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Short communication

Nano-sized clusters of a teicoplanin ψ-aglycon-fullerene conjugate. Synthesis, antibacterial activity and aggregation studies

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ABSTRACT

Glycopeptide antibiotic derivative teicoplanin ψ-aglycone has been bound covalently to a fullerenopyrrolidine derivative using azide-alkyne 1,3-dipolar cycloaddition reaction. The aggregation of the antibiotic-fullerene conjugate in aqueous solution has been studied. The conjugate exhibited antibacterial activity against enterococci resistant to teicoplanin.

1. Introduction

Antimicrobial drug resistance is a serious and recurring problem in the fight against infectious bacteria. Therefore, development of new antibiotics is an important field of medicinal chemistry. Glycopeptide type antibiotics vancomycin and teicoplanin are widely used for treating dangerous Gram-positive bacterial infections that are resistant to other antibiotics.

Since glycopeptide-resistant enterococci and *Staphylococcus aureus* as well as teicoplanin-resistant *Staphylococcus haemolyticus* have emerged, in the past two decades an intensive research has been directed towards the synthesis of new glycopeptide antibiotic derivatives [1–3].

One of the successful ways of obtaining new semisynthetic glycopeptide antibiotic derivatives, active against resistant bacteria, is the introduction of lipophilic side chains into the antibiotic molecules [4–6]. Recently, in a study of semisynthetic derivatization of vancomycin, ristocetin and teicoplanin aglycons [7–9] we have also found that attachment of lipophilic groups to those molecules results in derivatives with high antibacterial and in some cases anti-influenza virus activity. We have also reported [9] that such compounds form nanosized aggregates in water solution. The glycopeptide-type antibiotics inhibit cell-wall biosynthesis of Gram-positive bacteria by forming a stable hydrogen-bonded complex with the terminal D-alanyl-D-alanine sequence of the muramyl pentapeptide intermediate formed during the biosynthesis of mureide peptidoglycan [3]. Seven types of vancomycin resistance in enterococci are known, VanC is an intrinsic resistance phenotype in *Enterococcus casseliflavus* and *Enterococcus gallinarum*, the other six resistance mechanisms (VanA, B, D, G, L) are acquired. The various acquired glycopeptide resistance genotypes are associated with mobile genetic elements, which allow resistance to spread not only clonally but laterally. In Europe the most prevalent genotypes of glycopeptide resistance are vanA and vanB, resulting resistance to vancomycin and teicoplanin, or only to vancomycin, respectively [10,11]. The resistance results from a change in the peptidoglycan termini from D-Ala-D-Ala to D-Ala-D-lactate [12] thus weakening the complex formation with the antibiotics. Since the D-Ala-D-Ala is a repeating unit of the bacterial peptidoglycan it can be assumed that its multivalent interactions with multimeric glycopeptide antibiotics can enhance the binding potency and the antibacterial effect. Indeed, covalent dimers

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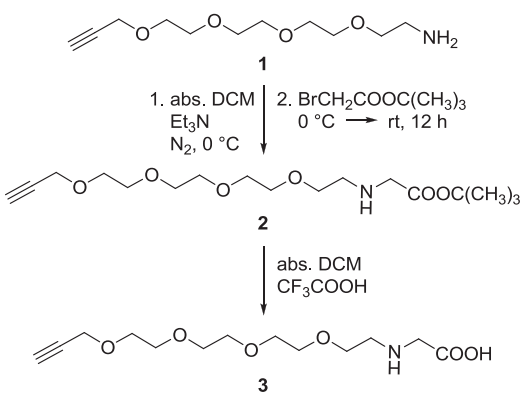
[13,14], trimers [15] and polymers [16] of vancomycin exhibited higher antibacterial activity against resistant strains. Since multivalency can be achieved by self-assembled clusters of molecules, we postulated that the very potent antibacterial activity of our recently published, aggregating teicoplanin-Ψ-aglycon derivatives [9] originates from a cluster effect, from a multivalent interaction of the antibiotic aggregate with the bacterial cell-wall repeating units. Those derivatives contained lipophilic groups such as n-decyl or biphenyl substituents. It was obvious for us to extend this principle to other glycopeptide derivatives, introducing larger lipophilic substituents into them. It is well known that fullerene (C_{60}) derivatives can form self-assembled supramolecular nanostructures in water [17]. Since derivatization of fullerene molecule is well established [18], the biomedical applications of functionalized fullerene nanomaterials are an intensively studied area of research [19]. It is assumed that covalent attachment of the bulky, very lipophilic fullerene to the teicoplanin pseudoaglycone molecule will result in amphiphilic adducts forming large clusters in water solution and hopefully exhibiting antibacterial activity against vancomycin resistant enterococci. Here we report the synthesis and studies of cluster formation and evaluation of antibacterial activity of such an antibiotic-C_{60} conjugate.

