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

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Increasing numbers of 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]fullerenyldihydropyrrolecarboxylic 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 healthcare issues posed by multidrug-resistant human pathogenic bacteria.[1,2] Of particular concern is the emergence of Gram-positive bacteria, for example, *Staphylococcus aureus* and *Enterococcus faecium*, that are resistant to the glycopeptide antibiotic, vancomycin.[3–5] Recently, a new variation to the peptide class of antibiotics has emerged, 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 peptidoglycan cell wall moiety[7] in vancomycin-resistant bacteria.[8] Furthermore, there is significant antimicrobial activity in some biaryl-based cyclic β-hairpin cationic peptidomimetics.[9] Whereas 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 investigated approaches reduce 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 that 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.

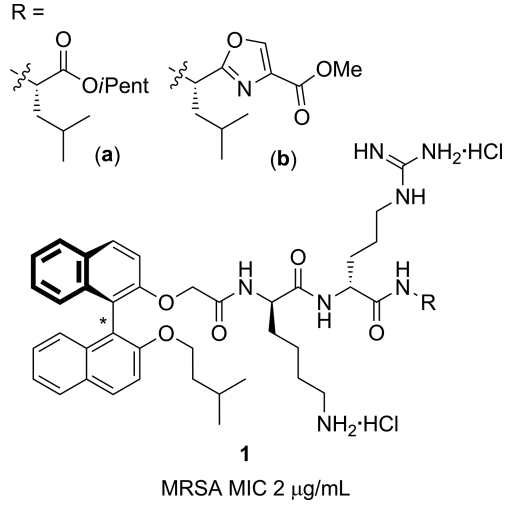

Figure 1. Binaphthyl-anchored peptide antibiotic agents.

The fullerene moiety is becoming increasingly prominent in biological applications,[18] 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] with the later being of significance to this project. To date, such systems have been the focus of a variety of materials and medicinal chem-

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istry 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 into the peptide chain. Additionally, the peptide chain will be rich in arginines and lysines, which are amino acids that are 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, which could be beneficial for the delivery of bioactive molecules.

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 trifluoroacetic acid (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 has been identified, 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).

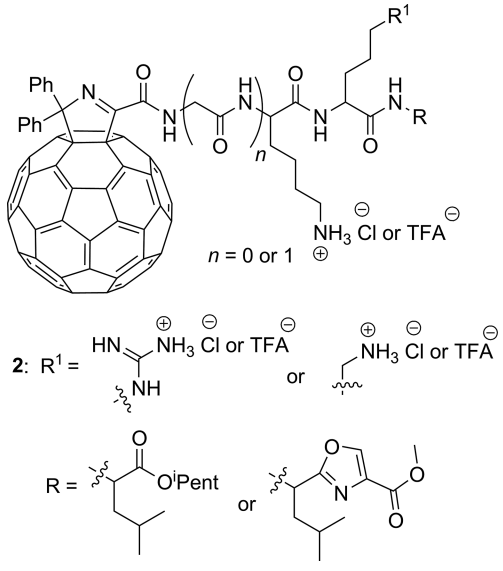
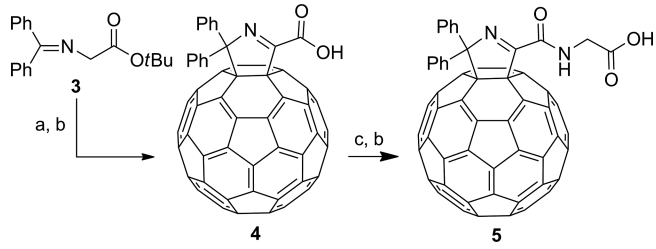


Figure 2. Structure of the target [60]fullerene-based dicationic peptoids **2**.

