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Synthesis of a proline-rich [60]fullerene peptide with potential biological activity

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Dedicated to Dr. Ulf Ragnarsson, from the Department of Biochemistry, University of Uppsala, Biomedical Center, Uppsala, Sweden, on the occasion of his retirement

Abstract—A proline-rich [60]fullerene peptide was synthesized by use of (i) a 1,3-dipolar cycloaddition of an N-substituted glycine derivative to [60]fullerene, (ii) esterification of the isolated alcohol with the C-terminal amino acid of the desired peptide sequence, and finally (iii) coupling of the remaining hexapeptide to give the final product **8** as a TFA salt, with oxidized methionine. Product **8** was found to be biologically active against sera from MCTD and SLE patients (ELISA experiment).

1. Introduction

Considerable effort in fullerene chemistry has been directed to establish this novel form of carbon as a standard building block in organic synthesis.[1–3] Especially, the most abundand member of the fullerene family, C_{60}, has received the highest attention as C_{60}-based molecules display a wide range of interesting features, which include nonlinear optical properties and superconductivity.[4] The exceptionally hydrophobic nature and spheroidal shape of C_{60} make it very interesting for its potential use in medicinal chemistry.[5] A series of [60]fullerene derivatives displays a wide range of biological properties, including neuroprotective, enzymatic, antiapoptotic, antibacterial, DNA photocleaving, nitric oxide synthase inhibiting, and chemotactic activities.[5] Among the different classes of derivatives, fullerene-based amino acids and peptides are particularly interesting, both for structural studies and biological applications.[5a] For example, C_{60}-based 3,4-fullero-proline (Fpr), which is the fullerene homologue of the natural proline residue, has been inserted into small peptides for studying its propensity to induce β-turn conformations and to influence the *cis–trans* equilibrium around the tertiary amide bond.[5a,6] Fulleroproline amino acid derivatives are also shown to interact with different hydrolytic enzymes in model transesterification reactions, and to form supramolecular complexes with, and selectively discriminate between, different size calix-[*n*]arenes, cyclodextrines, and other rationally designed peptides forming cavities.[7] Incorporation of the C_{60} moiety into biologically active peptides is thus desirable to possibly alter both the structure and the biological activity of the parent peptide.

The synthesis of the first [60]fullerene peptide was reported in 1993,[8] where a methanofullerene was linked to the terminal part of a pentapeptide with an alternating-Aib (α-amino isobutyric acid)-Ala- sequence. This model fullero-peptide was able to adopt a 3_{10}-helical structure.[9] Today a few examples of fullero-peptides are known, prepared under solution chemistry conditions.[10] More recently, the first example of solid-state fullero-peptide synthesis has been reported.[11] Here we wish to report the synthesis of a new proline-rich [60]fullerene peptide, which contains a solubilizing appendage (ethyleneglycol chain), covalently attached between the fullerene moiety and a heptapeptide, namely H-PPGMRPP-OH, which has antigenic properties.[12] The above proline-rich heptapeptide, found to be present in several copies in Sm and U1RNP autoantigens, is the main target of the *anti*-Sm and *anti*-U1RNP autoantibodies in sera of patients with autoimmune diseases such as Systemic Lupus Erythematosus (SLE) and

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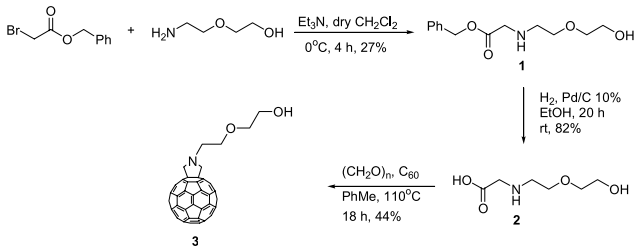
Mixed Connective Tissue Disease (MCTD). It was also found that the H-PPGMRPP-OH epitope is recognized by *anti*-Ro/La positive sera, although they are negative for *anti*-Sm and *anti*-U1RNP.[13] More recently, it has been demonstrated that conversion of the C-terminal carboxylate group of the parent peptide into the amide form resulted in a substantial decrease of the *anti*-Ro/La recognition, probably due to the predominance of one unfavorable conformer.[14,15]

