Synthesis of mono and bis[60]fullerene-based dicationic peptoids

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Keywords
Medicinal chemistry, antibiotics, amino acids, peptides, fullerenes, template synthesis

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Synthesis of Mono and Bis[60]fullerene Based Di-Cationic Peptoids


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Abstract: Increasing biological applications of fullerenyl amino acids and their derivatives encouraged us to synthesise [60]fullerenylidihydropyrrole peptides, prepared from the coupling of mono and bis [60]fullerenylidihydropyrrole carboxylic acids 4, 5 and 41 with presynthesised peptides 13, 16, 24, 28, 29 and 46. The resulting hydrophobic scaffolded di and tetra cationic derivatives were tested against Staphylococcus aureus NCTC 6571 and Escherichia coli NCTC 10418. The synthesis, characterisation and biological results are discussed in this paper.

Introduction

There are significant health care issues posed by multidrug resistant human pathogenic bacteria.[1,2] Of particular concern is the emergence of Gram-positive bacteria, e.g. Staphylococcus aureus and Enterococcus faecium, resistant to the glycopeptide antibiotic, vancomycin.[3-5] Recently, there emerged a new variation to the peptide class of antibiotics, exemplified by 1 (Figure 1).[6] These dicationic binaphthyl-templated linear peptides possess some similarity to structural aspects of vancomycin and could potentially act in a similar way but have added flexibility for interacting strongly with the changed peptido-glycan cell wall moiety[7] in vancomycin-resistant bacteria.[8] Also, there is significant antimicrobial activity in some biaryl-based cyclic β–hairpin cationic peptidomimetics.[9] While medicinal chemistry investigations of 1 as antibacterial agents made significant progress with the evolution of the amino acids required[10] and the better termini[6] early investigations into alternatives to the binaphthyl unit were restricted to macrocycles anchored by hydrophobic scaffolds based on 3,3′-amino acid linked 1,1′-binaphthyls,[11] carboazoles,[12-13] indoles,[14] benzenes[15] and benzo[b]thiophene.[16] The outcomes of these studies indicated that changing from the binaphthyl hydrophobic scaffold to smaller units severely decreased activity to the point of complete inactivity. Therefore, the hydrophobic bulk of the scaffold is critical in the structure-activity relationships of this class of compounds. Considering that all of these approaches investigated reducing the size of the hydrophobic anchoring unit, we thought that alternative hydrophobic units could match the space occupied by the binaphthyl unit. We have had considerable experience in the synthesis and use of amino acid substituted [60]fullerenes[17] and given the diameter of [60]fullerenes is 6.8 Å and the distance from distal points of the binaphthyl moiety is 8.3 Å, we considered the possibility of replacing the binaphthyl unit with a [60]fullerene. The synthesis of these molecules and their antibacterial activities are reported here.

The fullerene moiety is becoming increasingly prominent in biological applications[18], both as a ‘substituent’ but also as templates for the construction of macromolecules. Examples of mixed fullerene macrocycles include glycoconjugates[19] and fullerene amino acids[20] – it is the later which is of significance to this project. To date, such systems have been the focus of a variety of materials and medicinal chemistry projects and thus far take the form of fullerene pendants to amino acid oligomers with linkers, fullereryl-capped amino acids and the most prevalent, the incorporation of the fulleroproline moiety. In most instances, the C₆₀ unit is an anchor,
not representative of a true \( \alpha \)-amino acid side chain, with linker units providing space between the two moieties. The fullerenoamino acids provide examples closer to the incorporation of fullerenoamino acids in peptide chains. In our proposed target molecules, the fullerene moiety will be a true fullerene \( \alpha \)-amino acid and will be incorporated directly to the peptide chain. Additionally, the peptide chain will be rich in arginines and lysines, amino acids not necessarily prevalent with known fullerene peptides. However, recent examples of cationic fullerene conjugates\(^{[21]}\) suggest that the incorporation of protonated 'basic' side chains should pose no issues. Therefore, the combination of the hydrophobic \( \mathrm{C}_{60} \) unit and the protonated basic side chains has the potential for interesting solubility issues, possibly beneficial for the delivery of bioactive molecules.

