Urea based Foldamers

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ABSTRACT

N,*N*'-linked oligoureas are a class of enantiopure, sequence-defined peptidomimetic oligomers without amino acids that form well-defined and predictable helical structures akin to the peptide α-helix. Oligourea based foldamers combine a number of features – such as synthetic accessibility, sequence modularity, and folding fidelity – that bode well for their use in a range of applications from medicinal chemistry to catalysis. Moreover, it was recently recognized that this synthetic helical backbone can be combined with regular peptides to generate helically folded peptide-oligourea hybrids that display additional features in terms of helix mimicry and protein-surface recognition properties. Here we provide detailed protocols for the preparation of requested monomers and for the synthesis and purification of homo oligoureas and peptide-oligourea hybrids.

CONTENTS

KEYWORDS

Foldamers, Oligourea, Peptide mimicry, Helix, Protein surface recognition, Protein-protein interactions, Chimeric peptide/oligourea helices, Solid-phase synthesis

1. INTRODUCTION

Advances in foldamer research have led to the discovery of a wide range of non-natural synthetic oligomers predisposed to adopt well-defined folded structures akin to those found in proteins and nucleic acids(Gellman, 1998; Goodman, Choi, Shandler, & DeGrado, 2007; Gilles Guichard & Huc, 2011; Horne & Grossmann, 2020; Johnson & Gellman, 2013; Seebach, Beck, & Bierbaum, 2004). These synthetic sequence-specific backbones inspired by biopolymers obey different folding and self-assembly rules and provide enticing opportunities to explore entirely new chemical spaces. Although the creation of synthetic folded architectures was initially largely driven by curiosity, with a focus on exploration and conformational analysis of new backbones, it quickly turned out that the ability to synthesize sequence-based oligomers that fold with high fidelity raised the possibility of creating molecules with defined functions. In particular, it has been shown that biopolymer mimicry with foldamers can provide unique tools to study biology and may lead to the elaboration of novel diagnostic/therapeutic agents (Azzarito, Long, Murphy, & Wilson, 2013; Checco & Gellman, 2016; Gopalakrishnan, Frolov, Knerr, Drury, & Valeur, 2016; Johnson & Gellman, 2013; Pasco, Dolain, & Guichard, 2017). For example, α/β-peptides which are faithful α-helix mimics have been developed as inhibitors of protein-protein interactions or as receptor ligands with improved pharmacological properties, increased resistance to proteolytic degradation and in some cases prolonged duration of action in vivo(Boersma et al., 2011; Checco et al., 2015; Cheloha, Maeda, Dean, Gardella, & Gellman, 2014; Horne et al., 2009; Johnson et al., 2014; Johnson & Gellman, 2013; Kritzer, Hodsdon, & Schepartz, 2005; Kritzer, Stephens, Guarracino, Reznik, & Schepartz, 2005; Lee et al., 2009; Liu, Cheloha, Watanabe, Gardella, & Gellman, 2018; Liu et al., 2019; Michel, Harker, Tirado-Rives, Jorgensen, & Schepartz, 2009). Furthermore, the ability to link sequence and folding within non-biological synthetic foldamers can be exploited to create architectures reaching the size and complexity of small proteins (tertiary and quaternary structures) (Horne & Grossmann, 2020; Pappas et al., 2020) or molecular strands that display molecular recognition properties and functions beyond those found in nature(Ferrand & Huc, 2018; Gan et al., 2017).

Besides aliphatic and aromatic oligoamide foldamers which have been extensively studied, a few other backbones that do not contain an amide linkage and show high folding propensity have emerged as potentially useful proteinomimetics. This is the case of aliphatic *N*,*N*'-linked urea oligomers(Burgess, Shin, & Linthicum, 1995) which were found to form surprisingly well-defined and stable helical secondary structures reminiscent of the α-helix(Lucile Fischer et al., 2010; Lucile Fischer & Guichard, 2010; Semetey, Rognan, et al., 2002). Chiral oligourea foldamers combine a number of unique and positive features – such as synthetic accessibility, sequence modularity, and high folding fidelity – that bode well for their use in a range of applications from medicinal chemistry to catalysis.

2. HELICAL STRUCTURES OF *N*,*N*'-LINKED OLIGOUREAS AND OLIGOUREA/PEPTIDE CHIMERAS

In solution and in the crystal, enantiopure aliphatic *N*,*N*'-linked oligoureas adopt a helical fold, termed a 2.5-helix, with 2.5 residues per helical turn and a pitch of 5.1 Å stabilized by intramolecular three-centered H-bonds closing 12- and 14-membered H-bonded pseudorings (Fig. 1)(G. W. Collie, K. Pulka-Ziach, & G. Guichard, 2016; Lucile Fischer et al., 2010; A. Violette et al., 2005).

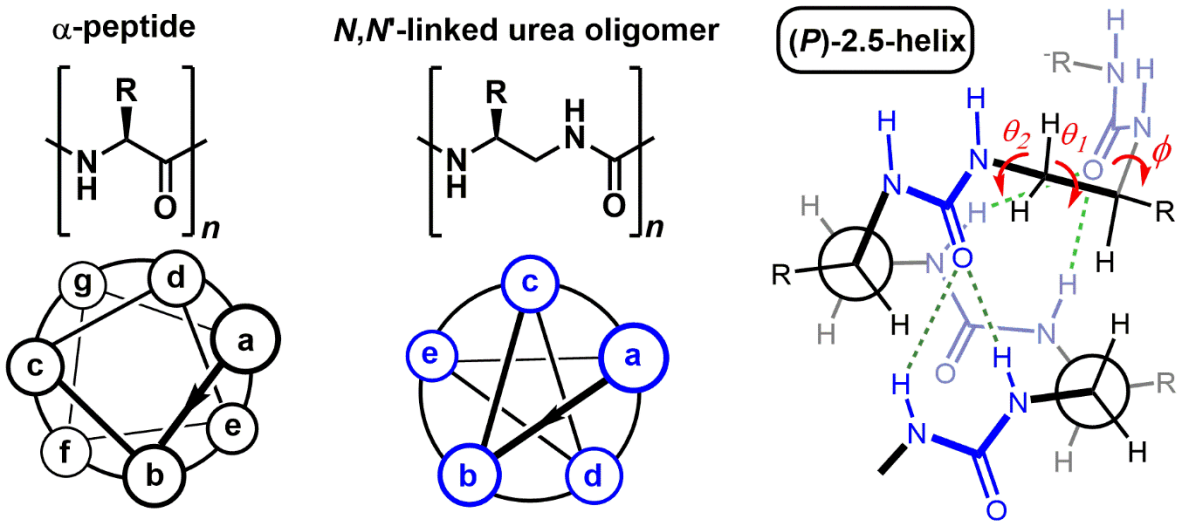


Fig. 1. Comparison of α-peptide $(Xaa)_n$ and *N*,*N*'-linked oligourea $(Xaa^u)_n$ backbones and representation of the 2.5-helical conformation of oligoureas showing the three center H-bond network. Ureido residues are denoted Xaa^u (X^u) by analogy to the three (one) letter code of α-amino acids

The helicity of urea oligomers is largely unaffected by the nature of the side chains in the sequence (in contrast to α-peptides), which makes these foldamers robust and tunable, allowing the different side chains (including polar ones) to be faithfully displayed at the helix surface. The chemical accessibility of the chiral ethylene diamine based monomers permits the synthesis of urea oligomers that bear, yet are not limited to, any of the 20 naturally occurring amino-acid side chains. Over the years, we and others have continuously expanded the repertoire of monomers(Douat-Casassus, Pulka, Claudon, & Guichard, 2012; Legrand et al., 2012; Y.-R. Nelli et al., 2012; Pendem, Douat, et al., 2013; Wechsel, Raftery, Cavagnat, Guichard, & Clayden, 2016) and improved oligomer synthesis by generalizing solution(Fremaux, Fischer, Arbogast, Kauffmann, & Guichard, 2011) and solid-phase methods(Burgess et al., 1995; Douat-Casassus et al., 2012; Gilles Guichard, Semetey, Rodriguez, & Briand, 2000; Kim, Bi, Paikoff, & Schultz, 1996) (see **section 4** "Main strategies to synthesize oligourea based foldamers").

Thorough exploration of the requirements for secondary structure formation has shown how variation of substitution patterns (*e.g. N*-pyrrolidine units (proline analogue)(Fremaux et al., 2011; Fremaux, Kauffmann, & Guichard, 2014), constrained *cis*-cyclohexyl diamine units(Pendem, Nelli, et al., 2013; Wechsel et al., 2016) and even bulkier 1,2-diaminobicyclo[2.2.2]octane bicyclic units(Legrand et al., 2012; Milbeo et al., 2020)), shifted side chain from $^{\beta}C$ to $^{\alpha}C$ with inversion of stereochemistry; Fig. 2A-C)(Pendem, Nelli, et al., 2013) and isosteric backbone modifications (*e.g.* insertion of γ-amino acid residues, carbamate or thiourea linkages; Fig. 2D-G)(Y. R. Nelli et al., 2015; Y. R. Nelli, Fischer, Collie, Kauffmann, & Guichard, 2013; Pendem, Nelli, et al., 2013) can be used to modulate helix properties and generate different spacing and orientation of side chains at the surface.