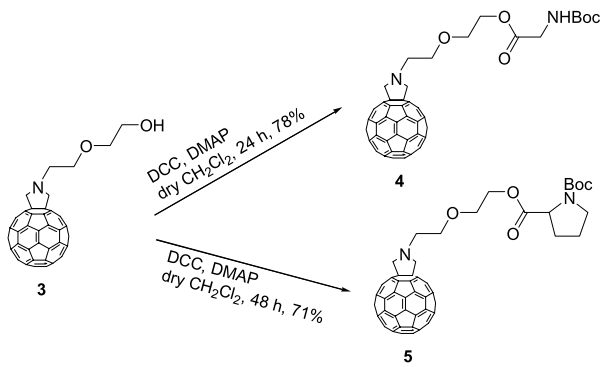
2. Results and discussion

2.1. Chemistry and spectroscopy

The synthesis of compound **8**, was performed according to Schemes 1 and 2. Accordingly, aminolysis of benzyl 2-bromo-acetate by 2-(2-aminoethoxy)ethanol in dry CH_2Cl_2 in the presence of Et_3N, afforded **1**, which upon catalytic hydrogenolysis with Pd/C 10% in ethanol, gave the N-substituted glycine **2**. 1,3-Dipolar cycloaddition of the azomethine ylide generated by condensation of **2** with formaldehyde to C_{60} led to good yields (~44%) of fulleropyrrolidine **3**, N-substituted with an ethyleneglycol chain (Scheme 1). This product gave correct ^{1}H NMR and ESI MS spectra. In particular, ^{1}H NMR spectra showed five signals for the five different types of protons in **3**.



Scheme 1. Synthesis of derivative **3**.

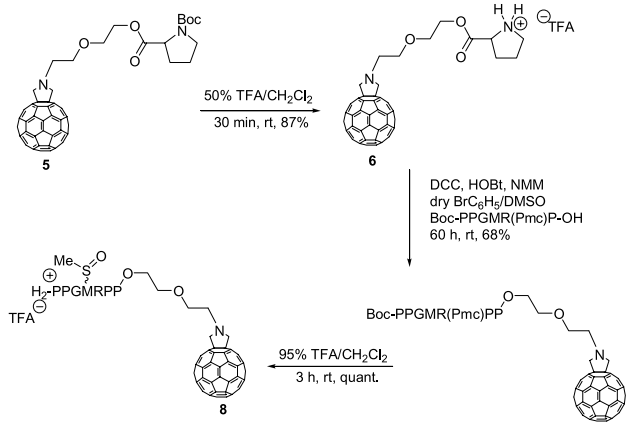


Scheme 2. Esterification of derivative **3** with Boc-protected amino acids.

At this stage it should be mentioned that attempts to couple derivative **3** with the protected Fmoc-PPGMR(Mtr)P-OH hexapeptide in one step were unsuccessful, (Fmoc-: 9-Fluorenylmethoxycarbonyl-, Mtr-: 4-methoxy-2,3,6-trimethylbenzenesulfonyl-). Therefore, we turned our efforts to introduce first a Boc-protected amino acid (Boc-: *tert*-butoxycarbonyl-) via an esterification reaction at the terminal hydroxyl group of derivative **3**. This was done successfully with both Boc-glycine and Boc-proline, affording as products synthetic intermediates **4**, and **5**, respectively, in relatively high yields (Scheme 2).

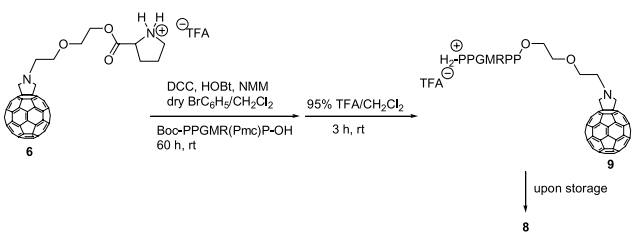
Both of the above amino acid-containing [60]fullero derivatives, were characterized by ^{1}H NMR and ESI MS spectroscopies, gave the correct spectra with regard to their chemical structure. Because we were interested in a covalent connection of derivative **3** to the known peptide H-PPGMRPP-OH, we decided to proceed with the proline connected fullero-derivative **5**, in that it contains the C-terminal amino acid of the parent heptapeptide sequence.

