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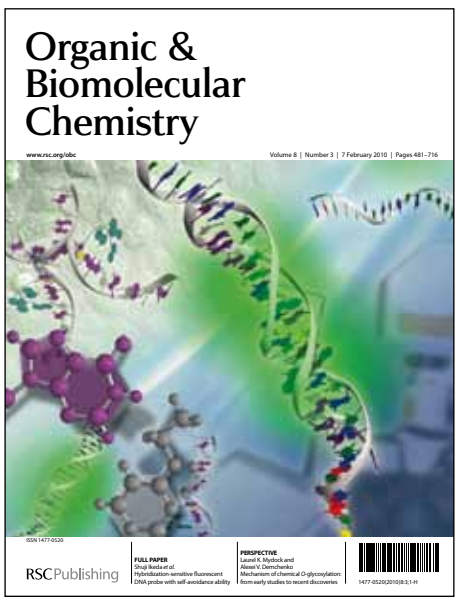

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Communication

Novel porphyrin-psoralen conjugates: synthesis, DNA interaction and cytotoxicity studies

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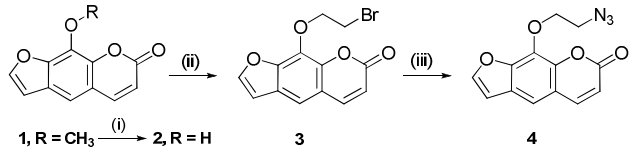
A Cu(I)-catalyzed azide-alkyne cycloaddition reaction (CuAAC) has been utilized to prepare novel triazole–linked cationic porphyrin–psoralen conjugates that exhibited significant photocytotoxicity against A549 cancer cells (IC_{50} = 84 nM).

Since many years photodynamic therapy (PDT) has emerged as an effective modality for the selective treatment of tumors and neoplastic lesions.[1] The mechanism of the tumor destruction is interconnected through several biological responses and still needs to be fully understood. However, a key step in PDT requires the accumulation and retention of a photosensitizing agent in the neoplastic tissue to generate cytotoxic species upon light exposure.[2] Number of strategies have been examined to improve selectivity and specificity of photodynamic sensitizers towards tumor cells.[3] The use of porphyrin hybrids has emerged as one of the attractive methods.[3a,c,d] Coupling of porphyrin with carbohydrates,[4] monoclonal antibodies,[5] oligo-nucleotides,[6] peptides,[7] has been reported in the recent past. Linking of porphyrin with synthetic compounds or natural products with established biological significance is found to give better efficacy, selectivity, and stability as compared with porphyrin alone.[8,9] Moreover, the structural diversity that can be introduced on porphyrin is limited, whereas any number of hybrids as combinations of parts of different biologically potent motifs can be prepared. The idea of synthetic porphyrin conjugates as a treatment modality is still emerging and its potential remains to be explored.

The photosensitizing 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), and congeners are widely used in psoralen plus ultraviolet-A radiation (PUVA) therapy as an effective treatment for various skin diseases and, recently, for T-cell lymphoma by means of photochemotherapy. The biological activity of these derivatives has been attributed to their ability to photoreact with DNA.[10,11] The photoadducts were found to be effective towards the inhibition of DNA synthesis through photoinduced mutagenicity.[12] In spite of this, PUVA therapy has some adverse effects such as photosensitive skin, formation of cutaneous tumors and induced genotoxicity.[13] Some studies revealed that PUVA induced apoptosis in cultured lymphoma cell lines, however, it was found to be less effective than the existing anticancer drugs and radiation therapy in terms of killing carcinoma cells. Affinity ligands based on antibodies, protein cage, polypeptide, nanoparticles, and bacteriophage have been successfully reported to direct lethal photosensitizers to cancer cells.[14] For example, conjugates porphyrin–platin and porphyrin–paclitaxel exhibited better efficacies and selectivity towards cancerous cells over normal cells as compared to parent drugs.[15a-c] These studies showed that "dual" approach has higher therapeutic effect and improved cellular uptake than single drug therapy.[15c-e]

