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Leveraging Synthetic Chlorins for Bio-imaging Applications

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Synthetic chlorins are not only fluorescent, the modulation of the tetrapyrrole system can also chelate metal ions. Conjugation of linkers at their pyrrolidines allows for conjugation to bio-molecules to create target specificity. By altering these chemo-photophysical properties, this work facilitates the use of chlorins in fluorescent imaging and positron emission tomography (PET).

Synthetic chlorins have the intrinsic ability to serve as multimodal imaging agents.[1] Chlorin dyes present several beneficial photophysical properties for exploration in the imaging field, for example in *in vivo* imaging of animals[2, 3] fluorescent-guided surgery of cancer resections,[4] potential fluorescent detection of viral and bacterial infections[5, 6] and nerve-related illnesses.[7] These compounds have also attracted researchers' attention due to their reputed attributes as an alternative for drug-resistance[8] and as chelators of metals and radionuclides.[9] Since the first synthesis of Woodward's chlorin e_6,[10] no strategic design for their use in the imaging field as contrast agents has been proposed. Most chlorins presented in the literature (i) require tedious *de Novo* synthesis to diversify the chemistry of conjugations and (ii) are reduced products at β-pyrrole positions on the porphyrin system which and results in mixtures of enantiomeric chlorins that will anticipate purity issues if translated.[11]

The hallmark of chlorin is its 650 nm absorption signal, which makes the chlorin a red absorbing dye— but with far more advantages and favourable bio-physical features over porphyrins which lack fluorescence for imagining, present limited functionalization for conjugation and are restricted to metalations.[12] In fact, through their macrotetracycles, these light driven systems use their absorbed energy more diversely and efficiently than their parent compound porphyrin,[13] an improvement that stems from chlorin's altered symmetry and pathway of conjugation.[14] This relationship between chlorins and absorbed energy offers the former one a menu of photophysical manifestations to choose, observed as fluorescence, phosphorescence and/or thermal dissipation.

Due to their toxicity, chlorins could be used in many forms of cancer,[15] infections[5] and in other photonic bioanalytical applications.[13] But, similarly to develop chlorins as imaging agents and to take maximum advantage of their properties, they need to be conjugated to a targeting-molecule, for instance: sugars (e.g. galactose),[16] peptides (e.g. spider venoms and antibodies)[17] or DNA (e.g. aptamers).[18] With a modular system in hand, the adjustment of each component is feasible, for instance if different linkers are employed, the use of distinct dyes and bio-active substance is possible resulting in higher leverage of photonic attributes, affinity and pharmacokinetics. Despite of their potential features, chlorins and their multi-modal properties remain unexplored.[1] In fact, chlorins' use has been limited to single applications due to complicated chemical approaches and perhaps a lack of a facile synthesis method.

In 2017, we reported initial findings on the development of chlorins as imaging agents. We synthesized chlorins using a new alcoholic ylide[19] moiety and the insertion of a pyrrolidine ring exhibiting a nucleophilic anchor for the subsequent appending of linkers was obtained.[13] Inspired by these encouraging results and to provide a modular, easily accessible chlorin platform, we hypothesized that chlorins with conjugatable functional groups could be used as fluorescent sensors to be delivered to physiologically relevant biomarkers sites in *in vitro* and in *in vivo* experiments. After testing the potential of chlorins as fluorescent tags and in the same line of imaging, we envisioned and explored conjugatable chlorins as suitable chelators for radionuclides, which would enable Cerenkov Luminescence and PET, this

