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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*]fullerenyldihydropyrrole peptides, prepared from the coupling of mono and bis [*60*]fullerenyldihydropyrrole 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] carbazoles,[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 prominate in biological applications[18], both as a 'substituent' but also as templates for the construction of macromolecules. Examples of mixed fullerenyl macrocycles include glycoconjugates[19] and fullerenyl 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 pendents to amino acid oligomers with linkers, fullerenyl-capped amino acids and the most prevalent, the incorporation of the fulleroproline moiety. In most instances, the C_{60} unit is an anchor, and

not representative of a true α-amino acid side chain, with linker units providing space between the two moieties. The fullerenoprolines provide examples closer to the incorporation of fullerenoamino acids in peptide chains. In our proposed target molecules, the fullerene moiety will be a true fullerenyl α-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 fullerenyl peptides. However, recent examples of cationic fullerenyl conjugates[21] suggest that the incorporation of protonated 'basic' side chains should pose no issues. Therefore, the combination of the hydrophobic C_{60} unit and the protonated basic side chains has the potential for interesting solubility issues, possibly beneficial for the delivery of bioactive molecules.
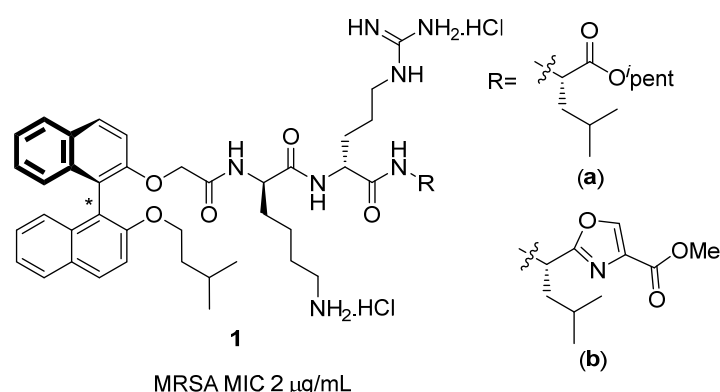

Figure 1. Binaphthyl-anchored peptide antibiotic agents.

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).
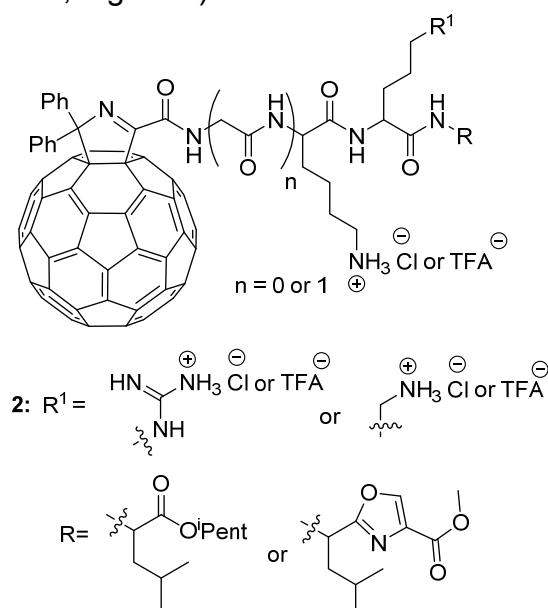

Figure 2. Structure of the target [60]fullerene based dicationic peptoids **2**.