**Figure 1.** Binaphthyl-anchored peptide antibiotic agents.

**Results and Discussion**
The target compounds contain \([60]\)fullerene anchoring cationic peptide chains and are exemplified by the general structure 2 (Figure 2). Significant SAR studies have been established with respect to the cationic amino acid side chains. Therefore, this study focused on a short linker between the fullerene moiety with a peptide of variable length, but restricted to either \( n = 0 \) or 1. The amino acid side chains will be either Lys or Arg, or combinations thereof – these would be tested as the dicationic salts, either chlorides or TFA salts. We have previously reported that despite the multiple stereogenic elements present in the molecule, different diastereomers of the same molecules had little difference in their antibacterial activity.\(^{[6]}\) Therefore, no ideal stereochemical combination exists, prompting us in this study to investigate a range of different stereochemical possibilities. The peptide chains are then capped with a variety of small terminal hydrophobic units – here, we targeted the ester and oxazole termini (R in structure 2, Figure 2).

**Figure 2.** Structure of the target \([60]\)fullerene based dicationic peptoids 2.
Chemistry

The synthesis of the target compounds began with the generation of the [60]fullerenyl amino acid scaffold 5, which was achieved using known chemistry to generate the acid 4. Therefore, the reaction of commercially available tert-butyl 2-((diphenylmethylenel)amino)acetate 3 with [60]fullerene under Bingel reaction conditions produced a [60]fullerenylidihydropyrrole tert-butyl ester derivative in 60% yield, which was treated with TFA to give the fullerenyl acid 4 (90%) (Scheme 1). This acid was reacted with tert-butylglycinate under EDCI/HOBt or HBTU peptide coupling conditions, followed by TFA treatment to give the acid compound 5 (48%). This second step adds the short linker to the molecule in the form of a Gly unit.

The next stage towards [60]fullerenyl-anchored peptide anti-bacterial molecules is the synthesis of the peptide moieties. In this approach, we wanted to make a number of derivatives to encompass a range of analogues without necessarily making systematic one-step changes, but instead, encompassing a range of derivatives. Scheme 2 outlines the synthesis of the tripeptide (e.g. 13 and 14) and tetrapeptide (e.g. 16) moieties for linking to the fullerenyl hydrophobic scaffold. Therefore, the free amine 6, containing an isopentylester was synthesised by reported procedures,[6] and coupled with Fmoc-D-Arg-OH (R = Pmc or Pbf) to produce dipeptides 7 (R = Pmc) and 8 (R = Pbf) (Scheme 2). These differ only in the protecting group and both were made due to ease of availability of starting materials at the time. Subsequent Fmoc deprotection and coupling with D or L-(Boc)-Lys-OH resulted in tripeptides 11 and 12, which upon Fmoc deprotection and gave the free amines 13 and 14, respectively. The reaction of 14 with Fmoc-Gly-OH produced the tetrapeptide 15, which was Fmoc deprotected to yield 16. This sequence produced two tripeptides (13 and 14) and a tetrapeptide (16) with a terminal isopentyl ester and a free amino substituent, ready for peptide coupling with a fullerenyl amino acid.

Scheme 1: Synthesis of the fullerenyl amino acid hydrophobic anchor: Reagents and conditions: (a) DBU, CBr₄, toluene, 6 h, rt, C₆₀, 60%. (b) TFA, CH₂Cl₂, rt, 3 h, 90%. (c) tert-butyl glycinate, CH₂Cl₂, EDCI/HOBt or HBTU, rt, 5 h, 48%.

The synthesis of the peptide moieties containing an oxazole terminus started with the synthesis of 17 by reported methods[22] (Scheme 2). This oxazole 17 then underwent a typical coupling with N,N'-diprotected forms of either L-Arg or D-Lys to produce 18 and 19, which were deprotected to the free amines 20 and 21, respectively. Reaction of these amines with Fmoc-D-Lys-(Boc)-OH gave 22 and 23, which were Fmoc deprotected giving 24 and 25, respectively - a final coupling with Fmoc-Gly-OH gave the tetrapeptides 26 and 27 and after Fmoc deprotection provided the free amines 28 and 29 for further coupling reactions to hydrophobic scaffolds.