2. Results and discussion

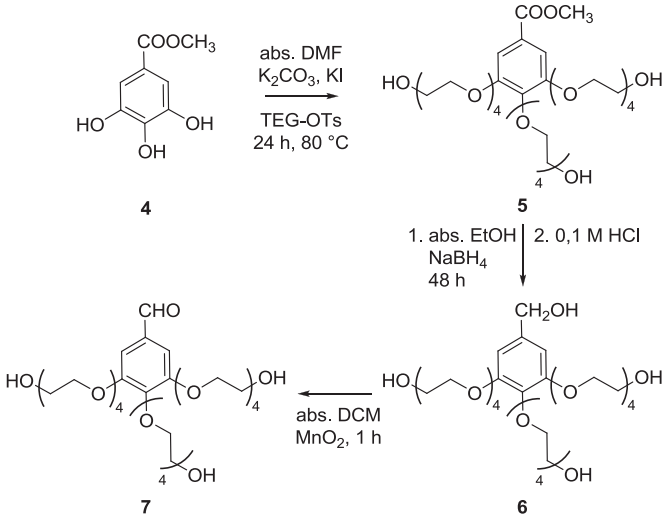
2.1. Chemistry

2.1.1. Preparation of teicoplanin Ψ-aglycone-fullerene conjugate

For the conjugation of the antibiotic molecule to fullerene we chose the copper(I) catalyzed 1,3-dipolar cycloaddition reaction of a terminal alkyne with an azido group known as “click” reaction [20,21]. Since the water solubility of the aglycon is rather low we introduced some hydrophilic tetraethylene glycol moieties on the fullerene derivative bearing the terminal alkyne group. For the derivatization of the fullerene molecule the versatile Prato reaction was chosen [22], which is a 1,3-dipolar cycloaddition of an azomethine ylide generated by the thermal reaction of an *N*-alkyl glycine with an aldehyde. For this purpose tetraethylene glycol (TEG) derivative **1** [23] was alkylated with *t*-butyl bromoacetate (**2**) and the acid protecting group was removed by hydrolysis to give **3** glycine derivative. (Scheme 1). For obtaining the aldehyde reaction partner methyl gallate (**4**) was alkylated with tetraethylene glycol monotosylate [24] resulting in **5**. The methoxycarbonyl group was then reduced (**6**) and the alcohol was oxidized with MnO_2 affording the **7** benzaldehyde derivative (Scheme 2). Prato reaction of **3**, **7** and fullerene resulted in the pyrrolidine derivative **8** with four TEG chains and one of them



Scheme 1. Synthesis of tetraethylene glycol precursor **3**.



Scheme 2. Synthesis of 3,4,5-trisubstituted benzaldehyde building block **7**.

carrying a propargyl group, ready for the subsequent click reaction with **9** azido-aglycon derivative. Copper(I)-catalyzed 1,3-dipolar cycloaddition (click) reaction of **8** with compound **9** resulted in the final product **10** (Scheme 3).