To attain highly efficient DNA photocleavge activity we designed novel water-soluble cationic porphyrin–psoralen conjugates. Porphyrin, a commonly used photosensitizer due to its clinical significance in both PDT and photodynamic diagnosis (PDD), was chosen as a high affinity DNA binding agent to design psoralen conjugate **11**. Also, motivated with the encouraging results of cationic porphyrin conjugates at our hands[16] and effectiveness of psoralen scaffolds against cancers, we prepared water-soluble novel cationic porphyrin–psoralen conjugates using click chemistry approach. Click chemistry is the new concept developed by Sharpless and co-workers that has attracted significant interest due to its versatility, high yield, simple reaction conditions and their tolerance of water and oxygen.[17] Click reaction involves the copper-mediated cycloaddition of organic azides and alkynes leading to 1,2,3–triazoles. It is a powerful linking reaction which has quickly found numerous applications in chemistry, biology, and materials science.[17]



Scheme 1 (i) $AlCl_3$, CH_2Cl_2, rt, 5 h; (ii) 1,2-dibromoethane, K_2CO_3, DMF, rt, 6 h; (iii) NaN_3, DMF: H_2O (3: 1 *v/v*), 40 °C, 8 h.

We envisaged the synthesis of porphyrin conjugate **11** from psoralen azide **4** and (propargyloxyphenyl)porphyrin **7**. The psoralen azide **4** was prepared as described in Scheme 1. Demethylation of the 8-methoxy psoralen **1** with anhydrous aluminium chloride at room temperature produced exclusively

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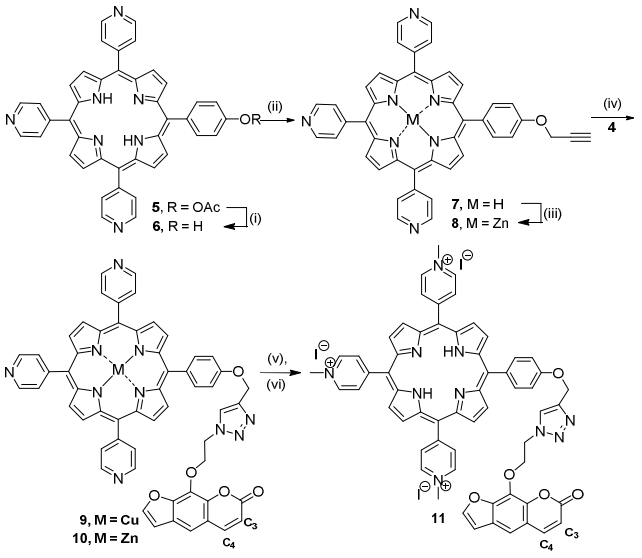
hydroxypsoralen **2**.[18] The alkylation of **2** with dibromoethane led to **3**, which upon treatment with sodium azide in DMF afforded psoralen azide **4** in good yield (70%).

We next prepared the porphyrin **5** by using Adler–Longo protocol.[19] Hydrolysis of **5** with potassium hydroxide produced 5–(4′–hydroxylphenyl)–10,15,20–tri(4'–pyridyl)porphyrin **6** in excellent yield (85%). Treatment of **6** with propargyl bromide (1.2 equiv.) in the presence of fused K_2CO_3 in dry DMF afforded 5–(4′–propargyloxyphenyl)–10,15,20–tri(4'–pyridyl)porphyrin **7** in 53% yield (Scheme 2). The ^{1}H NMR spectrum of **7** showed two characteristic singlets at δ 5.10 (OCH_2) and δ 2.88 (C≡CH) along with other protons. The coupling reaction of **7** with psoralen azide **4** (3.0 equiv.) in the presence of $CuSO_4.5H_2O$ (1.0 equiv.) and sodium ascorbate (2.0 equiv.) in DMF: H_2O (1: 1 *v/v*) at 80 °C for 36 h afforded metallated porphyrin–psoralen conjugate **9**. We attempted demetallation of compound **9** in 10% TFA/H_2SO_4 but the reaction afforded (4′-hydroxypheny)-10,15,20–tri(4'–pyridyl)porphyrin **6** as the only isolable product with the cleavage of propargyloxy group. So to avoid metallation of porphyrin core during the CuAAC reaction we converted the free base porphyrin **7** to zinc metallated porphyrin **8** by treatment with $Zn(OAc)_2$ in chloroform–methanol. Cycloaddition reaction of **8** with **4** (3 equiv.) using $CuSO_4.5H_2O$ (4 equiv.) and sodium ascorbate (8 equiv.) at 80 °C in DMF: H_2O for 168 h gave porphyrin–psoralen conjugate **10** (Scheme 2). With lesser equivalents of $CuSO_4.5H_2O$ and sodium ascorbate, and at lower temperatures, the reaction was very sluggish which led to decomposition of psoralen azide **4** and poor yield of the desired product.