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**. [17] Therefore, the reaction of commercially available *tert*-butyl 2-((diphenylmethylene)amino)acetate **3** with [*60*]fullerene under Bingel reaction conditions produced a [*60*]fullerenyldihydropyrrole *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 tripepetides (**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.
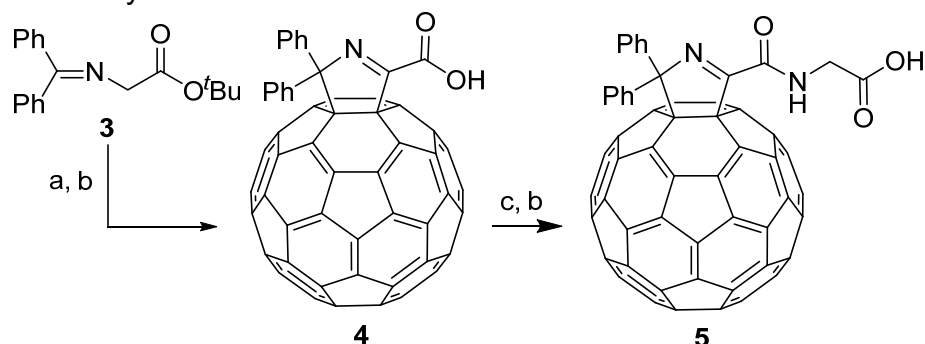
Scheme 1: Synthesis of the fullerenyl amino acid hydrophobic anchor: **Reagents and conditions:** (a) DBU, CBr$_4$, toluene, 6 h, rt, C$_{60}$, 60%. (b) TFA, CH$_2$Cl$_2$, rt, 3 h, 90%. (c) *tert*-butyl glycinate, CH$_2$Cl$_2$, EDCI/HOBt or HBTU, rt, 5 h, 48%.
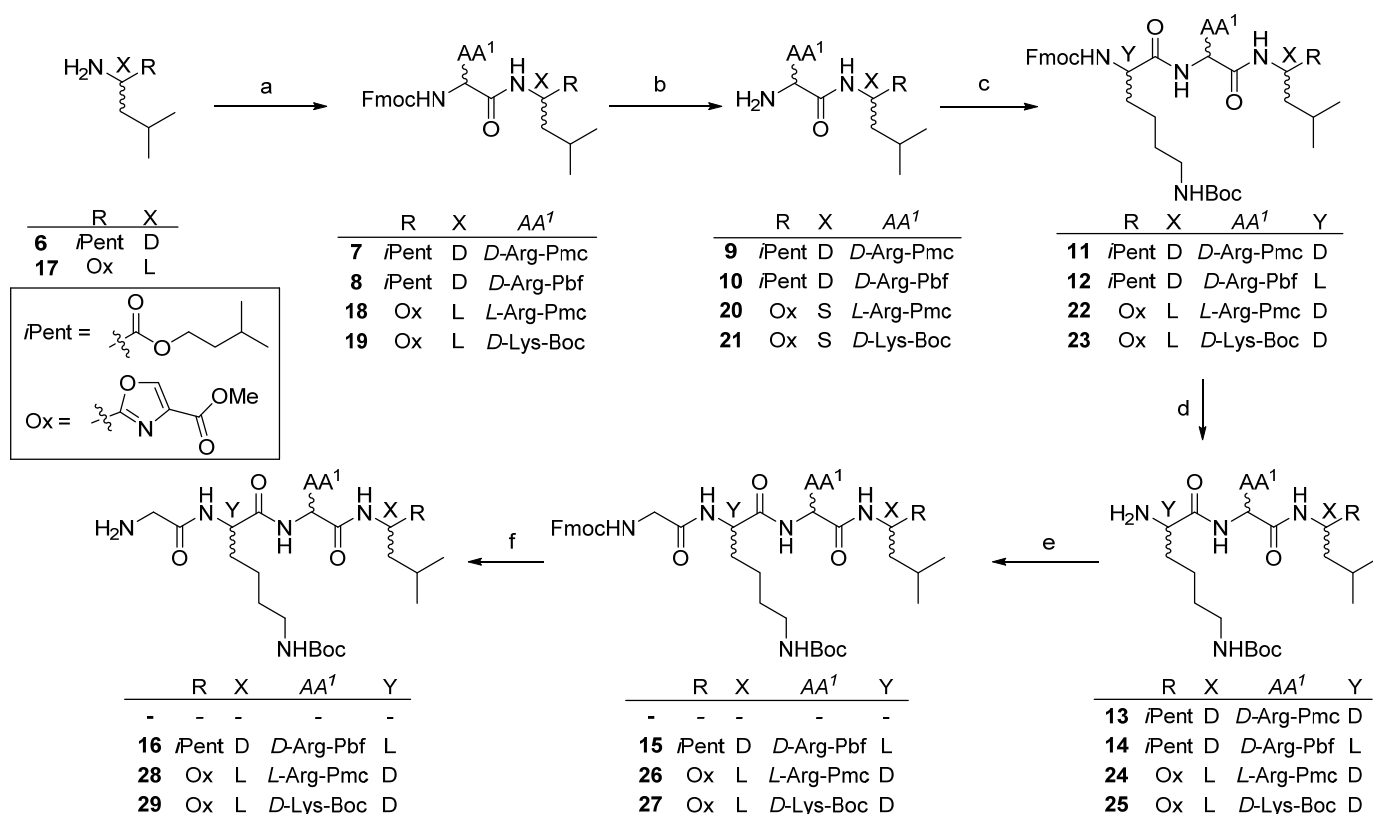
The synthesis of the peptide moieties containing an oxazole terminus started with the synthesis of **17** by reported methods[22] (Scheme 