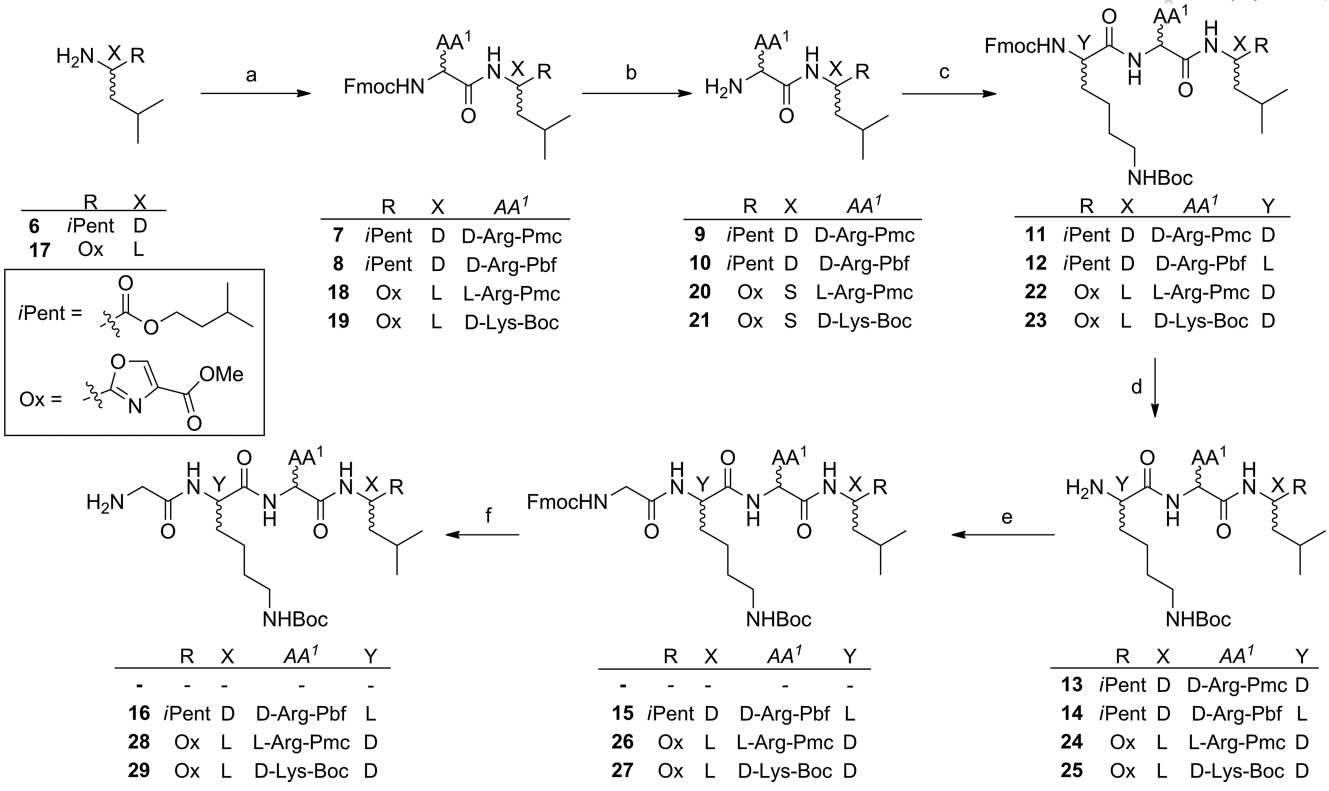
Chemistry

The synthesis of the target compounds began with the generation of the [60]fullerenyl amino acid scaffold **5**, which was achieved by applying known chemistry to generate the acid **4**.[17] Thus, 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 fullerenyl acid **4** (90%; Scheme 1). This acid was treated with *tert*-butylglycinate under EDCI/HOBt or HBTU peptide coupling conditions, followed by TFA treatment to give acid **5** (48%). This second step adds the short linker to the molecule in the form of a Gly unit.



