM = 0.13 ppm. Compound **14**, 6.5 mg (10% based on the initial loading given by the commercial supplier). Analytical $HPLC_{254\,nm}$: >95% (t_R = 19.2 min); HRMS (m/z): $[M]^{2+}$ calcd for $C_{66}H_{83}N_7O_6$, 534.81969; found, 534.81965; ΔM = 0.08 ppm).

CD spectroscopy

Spectra were acquired with an Olis DSM 10 CD spectrophotometer (Olis Inc., Bogart, GA, USA), equipped with a Quantum Systems temperature control module, using 1 mm quartz cuvettes at 25 °C. Samples (60 µM) were prepared in the appropriate solvents immediately prior to recording. Compound concentrations were determined using dry weight of the lyophilized material. The data are averages of 4–20 successive accumulations. Spectra were recorded in millidegree units, corrected for solvent contributions, and normalized to mean residue ellipticity $[\theta] = 100\psi/lcn$, where ψ is the signal in millidegrees, l is the path length in cm, c is the concentration in mM, and n is the number of residues.

Results and discussion

In order to test the folding capability of β-peptoids, the (*R*)- and (*S*)-1-phenylethyl groups were chosen as chiral *N*-alkyl side chains in β-alanine oligomers. Initially, the Michael addition-based method (Hamper et al., 1998) was applied in the synthesis of pentameric β-peptoids **10** and **11** as depicted in Scheme 1. The obtained yields were



Scheme 2. SPPS of compounds **13** and **14**. (a) 30% piperidine-DMF, 2 × 10 min; (b) **12** (5 equiv), TFFH (5 equiv), *i*Pr_2EtN (10 equiv), DMF, 2 h; (c) 30% piperidine-DMF, 2 × 15 min, 50 °C; (d) TFA-CH_2Cl_2 95:5, 50 min

not impressive (Fig. 2); however, preparative RP-HPLC furnished sufficient amounts of **10** and **11** as well as their truncated homologues to allow analytical HPLC, high-resolution mass spectrometry (HRMS), and CD spectroscopy to be performed. Independently, the sub-monomer approach has been optimized by Arvidsson and co-workers to afford good yields by performing the Michael addition step in water–tetrahydrofuran using a Tentagel resin.