Scheme 2 (i) KOH, MeOH: H_2O, reflux, 5 h; (ii) propargyl bromide (1.2 equiv), K_2CO_3 (1.2 equiv), DMF, 0–27 °C, 24 h; (iii) $Zn(OAc)_2$, $CHCl_3$: MeOH, reflux, 2 h; (iv) $CuSO_4.5H_2O$, sodium ascorbate, DMF: H_2O (1: 1 *v/v*), 80 °C, 32–168 h; (v) **10**, $CHCl_3$, aq HCl (25%), 1 h; (vi) MeI (120 equiv), DMF, rt, 36 h.

The proton NMR spectrum of porphyrin–psoralen conjugate **10** showed a singlet at δ 5.77 (–$C_6H_4OC\mathbf{H}_2$–*N*), two triplets at δ 5.00 (triazolyl-*N*$\mathbf{CH_2}$) and δ 3.76 (triazolyl-*N*$CH_2\mathbf{CH_2}$O-) along with two doublets at δ 8.17 and 6.82 for C_3–H and C_4–H protons of psoralen, respectively. Mass spectrum of conjugate **10** displayed a peak at *m/z* 1004 for $[M]^+$ ion that corresponds to the molecular formula $C_{57}H_{36}N_{10}O_5Zn$.

Porphyrin–psoralen conjugate **10** was demetallated using aqueous HCl in chloroform and treated with excess of methyl iodide to afford water–soluble cationic porphyrin–triazole **11**. The structure of conjugate **11** was elucidated by ^{1}H NMR and MALDI–TOF spectral data. The ^{1}H NMR spectrum of **11** showed a characteristic singlet at δ 4.73 for –N^+CH_3 protons. The *β*–pyrrolic and *N*–methylpyridinium protons (H–2' & H–6') in **11** were shifted downfield when compared to porphyrin conjugate **10**.[20] The HRMS (MALDI–TOF) spectrum of **11** displayed a peak at *m/z* 987.2841 that is in agreement with calculated mass 987.2814 for $C_{60}H_{47}N_{10}O_5$ (M^+–3I^-). The purity of the isolated porphyrin conjugate **11** was greater than 99% as evaluated by HPLC analysis.[20]

The interactions of cationic porphyrin conjugate **11** and 5,10,15,20-tetrakis(*N*-methylpyridinium-4-yl)porphyrin (TMPyP) with calf thymus DNA (ctDNA) were studied using UV-vis spectroscopy (Fig. 1). The absorption spectra of cationic porphyrin conjugate **11** (2 μM) was recorded with increasing

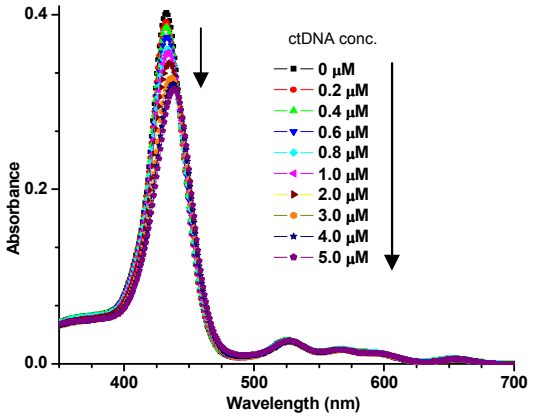


Fig. 1 Absorption spectra of conjugate **11** (2 μM) with increasing ctDNA concentrations (5 mM Tris-HCl, 0.1 M NaCl, pH 8.0, 25 °C).