Scheme 1. Synthesis of the fullerenyl amino acid hydrophobic anchor. Reagents and conditions: (a) DBU, CBr_4, toluene, 6 h, room temp., C_{60}, 60%; (b) TFA, CH_2Cl_2, room temp., 3 h, 90%; (c) *tert*-butyl glycinate, CH_2Cl_2, EDCI/HOBt or HBTU, room temp., 5 h, 48%.

The next stage towards [60]fullerenyl-anchored peptide 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; instead, we wished to encompass 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. Thus, free amine **6**, containing an isopentyl ester, 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 because of the ready 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, gave the free amines **13** and **14**, respectively. The reaction of **14** with Fmoc-Gly-OH produced 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



	R	X
6	*i*Pent	D
17	Ox	L

	R	X	AA^1
7	*i*Pent	D	D-Arg-Pmc
8	*i*Pent	D	D-Arg-Pbf
18	Ox	L	L-Arg-Pmc
19	Ox	L	D-Lys-Boc

	R	X	AA^1
9	*i*Pent	D	D-Arg-Pmc
10	*i*Pent	D	D-Arg-Pbf
20	Ox	S	L-Arg-Pmc
21	Ox	S	D-Lys-Boc

	R	X	AA^1	Y
11	*i*Pent	D	D-Arg-Pmc	D
12	*i*Pent	D	D-Arg-Pbf	L
22	Ox	L	L-Arg-Pmc	D
23	Ox	L	D-Lys-Boc	D

	R	X	AA^1	Y
13	*i*Pent	D	D-Arg-Pmc	D
14	*i*Pent	D	D-Arg-Pbf	L
24	Ox	L	L-Arg-Pmc	D
25	Ox	L	D-Lys-Boc	D

	R	X	AA^1	Y
-	-	-	-	-
15	*i*Pent	D	D-Arg-Pbf	L
26	Ox	L	L-Arg-Pmc	D
27	Ox	L	D-Lys-Boc	D

	R	X	AA^1	Y
-	-	-	-	-
16	*i*Pent	D	D-Arg-Pbf	L
28	Ox	L	L-Arg-Pmc	D
29	Ox	L	D-Lys-Boc	D

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, room temp., 4 h, 66% for **7**, 75% for **8**, 65% for **18** and 68% for **19**; (b) piperidine, CH_3CN, room temp., 4 h, 77–88%; (c) Fmoc-D or L-Lys-(Boc)-OH, EDCI, HOBt, CH_2Cl_2, room temp., 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, room temp., 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**.