The protected fullerenyl peptoids 30-33 were successfully synthesised by coupling [60]fullerenylidihydropyrrole acid 4 with the presynthesised peptides 16, 24, 28 and 29 under EDCI/HOBt peptide coupling conditions (Scheme 3). In a typical example, the tetrapeptide 16 was added dropwise to a solution of acid 4 and HOBt in CH₂Cl₂, which was sonicated for 15 minutes to aid solubility. EDCI was added at the same temperature and the reaction mixture was stirred for four hours at room temperature. As the reaction progressed, the turbid solution
transformed to a clear brown solution and upon workup, the first-in-class [60]fullerenyl anchored tetrapeptide 30 was isolated. Synthesis of compound 31 was possible by coupling with the oxazole terminated tripeptide 24, and the tetrapeptide version of 28, with the extra glycine amino acid in to the peptide chain, was responsible for producing 32. Coupling the acid 4 with peptide 29, which contained two Boc protected lysines with an oxazole terminus, yielded 33. This reaction used CHCl₃:CH₂Cl₂ (1:1) as a solvent due to better solubility of the diBoc protected lysine containing peptide whereas the synthesis of compounds 31 and 32 only required CH₂Cl₂ as the single solvent.

Analysis of the ¹H NMR of fullerenyl amino acid 34 indicated doublet of doublets centred at 7.36 ppm and 7.47 ppm, assigned to the para and meta protons respectively of the gem diphenyl substituents of the proline moiety, with the corresponding ortho protons assigned to the doublet at 8.03 ppm. The methylene of the glycine unit was assigned to the broad singlet at 3.26 ppm. Analysis of the HR ESI MS indicated a peak at m/z 1436.3822, assigned to the mono cationic species of 34, C₉₇H₄₉N₉O₆.

An additional antibiotic target molecule to consider is the [60]fullerene that is doubly substituted with peptides chains. Therefore, the previously unknown diacid 41 was synthesised from the reported bis-adduct 40,[17a] by using trimethyltin hydroxide[23] at 80 °C or borontribromide at -10 °C to room temperature - the former conditions resulted in a better yield of 65% compared to the latter of 50% (Scheme 4). As expected, attempts to synthesise [60]fullerene bis peptoid 42 using diacid 41 and peptide 13 under the standard coupling conditions used previously in this study failed, due to poor solubility. No progress in the coupling reaction was observed even after longer reaction times and an increase in reaction temperature to 60 °C. Therefore, a change in solvent to a 1:2 ratio of pyridine/chloroform was used generating a successful outcome with a 50% reaction yield of 42 (Scheme 4).

Unfortunately, attempted deprotection of 42 with TFA gave an insoluble product that could not be characterised. Analysis of the ¹H NMR spectra of 42 showed a singlet (18H) at 1.28 ppm, assigned to the two Boc groups attached to the lysine side chains. The presence of a single peak for these two groups indicates that both peptide side chains are in an equivalent environment and this is consistent with the expected C₅ symmetry of the trans-4 disubstituted [60]-fullerene.[24] The presence of 29 sp² resonances confirms this symmetry. Analysis of the HR ESI MS indicated a peak at m/z 2884.1675, assigned to the sodiated species of 42, C₁₇₄H₁₆₄N₁₆O₂₅S₂Na.
Scheme 2: Reagents and conditions: (a) Fmoc-D-arg-(Pmc/Pbf)-OH, Fmoc-L-arg-(Pmc)-OH or Fmoc-D-lys(Boc)-OH, EDCI, HOBT, CH₂Cl₂, rt, 4 h, 66% for 7, 75% for 8, 65% for 18 and 68% for 19; (b) piperidine, CH₃CN, rt, 4 h, 77-88%; (c) Fmoc-D or L-Lys-(Boc)-OH, EDCI, HOBT, CH₂Cl₂, rt, 4 h, 79% for 11, 80% for 12, 86% for 22 and 72% for 23; (d) Same conditions as (b) 88% for 13, 77% for 14, 84% for 24, and 85% for 25; (e) Fmoc-Gly-OH, EDCI, HOBT, CH₂Cl₂, rt, 4 h, 75% for 15, 71% for 26 and 82% for 27; (f) Same conditions as (b) 80% for 16, 88% for 28, and 86% for 29.
Scheme 3: Synthesis of C$_{60}$ fullerenyl anchored amino acid oxazoles and isopentyl esters.
Reagents and conditions: (a) EDCI, HOBt, CH$_2$Cl$_2$, rt, 4 h; (b) TFA/CH$_2$Cl$_2$ (1:1), rt, 16 h; (c) 1 M HCl/Et$_2$O, 0-rt 0.5 h.