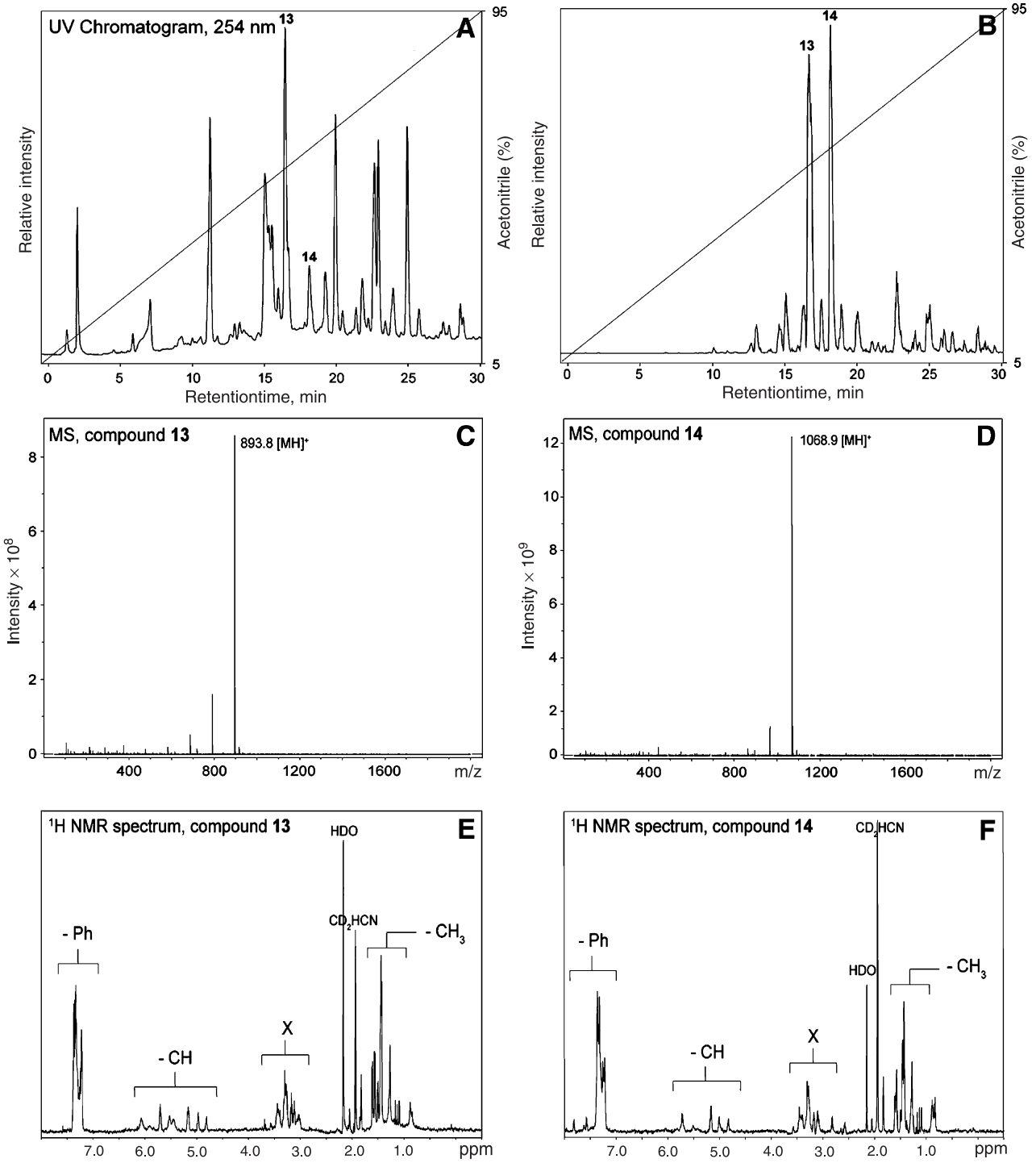


Fig. 3. HPLC-SPE-NMR/MS data for crude mixture of synthesis performed with PyBOP. (**A**) HPLC trace, UV detection. (**B**) HPLC trace, MS detection. (**C**) ESI-MS of compound **13**. (**D**) ESI-MS of compound **14**. (**E**) ^{1}H NMR spectrum of compound **13** obtained in the HPLC-SPE-NMR mode. (**F**) ^{1}H NMR of compound **14** obtained in the HPLC-SPE-NMR mode

This modified procedure resulted in significantly higher conversions and consequently more homogeneous crude products (Norgren et al., 2006).

Our approach to circumvent the initial inefficiency of the Michael addition approach was to use the Fmoc-protected building block **12** in solid-phase peptide synthesis (SPPS), which afforded the pentamer **13** and the hexamer **14** in yields suitable for initial evaluation. The SPPS approach on Rink amide resin employing PyBOP as the coupling reagent furnished a crude mixture containing **13** and **14** in a ratio of 3:1. While SPPS with repeated couplings using PyBOP afforded a similar mixture, the use of TFFH (Carpino and El-Faham, 1995) as the coupling reagent and Fmoc deprotection at elevated temperature furnished **14** in 10% isolated yield (Scheme 2). These cleavage mixtures were conveniently analyzed by HPLC-SPE-NMR, a novel hyphenated technique for ^{1}H NMR analysis of mixtures (Jaroszewski, 2005), prior to preparative-scale separation (Fig. 3). HPLC-SPE-NMR is thus a convenient means of characterization of crude reaction products and optimization of synthesis of peptidomimetics. It should be pointed out that a similarly low Fmoc SPPS efficiency has previously been encountered in preparation of α-peptoids due to the steric congestion of the secondary amino group (Fara et al., 2006). Possible improvements by microwave-assisted (Kappe, 2004) submonomer Michael additions or peptide couplings (Fara et al., 2006; Murray and Gellman, 2005; Olivos et al., 2002), as well as methodologies involving N-alkylation on solid phase (Olsen et al., 2005), are currently under investigation in our laboratory and will be reported in due course.