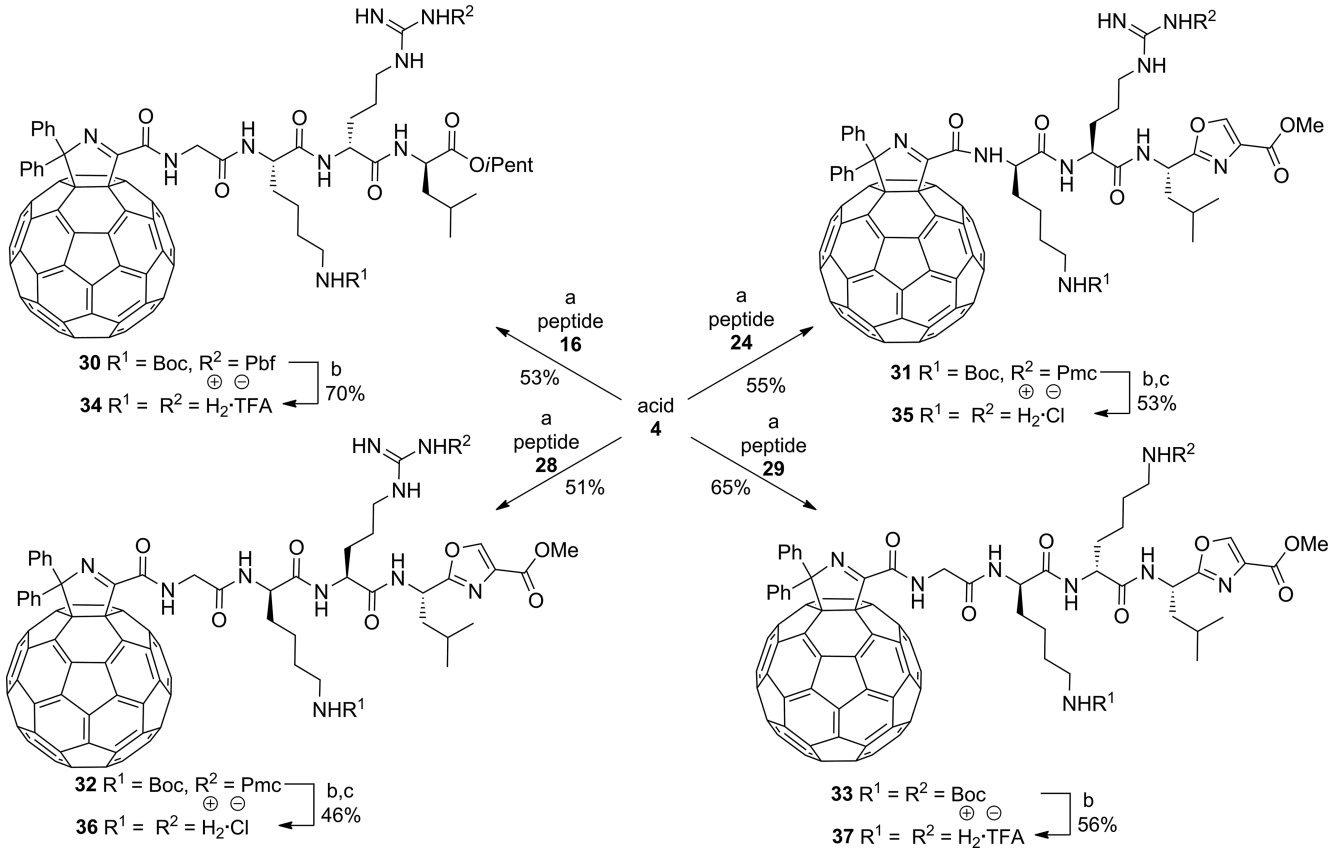
substituent, ready for peptide coupling with a fullerenyl amino acid.

The synthesis of the peptide moieties containing an oxazole terminus started with the synthesis of **17** by reported methods[22] (Scheme 2). Oxazole **17** then underwent standard 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 to give **24** and **25**, respectively; a final coupling with Fmoc-Gly-OH gave tetrapeptides **26** and **27** and, after Fmoc deprotection, free amines **28** and **29** were obtained 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_2Cl_2, which was sonicated for 15 min 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 into 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 the peptide chain, was used to produce **32**. Coupling the acid **4** with peptide **29**, which contained two Boc-protected lysines with an oxazole terminus, yielded **33**. The reaction required the use of $CHCl_3/CH_2Cl_2$ (1:1) as solvent mixture because of the better solubility of the di-Boc protected lysine containing peptide, whereas the synthesis of compounds **31** and **32** required only CH_2Cl_2 as a single solvent. Deprotection of **30**–**33** with TFA gave the corresponding dication TFA or HCl salts after anion exchange (Scheme 3).