2.2. Studies on aggregation of *10*

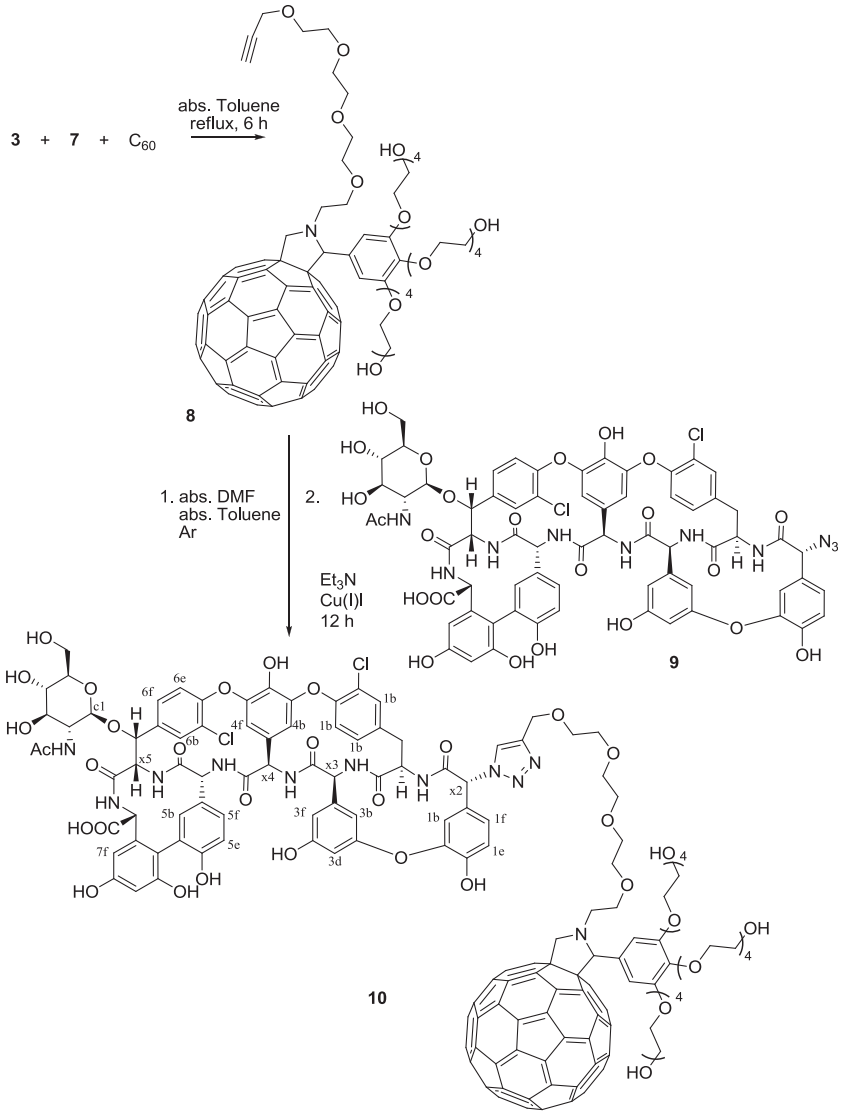
Since we expected that a possible antibacterial activity of compound **10** against resistant bacteria could be explained by formation of a self-assembled multivalent ligand from the amphiphilic molecule of **10**, the examination of its cluster formation properties were obvious.

According to our dynamic light scattering studies the effective diameter of the aggregates formed in the solution of **10** in the concentration range 0.01–0.1 mg/ml increased considerably with the increasing concentration. The effective diameter of aggregates at a concentration of 0.1 mg/ml was found to be 134 nm and the particle size increased up to 316 nm when the concentration was raised to 0.1 mg/ml (Fig. 1). These observations indicate that concentrated solutions facilitate for the formation of larger-sized aggregates.