amounts of ctDNA (0-55 μM). Absorption spectra of **11** showed a red shift of 12 nm and hypochromicity of 27% indicating intercalative binding towards ctDNA. Whereas, TMPyP showed a redshift of 16 nm and hypochromicity of 41%. Further, we carried out competitive binding study for the conjugate **11** using the emission intensity of DNA intercalator ethidium bromide (EB).[21] EB bound to ctDNA exhibits several fold enhancement in its fluorescence intensity which can be quenched by the addition of another DNA binding molecule. As shown in Fig. 2 with the increasing amounts of conjugate **11** an appreciable reduction in the fluorescence intensity of the ctDNA-bound EB was observed. The quenching plot of I_0/I *vs* [Por **11**]/[DNA] was found to be in good agreement with the linear Stern–Volmer equation with a slope of 3.31 for **11** (ESI, Fig. S1).[22] The decrease in fluorescence intensity could be attributed to the appreciable replacement of the EB bound to ctDNA by the intercalation of conjugate **11**.

The DNA cleavage activities of the porphyrin conjugate **11** were examined by its effectiveness in converting circular

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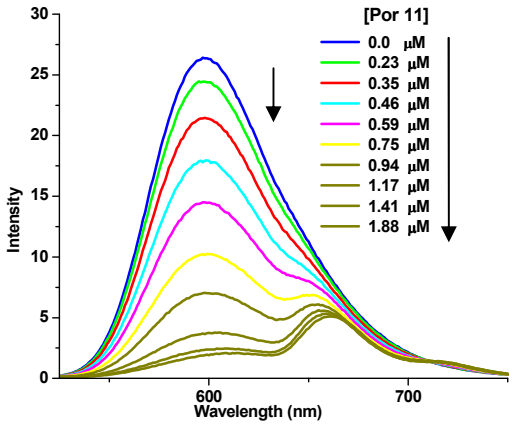

Fig. 2 Emission spectra of EB (5 μM) bound to DNA (12 μM) in 5 mM Tris-HCl, 0.1 M NaCl, (pH 8.0) at λ_{ex} = 510 nm in the absence and presence of varying concentrations of **11**.

supercoiled DNA (form I) to circular relaxed (form II) DNA (Fig. 3). The conjugate **11** (1-25 μM) and plasmid ΦX174 DNA (0.5 μg) were incubated in Tris-HCl (20 mM) buffer solution (pH 7.2) at ambient temperature for 30 min, then exposed to UV–A (310–390 nm, 4 mW) or visible (400–800 nm, 2 mW) light. As shown in Fig. 3, significant single strand cleavage was observed for the

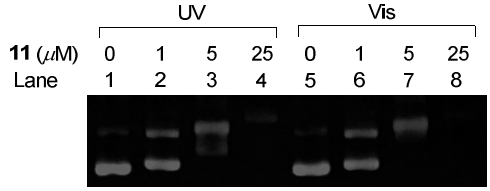

Fig. 3 Photoinduced DNA cleavage by **11**. ΦX174 supercoiled DNA (0.5 μg) was incubated with **11** (0-25 μM) in Tris–HCl (20 mM, pH 7.2) containing NaCl (20 mM), DMSO (2.5 vol%) at ambient temperature in the dark for 30 min, and then exposed to (lanes 1–4) UV–A or (lanes 5–8) visible light for 10 min.

UV and visible–light exposed **11**. It should be noted that high concentrations of **11** caused mobility change and darkening of the DNA bands, probably due to intercalation of **11** into plasmid DNA. It has been reported that psoralen intercalates into DNA duplex and forms interstrand cross-links in DNA upon UV-irradiation.[23] We performed restriction enzyme assay for the photo-irradiated plasmid DNA samples and confirmed that counjgate **11** could induce interstand crosslinking (See ESI, Fig S5,6). It is thus likely that the cross-link formation between **11** and DNA lowered mobility of the products.

The photocytotoxicities of **9** and **11** towards A549, a human epithelial cell line derived from a lung carcinoma, were determined by WST assay. The cell viabilities were ploted against the concentrations of conjugates (Fig. 4) and the results are listed in Table 1, in which cytotoxicities are expressed as the concentration of the drugs that inhibit 50% of cell proliferation (IC_{50}). Interestingly, **9** and **11** showed potent toxicities with IC_{50} values of 3.18 μM and 84 nM, respectively after UV–A or visible light exposure; whereas both conjugates are nontoxic (IC_{50} >100 μM) in the dark.