Scheme 4: Reagents and conditions: (a) (tert-butoxycarbonyl)glycine, THF, DMAP, CH$_2$Cl$_2$, rt, 40 h, 80%. (b) TFA, CH$_2$Cl$_2$, rt, 3 h, 90%. (c) CH$_2$Cl$_2$, diphenylmethanimine, 24 h, rt, 75%. (d) DBU, CBr$_4$, rt, Toluene, C$_{60}$, 6 h, 45%. (e) (CH$_3$)$_3$SnOH, DCE, 80 °C, 24 h, 65%. (f) BBr$_3$, CH$_2$Cl$_2$, -10 °C - rt, 18 h, 50%. (g) pyridine/chloroform (1:2), EDCI, HOBt, 8 h, 50%.

The synthesis of the fullerenyl peptide 50 was achieved using a modified route (Scheme 5). Initially, the dipeptide 46 was synthesised from intermediates 43 and 44 under standard coupling-deprotection conditions. The dipeptide 46 was then coupled to the fullerenyl amino acid 5 yielding 47 in a 67% yield. Subsequent deallylation followed by coupling to isopentyl-L-leucinate gave the tetrapeptide coupled [60]fullerenyl peptide 49 in 62% yield. Final side chain
deprotection under standard conditions followed by salt formation yielded the final product 50 in 70% yield.

Scheme 5: Reagents and conditions: (a) EDCI, HOBT, TEA, CH₂Cl₂, rt, 4 h, 85%; (b) TFA: CH₂Cl₂ (4:6), rt, 4 h, 90%; (c) HBTU, TEA, CH₂Cl₂, rt, 4 h, 67%; (d) (CH₃)₃SnOH, 1,2-DCE, 80 °C, 6 h, 84%; (e) isopentyl L-leucinate hydrochloride, EDCI, HOBT, TEA, CH₂Cl₂, rt, 4 h, 62%; (f) piperidine, CH₃CN, rt, 4 h, 72%; (g) 1 M HCl.Et₂O, 0 °C-rt, 0.5 h, 70%.

To improve the solubility of the [60]fullerene based peptides for antibacterial testing, it was necessary to use them as dicationic salts, which were prepared using either TFA and/or 1 M HCl in ether. Peptides 31, 32 and 49 were made as the HCl salts 35, 36 and 50, respectively by treating with TFA/CH₂Cl₂ (1:1) to deprotect the Pmc/Pbf and N-Boc functional groups then after removing the solvents under reducing pressure, the residue was resuspended in a minimal volume of CH₂Cl₂ and treated with an excess amount of 1 M HCl in ether to obtain the targeted HCl salts. The peptides 30 and 33 were made as their TFA salts 34 and 37 by treating with TFA/CH₂Cl₂ (1:1) to deprotect the Pmc/Pbf and N-Boc groups and at the same time forming the TFA salts. All salts were characterised by HRMS, and additionally, 34, 35 and 36 returned ¹H NMR spectra of good quality. Unfortunately, due to limited solubility of these salts, we were unable to obtain adequate ¹³C NMR spectra.
Antimicrobial Testing