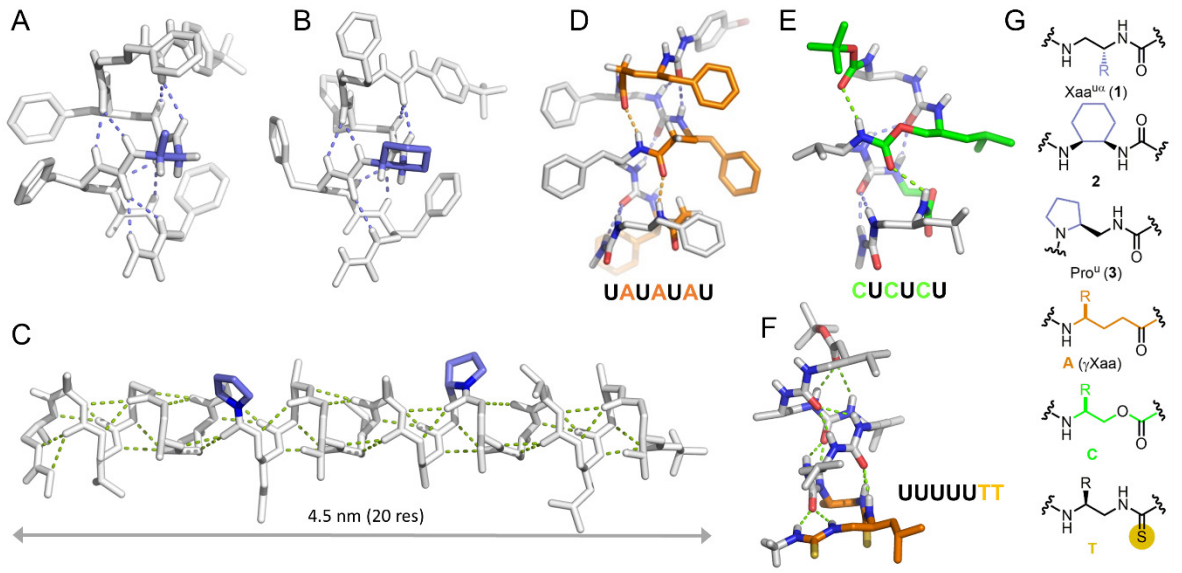


Fig. 2. Crystal structures of a pentaurea with a (A) shifted substitution pattern and a (B) constrained cyclohexyl ring at central residue;(Pendem, Douat, et al., 2013), (C) crystal structure of a 20-mer oligourea with two pyrrolidine units forming a long (45 Å) helical segment;(Fremaux et al., 2011); crystal structures of heterogeneous backbones consisting of (D) alternating urea/amide units, (E) alternating urea/carbamate units(Pendem, Nelli, et al., 2013) and (F) mixed urea/thiourea linkages.(Y. R. Nelli et al., 2015); (G) Example of monomers with non-canonical substitution patterns (Xaa$^{u\alpha}$(**1**) *cis*-cyclohexyl (**2**), Pro^{u} (**3**) and urea isosteric units (γ-amino acid (γXaa) **A**, carbamate **C** and thiourea **T** linkages) compatible with helix formation.

The similarities in screw sense, pitch and polarity between peptide α-helices and oligourea 2.5-helices also suggested to us that these two backbones could be combined, thus permitting key beneficial features of both species—such as natural epitope recognition of α-peptides and the innate helical stability of oligoureas—to be exploited in single chimeric constructs. We first investigated model chimeric α-peptide/oligourea and oligourea/α-peptide sequences to define the rules that govern helix formation in these sequences. The resulting chimeras (*i.e.* "block cofoldamers") with aliphatic oligoureas fused to short peptide segments (either at the N- or C-terminus or at both termini) were found to adopt a continuous helical structure in the solid state as well as in polar organic solvents, the peptide and oligourea helices being connected by a unique H-bond network(Fremaux et al., 2015; Mauran, Kauffmann, Odaert, & Guichard, 2016). The finding that oligourea foldamers can be interfaced with natural peptide helices and that the two helical forms do communicate within a single strand is

of particular significance for applications of foldamers in biology. Possible benefits of generating such chimeric helices include improved nucleation of α-helical peptide segments, increased resistance to proteolysis and prolonged activities *in vivo*.

3. APPLICATIONS OF UREA-BASED FOLDAMERS

The ability to synthesize diverse, sequence-specific oligoureas that fold with a high degree of predictability has stimulated the interest of our group and others to endow urea-based foldamers with function. Examples of applications reported for urea-based foldamers range from bioactive peptide mimics(Fremaux, Venin, Mauran, Zimmer, Guichard, et al., 2019) including modulators of protein-protein interactions(Cussol et al., 2021) to quaternary structures(Collie et al., 2015) and from electron transfer(Pulka-Ziach & Sęk, 2017) to catalysis(Bécart et al., 2017). Some of these applications are discussed in more detail below.

3.1 Cationic amphiphilic foldamers as host-defense peptide mimics and for the delivery of nucleic acids

Short chain oligoureas designed to mimic globally amphiphilic cationic α-helical host-defense peptides have been found to be active against a range of Gram-negative and Gram-positive bacteria(Claudon et al., 2010; Aude Violette et al., 2006). These oligoureas which display a high helical content in the vicinity of phospholipid membranes are thought to be active by a mechanism involving permeabilization of the bacterial membrane. One such foldamer was found to be active against bacterial forms of *Bacillus anthracis* encountered *in vivo* (*i.e.*, germinating spores, encapsulated and non-encapsulated bacilli) and to exert partial protection in cutaneous and inhalational models of infection with *B. anthracis*(Teyssières et al., 2016).

Sequence variations such as pH-responsive side chains and dimerization were introduced to convert these antimicrobial foldamers into gene transfection agents. Bioreducible helical cell penetrating foldamers (CPFs) with high capacity to assemble with plasmid DNA and to deliver nucleic acids into the

cell were finally obtained by thiol-mediated dimerization of a short (8-mer) amphipathic helical oligourea bearing His and Arg side chains. The best compound in this series was found to compare favorably in terms of transfection efficiency with LAH4, a His-rich peptide CPP with high transfection ability(Douat et al., 2015). New CPF sequences with activity in culture medium containing up to 50% of serum as well as sequences for siRNA delivery were reported recently(Bornerie, Brion, Guichard, Kichler, & Douat, 2021; Douat, Bornerie, Antunes, Guichard, & Kichler, 2019).

3.2 Nanostructures by self-assembly of amphiphilic water soluble oligoureas

A recent extension of this early work is the design of amphiphilic water-soluble foldamer sequences for the precise construction of nanometer scale assemblies mimicking protein quaternary structures(Gavin William Collie, Karolina Pulka-Ziach, & Gilles Guichard, 2016; Collie et al., 2015; Caterina M. Lombardo et al., 2016; Yoo, Collie, Mauran, & Guichard, 2020). We have shown that it is possible to orient the assembly process towards compact (bundles) or extended nanostructures (fibrils, nanotubes) in aqueous conditions by sequence manipulation and redistribution of charged side chains (Fig. 3B). Importantly, we found that compact nanostructures obtained by the assembly of designed amphiphilic oligourea helices possess isolated internal cavities large enough to host guest molecules(Collie et al., 2017).

3.3 Chimeric helices as inhibitors of protein-protein interactions and as receptor ligands

The structural similarity between oligourea and peptide helices suggests that the introduction of oligourea inserts into bioactive peptides could be used to generate effective inhibitors of protein-protein interactions or receptor ligands with a reduced peptide character (Fig. 3C). However, one difficulty associated with the use of foldamers as α-helix mimics is to select and arrange key side chains on the surface of the foldamer so as to maintain key contacts with the target. By selecting ubiquitin ligase MDM2, vitamin D receptor and histone chaperon ASF1 as targets, we have designed peptide/oligourea chimeras that retain affinity for their protein target while showing increased resistance to proteolysis(Cussol et al., 2021; Mbianda et al., 2020). X-ray structure analysis of several

of these peptide–oligourea hybrids bound to their respective protein targets confirms the high degree of α-helix mimicry that can be achieved with oligoureas and reveals general principles that should enable the design of more potent peptide-based inhibitors of protein–protein interactions.

Peptide-oligourea chimeras have also been used to generate mimics of Class-B GPCR ligands with increased resistance to proteolytic degradation. In particular, we have shown that oligourea foldamers are effective tools to improve the pharmaceutical properties of GLP-1, a 31-amino acid peptide hormone involved in metabolism and glycemic control. Our strategy consisted in replacing four consecutive amino acids of GLP-1 by three consecutive ureido residues by capitalizing on the structural resemblance of oligourea and α-peptide helices. The efficacy of the approach was demonstrated with three GLP-1-oligourea hybrids showing prolonged activity *in vivo*(Fremaux, Venin, Mauran, Zimmer, Guichard, et al., 2019). We have also shown that modified GLP-1 analogues with a single ureido residue replacement at position 2 exhibit antidiabetic properties and longer duration of action via selective enhancement of G protein-dependent cAMP signaling and altered GLP-1R trafficking(Fremaux, Venin, Mauran, Zimmer, Koensgen, et al., 2019).