CD spectra in TFE of β-peptoids phenylacetylated at the N-terminal showed mirror-image CD curves for compounds containing side chains of opposite chirality

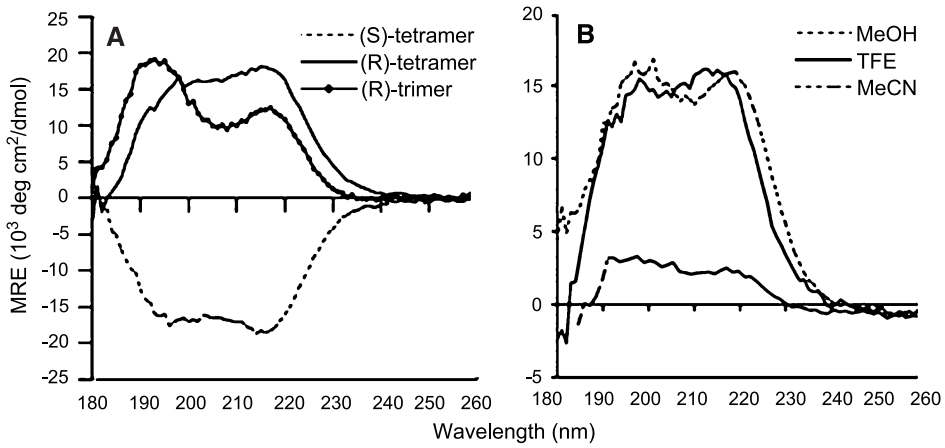


Fig. 4. (**A**) CD spectra of the β-peptoid homologues $PhCH_2CO$-(β-*N*spe)$_4$-NH_2, $PhCH_2CO$-(β-*N*rpe)$_4$-NH_2, and $PhCH_2CO$-(β-*N*rpe)$_3$-NH_2 obtained in TFE. (**B**) CD spectra of **10** in *Me*OH, TFE, and *Me*CN. The spectra represent averages of 4 successive accumulations. β-*N*spe = (*S*)-*N*-(1-phenylethyl)-β-alanine. β-*N*rpe, (*R*)-*N*-(1-phenylethyl)-β-alanine. MRE, Mean residue ellipticity

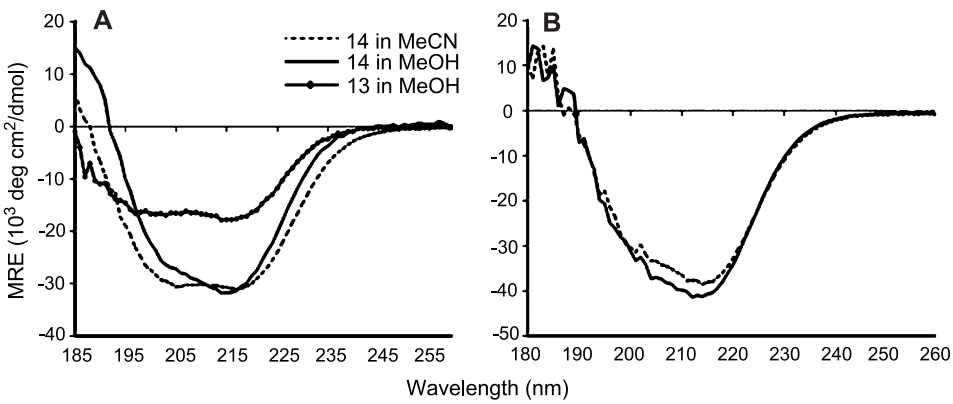


Fig. 5. (**A**) CD spectra of **13** and **14** in *Me*OH as well as **14** in *Me*CN; averages of 20 accumulations. (**B**) CD spectra of **14** in TFE at 20 °C (full) and 50 °C (dashed); averages of 4 accumulations