To this end, derivative **5** was first deprotected with a 50% solution of 2,2,2-trifluoroacetic acid (TFA) in CH_2Cl_2 and the resulted TFA salt was subjected to coupling with the protected hexapeptide Boc-PPGMR(Pmc)P-OH[10a] (Pmc-: 2,2,5,7,8-pentamethylchroman-6-sulfonyl-) (Scheme 3). Periodically the reaction progress was checked by TLC (PhMe/MeOH). After 60 h stirring at room temperature (rt), the reaction was stopped and product **7** was isolated by column chromatography on SiO_2, with PhMe/MeOH 6:1 v/v solvent mixture as eluant. The final step was the simultaneous removal of the protecting groups in **7** with a 95% solution of TFA in CH_2Cl_2. The TFA salt of the fullero-peptide derivative **8** was isolated, as a dark brown powder in 42% overall yield starting from **3**.



Scheme 3. Synthesis of [60]fullero-peptide **8**.

The ESI MS spectra of **8** showed a $[M+2H]^{2+}$=801.7 signal which corresponds to the compound with the oxidized methionine thioether group (i.e., containing a sulfoxide group). When the coupling reaction of the protected hexapeptide was performed in dry BrC_6H_5/CH_2Cl_2 the final deprotected [60]fullero peptide **9**, after chromatographic purification and deprotection steps as above,



Scheme 4. Synthesis of compound **9**, that gradually oxidized to **8**.

showed a correct ESI MS molecular ion signal of $[M+2H]^{2+}=792.98$ (Scheme 4).

This second sample **9**, gradually oxidized to compound **8** while in the solid state, even under an Ar atmosphere. After a month of storage it gave almost a complete oxidation of methionine –SMe group to –S(=O)Me (the final product showed an ion signal of $[M+2H]^{2+}=800.81$). From our experience we know that DMSO causes methionine oxidation to a small percentage after a long period of time.[16] Also, C_{60} itself or fullerene-containing derivatives are known to be effective photosensitizers for singlet oxygen, $^1O_2$, production.[17] We are of the opinion that the combination of both the above factors are the reason for the pronounced oxidation of methionine in derivative **8** (due to O_2 traces contained in the reagents/solvents). To further verify the above reasoning we stirred a solution of the protected peptide PPGMR(Pmc)P-OH in DMSO for 24 h. The ESI MS spectra showed only a small peak corresponding to the oxidized product, $[M-H]^{-1}=1034.71$, whereas the major peak, $[M-H]^{-1}=1018.75$, corresponded to the normal protected peptide. When the same conditions were applied in CH_2Cl_2 in the presence of a catalytic quantity of C_{60}, after 24 h the ESI-MS spectra showed two peaks, $[M+H]^{+}=1036.66$ and $[M+Na^{+}]^{+}=1058.59$, both of them corresponding to the protected peptide with oxidized methionine.

All of the proton resonances and the amino acid sequence of the oxidized TFA salt **8** were identified by combining COSY, TOCSY and NOESY 2D NMR experiments in DMSO-d_6. In agreement with our previously reported results,[12,13] two *cis–trans*-conformers out of the eight theoretically possible, due to the presence of four proline residues, were detected. All of the X-Pro peptide bonds of the major conformer were identified in the *trans* form. The assignment was based on either the presence of the X-$C^{\alpha}H$/P-$C^{\alpha}H$ and/or the absence of the X-$C^{\alpha}H$/P-$C^{\alpha}H$ NOE effects. However, the localization of the *cis* X-Pro peptide bonds was not possible in the minor conformer due to its low percentage (<10%). Table 1 summarizes the proton chemical shift values for the major conformer of **8**.

Table 1. Proton chemical shifts (ppm) of **8** in DMSO-d_6 at 303 K

Amino acid	NH	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	Other protons
Pro[1]	Nd[a]	4.19	2.50	Nd[a]	3.79	
Pro[2]	—	4.38	2.13	1.91	3.62	
					3.46	
Gly[3]	8.27	3.68	—	—	—	
Met[4]	7.89	4.43	1.94	2.68	—	
			1.84			
Arg[5]	8.28	4.43	1.68	1.52	3.07	7.50 ($N^{\varepsilon}H$)
						7.28 ($N^{\eta}H$)
						6.87 ($N^{\eta}H$)
Pro[6]	—	4.58	2.18	1.95	3.71	
					3.51	
Pro[7]	—	4.32	1.91	2.17	3.82	
					3.62	

[a] Not detected.