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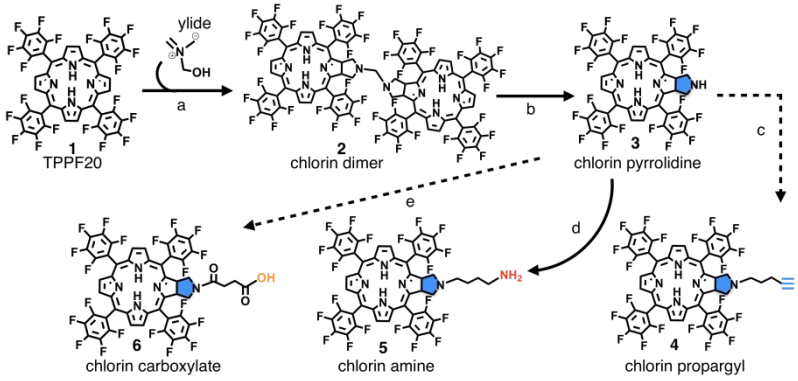
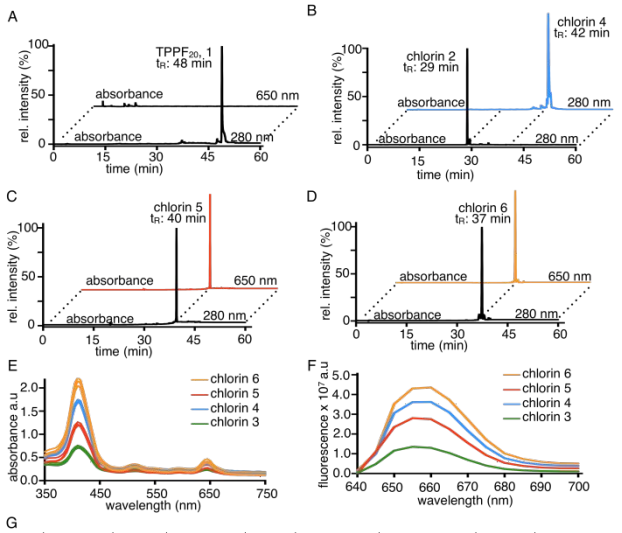


Scheme 1. Synthetic scheme for chlorins followed these steps. a. Mixed-grinded ylide, glycine/paraformaldehyde (1:1), added in aliquots in chlorobenzene every 6 h, 120 °C, 24 h, 35% yield. b. 5 equiv. trifluoroacetic acid (TFA), H_2O/DMSO, 120 °C, 24 h, 50% yield. c. 5 equiv. 5-Iodo-1-pentyne, K_2CO_3, ACN, 80°C, 4 h, 70% yield. d. 5 equiv. 4-bromobutyronitrile, K_2CO_3, ACN, 80°C, 2 h, 80% yield. Then, a water work-up and solvent evaporation, 10 equiv. $LiALH_4$, THF, 24 h, 30%. e. 5 equiv. succinic anhydride, DCM, NEt_3, room temperature (r.t.), 10 min, 90% yield.

approach if successful would allow our light driven systems to act as chelators and function as radio-tracers for the first time, a reputed but yet unexplored property for chlorins. Concisely, this study leveraged the fluorescent and PET properties of chlorins in their development as imaging agents with the potential to translate.

In this work, we synthesized 5 new chlorins in our continuous effort to develop molecularly targeted fluorescent/PET chlorins.



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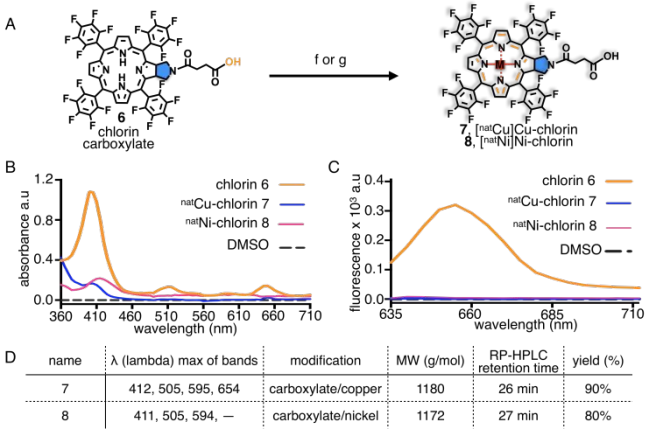
name	absorbance max.DMSO	emission max.DMSO	extinction co-efficient (ε)	Φ_F	RP-HPLC retention time	gradient	flow rate	yield (%)
1	410 nm	—	—	0.05	48 min	0-95% solvent B	1 mL/min	—
2	412 nm	660 nm	6.0 x 10^4	0.46	29 min	0-95% solvent B	1 mL/min	35%
3	410 nm	660 nm	3.3 x 10^4	0.44	44 min	0-95% solvent B	1 mL/min	50%
4	412 nm	662 nm	1.3 x 10^4	0.40	42 min	0-95% solvent B	1 mL/min	70%
5	412 nm	662 nm	4.3 x 10^4	0.39	40 min	0-95% solvent B	1 mL/min	30%
6	412 nm	660 nm	7.8 x 10^4	0.42	37 min	0-95% solvent B	1 mL/min	90%