3.4 Composite proteins containing foldamer segments

Encouraged by the similarity between peptide and oligourea helices, we have explored the chemical synthesis, folding, and function of composite proteins created by substituting one α-helical regions of a target protein by non-peptide helical segments (urea-based foldamers; Fig. 3D). Such composite proteins may prove useful to interrogate protein folding, address the role of individual folded segments and modulate protein function and properties. We moved a step towards the creation of such composite proteins by replacing the 10-residue long original α-helical segment in the Cys2His2 zinc finger 3 of transcription factor Egr1 (also known as Zif268) by an oligourea sequence bearing two appropriately spaced imidazole side chains for zinc coordination(C. M. Lombardo et al., 2019). Cys2His2 zinc fingers are recurrent protein motifs involved in specific duplex DNA recognition of about 30 residues, which consist of two antiparallel strands of β-sheet connected by a turn, packed against

an α-helix(Wolfe, Nekludova, & Pabo, 2000). This archetypal ββα fold is stabilized by a single zinc ion coordinated by a pair of cysteine residues located in the β-sheet and a pair of histidine residues located at the C-terminus of the α-helix in a tetrahedral geometry. We have shown by spectroscopic techniques and mass spectrometry analysis under native conditions that the ability of the peptide/oligourea hybrid to coordinate zinc ion was not affected by the introduction of the foldamer insert. Moreover, detailed NMR analysis provided evidence that the engineered zinc finger motif adopts a folded structure in which the native β-sheet arrangement of the peptide region and global arrangement of DNA binding side chains are preserved. Titration in the presence of Egr1 target DNA sequence also supported binding to GC bases as reported for the wild type motif.

3.5 Anion recognition, organocatalysis and electron transfer

Oligoureas typically mediate interactions through their side chains but the main chain ureas also display interesting molecular recognition properties. We have shown that the helical oligourea backbone is well preorganized to bind small guest molecules such as anions. ^{1}H NMR studies in various organic solvents including DMSO revealed that anions such as carboxylate bind at the positive end of the helix macrodipole without causing helix unfolding(Diemer, Fischer, Kauffmann, & Guichard, 2016). This property was exploited to induce screw-sense preference of achiral oligourea helices consisting of meso-cyclohexane-1,2-diamine monomers by selective formation of a 1:1 hydrogen-bonded complex with a chiral carboxylate anion(Wechsel et al., 2016; Wechsel, Žabka, Ward, & Clayden, 2018).

Taking inspiration from these studies, we envisioned that helical oligo(thio)urea foldamers could be designed to catalyze enantioselective C-C bond formation. The catalytic system we designed is composed of two modules, including an oligourea hexamer as the H-bonding chiral component (Fig. 3E) and a tertiary amine as a base component. We have shown that this system promotes the Michael reaction between enolizable carbonyl compounds and nitroolefins at remarkably low chiral catalyst loading with high enantioselectivity(Bécart et al., 2017). Structure-activity relationship studies revealed a strong correlation between the oligomer catalyst efficiency and its folding propensity. This

catalytic system can be readily optimized, as each component can be separately fine-tuned to increase reaction rates, selectivity, and other properties.

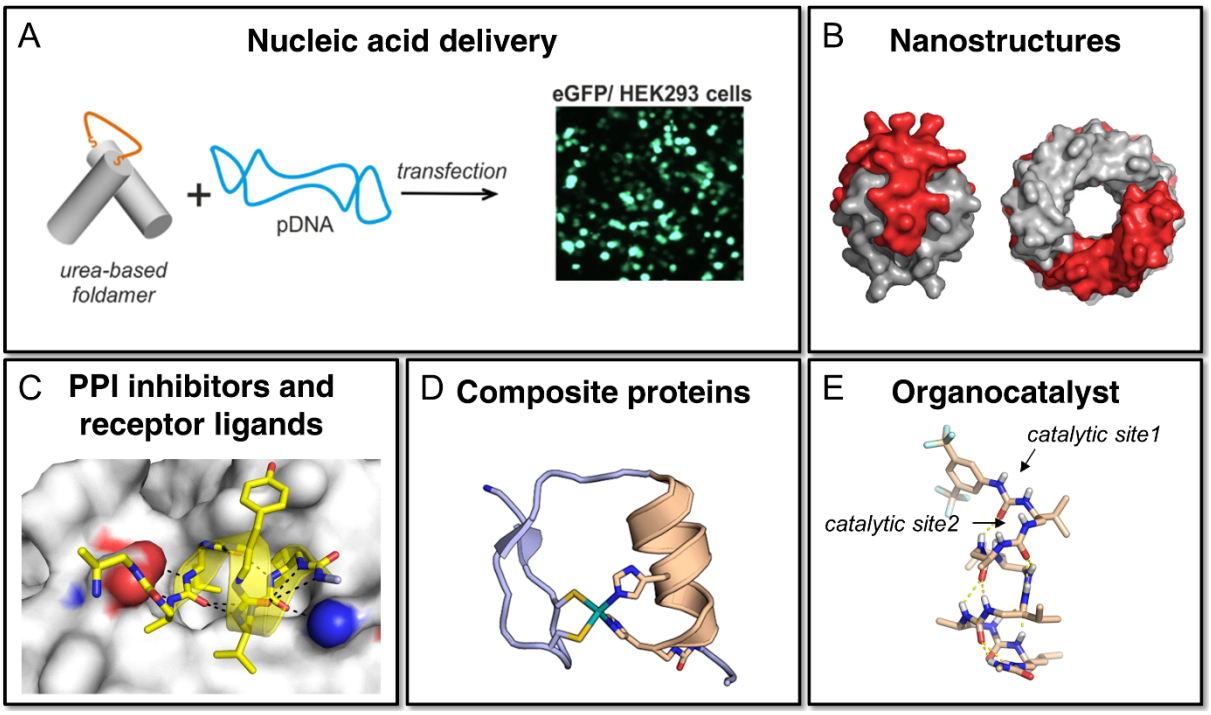


Fig. 3. Some Applications of urea-based foldamers. (A) pH-responsive dimeric urea-based foldamers to enhance cellular uptake of nucleic acids. (B) X-ray structures of a hexameric bundle (left) and a helical nanotube (right) formed by self-assembly of designed amphiphilic oligourea helices. (C) X-ray structure of a short oligourea helix (yellow) mimicking key residues of the steroid receptor coactivator SRC2-3 in complex with the vitamin D receptor. (D) NMR-derived low energy structure of a composite zinc finger containing a nonpeptide foldamer helical domain (peptide in light blue and oligourea in salmon). (E) Atomic structure of a hydrogen bonding oligourea-based chiral catalyst. *Sources* : (A) Reprinted with permission from . Copyright 2015 John Wiley & Sons Inc. (B) : Reprinted with permission from . Copyright 2015 Springer Nature. (C) Reprinted with permission from . Copyright 2021 John Wiley & Sons Inc. (D) Reprinted with permission from . Copyright 2019 American Chemical Society. (E) Reprinted with permission from . Copyright 2017 American Chemical Society.

4. MAIN STRATEGIES TO SYNTHESIZE OLIGOUREA BASED FOLDAMERS

Both solution and solid phase methods have been developed to synthesize enantiopure aliphatic *N*,*N*'-linked oligoureas. They are based on a sequential coupling of monoprotected chiral 1,2-diamine monomers activated as *O*-succinimidyl carbamates. With six positions for substituents (compare **1**, **2** and **3** in Fig. 2), these building blocks are incredibly diverse (for comparison α-amino acids contain three positions for substituents whereas β-amino acids have five). Monomers substituted at either the

$^{\alpha}$C or the $^{\beta}$C position can be conveniently prepared in a few steps from α-amino acids bearing any of the 20 naturally occurring amino-acid side chains (Fig. 4).

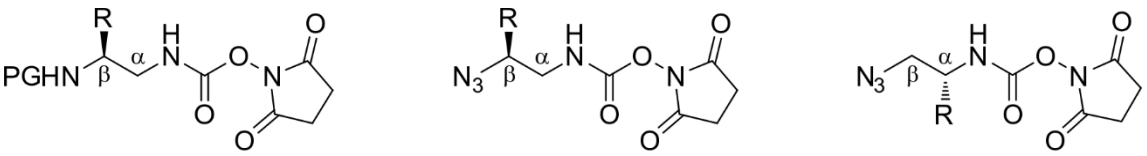


Fig. 4. Chemical structures of monoprotected chiral 1,2-diamine monomer and azide-type monomers, activated as *O*-succinimidyl carbamates. Ureido residues are denoted Xaa^{u} by analogy to the three-letter code of α-amino acids, and Xaa$^{u\alpha}$ related to shifted side chain from $^{\beta}$C to $^{\alpha}$C.

4.1 Synthesis of oligoureas in solution

The synthesis of short oligourea sequences can be efficiently performed in solution using Boc-protected monomers by iterative coupling and deprotection steps(G. Guichard et al., 1999). The reaction of the activated carbamate with a primary or secondary amine proceeds rapidly in presence of *Hünig*'s base at room temperature, and the only by-product formed – *i.e. N*-hydroxysuccinimide, is easily removed by aqueous workup. A short purification by flash chromatography or recrystallization affords the pure ureido derivative in good yields. The Boc-deprotection step is performed in the presence of TFA at 0°C, and the resulting trifluoroacetate salt which is frequently recovered after Et_2O precipitation is used without further purification. Oligomers bearing up to 9 urea residues were obtained in high yields and in a sequence controlled manner using this synthesis route. However, to overcome solubility issues, and increase the efficacy of synthesis in the case of longer and diverse oligourea sequences, other strategies, such as convergent fragment condensation or stepwise solid-phase synthesis techniques were developed.

4.2 Fragment condensation

Convergent coupling is a useful alternative to stepwise synthesis – whose efficacy starts to decrease beyond 10 residues due to solubility and/or purification issues – for accessing longer oligomers. Fragment condensation requires an oligourea segment to be activated as a succinimidyl carbamate at

one end, prior to coupling to the terminal amine of a second oligourea segment. However, the activation of oligoureas is impaired by the formation of a cyclic biuret resulting from the attack of the formed succinimidyl carbamate by the nearest urea NH (Fremaux et al., 2011; Semetey, Didierjean, Briand, Aubry, & Guichard, 2002). The introduction of a protecting group on this specific NH or the use of a *N*-alkylated terminal residue instead (*e.g.* a proline derived monomer) circumvented this synthetic issue allowing activated oligoureas to be readily prepared. The fragment condensation step was found to proceed in good to high yield when using a pyrrolidine ring at the segment junction and long helical oligoureas up to 20 residues were obtained by iterative segment couplings(Fremaux et al., 2011).