2.2. Biological activity

[60]Fullerene peptide **8**, with oxidized methionine, was evaluated for its ability to recognize *anti*-Sm and *anti*-U1RNP autoantibodies in SLE and MCTD patients' sera, (ELISA experiment). In Table 2, the reactivities of derivative **8**, as tested against *anti*-Sm/U1RNP sera from SLE and MCTD patients with Sjogren's syndrome, are listed in comparison with the parent peptide in its free and C-terminal amide form.

Table 2. Reactivity of sera containing various auto-antibodies against [60]fullerene derivatives **8** and **3**

Derivative	*Anti*-Sm/U1RNP (+) *anti*-Ro/La (−) (%)	*Anti*-Ro/La (+) *anti*-Sm/U1RNP (−) (%)
H-PPGMRPP-OH[a]	75	40
H-PPGMRPP-NH_2[a]	75	17
[60]Fullerene derivative **8**	92	100
[60]Fullerene derivative **3**	0	0

[a] From Refs. 13,14.

As can be seen from Table 2, derivative **8** strongly recognizes *anti*-Sm/U1RNP specificities. Surprisingly, derivative **8** is fully recognized by *anti*-Ro/La positive sera, which are negative to *anti*-Sm/U1RNP sera. Derivative **3**, showing no recognition at all, was taken as a control. Taking into account that derivative **3** is not reactive at all, one could hypothesize that conversion of the carboxylic end of the parent peptide to an ester functionality in derivative **8** would be responsible for the decrease in disease specificity. This effect could be attributed to the predominance of a different conformer with respect to that prevailing in H-PPGMRPP-NH_2. In addition, the oxidized form of the thioether group of methionine may also affect the specificity of the compound.

3. Conclusions

The present work describes the synthesis of a [60]fullero-peptide which contains an ethyleneglycol chain between the fullerene and biologically active peptide moieties. The fullero-peptide was characterized with NMR, ESI MS, and MALDI-TOF MS spectroscopies. In addition, it showed strong recognition against *anti*-Sm/U1RNP sera from SLE and MCTD patients. This encouraging result could be of help for the design and synthesis of new derivatives that could be more potent and more disease selective. More detailed conformational studies of derivative **8** by 2D NMR spectroscopy are currently underway.

4. Experimental

4.1. General

All NMR spectra were taken in $CDCl_3$ 98% D, unless otherwise noted, on a Bruker 400 MHz AMX instrument. ESI MS spectra were taken on a quadrapole Micromass Platform LC, model MassLynx v3.3. MALDI-TOF MS spectra were taken on a Voyager DE-STR (Applied Biosystems, Foster City, CA) Spectrometer, with DHB (2,5-dihydroxybenzoic acid) as matrix. Spectra were aquired at 20 kV acceleration voltage, in the reflector mode.

[C_{60}]Fullerene was purchased from Materials and Electronical Research Corporation (Tucson, AZ, USA). All reagents and solvents were obtained from commercial suppliers and used without further purification. Dry quality solvents were obtained according to literature procedures,[18] and kept in MS 4A under Ar atmosphere. Thus, CH_2Cl_2 distilled from P_2O_5; PhMe, distilled from Na with benzophenone as an indicator; DMSO, stirred with NaOH for 24 h and then distilled at 2–3 mm Hg under continuous flow of N_2; pyridine, pre-dried with $MgSO_4$, and then distilled from BaO; *N*-methyl-morpholine distilled from Na; bromo-benzene distilled in vacuo; DMF pre-dried with KOH, refluxed for 1 h in the presence of ninhydrin and then distilled from BaO.