Fig. 1 Photochemical profiles of synthesized chlorins. (A), (B), (C) and (D): HPLC of porphyrin (1) and chlorins (2, 4, 5, 6) showing absorbances at 280 nm and 650 nm with retention times observed at 48, 29, 42, 40 and 37 mins, respectively. (E) Excitation and (F) emission spectra of chlorins 3-6. (G) Table comprising photo-chemical properties of compounds 1-6.

Furthermore, we provide an optimized synthesis that affords a dimer chlorin, which is rapidly converted to a chlorin with a pyrrolidine for the conjugation of linkers. Concisely, this work renders two new conjugatable chlorins with different anchors (e.g. primary amine and propargyl) and a chlorin carboxylate[13] to enable different types of click chemistries for bioconjugation; and two chlorins as chelators of nickel and copper. Convenient and successfully, the labelling of chlorin with copper-64 was performed together with proof-of-principle *in vitro* Cerenkov/fluorescence. *In vitro* experiments showed high fluorescence signals coming from small-cell lung cancer (SCLC), H-82 cancer cell lines. This work not only expands our growing library of chlorin-based imaging agents and serves as a platform through which we can potentially explore imaging of tumours, infections and nerve structures[7, 20] *in vivo*, (Fig. ESI) but also confirms a reputed attribute/application of synthetic chlorins in the radiochemical field.

The synthesis (**scheme 1**) and basis of these light driven systems is $TPPF_{20}$ tetrakis-(pentafluorophenyl) porphyrin **1**, a chemical synthesis which was developed using a cycloaddition reaction that afforded a key chlorin dimer **2** in 35% yield and 92%



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name	λ (lambda) max of bands	modification	MW (g/mol)	RP-HPLC retention time	yield (%)
7	412, 505, 595, 654	carboxylate/copper	1180	26 min	90%
8	411, 505, 594, —	carboxylate/nickel	1172	27 min	80%

Fig. 2 Synthesis of chlorins with metals and their standard features. (A) Synthesis of chlorins 7 and 8. For 7, (f) 5 equiv. $CuCl_2$, MeOH, 25 °C, 1 h, 90% and for 8, (g) 5 equiv. $NiCl_2$, DMF, 120 °C, 24 h, 80%. (B) Absorbance and (C) and Fluorescence spectra of 0.1 µM chlorin 6 (orange), 0.1 µM Cu-chlorin 7 (blue), 0.1 µM Ni-chlorin 8 (pink) and 100 µL of DMSO (black) observed from 360–710 nm, where the 650 nm chlorin peak was observed. (D) Table comprising photo-chemical properties of chlorins 7 and 8.

purity. Intriguingly, compound **2** was polar in comparison to

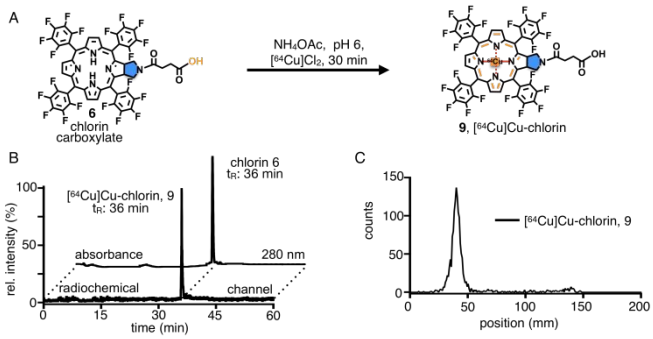
other chlorins we made in the past. With the help of a RP-HPLC column (Atlantis T3 C18) using a gradient of 0-95% B over 60 min (A: H_2O/0.1% TFA, B: 99.9% AcN/0.1% TFA) compounds **1** and **2** resulted in retention times of 48 min and 29 min, respectively (Figs. 1A, 1B). We think that apart from the insertion of the two nitrogen atoms in **2**, the polarity increases because the molecule is positively charged.[19] This result was observed again on thin-layer chromatography (TLC), with a retention factor of 0.4 for compound **2** (Fig. ESI).