As an initial screen for biological activity, the [60]fullerenyl peptides 30-33, 42, 47 and 49 and
the dicationic salts 34-37, and 50 were subjected to in vitro antibacterial testing. These twelve
[60]fullerene derivatives were tested using the broth microdilution method against
Staphylococcus aureus NCTC 6571 and Escherichia coli NCTC 10418. MICs for antibiotic
positive controls tested simultaneously were 4 µg/mL for kanamycin and 2 µg/mL for
chloramphenicol for E. coli and for S. aureus were 4 µg/mL for kanamycin and 1 µg/mL for
vancomycin. Unfortunately all fullerennyl-based compounds were inactive, even at higher
concentrations, with minimum inhibitory concentrations (MICs) greater than 100 to 340
(µg/mL).

Conclusions

The synthesis of peptides anchored by [60]fullerenes was successful with a series of mono
substituted and disubstituted fullerene derivatives were synthesised. Although these
derivatives did not show any anti-bacterial activity, their synthesis has progressed the
development of the important field of fullerenyl amino acids and peptide derivatives, in
particular, their synthesis and isolation as peptide salts. This is highlighted by the reasonable
solubility of the deprotected compounds in DMSO/water and the relative ease of purification of
the protected fullerenyl peptides by column chromatography. Further, these fullerenyl amino
acids have a more rigid, and thus more defined, tethered structure than most other fullerenyl
peptides reported, which traditionally employ a flexible fullerene-peptide linker. These more
rigid properties could become increasingly important in the fields of medicinal chemistry or
materials science.

Experimental Section

1. General Synthetic Procedures

Protocol 1: Peptide coupling
To a solution of the acid in dichloromethane or chloroform (10 mL/0.10 mmol) at room
temperature was added EDCI/HBTU (1.2 equiv.), HOBT (1.2 equiv.), and the amine (1 equiv.).
If the amine was a hydrochloride salt, DIPEA (1.2 equiv.) or TEA (1.2 equiv.) was also added.
After stirring for 3-6 h, the solvent was removed under reduced pressure, and then the
resulting residue was subjected to silica gel column chromatography (1-4% MeOH/CH2Cl2 as
the eluent) to afford the coupled product.

Protocol 2: N-Fmoc deprotection
The Fmoc-protected amine was stirred in 1 equiv. of piperidine/acetonitrile (5 mL/0.10 mmol)
overnight at rt, unless otherwise stated. The solvent was removed under reduced pressure
and the crude product was purified by column chromatography, using 5% MeOH/CH2Cl2 to
yield the free amine.

Protocol 3: N-Boc, Pbf and Pmc deprotection
The N-Boc, Pbf or Pmc protected amine was stirred for 1 h (for Boc) or overnight (for Pbf and
Pmc) in 1:1 CH2Cl2/TFA (6 mL/0.10 mmol) solution at rt. The solvent was removed under reduced pressure
and the residue was resuspended in a minimal volume of CH2Cl2. The
solution was then treated with an excess of 1 M HCl/ether (2 mL/0.01 mmol) solution and the
solvent evaporated. The crude product was purified by precipitation from CH2Cl2, with hexane
and diethyl ether.

Protocol 4: Allyl and bis-1,3-benzyl ester hydrolysis
To a solution of the ester in 1,2-dichloroethane (10 mL/0.10 mmol) at rt was added
Sn(CH3)3OH (4 equiv.) and the solution was heated at 80 °C for 4 h, then 4 more equiv. of
Sn(CH3)3OH was added at the same temperature and the reaction was continued for further 4
h. The reaction mixture was evaporated under reduced pressure and the resulting residue was
taken up in dichloromethane (15-20 mL). The organic layer was washed with 5% HCl (3 x 10-
15 mL), brine (3 x 10 mL) and dried over Mg\textsubscript{2}SO\textsubscript{4} and evaporated under reduced pressure to yield corresponding acid.

**Supporting Information**

Full experimental procedures and spectral data, and copies of \textsuperscript{1}H and \textsuperscript{13}C NMR spectra. Procedures for antibacterial testing and table of outcomes for individual molecules.

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**Keywords:** [60] Fullerene/Amino Acid/Anti-Infectives/Cationic Peptoids