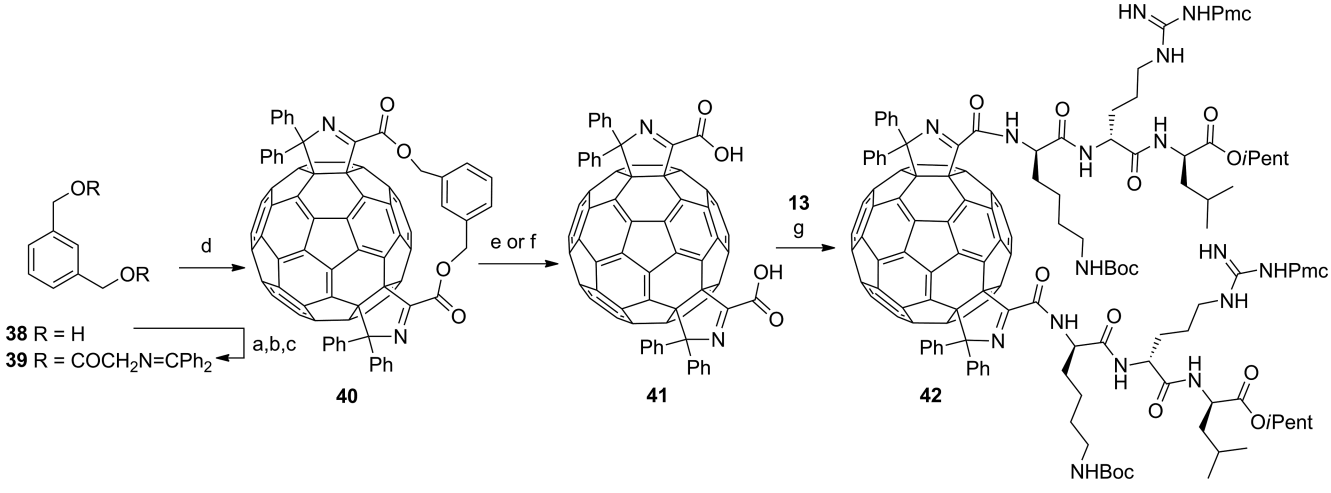
Analysis of the ^1H NMR spectrum of the fullerenyl dication peptide **34** revealed a doublet of doublets centred at δ = 7.36 and 7.47 ppm, assigned to the *para* and *meta* protons, respectively, of the *gem* diphenyl substituents of the proline moiety, with the corresponding *ortho* protons assigned to the doublet at δ = 8.03 ppm. The methylene moiety of the glycine unit was assigned to the broad singlet at δ = 3.26 ppm. Analysis of the HRMS (ESI) revealed a peak at *m*/*z* 1436.3822, assigned to the monocationic 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. Thus, the previously unknown diacid **41** was synthesised from the reported bis-adduct **40**,[17a] by using trimeth-



Scheme 3. Synthesis of C_{60} fullerenyl-anchored amino acid oxazoles and isopentyl esters. Reagents and conditions: (a) EDCI, HOBt, CH_2Cl_2, room temp., 4 h; (b) TFA/CH_2Cl_2 (1:1), room temp., 16 h; (c) 1 M $HCl{\cdot}Et_2O$, 0 °C to room temp., 0.5 h.

yltin hydroxide[23] at 80 °C or borontribromide at –10 °C to room temperature; the former conditions resulted in a better yield of 65% compared with the latter of 50% (Scheme 4). As expected, attempts to synthesise [60]fullerene bis-peptoid **42** by using diacid **41** and peptide **13** under the standard coupling conditions used previously in this study failed because of 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, leading to a successful outcome with 50% reaction yield of **42** (Scheme 4). Unfortunately, at-