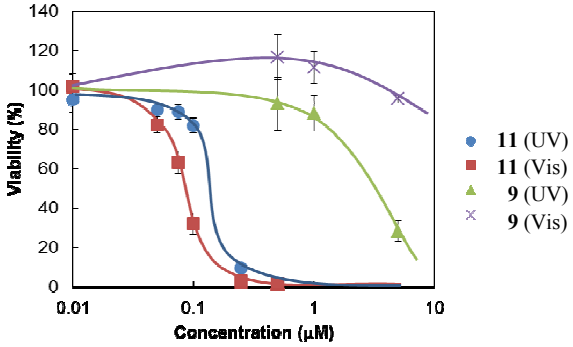

Fig. 4 Cell viablility data for porphyrin conjugates (**9** and **11**).

Table 1 Phototoxicity results of **2**, **9, 11** and TMPyP towards A549.

	IC_{50}[a]		
Porphyrin	Dark	Visible	UV-A
11	>100 μM	84 nM	146 nM
9	>100 μM	>10 μM	3.18 μM
TMPyP	>100 μM	171 nM	659 nM
2	>100 μM	>100 μM	>100 μM

[a] The results are the mean values of at least 3 independent experiments.

This enhanced photocytotoxicity relative to the dark control is an essential property for a photochemotherapeutic agent. As envisioned the cationic conjugate **11** exhibited higher phototoxicity than TMPyP (IC_{50} 171–659 nM), which has been extensively studied as a tumor–localizing, potent photosensitizing drug.[24]

To visualize nuclear changes and apoptotic body formation that are characteristic of apoptosis, A549 cells were treated with **11** (0.1 μM) for 24 h, washed with phosphate buffered saline, and

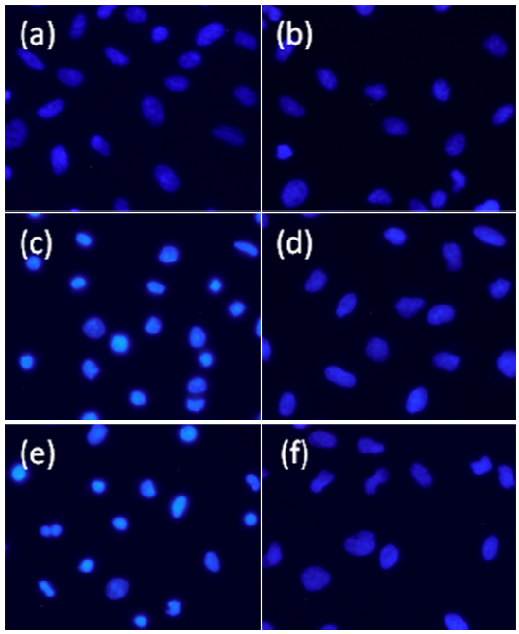

Fig. 5 Fluorescence microscopic images of Hoechst 33342-stained A549 cells. Normal appearance of nuclei in control cells (a); Cells were incubated in the presence of **11** (0.1 μM) in the dark (b); Cells were exposed to UV-light (4 mW, 10 min) (c, d) or visible-light (2 mW, 10 min) (e, f) in the presence (c, e) or absence (d, f) of **11** (0.1 μM).

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then were exposed to UV or visible light for 10 min. After further incubation for 24 h, cells were stained with Hoechst 33342 (Fig. 5). The non-irradiated cells (Fig. 5d & 5f) showed no remarkable changes as compared to the control (Fig. 5a), while after the cells were exposed to UV or visible light (Fig. 5c & 5e), a number of bright spots appeared in the cells. Such morphological changes of nuclei are often observed in apoptotic cells, and thus we preliminarily conclude that the cationic porphyrin conjugate **11** may induce cell death in A549 cells through an apoptotic pathway.

In summary, we have prepared a novel water soluble cationic porphyrin–psoralen conjugate which exhibited high photocytotoxicity towards A549 cancer cells when compared to a tumor–localizing and potent photosensitizing agent, TMPyP. This work demonstrates that porphyrin–psoralen conjugate **11** could be a potential candidate for PDT. Further mechanistic and structure-activity relationship studies of porphyrin–psoralen conjugates are underway in our laboratory and will be reported in due course of time.