On transmission electron microscopy (TEM) images we have detected aggregates with about 200 nm diameter (Fig. 2). Studying cluster–cluster aggregation phenomena of fullerene-cyclodextrin conjugates, Samal and Geckeler [25] reported an unexpected phenomenon: the formation of larger aggregates on dilution of aqueous solution of the conjugates. One year later Hallwass et al. [26] reported that their pulsed field gradient NMR studies were inconsistent with those results obtained by Samal and Geckeler. In water solution, in the minimal antibacterial concentration range, our teicoplanin-pseudoaglycon fullerene conjugate **10** formed on dilution definitely smaller aggregates than in higher concentration. On the TEM electron micrograph, aggregates of 200 nm clusters of **10** can be detected demonstrating the complexity of the aggregation phenomenon. So we can conclude that at least for this particular fullerene derivative we could not observe the unexpected solute aggregation phenomenon on dilution reported by Samal and Geckeler.

2.3. Antibacterial activity of antibiotic-fullerene conjugate *10*

Although there is an intensive research activity in the domain of biomedical applications of functionalized fullerene-based



Scheme 3. Synthesis of the aimed fullerene derivative **10**.

nanomaterials [19], our compound is the first antibiotic derivative of fullerene. The antibacterial activity was evaluated on a panel of various Gram-positive bacteria and is summarized in Table 1. Compound **10** showed moderate activities against MSSA, MRSA, lower than teicoplanin but exhibited activity against *Enterococcus faecalis* 15376 VanA+, which is completely resistant to vancomycin and teicoplanin. This observation supports our hypothesis: the aggregation of the antibiotic forms a multivalent ligand for the repeating terminal D-Ala-D-Ala sequence of the bacterial cell wall peptidoglycan. This multivalent interaction can form stronger complexes. Glycopeptide resistance of enterococci and in some cases of *S. aureus* is based on the replacement of the acyl-D-Ala-D-Ala terminus with an acyl-D-Ala-D-lactate terminus. In this way one of the hydrogen bonds between the bacterial peptidoglycan and the antibiotic is abolished, and the interaction decreases by 3 orders of magnitude. We suppose that in the case of compound **10** the multivalency can overcome this resistance mechanism as it has been demonstrated for *E. faecalis* 15376 *vanA*+. The higher activity of **10** compared to teicoplanin in case of various *Staphylococcus epidermidis* strains is also remarkable.

3. Conclusions

We suppose that in the case of compound **10** the multivalency can overcome the known resistance mechanism towards glycopeptides as it has been demonstrated for *E. faecalis 15376* VanA+. The higher activity of **10** compared to teicoplanin in case of various *S. epidermidis* strains is also noteworthy. In summary, we can conclude that searching for new glycopeptide antibiotic analogs capable to form aggregates in solution is a promising way of obtaining new compounds for fighting against resistant bacteria.

4. Experimental section

4.1. Materials

Teicoplanin pseudoaglycon azido derivative was prepared as described in our previous work [9]. Fullerene was purchased from SES Research, Houston, Texas. Azido derivative of teicoplanin pseudoaglycon was prepared as described in ref 9. Tetraethylene glycol, toluenesulfonyl chloride, sodium hydride, propargyl bromide, trifluoroacetic acid were purchased from Aldrich. All