2). This oxazole **17** then underwent a typical coupling with *N,N'*-diprotected forms of either *L*-Arg or *D*-Lys to produce **18** and **19**, which were deprotected 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*]fullerenyldihydropyrrole 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 CH$_2$Cl$_2$, 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
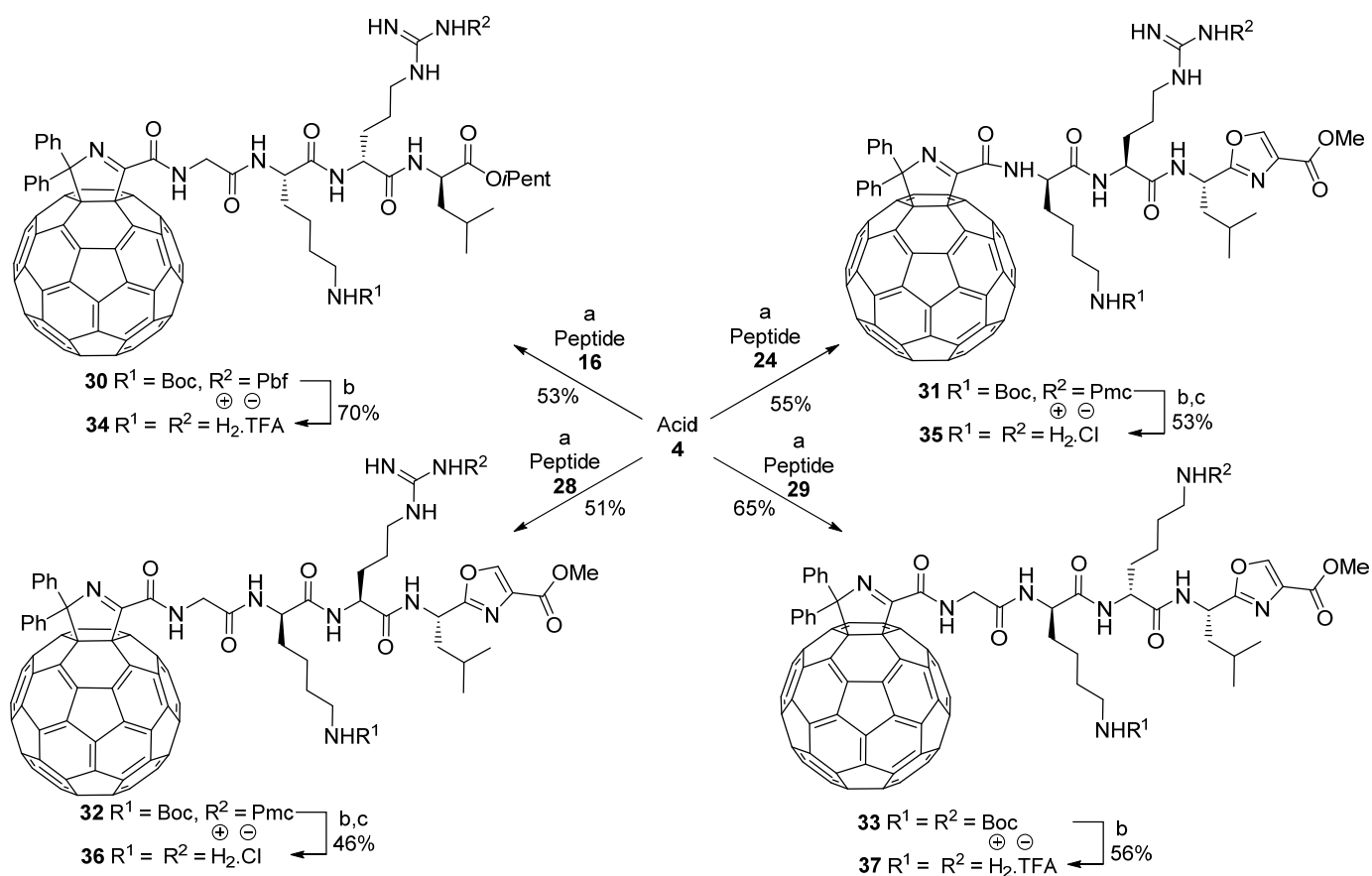
transformed to a clear brown solution and upon workup, the first-in-class [*60*]fullerenyl anchored tetrapeptide **30** was isolated. Synthesis of compound **31** was possible by coupling with the oxazole terminated tripeptide **24**, and the tetrapeptide version of **28**, with the extra glycine amino 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 $CHCl_3:CH_2Cl_2$ (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 CH_2Cl_2 as the single solvent.