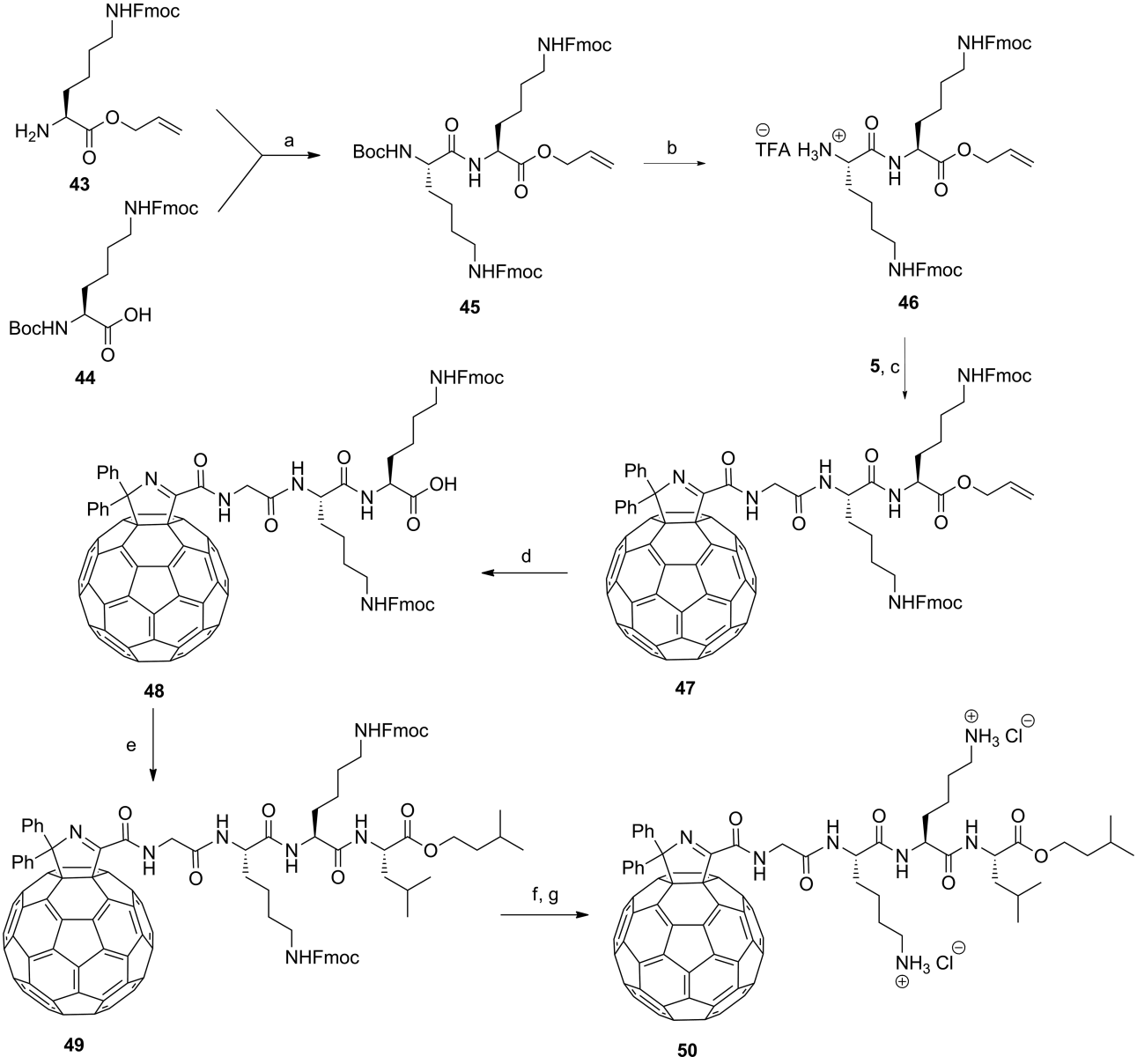


Scheme 4. (a) (*tert*-butoxycarbonyl)glycine, THF, DMAP, CH_2Cl_2, room temp., 40 h, 80%. (b) TFA, CH_2Cl_2, room temp., 3 h, 90%. (c) CH_2Cl_2, diphenylmethanimine, 24 h, room temp., 75%. (d) DBU, CBr_4, room temp., toluene, C_{60}, 6 h, 45%. (e) $(CH_3)_3$-SnOH, DCE, 80 °C, 24 h, 65%. (f) BBr_3, CH_2Cl_2, –10 °C to room temp., 18 h, 50%. (g) pyridine/chloroform (1:2), EDCI, HOBt, 8 h, 50%.

tempted deprotection of **42** with TFA gave an insoluble product that could not be characterised. Analysis of the ^{1}H NMR spectrum of **42** showed a singlet signal (18 H) at δ = 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, which 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 HRMS (ESI) indicated a peak at *m*/*z* 2884.1675, assigned to the sodiated species of **42** ($C_{174}H_{164}N_{16}O_{20}S_2Na$).