The cleavage of compound **2** (chlorin dimer) used trifluoro-acetic acid (TFA) diluted in a 20% water, 80% dimethylsulfoxide (DMSO) solution, and resulted in a fused pyrrolidine ring, compound **3** (chlorin pyrrolidine), featuring a free nitrogen base that is used as a nucleophile to append tethers to the chlorin core and which enables bioconjugation via nucleoplilic substitution. Once chlorin **3** (Fig. ESI) with a free nitrogen base was synthesized; the transformation to reactive functional groups (e.g. as nucleophilic or electrophilic moieties) was possible. Given this convenient scenario, we were able to prepare conjugatable chlorins, with distinct linkers, including a propargyl, primary amine and our previously reported carboxylate chlorin and their standard characterization obtained. The substitution reaction to afford **4** (chlorin propargyl) used 5-iodo-1-pentyne in ACN, and resulted in a chlorin with a tether featuring a triple bond which enables Cu-chemistry. Similarly, a RP-HPLC column was used to isolate compound **4**, which showed a retention time of 42 min (Fig. 1B), 70% yield and 94% purity. Again, a substitution reaction with 4-bromobutyronitrile followed by a $LiAlH_4$ nitrile reduction afforded compound **5** (chlorin amine) which had a retention time of 40 min (Fig. 1C), 30% yield and 92% purity. Chlorin **5** with a primary amine tether can be used as a nucleophile in NHS-ester click chemistry. A well-characterized compound **6** was used as our reference in this study, a RP-HPLC column was used to isolate **6**, which showed a retention time of 37 min (Fig. 1D), 90% yield and 95% purity.

LC/MS spectra of compounds **4-6** showed clean ionic peaks; the observed masses were 1084, 1089 and 1118, respectively (Fig. ESI). Absorption and fluorescence spectra for **4-6** were also determined and the characteristic 650 nm absorption signal for the chlorin family was detected in all functionalized compounds while no other type of chromophore signal was observed (Figs. 1E, 1F). The fluorescence emission maximum was found at 660-662 nm for **4**, **5** and **6**. The fluorescent quantum yield of compounds **4**, **5** and **6** were 0.40, 0.39 and 0.42, respectively. There is an increase in the fluorescence quantum yields of 8, 7.8 and 8.4-fold for chlorins **4**, **5** and **6** respectively, in comparison to the parent porphyrin.[16] A collection of photo-chemical features for chlorins **4-6** are comprised in Fig. 1G.

The identity of the three conjugatable chlorins **4-6** was also confirmed using ^{1}H NMR experiments, the aromatic resonances appear at around 8.6-8.3 ppm, pyrrolidine resonances appear at around 5.2 ppm for the β-pyrrole protons and at around 2.3 and 3.2 ppm for the methelene protons of the inserted ring for **4-6**. ^{13}C NMR spectra showed clearly multiple resonances arising from the chlorins, the following pattern was observed, 8 resonances for the quaternary carbons of chlorin core, 6 resonances for carbons attached to F-atoms, 3 aromatic resonances for the pyrroles, 2 aliphatic resonances for the pyrrolidine and 3, 4 and 2 aliphatic resonances of the added linkers for chlorins **4**, **5** and **6**, respectively.