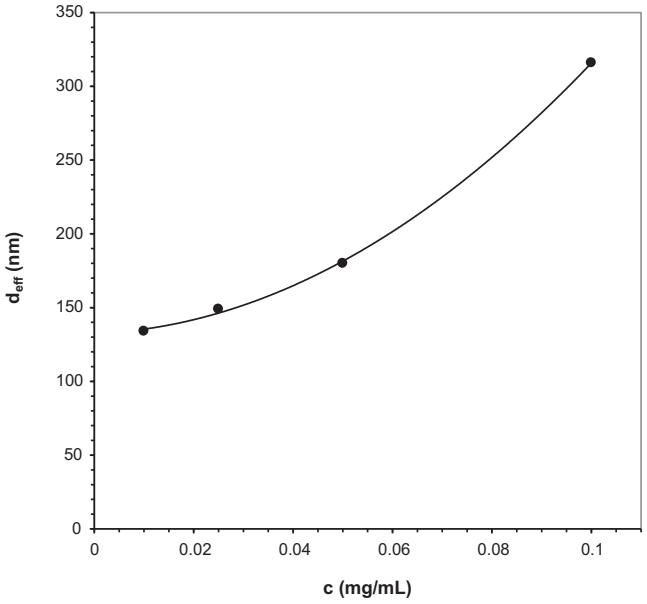


Fig. 1. Dynamic light scattering results for compound **10**.

solvents and reagents were purchased from commercial sources and were used as received.

4.2. Instrumental methods

NMR spectra were recorded with Bruker DRXII-500 and DRX-400 spectrometers in $CDCl_3$ or DMSO-d_6 solutions. Chemical shifts have been reported in parts per million (ppm) downfield from tetramethylsilane. The assignment of the final product was performed on the basis of previously reported full assignation of teichoplanin aglycon using 1D and 2D NMR techniques (HSQC) [7]. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis of products was carried out using Bruker BIFLEX III mass spectrometer (Bruker Daltonik GmbH, Germany).

4.2.1. Size distribution using dynamic light scattering

The solutions of compound **10** in water in a concentration range of 0.01–0.1 mg/ml were studied by dynamic light scattering (DLS) performed on a Brookhaven Light Scattering instrument equipped with a BI-9000 digital dynamic correlator. The laser light source was a solid state, vertically polarized laser operating at 533 nm. DLS measurements were performed at 25 °C at scattering angle $\theta = 90°$. The autocorrelation function of the scattered light intensity was acquired in the homodyne mode. For the determination of particle sizes and particle size distributions the NNLS (Non-Negative Constraint Least Squares) calculation combined with multiple pass analysis was applied.

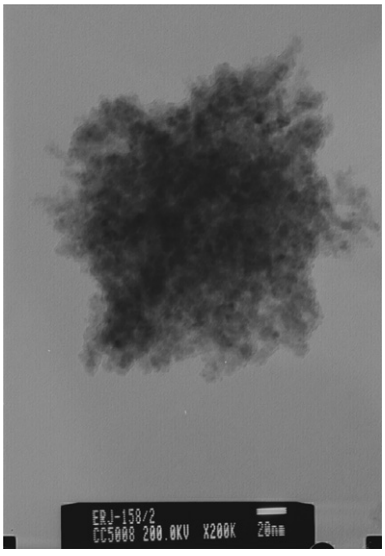


Fig. 2. Transmission electron microscopy image of the cluster of compound **10**.

Table 1
Antibacterial activity of compound **10**.

Bacteria	Teicoplanin	Compound **10**
	MIC (μg/ml)	
Bacillus subtilis ATCC 6633	0.5	16
Staphylococcus aureus MSSA ATCC 29213	0.5	8
Staphylococcus aureus MRSA ATCC 33591	0.5	4
Staphylococcus epidermidis ATCC 35984 biofilm	4	2
Staphylococcus epidermidis mecA	16	1
Enterococcus faecalis ATCC 29212	1	6
Enterococcus faecalis 15376 VanA	256	16
Enterococcus faecalis ATCC 51299 VanB	0.5	16

MIC: Minimum Inhibition Concentration, ATCC: American Typed Culture Collection, MRSA: Methicillin Resistant *Staphylococcus aureus*, vanA: vanA gene positive, vanB: vanB gene positive.

4.2.2. Transmission electron microscopy image analysis

TEM measurements were performed using a Jeol 2000FX-II equipment operating at 200 kV accelerating voltage. Sample preparation: the sample solutions were dried on 400 mesh copper microgrids covered by amorphous carbon substrate layer.