4.3 Solid phase synthesis of oligoureas

We initially used Fmoc-protected monomers and standard protocols adapted from solid-phase peptide synthesis (SPPS) to prepare oligoureas on solid support(Gilles Guichard et al., 2000). Although short (up to seven residues) oligourea sequences were obtained in good yields and purities, the coupling steps required long reaction times (several hours) and a large excess of monomers (at least double couplings with 3 equiv. of monomers). Next, we thought to employ microwave irradiation, a widespread technique in SPPS to increase coupling rates and accelerate synthesis. However, we found that the Fmoc-protected monomers activated as *O*-succinimidyl carbamates were not compatible with microwave irradiation leading to uncontrolled oligomerization on resin. The SPS of oligoureas using Boc-protected monomers and microwave assistance proved to be a much more robust strategy. Because the urea linkage formed by anchoring the first residue on MBHA-type resins was found to be sensitive to the acidic conditions required to cleave the Boc group, we introduced an isosteric γ^4-amino acid as the first residue to enable the formation of a stable amide bond onto the resin, prior to elongation of the oligourea sequence (Claudon et al., 2010). Excellent results in terms of synthesis efficiency (excess of monomers was substantially reduced and speed of synthesis was increased), and purity of crude products have been obtained. Yet the final HF cleavage from the resin renders this strategy not practical for routine syntheses in the laboratory.

To combine both the convenient use of standard TFA-labile resins and the efficiency of microwave-assisted coupling steps, we finally turned to monomers bearing azide as a masked amine for the SPS of oligoureas (Douat-Casassus et al., 2012). Thus far, the best conditions identified for on-resin reduction of azido-terminated oligoureas involve the use of trimethylphosphine with microwave assistance. This method is compatible with automation and parallel synthesis and is now routinely used in our laboratory to prepare oligoureas and related peptide-oligourea hybrids(Antunes, Douat, & Guichard, 2016; Cussol et al., 2021). The detailed synthetic procedures are reported in **section 5.3**.

5. SYNTHETIC PROTOCOLS

5.1 Materials

5.1.1 Reagents

5.1.1.1 Chemicals

Fmoc α-amino acids

Isobutyl chloroformate (IBCF)

N-Methylmorpholine (NMM)

Sodium borohydride ($NaBH_4$)

1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)

Trifluoroacetic acid (TFA)

Triphenylphosphine (PPh_3)

Phthalimide

Diisopropyl azodicarboxylate (DIAD)

Methanesulfonyl chloride (MsCl)

N,*N*'-disuccinimidyl carbonate (DSC)

328 Imidazole-1-sulfonyl azide hydrochloride ($N_3SO_2Im{\cdot}HCl$) (prepared as described in literature(Goddard-

329 Borger & Stick, 2007, 2011)

330 Copper (II) sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$)

331 Triethylamine (Et_3N)

332 Sodium azide (NaN_3)

333 Iodine (I_2)

334 Imidazole

335 Sodium chloride (NaCl)

336 Sodium bicarbonate ($NaHCO_3$)

337 Potassium carbonate (K_2CO_3)

338 Potassium hydrogen sulfate ($KHSO_4$)

339 Magnesium sulfate ($MgSO_4$)

340 *N*,*N*-diisopropylethylamine (DIEA)

341 Isopropyl isocyanate

342 Triisopropylsilane (TIS)

343 Piperidine

344 *N*,*N'*-diisopropylcarbodiimide (DIC)

345 Ethyl-2-cyano-2-(hydroxyimino)acetate (Oxyma)

346 Trimethylphosphine solution (1M in tetrahydrofuran).

347 *5.1.1.2 Resins*

348 Rink Amide MBHA resin (Novabiochem #8550030005, loading of 0.52 mmol/g).

5.1.1.3 Solvents

Dry DCM and THF are obtained by filtration through activated alumina using a dedicated purification system (MBRAUN SPS-800) and should be used immediately. The other solvents are purchased from commercial sources and used without any further purification. Specific solvent grades are recommended for the solid phase synthesis: *N,N*-dimethylformamide (DMF, Carlo Erba #P0343521, for peptide synthesis), dichloromethane (DCM, Carlo Erba #412622000, HPLC grade), 1,4-dioxane (Carlo Erba #338003) and acetonitrile (MeCN, Carlo Erba #412409, HPLC grade), water (mQ grade).

5.1.2 Equipment

Magnetic stirrer with temperature sensor (Heidolph), Balance (Mettler Toledo), UV lamp (Vilber), Rotary evaporator (Buchi or Heidolph), Bath sonicator (Bioblock Scientific), High vacuum pump (RZ6, Vacuubrand) with a glass vacuum manifold and a cold finger, Combiflash purification system (Teledyne ISCO), pH 0-11 test paper (Labomoderne), CEM Discover Bio (manual microwave peptide synthesizer, CEM Corporation), CEM Liberty Blue (automated microwave peptide synthesizer, CEM Corporation), CEM polypropylene SPS reaction vessel (25 mL, CEM Corporation), plastic syringes (1, 2 and 5 mL), micropipette (100 µL, Eppendorf), frit column plate (Roland vetter laborbedarf OHG), Eppendorf tube (1.5 mL), falcon centrifuge tube (15 mL), centrifuge (Mega star 600R, VWR), vortex mixer (Top mix FB 15024, Fisher scientific), freeze dryer (VirTis BenchTop Pro, SP Scientific) with a vacuum pump (RC6, Vacuubrand), flask shaker (SF1, Stuart). RP-HPLC (reverse phase high performance liquid chromatography) to check purity was carried out on a Macherey-Nagel Nucleodur 100-3 C_{18}ec column (3 µm, 100 x 4 mm) at a flow rate of 1 mL/min with a binary eluent system (solvent A: MilliQ water containing 0.1 % (v/v) TFA and solvent B: acetonitrile containing 0.1 % (v/v) TFA).

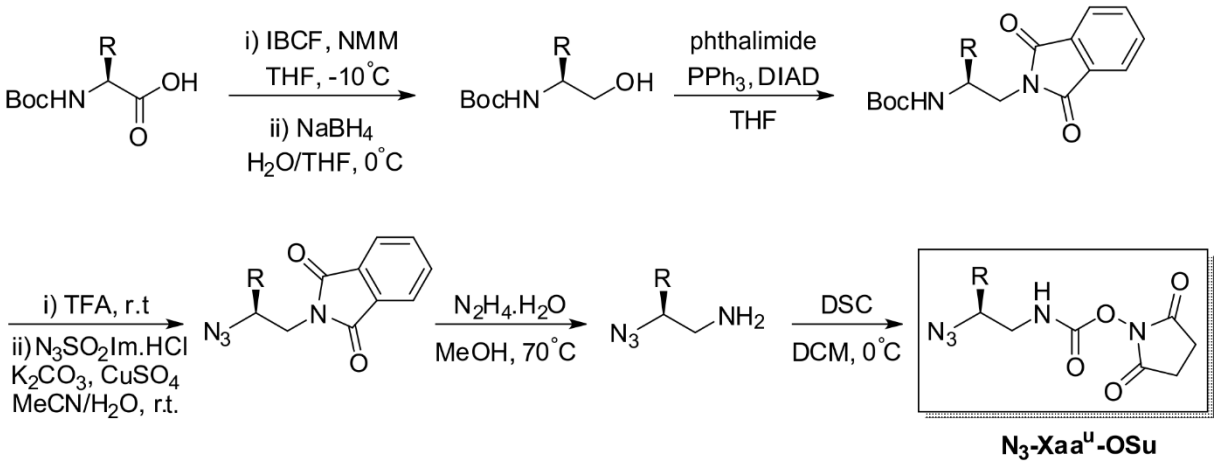
5.2 Monomer preparation

5.2.1 Azide type monomers bearing the side chain at the $^{\beta}$C position (N_3-Xaa^{u}-OSu)

The azide-type monomers bearing proteinogenic side chains are synthesized in 6 steps from the corresponding α-amino acids as starting material, as described in the general scheme (Fig. 5). Two

pathways have been developed depending on the nature of the side chain: path A is recommended for monomers without acid labile protecting group on the side chain; path B should be followed otherwise. These monomers are usually synthesized in our laboratory on a 20 to 30 mmol scale.

path A



path B

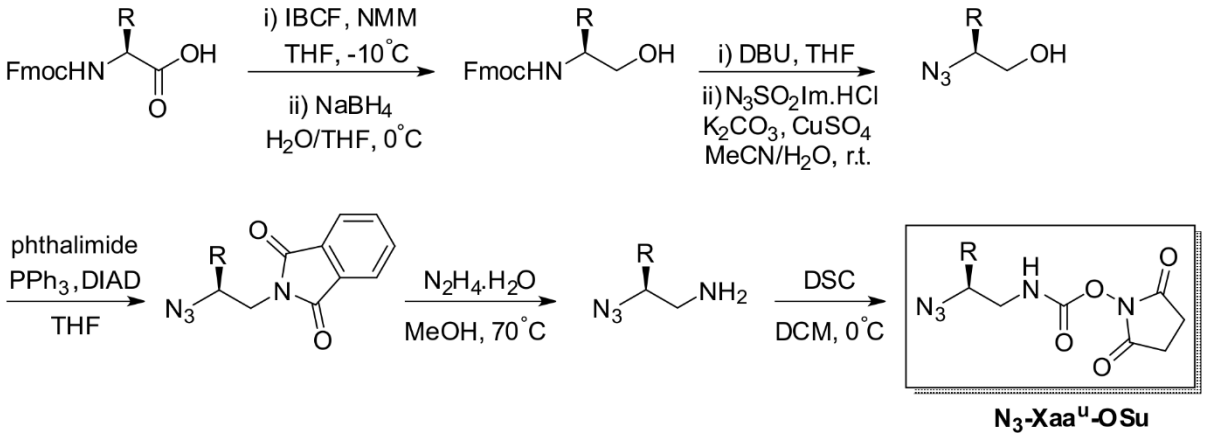


Fig. 5. General synthetic procedure for the preparation of azide type monomers bearing side chain at the $^{\beta}$C position, depending on the nature of the side chain.