(Fig. 4A), as also observed for peptoid secondary structures (Sanborn et al., 2002; Wu et al., 2001a, b). The CD spectrum obtained in *Me*OH resembled that in TFE, whereas the spectrum obtained in *Me*CN was considerably less intense (Fig. 4B), which would indicate collapse of a given secondary structure in this aprotic solvent.

The shapes of the CD curves of purified hexamer **14** in *Me*OH (Fig. 5A) and TFE (Fig. 5B) resembled those of the N-terminal acylated analogues, with a minimum around 215 nm and a zero-crossing around 192 nm, albeit with a higher intensity. The spectrum of the shorter oligomer (**13**) was considerably less intense per residue as compared to that of **14**, which is indicative of a higher degree of secondary structure in the latter case (Fig. 5A). Importantly, the N-phenylacetylated trimer gave rise to a differently shaped CD curve than the corresponding tetramer (Fig. 4A), suggesting that at least four residues are required to obtain the fingerprint CD curves with a minimum around 215 nm. Furthermore, the CD spectra showed only a weak temperature dependency upon heating (Fig. 4B), which parallels previous results with peptoid foldamers (Kirshenbaum et al., 1998). Finally, a comparison of the CD spectra in *Me*OH of **10** (N-acylated pentamer; Fig. 4B) and **13** (non-acylated pentamer; Fig. 5A) indicated that the N-terminal acylation slightly destabilizes the secondary structure.

Although no exact structural information is available for this β-peptoid design at present, our observation that the mean per-residue ellipticity is chain-length-dependent as well as solvent-dependent provides firm evidence of the presence of secondary structure in solution. The shapes of the CD curves shown herein resemble those recently published by Arvidsson et al. for (β-*N*spe)$_n$-OH oligomers, where a similar trend in solvent-dependency of CD spectra was observed (Norgren et al., 2006). However, no significant chain-length dependency of CD spectra intensity was reported for those analogues, in contrast to the (β-*N*spe)$_n$-NH_2 oligomers and the *N*-phenylacetylated analogues reported in the present work. Such difference is not unprecedented in the literature, as the presence of a helix macrodipole in peptides as well as in β^3-peptides (Hart et al., 2003) is known to play an important role for helix stability.

Conclusion

In summary, β-peptoids exhibiting CD behaviour resembling that of known foldamers have been prepared by solid-phase synthetic methods. The trend in mean residue ellipticity observed for these analogues is noteworthy, because β-peptoids are not only devoid of hydrogen bonds (which stabilize secondary structures of peptides and β-peptides), but the chiral side chains are separated by an additional methylene group as compared with peptoids, thus resulting in an additional rotatable bond in the backbone. We interpret these results as a proof of concept that the chiral β-peptoids represent a novel peptidomimetic foldamer design, which merits further structural investigation. Since foldamers have been shown to exhibit valuable biological activities (Kritzer et al., 2004; Sadowsky et al., 2005; Seurynck et al., 2005) including mimicry of natural host-defense peptides with non-hemolytic antimicrobial activity (Arvidsson et al., 2005; Hamuro et al., 1999; Patch and Barron, 2003; Porter et al., 2000), we envisage that chiral β-peptoids may likewise prove valuable as scaffolds in the design of biologically active ligands. We have already demonstrated that de novo designed oligomers of β-peptoid residues within a chimeric framework including α-amino acids can furnish potent (low μM), non-hemolytic, and protease-stable antimicrobials (Olsen et al., 2007).

Acknowledgements

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