4.3. Antibacterial activity

The efficacy of the prepared compounds was determined with the broth micro dilution method according to the CLSI guideline. Bacterial strains were grown on 5% bovine blood agar plates at 35 °C overnight. Appropriate numbers of colonies were suspended in physiological saline in order to reach the density of 0.5 McFarland for inoculation.

Stock solutions containing different concentrations of the substances were prepared in either distilled water or H_2O and methanol (1:1) or H_2O and DMSO (1:1), respectively, depending on the solubility of the given preparation. These were two-fold serially diluted from 256 to 0.5 μg/ml in cation-adjusted Mueller-Hinton broth, and then 100 μl of each dilution was transferred into microplate holes. Inoculation was carried out with 10 μl of each bacterial suspension. Incubation was performed at 35 °C for 18 h and determination of the minimal inhibitory concentration (MIC) was made with the naked eyes on a mirror.

4.4. Synthesis of the teicoplanin-ψ-aglycon-fullerene conjugate

4.4.1. Synthesis of ***2***

To the solution of **1** (763 mg, 3.3 mmol) in dry dichloromethane (30 ml) triethylamine (555 μl, 4.0 mmol) was added, and the mixture was cooled to 0 °C and stirred under N_2 atmosphere. *t*-Butyl bromoacetate (536 μl, 3.63 mmol) was added dropwise to the solution and it was stirred for overnight at rt. Then the mixture was extracted with brine, the organic layer was dried (Na_2SO_4) and the solvent was evaporated in vacuum. The residue was purified by flash chromatography (CH_2Cl_2/CH_3OH 95:5) to yield compound **2** (46%). δ_H (400 MHz; $CDCl_3$; TMS) 1.41 (9H, s, C–$(CH_3)_3$), 3.11 (2H, m,

NH–C**H**$_2$), 3.28 (2H, m, C**H**$_2$–C≡), 3.47–3.70 (17H, m, CH_2CH_2, C≡C**H**, C**H**$_2$CO); δ_C (100 MHz; $CDCl_3$; TMS) 28.4, 59.8, 61.6, 70.0, 70.1, 70.2, 70.4, 70.5, 72.6, 80.3, 173.1;

MS (MALDI-TOF) *m/z* calcd for $C_{17}H_{31}NO_6Na^+$ $[M + Na]^+$: 368.21, found: 368.25.

4.4.2. Synthesis of ***3***

Compound **2** (528 mg, 1.53 mmol) was dissolved in dry dichloromethane (30 ml) and stirred with 10 ml of 80% trifluoroacetic acid overnight, then the mixture was concentrated under vacuum to yield compound **3** (87%).

δ_H (400 MHz; $CDCl_3$; TMS) 2.47 (1H, m, NH), 3.21 (2H, m, CH_2–C**H**$_2$–NH), 3.30 (1H, t, C≡CH), 3.47–3.94 (16H, m, CH_2CH_2), 4.18 (2H, m, CCH_2), 4.40–4.72 (2H, m, CH_2NH), 8.90 (1H, br s, COOH); δ_C (100 MHz; $CDCl_3$; TMS) 47.3, 58.2, 60.2, 68.7, 69.6, 70.0, 70.2, 70.6, 72.4, 75.0, 169.5.

4.4.3. Synthesis of ***5***

Compound **4** (920 mg, 5 mmol) was dissolved in dry dimethylformamide (35 ml) in the presence of K_2CO_3 (2.76 g, 20 mmol), KI (830 mg, 5 mmol) and tetraethylene glycole monotosylate (7.0 g, 20 mmol). The reaction mixture was stirred for 24 h at 80 °C. After the solvent was removed under vacuum, dichloromethane was added to the residue and it was extracted with brine (2 × 60 ml), and then washed with water (60 ml). The organic layer was dried (Na_2SO_4), then the mixture was concentrated under vacuum. The residue was purified by flash chromatography (CH_2Cl_2/CH_3OH 95:5) to yield compound **5** (48%).