5.2.1.1 Procedure for the preparation of N-protected β-amino alcohol

Timing: 8 h.

1. *N*-Boc (or Fmoc) α-amino acid was dissolved in anhydrous THF (at 0.5 M) under N_2 atmosphere and the solution was cooled down to -20 °C (in salt-ice bath). After addition of NMM (1.2 equiv.) and IBCF (1.2 equiv.), the mixture was stirred at -10 °C, for 30 min.
2. The resulting white suspension was then filtered off and washed with THF twice. The filtrate and washing solution were combined and added to a solution of $NaBH_4$ (1.2 equiv., 1.0 M) in water at 0 °C. The resulting mixture was stirred at room temperature for 2 h.
3. The reaction mixture was then quenched by adding a 1 M $KHSO_4$ solution. The THF was removed using rotary evaporation and the crude was dissolved in EtOAc. After phase separation, two additional extractions of the aqueous phase were performed with EtOAc. The organic layers were then combined and washed twice with a 1 M $KHSO_4$ solution, then twice with a saturated solution of $NaHCO_3$ followed by brine. The organic layer was then dried over $MgSO_4$ and concentrated under reduced pressure to give the corresponding alcohol.

Note: The addition of THF filtrate to aqueous $NaBH_4$ solution is accompanied by releasing of hydrogen gas.

5.2.1.2 Procedure for the conversion of the alcohol into the corresponding phthalimide

Timing: 12 -24 h.

Note: If you are following path B, the **section 5.2.1.3** should be performed before **5.2.1.2**.

4. Triphenylphosphine (1.2 equiv.) and phthalimide (1.2 equiv.) were dissolved in anhydrous THF (0.3 M), followed by dropwise addition of DIAD (1.2 equiv.) at 0°C under N_2 atmosphere. The mixture was stirred for 10 min.
5. *N*-Boc protected β-amino alcohol (in path A) (1.0 equiv.) or β-azido alcohol (in path B) (1.0 equiv.) was dissolved in anhydrous THF (0.5 M).
6. The THF solution in step 5 was added to the reaction mixture at 0°C and then the reaction was allowed to reach room temperature.

7. The reaction takes 2 h to overnight to go to completion (monitored by TLC). After completion, THF was evaporated under reduced pressure and the crude material was purified by flash chromatography.

5.2.1.3 Procedure for the introduction of the azido group

Timing: 1 day.

8. This step aims removing the protecting group on the amine. Please choose the suitable conditions according to the protecting group of the starting α-amino acid.

*Option **A** (removal of Boc group, path **A**)*: The *N*-Boc protected β-amino phthalimide (1.0 equiv.) was dissolved in pure TFA and reaction was stirred at room temperature for 1 hour. Crude was concentrated and directly used in the next step.

*Option **B** (removal of Fmoc group, path **B**):* The *N*-Fmoc protected β-amino alcohol (1.0 equiv.) was dissolved in THF (0.2 M) followed by addition of DBU (1.1 equiv.). After 1 hour, the solvent was concentrated and crude was directly used in the next step.

9. The free amine or the corresponding TFA ammonium salt previously obtained was dissolved in a 1:1 mixture of MeCN / H_2O (0.1 M). K_2CO_3 (≥ 1.5 equiv.), $CuSO_4{\cdot}5H_2O$ (0.01 equiv.) and $N_3SO_2Im{\cdot}HCl$ (1.2 equiv.) were added. Reaction was then stirred overnight at room temperature.

10. MeCN was evaporated and EtOAc was added to the remaining aqueous layer. The two layers were separated and the aqueous phase was extracted four times with EtOAc. Combined organic layers were washed with a 1 M $KHSO_4$ solution three times and one time with brine. The organic layer was then dried over $MgSO_4$ and concentrated under reduced pressure. The azide compound was then purified using flash column chromatography.

Caution: TFA is corrosive!!! Evaporation of TFA should be carried out on acid resistant equipment.

Note 1: The diazotransfer reagent Imidazole-1-sulfonyl azide hydrochloride ($N_3SO_2Im{\cdot}HCl$) is prepared according to the literature(Goddard-Borger & Stick, 2007). **Caution** should be taken when preparing and handling this reagent(Goddard-Borger & Stick, 2011).

Note 2: In step 9, the amount of K_2CO_3 should be adjusted to neutralize all the acid in the reaction mixture until a pH greater than 8 is reached.

5.2.1.4 Procedure for the removal of the phthalimide group

Timing: 8h.

11. To a solution of the phthalimide derivative in MeOH (0.1 M) was added hydrazine hydrate (3 equiv.). The reaction mixture was heated to reflux and heating was maintained for 4 h.

12. The white precipitate was filtered off and MeOH was evaporated using rotary evaporation. The remaining mixture was dissolved in EtOAc and precipitate was removed by filtration. The filtrate was extracted twice with a 1 M $KHSO_4$ solution from the organic phase, and the combined aqueous layers were washed twice with EtOAc.

13. Solid K_2CO_3 was then carefully added to the aqueous phase until pH 8 is reached. The basic amine is finally extracted with DCM four times, dried over $MgSO_4$ and concentrated under reduced pressure.

Caution: The monomers corresponding to Ala, Val and Leu side chains are volatile, do not concentrate to dryness.

5.2.1.5 Procedure for the preparation of succinimidyl azido-2-substituted-ethyl-carbamates

Timing: 5h.

14. Disuccinimidyl carbonate (1.1 equiv.) dissolved in anhydrous DCM (0.3 M) was cooled to 0°C. The amine obtained from the previous step was solubilized in anhydrous DCM (0.2 M) and added dropwise to the disuccinimidyl carbonate solution. Reaction was then stirred for 3 hours and allowed to reach room temperature.

15. After removal of DCM under rotary evaporation, the crude material was diluted in EtOAc, washed twice with a 1 M $KHSO_4$ solution and once with brine. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure.

16. The expected monomer was recovered as a solid after precipitation in a mixture Et_2O/pentane.

5.2.1.6 Example of monomer characterization

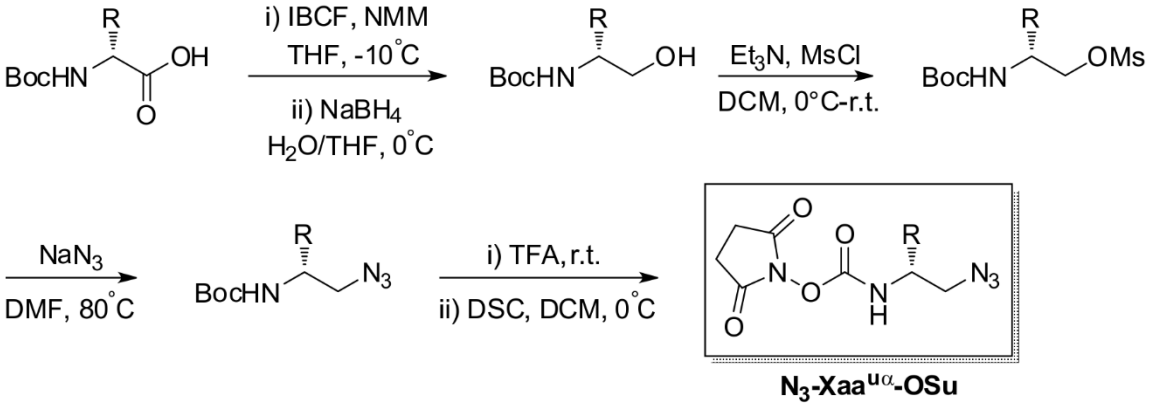
N_3-Leu^u-OSu : mp 92-94°C; $[\alpha]^{25}_D$ +49.3; t_R = 7.25 min (gradient 10 to 100% of MeCN in H_2O over 10 min); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.69 (t, J = 6.0, 1H), 3.65 – 3.56 (m, 1H), 3.46 (ddd, J = 13.9, 6.8, 3.7, 1H), 3.14 (ddd, J = 13.8, 8.2, 5.4, 1H), 2.83 (s, 4H), 1.87 – 1.70 (m, 1H), 1.51 (ddd, J = 14.6, 8.5, 6.2, 1H), 1.35 (ddd, J = 13.9, 8.1, 5.6, 1H), 0.96 (d, J = 6.6, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.97, 151.77, 60.12, 45.73, 40.74, 25.58, 25.00, 22.92, 22.19; HRMS (ESI-TOFMS) *m/z* cald for $C_{11}H_{17}N_5O_4Cl$ $[M+Cl]^-$ 318.0969, found 318.0978.