Analysis of the ^{1}H 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**, $C_{97}H_{49}N_9O_6$.
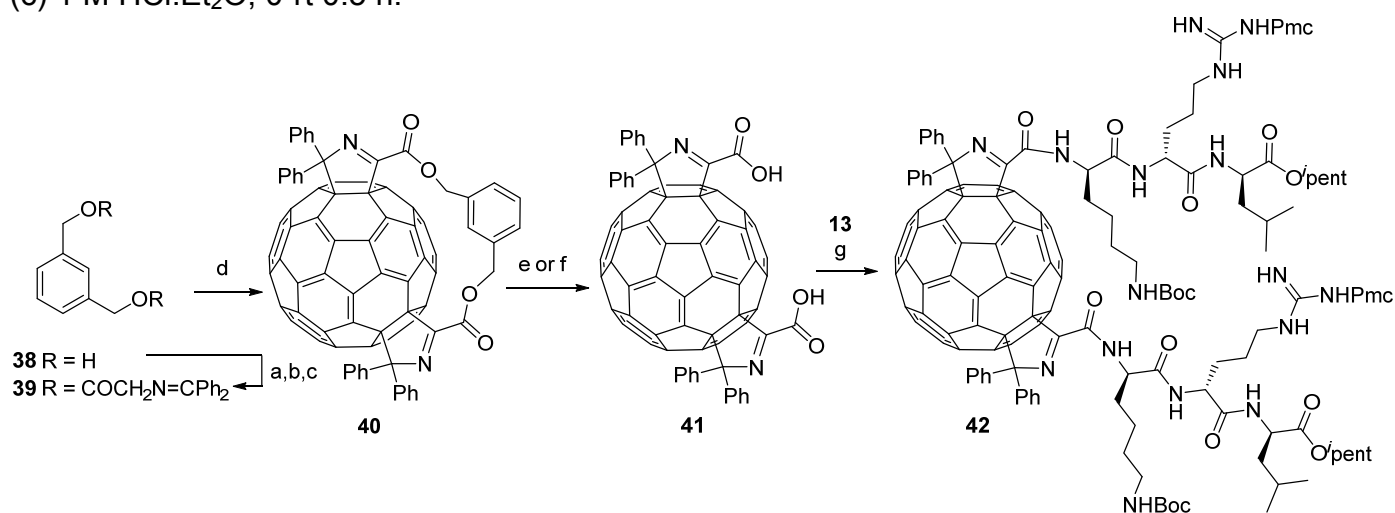
An additional antibiotic target molecule to consider is the [*60*]fullerene that is doubly substituted with peptides chains. Therefore, the previously unknown diacid **41** was synthesised from the reported bis-adduct **40**,[17a] by using trimethyltin hydroxide[23] at 80 °C or borontribromide at -10 °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 ^{1}H 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 C_s symmetry of the *trans-4* disubstituted [60]-fullerene.[24] The presence of 29 sp^2 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**, $C_{174}H_{164}N_{16}O_{20}S_2Na$.