δ_H (500 MHz; $CDCl_3$; TMS) 2.70 (3H, br s, OH), 3.55–3.75 (45H, m, CH_2CH_2, OCH_3), 3.80–3.90 (2H, 2t, $PhOCH_2$), 4.20–4.30 (4H, 2t, $PhOCH_2$), 7.30 (2H, s, C_6H_2); δ_C (100 MHz; $CDCl_3$; TMS) 52.7, 62.2, 69.4, 70.2, 70.9, 71.0, 71.2, 71.4, 73.1, 73.2, 109.5, 125.6, 152.8, 165.6; MS (MALDI-TOF) *m/z* calcd for $C_{32}H_{56}O_{17}Na^+$ $[M + Na]^+$: 735.34, found: 735.30.

4.4.4. Synthesis of ***6***

Compound **5** (977 mg, 1.37 mmol) and $NaBH_4$ (993 mg, 21 mmol) were dissolved in abs. ethanol and stirred for 48 h at rt. Then the precipitate formed after the addition of 0.1 M HCl was filtered, and the filtrate was concentrated in vacuum. The residue was dissolved in water (60 ml), and was stirred with Serdolit blue anion exchange resin for 15 min, then with Serdolit red cation exchange resin for another 15 min. After filtration the solvent was evaporated in vacuum, and the residue was purified by flash chromatography (CH_2Cl_2/CH_3OH 85:15) to a yield compound **6** (79%).

δ_H (500 MHz; $CDCl_3$; TMS) 2.60–2.90 (4H, m, OH), 3.55–3.75 (42H, m, CH_2CH_2), 3.75–3.85 (2H, 2t, $PhOCH_2$), 4.10–4.20 (4H, 2t, $PhOCH_2$), 4.55 (2H, s, $PhCH_2$), 6.60 (2H, s, C_6H_2); δ_C (100 MHz; $CDCl_3$; TMS) 68.7, 69.5, 70.2, 70.3, 70.5, 71.7, 71.4, 72.5, 72.6, 108.8, 124.9, 152.1; MS (MALDI-TOF) *m/z* calcd for $C_{31}H_{56}O_{16}Na^+$ $[M + Na]^+$: 707.35, found: 707.27.

4.4.5. Synthesis of ***7***

To compound **6** (700 mg, 1.02 mmol) dissolved in dry dichloromethane (70 ml), MnO_2 (3.5 g, 0.04 mmol) was added and the reaction mixture was stirred vigorously for 1 h. Then the mixture was filtered and the solvent was evaporated in vacuum to yield compound **7** (92%).

δ_H (500 MHz; $CDCl_3$; TMS) 2.18–2.27 (4H, br s, OH), 3.51–3.74 (42H, m, CH_2CH_2), 3.80–3.90 (2H, 2t, $PhOCH_2$), 4.19–4.31 (4H, 2t, $PhOCH_2$), 7.15 (2H, s, C_6H_2), 9.83 (1H, s, CHO); δ_C (100 MHz; $CDCl_3$; TMS) 61.3, 68.5, 69.2, 69.9, 70.0, 70.1, 70.2, 70.4, 72.2, 108.5, 131.2, 152.6, 190.6; MS (MALDI-TOF) *m/z* calcd for $C_{31}H_{54}O_{16}Na^+$ $[M + Na]^+$: 705.33, found: 705.30.

4.4.6. Synthesis of ***8***

Compound **3** (58 mg, 0.2 mmol) and compound **7** (136 mg, 0.2 mmol) were added to the solution of fullerene (72 mg, 0.1 mmol) in dry toluene (30 ml), and the mixture was stirred under reflux for 6 h. Then the solvent was evaporated in vacuum, and the residue was purified by flash chromatography (CH_2Cl_2/CH_3OH 9:1 and 0.1% triethylamine) to yield compound **8** (20%).