4.2. Peptide synthesis

Peptides were synthesized on a 2-chlorotrityl chloride resin following the Fmoc SPPS procedure.[19] Arginine was introduced as Fmoc-Arg(Mtr)-OH and Fmoc-Arg(Pmc)-OH, the N-terminal proline as Fmoc-Pro-OH and Boc-Pro-OH for the synthesis of Fmoc-PPGMR(Mtr)P-OH and Boc-PPGMR(Pmc)P-OH respectively, methionine as Fmoc-Met-OH, and glycine as Fmoc-Gly-OH. Fmoc groups were removed using 20% piperidine in DMF. Couplings were performed using an amino acid/TBTU/HOBt/DIEA/resin molar ratio of 3:2.9:3:3:1 (TBTU: *O*-benzotriazol-1-yl-*N*,*N*,*N*′, *N*′-tetra-methyluronium tetrafluoroborate, HOBt: 1-hydroxybenzotriazole, DIEA: *N*,*N*-diisopropylethyl-amine). DMF, used for couplings, was previously distilled in the presence of ninhydrin to remove traces of amines. The crude peptides were obtained by treatment of the peptidyl resin for 2 h with a mixture of acetic acid/2,2,2-trifluoro-ethanol/dichloromethane (2:2:6, v/v/v). The resin was removed by filtration, the filtrate was evaporated under reduced pressure, and the product precipitated with cold diethyl ether. The yields were 64 and 86% for Fmoc-PPGMR(Mtr)P-OH (ESI MS calculated molecular ion $[M-H]^{-1}$: 1087.30, found: 1087.17) and Boc-PPGMR(Pmc)P-OH (ESI MS calculated molecular ion $[M-H]^{-1}$: 1019.30, found: 1018.97) respectively. The purity of the peptides, assessed by analytical HPLC, ranged between 80 and 90%. They were used for covalent attachment to fullero-proline derivatives without further purification.

4.2.1. Synthesis of *N*-2-(2-aminoethoxy-ethanol-)glycine (2). The synthesis of **2** was performed in two steps. In the first step aminolysis of benzyl 2-bromoacetate with 2-(2-amino-ethoxy)-ethanol afforded ester **1**, which upon catalytic hydrogenolysis led to the N-substituted glycine (**2**).

4.2.1.1. Aminolysis of benzyl 2-bromoacetate. In a 250 mL, flame dried, two necked round-bottomed flask, equipped with a dropping funnel, and a magnetic stirring bar, under N_2, were placed 2.5 mL (25 mmol) of 2-(2-amino-ethoxy)-ethanol, and 2.5 mL of dry triethylamine diluted with 90 mL of dry CH_2Cl_2. The mixture was cooled at 0 °C, and 2.5 mL (15.9 mmol) of benzyl 2-bromoacetate (as a solution in 10 mL of dry CH_2Cl_2) were added over a period of 1 h. The reaction mixture was left at rt with stirring for 4 h. Then, the organic phase was washed with H_2O (three times) and then with brine (two times), and dried over Na_2SO_4. The solvent was removed with a rotary evaporator and the remaining material was chromatographed on a silica gel column (SiO_2) with an EtOAc/MeOH mixture, 99:1 v/v, as eluant. By this procedure 1.1 g (4.34 mmol) of the benzyl ester of N-substituted glycine **1** were isolated in 27% yield, as a pale yellow viscous oil. ^{1}H NMR (250 MHz, $CDCl_3$) δ 2.82 (t, 2H, J=5.0 Hz), 3.49 (s, 2H), 3.58 (m, 4H), 3.70 (t, 2H, J=4.5 Hz), 5.17 (s, 2H). ^{13}C NMR (62 MHz, $CDCl_3$) δ 48.8, 50.7, 61.8, 66.6, 70.4, 72.3, 128.3, 128.6, 135.5, 172.2. FT IR (KBr) ν_{max}: 700.72, 747.57, 1071.23, 1123.45, 1191.18, 1354.71, 1384.55, 1457.34, 1740.46, 2870.53, 3338.50 cm^{-1}. ESI MS calculated molecular ion $[M+H]^+$: 254.30, found: 254.75.