<Insert Table 1 here>

5.2.2 Azide type monomers bearing the side chain at the $^\alpha$C position (N_3-Xaa$^{u\alpha}$-OSu)

Two synthetic routes are used to prepare these α-substituted monomers, starting either from Boc-protected or Fmoc-protected amino acids (Fig. 6). Path A corresponds to the synthesis using Boc-protected amino acids which are recommended for monomer without acid labile protective groups on the side chain. Path B should be used otherwise. Both synthetic routes are robust, even if a flash column chromatography has to be used to remove impurities after the deprotection step with DBU for the path B, while a simple precipitation is required for the path A.

path A



path B

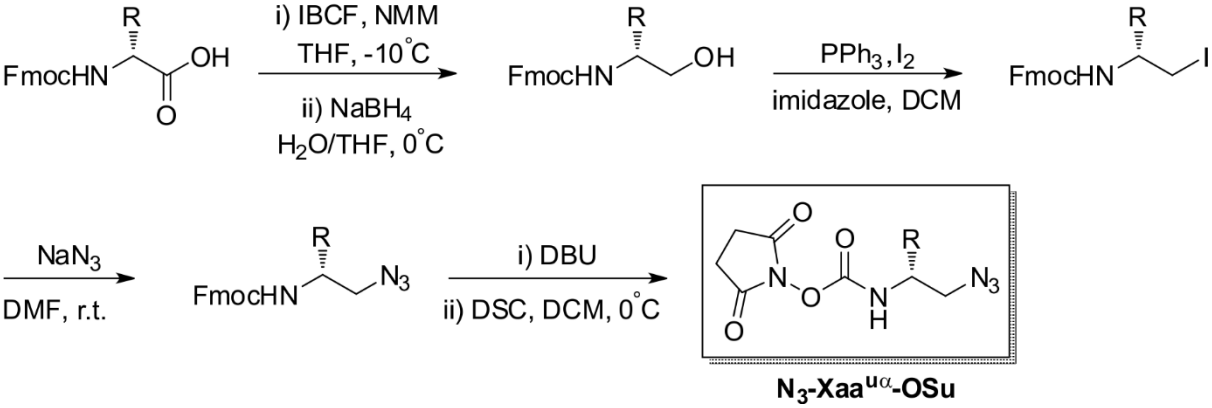


Fig. 6. General synthetic procedure for the preparation of azide type monomers bearing side chain at the $^{\alpha}$C position with inversion of stereochemistry, depending on the nature of the side chain.

It is worth noting that substituting monomers whose side chain has been shifted to the $^{\alpha}$C for canonical monomers (*i.e.* monomers bearing the side chain at the $^{\beta}$C) at discrete positions in an oligourea sequence is compatible with helix formation, provided that the two classes of monomers have opposite absolute configuration. Hence, the synthesis of Xaa$^{u\alpha}$ should start with α-amino acids of (*D*) configuration if the other Xaa^{u} monomers of the sequence have been prepared from α-amino acids of (*L*) configuration.

5.2.2.1 Procedure for the preparation of N-protected β-amino alcohol

Timing: 8 h

As previously described in **section 5.2.1.1**.

5.2.2.2 Procedure for the mesylation of N-Boc-β-amino alcohol (path A)

Timing: 24 h

17. The *N*-Boc protected β-amino alcohol (1.0 equiv.) was dissolved in DCM (0.5 M) and cooled to 0°C under Ar. Et_3N (2.0 equiv.) was added and the mixture was stirred for 15 min at 0°C. Then MsCl (1.1 equiv.) was added dropwise at 0°C and reaction mixture was allowed to reach room temperature and let to react overnight.

18. After removal of DCM by rotary evaporation, the crude material was diluted in EtOAc, washed successively twice with a 1 M $KHSO_4$ solution and once with brine, dried over $MgSO_4$ and concentrated under reduced pressure to afford the *N*-Boc protected β-amino methanesulfonate derivative.

5.2.2.3 Procedure for the preparation of tert-butyl (2-azido-1-substituted-ethyl)carbamate (Path A)

Timing: 8 h

19. The *N*-Boc protected β-amino methanesulfonate derivative (1.0 equiv.) was dissolved in DMF (0.4 M). After addition of NaN_3 (5.0 equiv.), reaction mixture was heated to 80°C and stirred for 5 h.

20. The reaction medium was then diluted with EtOAc, washed four times with water and once with brine. Organic layer was then dried over $MgSO_4$ and concentrated under reduced pressure. The *tert*-butyl (2-azido-1-substituted-ethyl)carbamate was obtained as a white solid after high vacuum drying.

5.2.2.4 Procedure for the preparation of succinimidyl (2-azido-1-substituted-ethyl)-carbamates (Path A)

Timing: 24 h

21. The *tert*-butyl (2-azido-1-substituted-ethyl)carbamate (1.0 equiv.) was dissolved in pure TFA and let to react 30 min.

22. TFA was removed using rotary evaporation, then the crude material was carefully neutralized using a saturated solution of $NaHCO_3$.

23. The amine compound was then extracted using EtOAc five times. The combined organic layers were dried over $MgSO_4$ and concentrated under reduced pressure to give the expected amine which was then directly used in the next step.

24. As previously described in **section 5.2.1.5**, the expected monomer was obtained as a solid.

Caution: TFA is corrosive!!! Evaporation of TFA should be carried out on acid resistant equipment.

5.2.2.5 Procedure for the preparation of N-Fmoc β-iodoamine derivative (Path B)

Timing: 8 h

25. I_2 (3.0 equiv.), PPh_3 (3.0 equiv.) and imidazole (5.0 equiv.) were dissolved in DCM (1.0 M) and flushed with N_2.

26. *N*-Fmoc protected β-amino alcohol was dissolved in DCM (1.0 M) and added to the reaction mixture. Reaction was then stirred for 5 h.

27. The reaction medium was then washed twice with an aqueous solution of $Na_2S_2O_3$ (0.5 M) and once with brine. After flash column chromatography, the *N*-Fmoc β-iodoamine derivative compound was recovered as a solid and used directly in the next step.

5.2.2.6 Procedure for preparation of N-Fmoc- amino azide compound (Path B)

Timing: 8 h

28. The *N*-Fmoc protected β-iodoamine (1.0 equiv.) was dissolved in DMF (0.4 M). After addition of NaN_3 (5.0 equiv.), reaction mixture was stirred for 5 h at room temperature.

29. The reaction medium was then diluted with EtOAc, washed four times with water and once with brine. Organic layer was then dried over $MgSO_4$ and concentrated under reduced pressure. No silica gel purification was required. The *N*-Fmoc protected amino azide was obtained as a solid after high vacuum drying.

5.2.2.7 Procedure for the preparation of succinimidyl 2-azido-1-substituted-ethyl-carbamates (Path B)

Timing: 24 h

30. (9*H*-fluoren-9-yl)methyl (2-azido-1-substituted-ethyl)carbamate was dissolved in EtOAc (0.1 M) and DBU (1.0 equiv.) was added. Reaction mixture was then stirred for 1 h.

31. The crude material was concentrated and purified by flash chromatography (100% EtOAc followed by DCM/MeOH/Et_3N 90:8:2). The expected amine was recovered and directly used in the next step.

32. Follow the description in **section 5.2.1.5** to give expected building blocks as a solid.

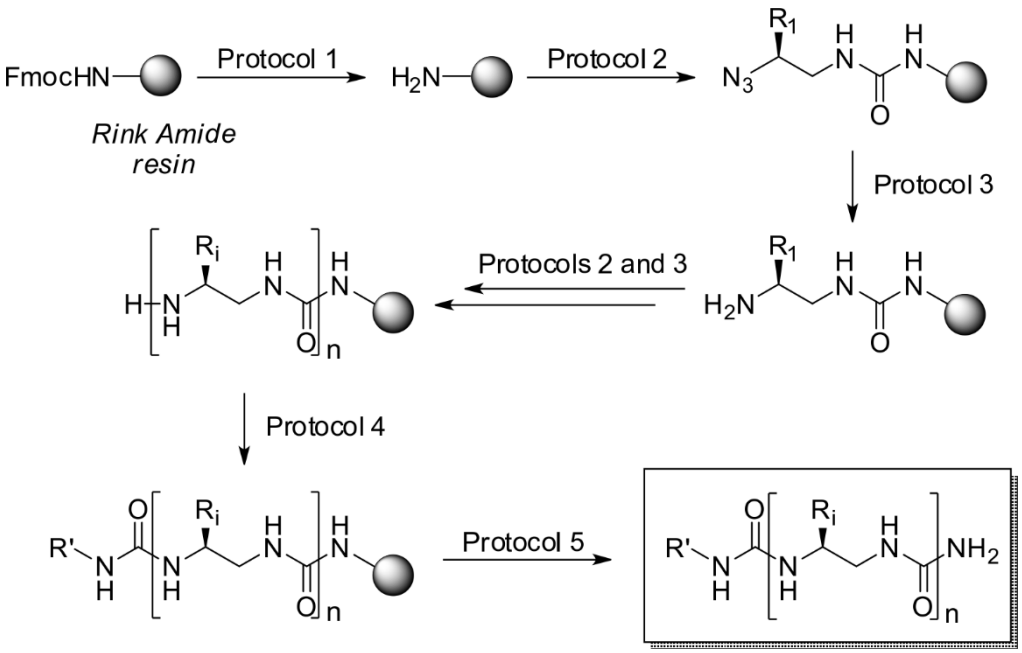
<Insert Table 2 here>

5.3 Synthesis of oligourea based foldamers on solid support

The methodology developed to synthesize water soluble oligourea based foldamers on solid support (homo-oligourea as well as hybrid peptide-oligourea or oligourea-peptide sequences) using azide-type monomers is reported here.