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 2: Reagents and conditions: (a) Fmoc-*D*-arg-(Pmc/Pbf)-OH, Fmoc-*L*-arg-(Pmc)-OH or Fmoc-D-lys(Boc)-OH, EDCI, HOBt, CH_2Cl_2, rt, 4 h, 66% for **7**, 75% for **8**, 65% for **18** and 68% for **19**; (b) piperidine, CH_3CN, rt, 4 h, 77-88%; (c) Fmoc-*D* or *L*-Lys-(Boc)-OH, EDCI, HOBt, CH_2Cl_2, 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, CH_2Cl_2, 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_2Cl_2, rt, 4 h; (b) TFA/ CH_2Cl_2 +(1:1), rt, 16 h; (c) 1 M $HCl.Et_2O$, 0-rt 0.5 h.

Structure labels (Scheme 3):

30 R^1 = Boc, R^2 = Pbf
34 R^1 = R^2 = H_2.TFA — b, 70%
Peptide **16**, a, 53%

31 R^1 = Boc, R^2 = Pmc
35 R^1 = R^2 = H_2.Cl — b,c, 53%
Peptide **24**, a, 55%

Acid **4**

32 R^1 = Boc, R^2 = Pmc
36 R^1 = R^2 = H_2.Cl — b,c, 46%
Peptide **28**, a, 51%

33 R^1 = R^2 = Boc
37 R^1 = R^2 = H_2.TFA — b, 56%
Peptide **29**, a, 65%

Scheme 4: Reagents and conditions: (a) (*tert*-butoxycarbonyl)glycine, THF, DMAP, CH_2Cl_2, rt, 40 h, 80%. (b) TFA, CH_2Cl_2, rt, 3 h, 90%. (c) CH_2Cl_2, diphenylmethanimine, 24 h, rt, 75%. (d) DBU, CBr_4, rt, Toluene, C_{60}, 6 h, 45%. (e) $(CH_3)_3SnOH$, DCE, 80 °C, 24 h, 65%. (f) BBr_3, CH_2Cl_2, -10 °C - rt, 18 h, 50%. (g) pyridine/chloroform (1:2), EDCI, HOBt, 8 h, 50%.

Structure labels (Scheme 4):