The synthesis of the fullerenyl peptide **50** was achieved by using a modified route (Scheme 5). Initially, 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 67% yield. Subsequent deallylation followed by coupling to isopentyl-L-leucinate gave tetrapeptide-coupled [60]fullerenyl peptide **49** in 62% yield. Final side chain deprotection under standard conditions followed by salt formation gave the final product **50** in 70% yield.

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 by using 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 treatment with TFA/CH_2Cl_2 (1:1) to deprotect the Pmc/Pbf and *N*-Boc



Scheme 5. Reagents and conditions: (a) EDCI, HOBt, TEA, CH_2Cl_2, room temp., 4 h, 85%; (b) TFA/CH_2Cl_2 (4:6), room temp., 4 h, 90%; (c) HBTU, TEA, CH_2Cl_2, room temp., 4 h, 67%; (d) $(CH_3)_3SnOH$, 1,2-DCE, 80 °C, 6 h, 84%; (e) isopentyl L-leucinate hydrochloride, EDCI, HOBt, TEA, CH_2Cl_2, room temp., 4 h, 62%; (f) piperidine, CH_3CN, room temp., 4 h, 72%; (g) 1 M HCl·Et_2O, 0 °C to room temp., 0.5 h, 70%.

functional groups then, after removing the solvents under reducing pressure, the residue was resuspended in a minimal volume of CH_2Cl_2 and treated with an excess of 1 M HCl in ether to obtain the target HCl salts. Peptides **30** and **33** were made as their TFA salts **34** and **37** by treatment with TFA/CH_2Cl_2 (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 **34**, **35** and **36** returned ^{1}H NMR spectra of good quality. Unfortunately, due to limited solubility of these salts, we were unable to obtain adequate ^{13}C 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 antibacterial testing in vitro. These twelve [60]fullerene derivatives were tested using the broth microdilution method against *Staphylococcus aureus* NCTC 6571 and *Escherichia coli* NCTC 10418. Minimum inhibitory concentrations (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 MIC greater than 100 to 340 (μg/mL).

Conclusions

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

Experimental Section

Protocol 1: Peptide Coupling: To a solution of the acid in either 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.). When 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 the resulting residue was subjected to silica gel column chromatography (MeOH/CH_2Cl_2, 1–4%) to afford the coupled product.

Protocol 2: *N*-Fmoc Deprotection: The Fmoc-protected amine was stirred in piperidine/acetonitrile (1 equiv., 5 mL/0.10 mmol) overnight at room temp., unless otherwise stated. The solvent was removed under reduced pressure and the crude product was purified by column chromatography(MeOH/CH_2Cl_2, 5%) to give 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 CH_2Cl_2/TFA (1:1, 6 mL/0.10 mmol) solution at room temp. The solvent was removed under reduced pressure, then the residue was resuspended in a minimal volume of CH_2Cl_2. The solution was treated with an excess of 1 M HCl in diethyl ether (2 mL/0.01 mmol) and the solvent was 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 room temp. was added $Sn(CH_3)_3OH$ (4 equiv.) and the solution was heated at 80 °C for 4 h, then further $Sn(CH_3)_3OH$ (4 equiv.) was added at the same temperature and the reaction was continued for 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 × 10–15 mL), brine (3 × 10 mL), dried with $MgSO_4$, and evaporated under reduced pressure to give the corresponding acid.

Acknowledgments

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