5.3.1 Solid phase synthesis of oligoureas

The synthesis of an amphiphilic oligourea foldamer designed to self-assemble in aqueous solution (**H1** : *i*Pr^{u}L^{u}E^{u}K^{u}L^{u}Y^{u}L^{u}E^{u}K^{u}L^{u}A^{u}L^{u}) (Collie et al., 2015) is exemplified on a 50 µmol scale (Fig. 7). Microwave assisted protocols (from 1 to 4) are carried out under N_2 atmosphere using CEM Discover Bio system equipped with a fiber optic sensor for temperature control. In our experience, the protocols can be applied up to 150 µmol scale with consistent efficiency.



553

554 **Fig. 7.** General synthetic procedure for SPS of oligourea foldamers.

555

556 *5.3.1.1 Protocol 1 : Fmoc deprotection to activate the resin*

557 **Timing**: 2 h 10 min

558 *1.* Add 96 mg of Rink Amide MBHA resin (50 µmol for 0.52 mmol·g^{-1} loading) to the CEM
559 polypropylene SPS reaction vessel.

560 *2.* Add 2 mL of DMF to the dried resin and wait 2 h for resin swelling.

561 *3.* Remove the solvent by filtration, add 3 mL of 20% piperidine/DMF (v/v), and then flush with
562 N_2.

563 *4.* Place the reaction vessel inside the microwave reactor and irradiate with microwaves (50 W
564 maximum power, ramp to 50°C, with 8 min hold time).

565 *5.* Take reaction vessel out of microwave reactor, filter the resin, and then wash the resin with
566 DMF (3 x 3 mL).

567 *5.3.1.2 Protocol 2 : Azide-type monomer coupling*

Timing: 1 h

6. Add 75 µmol of N_3-Xaa^u-OSu (1.5 equiv. relative to resin loading) to the 1.5 mL Eppendorf tube followed by adding 26 µL of DIEA (3 equiv. relative to resin loading) and 1.5 mL of DMF.

7. Sonicate and vortex the Eppendorf tube to solubilize N_3-Xaa^u-OSu well.

8. Transfer the N_3-Xaa^u-OSu solution to the reaction vessel, rinse the Eppendorf tube with 1.5 mL of DMF to transfer residual solution to the reaction vessel, and then flush the reaction vessel with N_2.

9. Place the reaction vessel inside the microwave reactor and irradiate with microwaves (25 W maximum power, ramp to 70°C, with 20 min hold time).

10. Take reaction vessel out of the microwave reactor, filter the resin, and then wash the resin successively with DMF (3 mL), DCM (3 x 3 mL) and DMF (2 x 3 mL).

11. Repeat Steps 6. to 10. once more.

Note: The completion of the coupling step can be assessed using a colorimetric test (chloranil test(Vojkovsky, 1995). The absence of free amino function should give a negative result with the resin beads being colorless.

5.3.1.3 Protocol 3 : Azide reduction

Timing: 50 min

12. Wash the resin with a 70% 1,4-dioxane/water solution (v/v) (2 x 3 mL).

13. Add 0.5 mL of 1 M trimethylphosphine in THF solution (10 equiv. relative to resin loading) to the resin followed by adding 2 mL of a 70% 1,4-dioxane/water solution (v/v), and then flush the reaction vessel with N_2.

14. Place the reaction vessel inside the microwave reactor and irradiate with microwaves (25 W maximum power, ramp to 70°C, with 15 min hold time).

15. Take reaction vessel out of microwave reactor, filter the resin, and then wash the resin with 70% 1,4-dioxane/water (v/v) (2 x 3 mL) and DMF (3 x 3 mL).

16. Repeat Steps 12. to 15. once more.

5.3.1.4 Protocol 4 (optional): isopropyl urea capping of the N-terminal amine

Timing: 40 min

17. Add 15 µL of isopropyl isocyanate (3 equiv. relative to resin loading) to the 1.5 mL Eppendorf tube followed by adding 44 µL of DIEA (5 equiv. relative to resin loading) and 1.5 mL of DMF.

18. Transfer the solution to the reaction vessel, rinse the Eppendorf tube with 1.5 mL of DMF to transfer residual solution to the reaction vessel, and then flush the reaction vessel with N_2.

19. Place the reaction vessel inside the microwave reactor and irradiate with microwaves (25 W maximum power, ramp to 70°C, with 10 min hold time).

20. Filter the resin and wash successively with DMF (3 mL), DCM (3 x 3 mL) and DMF (2 x 3 mL).

21. Repeat Steps 17. to 20. once more.

5.3.1.5 Protocol 5: Cleavage of the oligourea from the resin

Timing: 3 - 8 h

22. Transfer the resin into a 5 mL plastic syringe with a frit column plate and wash the resin with DCM (5 x 3 mL).

23. Close syringe with a cap, add 3 mL of the cleavage cocktail (TFA/TIS/H_2O=95/2.5/2.5 (v/v/v)) to the resin, and then gently close the syringe with its plunger.

24. Shake the syringe for 2h using mechanical shaker.

25. Filter the cleavage mixture to a round bottomed flask and rinse the resin with cleavage cocktail (2 x 0.5 mL).

613 *26.* Concentrate the combined filtrate in the round bottomed flask on a rotary evaporator with a
614 bath temperature of 40°C to obtain a viscous oil.

615 *27.* Add 5 mL of Et_2O into the oil to precipitate, and triturate the precipitate.

616 *28.* Transfer the mixture to a centrifugal tube and rinse the flask with Et_2O (2 x 3 mL) to transfer
617 residual mixture.

618 *29.* Centrifuge the mixture for 5 min with 4000 rpm and remove the supernatant.

619 *30.* Add 10 mL of Et_2O to disperse the precipitate, repeat Step 29. and dry the solid on a vacuum
620 manifold.

621 *31.* Dissolve the solid in 20% acetonitrile/water (v/v), and freeze-dry it.

622 **Note 1**: The cleavage time at Step 24. can vary from 2 hours to 6 hours depending on sequences.

623 **Note 2**: Solid-phase synthesis (assembly on the resin and cleavage) of **H1** was typically completed
624 within 28 working hours.

625 ***5.3.2 Solid phase synthesis of hybrid peptide-oligourea (PUH) or oligourea-peptide (UPH) sequences***

626 The synthesis of an amphiphilic peptide-oligourea block co-foldamer designed to self-assemble (**PUH** :
627 ALKEIAYAL^{u}E^{u}E^{u}L^{u}Q^{u}L^{u}) is exemplified here on a 50 µmol scale (Fig. 8). The elongation of the peptide
628 part of the hybrid sequences is accomplished under N_2 atmosphere using automated solid phase
629 peptide synthesizer with microwaves assistance (CEM Liberty Blue system) equipped with a fiber optic
630 sensor for temperature control. The peptide part can be incorporated regardless of its position in the
631 sequence (before (e.g. **UPH**), after (e.g. **PUH**) or between the oligourea part). For the oligourea
632 segment, the same protocols as described in the previous section are used. In our experience, the
633 protocols can be applied up to 150 µmol scale with consistent efficiency.

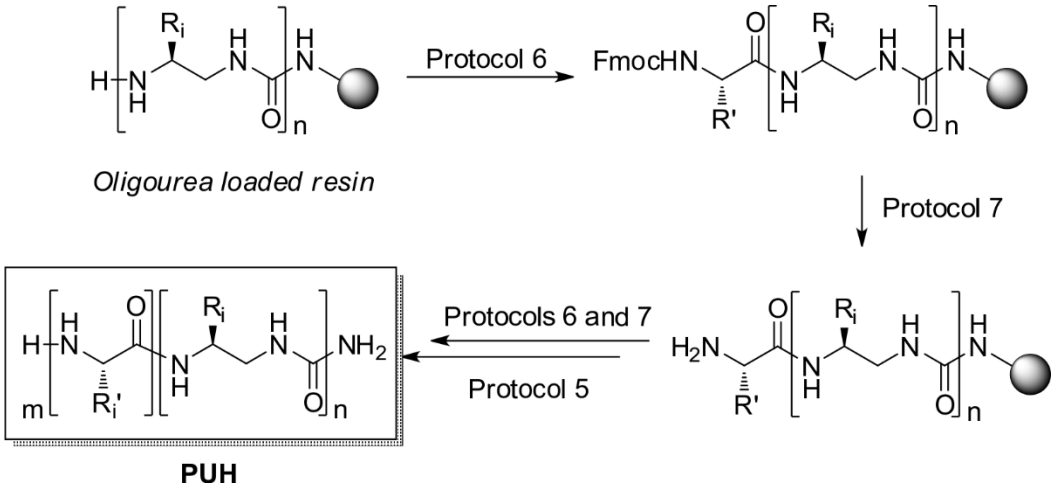


Fig. 8. General synthetic procedure for SPS of peptide-oligourea hybrid foldamers (**PUH**).

5.3.2.1 Protocol 6: Peptide coupling

***Timing**: 5 min*

32. Add 1.5 mL of 0.2 M Fmoc-α-Xaa-OH in DMF (6 equiv. relative to resin loading) to the resin followed by adding 0.6 mL of 0.5 M DIC in DMF (6 equiv. relative to resin loading) and 0.3 mL of 1 M Oxyma in DMF (6 equiv. relative to resin loading).

33. Irradiate with microwaves (170 W maximum power, ramp to 75°C, with 15 s hold time + 30 W maximum power, ramp to 90°C, with 110 s hold time)

34. Filter the resin and wash with DMF (2 mL).

35. Repeat steps 32. to 34. once more

5.3.2.2 Protocol 7: Fmoc deprotection

***Timing**: 5 min*

36. Add 3 mL of 20% piperidine/DMF (v/v) to the resin

37. Irradiate with microwaves (155 W maximum power, ramp to 75°C, with 15 s hold time + 30 W maximum power, ramp to 90°C, with 50 s hold time)

38. Filter the resin and wash with DMF (2 x 2 mL and 3 mL)

Note: Automated-assisted synthesis (assembly on the resin and cleavage) of **PUH** and **UPH** was typically completed within 16-20 working hours.