38 R = H
39 R = $COCH_2N=CPh_2$ — a,b,c

40, **41**, **42**

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.
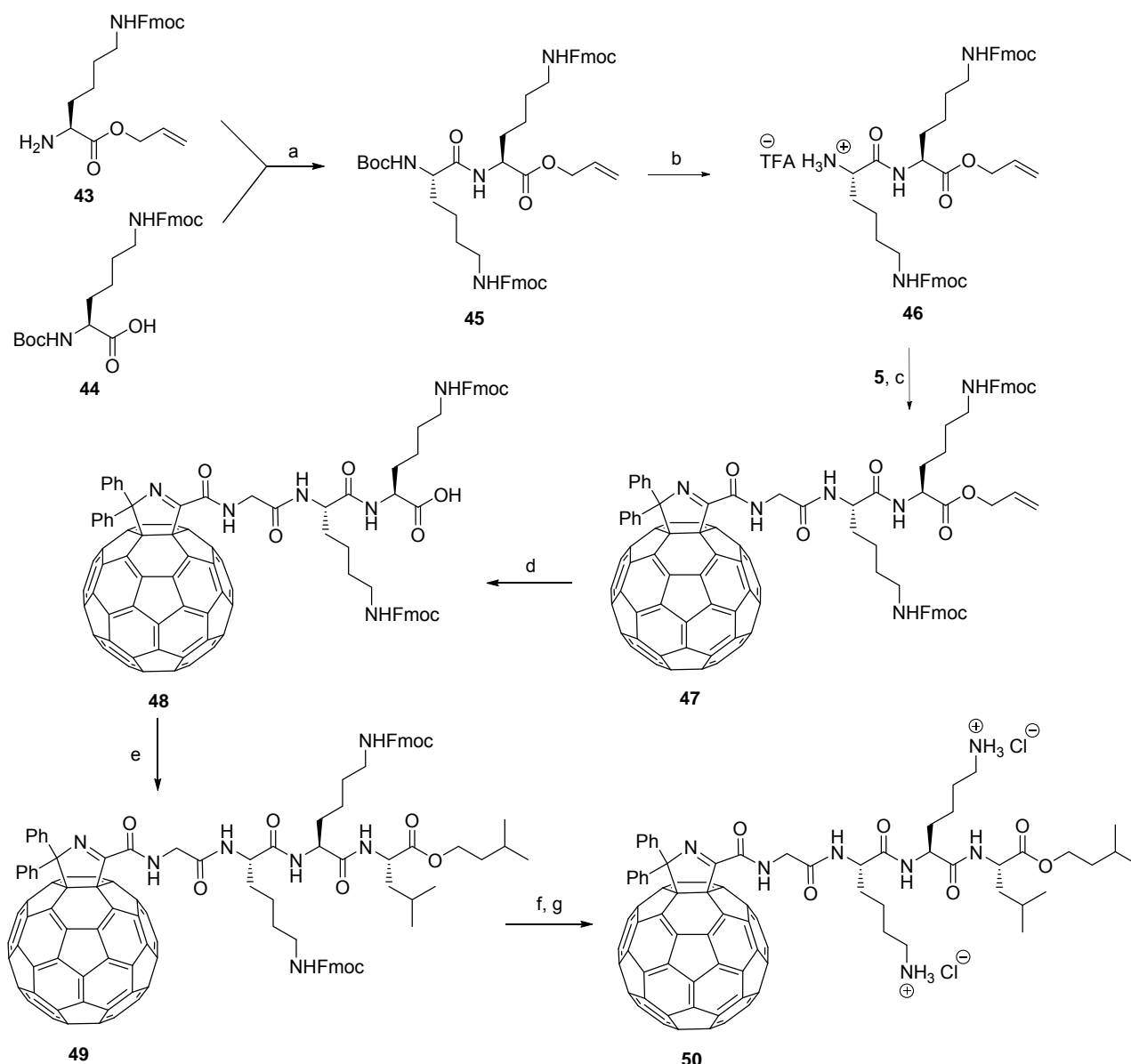
Scheme 5: Reagents and conditions: (a) EDCI, HOBt, TEA, CH₂Cl₂, rt, 4 h, 85%; (b) TFA: CH₂Cl₂ (4:6), rt, 4 h, 90%; (c) HBTU, TEA, CH₂Cl₂, rt, 4 h, 67%; (d) (CH₃)₃SnOH, 1,2-DCE, 80 °C, 6 h, 84%; (e) isopentyl *L*-leucinate hydrochloride, EDCI, HOBt, TEA, CH₂Cl₂, rt, 4 h, 62%; (f) piperidine, CH₃CN, rt, 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 fullerenyl-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/CH_2Cl_2 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/CH_2Cl_2 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 CH_2Cl_2/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 CH_2Cl_2. 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 CH_2Cl_2, 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 $Sn(CH_3)_3OH$ (4 equiv.) and the solution was heated at 80 °C for 4 h, then 4 more equiv. of $Sn(CH_3)_3OH$ 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_2SO_4 and evaporated under reduced pressure to yield corresponding acid.

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

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