4.2.1.2. Catalytic hydrogenolysis of 1 to give 2. In a 250 mL, one-necked round-bottomed flask equipped with a magnetic stirring bar, were placed 1.1 g (4.34 mmol) of **1**, dissolved in 124 mL of EtOH. The solution was degassed with an N_2 stream and then 62 mg of Pd/C 10% were added. Hydrogen was passed through the reaction mixture for 20 h at rt. Then, the catalyst was removed by filtration and the solvent was removed in a rotary evaporator, leaving 0.58 g (3.55 mmol) of **2**, (82% yield), as a yellow viscous oil, which was used in the next step without further purification. ^{1}H NMR (250 MHz, DMSO-d_6) δ 3.00 (t, 2H, J=5.2 Hz), 3.20 (s, 2H), 3.49 (m, 4H), 3.62 (t, 2H, J=5.2 Hz). ^{13}C NMR (62 MHz, $CDCl_3$) δ 168.8, 73.1, 67.0, 61.1, 50.8, 47.4. FT IR (KBr) ν_{max}: 481.89, 593.62, 692.76, 892.44, 1070.40, 1127.18 (C–O), 1324.87, 1400.78, 1632.29 (C=O), 2358.20, 2940.29, 3401.26 cm^{-1}. MALDI-TOF MS (matrix: DHB): calculated molecular ion $[M+H]^+$: 164.17, found $[M+H]^+$: 164.16, $[M+Na]^+$: 186.15.

4.2.2. Synthesis of N-substituted 3,4-fullero pyrrolidine (3). In a two-necked, 500 mL round-bottomed flask, were placed 250 mg (0.347 mmol) of C_{60} dissolved in 300 mL of PhMe. Then, to this solution 113 mg (0.692 mmol) of 2 (dissolved in a small amount of EtOH) were added at once followed by the addition of 52 mg (0.577 mmol) of $(CH_2O)_n$ with the help of a small quantity of PhMe. The resulting mixture was refluxed (~120 °C) with stirring for 18 h. After column chromatography on silica gel (SiO_2) with PhMe/EtOAc, 4:1 v/v, as eluant, 129.3 mg (0.152 mmol) of **3** were isolated in 44% yield, as a black powder. ^{1}H NMR (250 MHz, $CDCl_3$) δ 3.40 (t, 2H, J=5.5 Hz), 3.83 (m, 4H), 4.09 (t, 2H, J=5.6 Hz), 4.58 (s, 4H). ^{13}C NMR (62 MHz, $CDCl_3$) δ 54.2, 62.1, 68.2, 70.1, 70.3, 136–156 (C_{60} skeletal carbon signals). FT IR (KBr) ν_{max}: 526.62 (C_{60}), 1115.42 (C–O), 1183.12 (C_{60}), 1425.41 (C_{60}), 1629.45, 2776.11, 2865.67, 2926.00, 3447.83 (OH) cm^{-1}. ESI MS calculated molecular ion $[M+H]^+$: 852.82, found: 852.73. MALDI-TOF MS (matrix: DHB) found: 852.64. Elemental analysis calculated for $C_{66}H_{13}O_2N$: C, 93.07; H 1.54; N 1.64%, found: C 93.42, H 1.49, N 1.60%.

4.2.3. Synthesis of glycine [60]fullerene derivative 4. In a flame dried, 100 mL two-necked, round-bottomed flask, equipped with a reflux condenser and a magnetic stirring bar, under N_2 atmosphere, were placed 16.45 mg (0.094 mmol) of Boc-Gly-OH and 19.4 mg (0.094 mmol) of DCC (*N*,*N*′-dicyclohexylcarbodiimide), dissolved in dry CH_2Cl_2. The resulted mixture was stirred at rt for 30 min. Then, 20 mg (0.023 mmol) of **3** were added, followed by the addition of 1.15 mg (0.009 mmol) of 4-(dimethylamino)-pyridine (DMAP), both of them dissolved in dry CH_2Cl_2.