5.3.3 Purification and characterization of oligourea-based foldamers

Analytical RP-HPLC (Fig. 9A-B) and LC-MS (liquid chromatography-mass spectrometry) (Fig. 9C) are used to evaluate the purity of the crude product and confirm the identity of the expected compound after cleavage from the resin.

Purification via preparative RP-HPLC is performed on a Macherey-Nagel Nucleodur 100-5 C_{18}HTec column (5 µm, 250 x 21 mm) at a flow rate of 20 mL/min with the same binary eluent system as analytical RP-HPLC. Gradients and running time were optimized for each sequence. For example, **H1** is purified using a gradient of 30% to 70% solvent B over 20 min. In addition to the primary characterization using mass spectrometry, conformational analysis and structural characterization in solution or solid state can be accomplished using NMR, CD, crystallography and others.

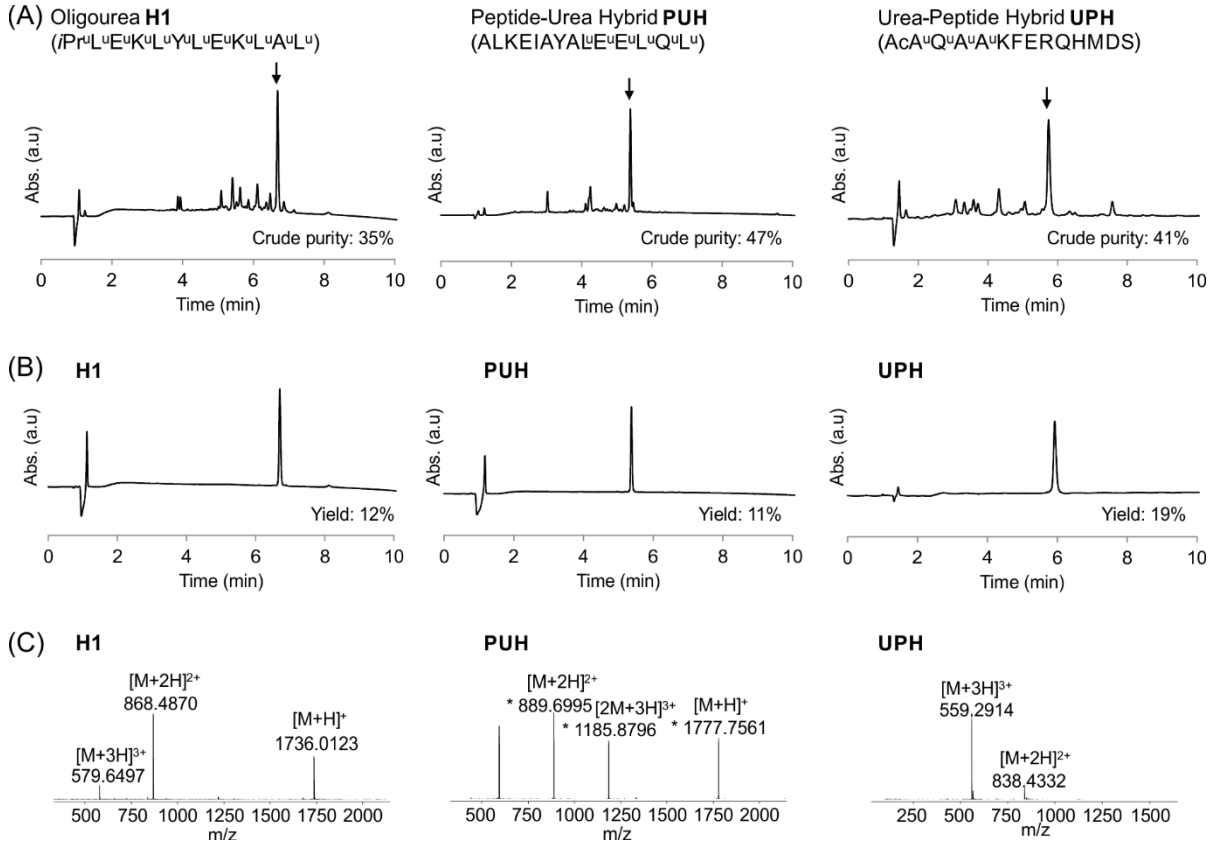


Fig. 9. Analytical HPLC profiles (A) before, and (B) after purification. (C) mass spectrometry analysis of foldamers **H1**, **PUH** and **UPH**. Crude purities are determined from peak area percentages. Arrows in (A) indicate peaks corresponding to the desired products. Gradients of 15% to 30% solvent B were applied for analytical HPLC of **UPH** while the standard gradient of 10% to 100% solvent B were applied for **H1** and **PUH**.

5.3.4 Troubleshooting

The above-mentioned protocols generally result in good to high crude purities as shown in Fig. 9A. However, in the specific case of oligourea-peptide hybrids (in contrast to peptide-oligourea hybrids), we observed that some sequences yield poor results with a low purity of the crude product. A more detailed analysis following intermediate cleavage at different steps of the synthesis reveals the formation of a byproduct after each azide reduction step, whose mass is higher than the expected amine by a value of 26 Da. The current hypothesis based on NMR and MS analysis is that a competitive cyclization occurs concomitantly with the reduction leading to a biuret formation, which ends further elongation of the sequence. Varying either the nature of the phosphine or the protecting group of the monomer (e. g. using Fmoc-protected monomer instead of azido-type) may help improving the overall

efficiency of the synthesis. Investigations are currently ongoing to better identify sequences that may cause problems, and develop a more robust protocol for such “difficult” sequences.

6. CONCLUDING REMARKS AND FUTURE DIRECTIONS

The access to a large repertoire of monomeric units whose molecular diversity extends beyond that of proteinogenic side chains, together with robust automated SPS methods have been instrumental in the development of oligourea foldamers and their applications (molecular recognition, catalysis, disruption of PPI, …). The combination of automation and microwave assistance considerably reduces the duration of the synthesis while enabling parallel synthesis and access to foldamer libraries. Moreover, the possibility to generate chimeric helices by combining peptide and oligourea backbones in a single strand further expands the range of applications of oligoureas as α-helix mimics. Although the reported protocols allow smooth syntheses of a large number of homooligourea and hybrid sequences, we identified some “difficult sequences” while preparing oligourea-peptide chimeras. Continuous improvement of the synthesis of hybrid foldamer-peptide sequences is justified by the recent finding that the replacement of a short α-helical segment within a bioactive peptide by an oligourea insert may yield peptide analogues with increased resistance to proteolytic degradation and prolonged duration of action *in vivo*. Finally, oligourea chemistry is versatile enough to be combined with other known peptide stabilization methods such as macrocyclization or lipidation to further increase helical content and potency of bioactive peptides.

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937

938 **TABLES**

939 **Table 1.** Azide type monomers bearing side chain at the $^{\beta}$C position prepared according to **Figure 4**:

940 details of pathway and overall yield.

Starting amino acid	Path[a]	Monomer	Yield[b] (%)
Boc-Ala-OH	**A**	N_3-Ala^{u}-OSu	21
Boc-Val-OH	**A**	N_3-Val^{u}-OSu	25
Boc-Leu-OH	**A**	N_3-Leu^{u}-OSu	28
Boc-Ile-OH	**A**	N_3-Ile^{u}-OSu	48
Boc-Phe-OH	**A**	N_3-Phe^{u}-OSu	31
Fmoc-Lys(Boc)-OH	**B**	N_3-Lys^{u}(Boc)-OSu	26
Fmoc-Glu(O^{t}Bu)-OH	**B**	N_3-Glu^{u}(O^{t}Bu)-OSu	37
Fmoc-Gln(Trt)-OH	**B**	N_3-Gln^{u}(Trt)-OSu	11
Fmoc-Thr(O^{t}Bu)-OH	**B**	N_3-Thr^{u}(O^{t}Bu)-OSu	16
Fmoc-Tyr(O^{t}Bu)-OH	**B**	N_3-Tyr^{u}(O^{t}Bu)-OSu	20
Fmoc-Trp(Boc)-OH	**B**	N_3-Trp^{u}(Boc)-OSu	17
Fmoc-Arg(Pbf)-OH	**B**	N_3-Arg^{u}(Pbf)-OSu	22

941 [a]See **Figure 4**. [b]The yield corresponds to the overall yield.
942

943 **Table 2.** Azide type monomers bearing side chain at the $^{\alpha}$C position prepared according to **Figure 5**:

944 details of pathway and overall yield.

Starting amino acid	Path[a]	Monomer	Yield[b] (%)
Boc-(D)-Ala-OH	**A**	N_3-Ala$^{u\alpha}$-OSu	28
Boc-(L)-Val-OH	**A**	N_3-Val$^{u\alpha}$-OSu	19
Boc-(L)-Lys(Alloc)-OH	**A**	N_3-Lys(Alloc)$^{u\alpha}$-OSu	37
Fmoc-(L)-Trp(Boc)-OH	**B**	N_3-Trp(Boc)$^{u\alpha}$-OSu	21
Fmoc-(L)-Arg(Pbf)-OH	**B**	N_3-Arg(Pbf)$^{u\alpha}$-OSu	11
Fmoc-(D)-Lys(Boc)-OH	**B**	N_3-Lys(Boc)$^{u\alpha}$-OSu	33

945 [a]See **Figure 5**. [b]The yield corresponds to the overall yield.