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20 **Preparation of cationic porphyrin–psoralen conjugate 11:** To a stirred solution of metallated porphyrin **8** (0.12 g, 0.163 mmol) in DMF:H_2O (1:1; 70 mL) was added $CuSO_4.5H_2O$ (0.163 g, 0.653 mmol), sodium ascorbate (0.259 g, 1.306 mmol) followed by psoralen azide **4** (0.132 g, 0.49 mmol). The reaction mixture was allowed to stir at 80 °C for 168 h. After completion of the reaction, the contents were diluted with chloroform (4 × 80 mL) and ammonia solution (20%, 30 mL). The resulting solution filtered over a thin celite bed and washed thoroughly with chloroform. The combined organic phase was washed with water, dried over anhydrous sodium sulfate and the solvents were evaporated *in vacuo*. The residue thus obtained was purified by column chromatography using chloroform/methanol (8:2) as eluent to afford pure porphyrin conjugate **10** (0.082 g, yield 52%) as a red solid. To a cooled solution (5-10 °C) of Zn(II) conjugate **10** (0.091 g) in chloroform (80 mL) was added aq. HCl (25%, 20 mL) and stirred for 1 h at 27 °C. After complete demetallation, the

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temperature was lowered to 0–2 °C and the reaction mixture was basified with ammonia solution, the organic layer was separated and dried over anhydrous sodium sulfate. The solvents were distilled off *in vacuo* to afford demetallated porphyrin triazole (0.085 g), which was dissolved in dichloromethane:methanol (9:1), precipitated by adding hexane, filtered, dried and used as such for further reaction. To a solution of demetallated porphyrin triazole (0.085 g, 0.091 mmol) in dry *N,N′*-dimethylformamide (20 mL) was added methyl iodide (0.75 mL, 11.73 mmol) and allowed to stir at room temperature until starting material was consumed completely (36 h). The solvent was removed *in vacuo* and methanol (5 mL) was added. The compound was precipitated by adding diethyl ether (15 mL), and the resulting solid was filtered. This procedure was repeated four times to afford compound **11** (0.071 g, yield 65%) as brown solid. m.p. > 300 °C; ^{1}H NMR (500 MHz, DMSO–d_6) δ 9.48–9.47 (m, 6H, 2′,6′ *Me*–$^+$*N*–*pyridiniumyl* H), 9.32–9.01 (m, 14H, 3′,5′ *Me*–$^+$*N*–*pyridiniumyl* H & *β–pyrrole* H), 8.25 (d, J = 9.9 Hz, 1H, psoralen $C_{3'}$H), 8.20 (d, J = 8.9 Hz, 1H, psoralen $C_{7'}$H), 7.88 (s, 1H, triazolyl $C_{5''}$H), 7.48 (d, J = 8.2 Hz, 2H, 2′,6′ *meso*-phenyl H), 7.33 (s, 1H, psoralen $C_{5'}$H), 7.24–7.23 (m, 3H, 3′,5′ *meso*–phenyl H & psoralen $C_{6'}$H), 6.75 (d, J = 9.9 Hz, 1H, psoralen $C_{4'}$H) 5.83 (s, 2H, –C_6H_4O**C**$\mathbf{H_2}$–triazol), 5.22 (t, J = 7.5 Hz, 2H, triazolyl–CH_2**C**$\mathbf{H_2}$O–), 4.73 (s, 9H, 3 × –N^+CH_3), 3.99 (t, J = 7.4 Hz, 2H, triazolyl–**C**$\mathbf{H_2}$$CH_2$O–), −3.01 (s, 2H, pyrrole NH); ^{13}C NMR (126 MHz, DMSO–d_6) δ 161.10 (O–**C**=O–, psoralen) 159.05, 151.66, 151.00, 148.58, 148.08, 147.10, 144.28, 143.27, 142.75, 140.44,138.45, 133.18, 132.13, 131.89, 131.18, 127.35, 126.94, 124.18, 122.49, 118.81, 116.11, 114.98. 114.88, 107.19, 67.21 (O**C**H_2–triazol), 56.97 (–O**C**H_2**C**H_2–), 47.30 (–N^+**C**H_3); MALDI–TOF: *m/z* calcd for $C_{60}H_{47}N_{10}O_5$ [M^+–3I]: calcd: 987.2814; found: 987.2841; HPLC purity: 99.24%.

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