The reaction mixture was left with stirring at rt under N_2 for 24 h. Column chromatography on silica gel (SiO_2), eluant: PhMe/EtOAc, 4:1 v/v, afforded 18 mg (0.018 mmol) of derivative **4**, (78% yield), as a dark brown powder. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.45 (s, 9H), 3.37 (t, 2H, J=5.4 Hz), 3.90 (t, 2H, J=4.7 Hz), 3.97 (d, 2H, J=5.5 Hz), 4.06 (t, 2H, J=5.4 Hz), 4.44 (t, 2H, J=4.7 Hz), 4.51 (s, 4H), 4.99 (s, 1H). ^{13}C NMR (62 MHz, $CDCl_3$) δ 170.4, 169.9, 155.0, 147.3, 146.2, 146.0, 145.7, 145.4, 145.3, 144.5, 143.1, 142.6, 142.2, 142.1, 141.9, 140.2, 136.2, 80.0, 70.8, 70.5, 68.9, 68.5, 64.4, 54.2, 42.5, 28.3. FT IR (KBr) ν_{max}: 527.30, 567.68, 597.52, 769.10, 1052.58, 1119.72, 1163.81, 1242.81, 1364.96, 1637.29, 1716.55, 1746.36, 2776.11, 2924.70, 3432.63 cm^{-1}. ESI MS calculated molecular ion $[M+H]^+$: 1009.99, found: 1009.68. FT IR (as a TFA salt after removal of −Boc group, KBr) ν_{max}: 527.03 (C_{60}), 1060.04, 1130.42 (C−O), 1201.37, 1427.12 (C_{60}), 1632.41, 1678.77, 1751.84, 2924.16, 3430.34 cm^{-1}. MALDI-TOF (of the TFA salt after removal of −Boc group), (matrix: DHB): calculated molecular ion $[M+H]^+$: 909.87, found: 909.59.

4.2.4. Synthesis of proline [60]fullerene derivative 5. In a flame dried, 100 mL two-necked round-bottomed flask, equipped with a reflux condenser and a magnetic stirring bar, under N_2 atmosphere, were placed 75.8 mg (0.35 mmol) of Boc-Pro-OH and 72.7 mg (0.35 mmol) of DCC, dissolved in dry CH_2Cl_2. The resulted mixture was stirred at rt for 30 min. Then, 30 mg (0.035 mmol) of 3 were added, followed by the addition of 4.28 mg (0.014 mmol) of DMAP, both of them dissolved in dry CH_2Cl_2. The reaction mixture was left with stirring at rt, under N_2, for 48 h. Column chromatography on silica gel (SiO_2), eluant: PhMe/EtOAc, 9:1 v/v, afforded 26.2 mg (0.025 mmol) of derivative **5**, (71% yield), as a dark brown powder. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.44 and 1.46 (two s, 9H trans/cis -Boc, 60/40), 2.00 (m, 2H), 2.17 (m, 2H), 3.36 (t, 2H, J=5.3 Hz), 3.52 (m, 2H), 3.88 (t, 2H, J=4.7 Hz), 4.05 (t, 2H, J=5.4 Hz), 4.27 (q, 1H, J=4.2 Hz), 4.40 (m, 2H), 4.51 (s, 4H). ^{13}C NMR (62 MHz, $CDCl_3$) δ 173.2, 172.9, 155.1, 155.0, 154.4, 153.7, 147.3, 146.2, 146.1, 145.7, 145.4, 145.3, 144.6, 143.1, 142.6, 142.2, 142.1, 141.9, 140.1, 136.2, 79.9, 79.7, 70.8, 70.6, 70.5, 69.1, 68.6, 68.5, 64.0, 59.1, 58.8, 54.3, 46.6, 46.4, 31.0, 30.1, 28.5, 28.4, 24.4, 23.7. FT IR (KBr) ν_{max}: 527.35, 720.61. 769.10, 1082.42, 1124.00, 1185.76, 1268.92, 1397.36, 1455.42, 1630.73, 1699.90, 1738.90, 2917.91, 2970.14, 3434.44 cm^{-1}. ESI MS calculated molecular ion $[M+H]^+$: 1050.08, found: 1049.80. MALDI-TOF MS (matrix: DHB) found: 1049.91.

4.2.5. Deprotection of 5 to give salt 6. In a 50 mL round-bottomed flask equipped with a magnetic stirring bar were placed 26.2 mg (0.025 mmol) of **5**, dissolved in 4 mL of CH_2Cl_2. Then 4 mL of TFA were added and the mixture stirred at rt for 30 min. Removal of the solvent (and the remaining TFA) with a rotary evaporator afforded 23 mg (0.022 mmol) of **6**, (yield 87%), as a brown powder. ESI MS calculated molecular ion $[M+H]^+$: 949.96, found: 950.03. MALDI-TOF MS (matrix: DHB) found: 950.01.