δ_H (500 MHz; $CDCl_3$; TMS) 2.04–2.39 (4H, m, OH), 2.44 (2H, s, N–C**H**$_2$–CH_2), 3.41–3.49 (2H, m, N–C**H**$_2$–C_{60}), 3.50–3.78 (42H, m, CH_2CH_2), 3.91–4.08 (2H, m, $PhOCH_2$), 4.09–4.57 (6H, m, $PhOCH_2$, N–CH_2–C**H**$_2$), 5.03 (1H, s, C≡CH), 5.19 (1H, s, N–C**H**–Ph), 5.22 (1H, s, N-C**H**-Ph), 7.18 (1H, s, aromatic H), 7.33 (1H, s, aromatic H); δ_C (100 MHz; $CDCl_3$; TMS) 52.7, 58.4, 63.8, 67.2, 68.7, 68.8, 69.1, 70.0, 70.1, 70.2, 72.2, 72.3, 74.3, 75.9, 76.4, 76.7, 82.0, 108.7, 108.8, 128.5, 132.3, 135.3, 135.5, 136.0, 136.2, 138.0, 139.2, 139.4, 139.7, 139.8, 141.2, 141.3, 141.4, 141.5, 141.7, 141.8, 141.9, 142.2, 142.3, 142.6, 142.8, 144.0, 144.1, 144.3, 144.4, 144.8, 144.9, 145.1, 145.4, 145.5, 145.6, 145.8, 145.9, 146.1, 146.5, 147.0, 153.0, 153.4, 153.7, 155.9; MS (MALDI-TOF) *m/z* calcd for $C_{103}H_{75}NO_{19}Na^+$ $[M + Na]^+$: 1652.55, found: 1652.48.

4.4.7. Synthesis of ***10***

Compound **8** (32.6 mg, 0.02 mmol) was dissolved in the mixture of dry dimethylformamide (4 ml) and dry toluene (1 ml) and stirred under Ar atmosphere. Compound **9** (28.4 mg, 0.02 mmol), triethylamine (6 μl, 0.04 mmol) and Cu(I)I (5 mg, 0.026 mmol) were added to the solution, and it was stirred overnight. It was filtrated, the solvent was evaporated in vacuum and the residue was purified by flash chromatography (first with toluene/methanol 6:4, then with toluene/dimethylformamide 1:1 and finally with dimethylformamide).

δ_H (500 MHz; DMSO-d_6) 2.87 (2H, s, N–C**H**$_2$–CH_2), 3.14–4.22 (58H, m, CH_2CH_2, $PhOCH_2$), 4.28 (1H, s, x5), 4.36 (1H, s, c1), 4.41–4.43 (2H, m, N–CH_2–C**H**$_2$), 4.81 (1H, s, x2), 5.05 (1H, m, 4f), 5.24 (2H, m, N–C**H**–Ph), 5.39 (1H,s, x3), 5.54 (1H, m, 4b), 5.61 (1H,s, x4), 6.30 (1H, m, 7f), 6.32 (1H, m, 3b), 6.34 (1H, m, 3d), 6.41 (1H, m, 3f), 6.48 (1H, m, 7d), 6.62 (1H, m, 5e), 6.63 (1H, m, 1b), 6.65 (1H, m, 5f), 7.03 (1H, m, 1e), 7.11 (1H, m, 5b), 7.16 (1H, m, 1f), 7.18 (1H, m, 2b), 7.19 (1H, m, 6e), 7.20 (1H, s, aromatic H), 7.21 (1H, s, aromatic H), 7.31 (1H, m, 2e), 7.46 (1H, m, 6f), 7.68 (1H, m, 2f), 7.71 (1H, m, 6b); MS (MALDI-TOF) *m/z* calcd for $C_{169}H_{131}Cl_2N_{11}O_{42}Na^+$ $[M + Na]^+$: 3079.88, found: 3079.53.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejmech.2012.06.054.

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