4.2.6. Synthesis of the [60]fullerene heptapeptide 8

4.2.6.1. Coupling of the protected hexapeptide Boc-PPGMR(Pmc)P-OH with the [60]fullerene derivative 6 (isolation of 7). In a 100 mL flame dried, two-necked, round-bottomed flask, equipped with a reflux condenser and a magnetic stirring bar, under N_2 atmosphere, were placed 38.8 mg (0.038 mmol) of the protected hexapeptide Boc-PPGMR(Pmc)P-OH, 10.2 mg (0.05 mmol) of DCC, and 7.6 mg (0.05 mmol) of HOBt, dissolved in 1 mL of dry DMSO. The reaction mixture was stirred at rt for 30 min. Then, 20 mg (0.019 mmol) of derivative **6**, after being dissolved in 3 mL of dry BrC_6H_5/DMSO 6:1 v/v, and neutralized with 4.18 μL (0.038 mmol) of *N*-methyl morpholine, were added to the solution. The reaction mixture was left with stirring at rt for 60 h. After column chromatography on silica gel, PhMe/MeOH 6:1 v/v as eluant, protected product **7** was isolated (25 mg, 0.013 mmol) in fairly good yield (68%), as a brown powder.

4.2.6.2. Deprotection of 7 to give 8. 14.5 mg (0.007 mmol) of the above isolated, protected fullerene peptide, dissolved in 5 mL of CH_2Cl_2, was placed in a 50 mL round-bottomed flask equipped with a magnetic stirring bar. Then, to the above solution 5 mL (65.34 mmol) of TFA were added, and the reaction mixture was left on stirring at rt for 3 h. The solvent and excess TFA were removed on rotary evaporator followed by high vacuum pump. By this procedure 12.5 mg (0.007 mmol) of **8** were isolated quantitatively, (42% overall yield starting from **3**). ESI MS calculated molecular ion for **9**, with non-oxidized methionine $[M+2H]^{2+}$: 793.35, found: 792.98. ESI MS calculated molecular ion for **8**, with oxidized methionine $[M+2H]^{2+}$: 801.25, found: 801.07. MALDI-TOF (matrix: DHB): calculated molecular ion $[M+H]^+$: 1601.71, found: 1602.04. FT IR (KBr) ν_{max}: 527.41 (C_{60}), 1124.77 (C−O), 1184.77 (C_{60}), 1449.01 (C_{60}), 1641.05, 1738.90, 2955.22, 3430.98 cm^{-1}.

4.3. ELISA test

All sera were initially tested for ANA (Antinuclear Antibodies) using Hep-2 cells as substrate. Antigen, 10 μg/mL in carbonate buffer pH 9.6, was coated to 96-well polystyrene cuvettes (NUNC, Denmark), 50 μL/well, and was incubated at 4°C overnight. The non-specific binding sites were blocked with 5% bovine serum albumin (BSA) in Tris (50 mM)−NaCl (0.9%)−NaN_3 (0.01%) pH 7.4 (100 μL/well), overnight at 4 °C. After washing with PBS, sera were incubated (50 μL/well) at rt for 1 h in 1:100 dilution in PBS/BSA (2%)/Tween 20 (0.05%). After washing with PBS, the antibodies bound to the peptide were detected with alkaline phosphatase conjugated to *anti*-human IgG (Seralas), which was incubated for 30 min at rt (1/2500 dilution, in PBS/BSA (2%)/Tween 20 (0.005%), 50 μL/well. Finally, the plates were washed with PBS and a solution of OPD (*o*-phenyl diamine in 10 mL of citrate buffer 0.05 M, pH 5, 5 μL H_2O_2, was added to the wells (50 μL/well). The enzymatic reaction was stopped after 5 min with the addition of 2 N HCl solution (50 μL/well), and the absorbance (A) was read at 405 nm. Positive values were considered those which were above the mean optical density of normals increased by three standard deviations.

4.4. NMR spectroscopy

Identification of amino acid spin systems and sequential assignment in **8**, was made using a combination of TOCSY

and NOESY experiments. One- and two-dimensional NMR spectra were recorded on a Bruker model AMX 400 MHz spectrometer, with DMSO-d_6 as the deuterated solvent and also as a reference.

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