Since chlorins strongly bind most metal ions, we assessed their properties as chelators. To this end, we have used nickel, copper and copper-64 to prepare ^{nat}Cu-chlorin **7**, ^{nat}Ni-chlorin **8** (Fig. 2A) and [^{64}Cu]Cu-chlorin **9**, respectively. For compound **7**, copper chloride, a basic buffer at r.t. conditions were required for the chelation of the chlorin to copper. For compound **8**, nickel chloride and high temperatures were needed for the chlorin to chelate nickel. A RP-HPLC column was used to determine that 26 min and 27 min were the retention times of **7** and **8** (Fig. ESI), which were obtained in 88% and 90% purities, respectively. For chlorins **7** and **8**, their chemical protocols were developed, their excitation and emission and chemo-photophysical properties presented in Figs. 2B, 2C and 2D. Interestingly, for the absorbance, we found that the 650 nm peak of **7** and **8** (light green colour to the naked eye), decreased 43-fold and 34-fold, respectively, in comparison to chlorin **6** (dark green colour to the naked eye) that did not have a metal. The same effect was observed for the fluorescence of chlorin **6** when metals were added. In all cases, this decrease in absorbance, which in the past has been associated to electron capture,[21] resulted in a reduction of fluorescence intensity. For [^{64}Cu]Cu-chlorin **9**, the labelling used a basic buffer at r.t. (Fig. 3A), the best radiochemical reaction yielded a conversion rate of more than 60% when 90 nmol of **6** were used. The product was obtained in



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name	moles	reaction volume	modification	solvent	purity	isolated yield
6	50 nmol	100 µL	carboxylate/copper-64	NH_4OAc buffer	90%	25%
6	90 nmol	100 µL	carboxylate/copper-64	NH_4OAc buffer	94%	45%
6	180 nmol	100 µL	carboxylate/copper-64	NH_4OAc buffer	90%	32%

Fig. 3 Radiolabeling and characterization of [^{64}Cu]Cu-chlorin. (A) Radiochemical scheme for the synthesis of chlorin 9. (B) Radio-HPLC chromatogram of a labelled [^{64}Cu]Cu-chlorin synthesized at 37 °C with a corresponding HPLC chromatogram of unlabelled chlorin 6 at 280 nm absorption. (C) Radio-iTLC of [^{64}Cu]Cu-chlorin 9, showing a peak at 40 mm. (D) Table comprising radio-chemical features of the radio-synthesis of 9.

>92% radiochemical purity and in 40-45% isolated radiochemical yield (n.c.d.). [^{64}Cu]Cu-chlorin **9** was found to have a retention time of 36 min in the radiochemical channel, which had the same retention time of cold chlorin **6** observed at 280 nm (Fig. 3B) using a RP-HPLC C18 column. The reaction was monitored by instant layer chromatography (iTLC) (Fig. 3C), the radio-chemical features of the radio-synthesis

reported (Fig. 3D) and the Cerenkov luminescence profile of **9** observed (Fig. ESI).

In vitro, fluorescence signal from chlorin **6** was observed in SCLC H-82 cancer cells; these cancer cells showed fluorescence after incubation for 2 h with chlorin **6** (67 nmol, 0.7 mM in 100 µL of PBS), moreover, fluorescent microscopy images of cancer cells (Fig. 4) are consistent with previous reports of chlorin fluorescence *in vitro*.[2, 16]

In summary, we further explored two central properties of chlorins: their potential to serve in conjugations as a molecularly targeted agents and their ability for fluorescence imaging and PET. We have synthesized two new conjugatable chlorins to diversify click chemistries of chlorins. Moreover, the syntheses of Ni-chlorin and Cu-chlorin were also shown, and the results suggest differences in comparison to metal-free chlorins, potentially opening the door to chlorins with improved chemo-photothermal properties. We also presented a chlorin radiotracer for Cerenkov luminescence and PET enabling a new feature for chlorins. Taken together, these results indicate that these chlorins may be further pursued for the imaging of tumours and nerve tissue or the detection of bacterial or viral infections. Future research will seek to expand this work into other chlorins — such as bacteriochlorin and isobacteriochlorin, both with extra pyrrolidines attached — taking advantage of their photophysical properties for use as research tools. Additionally, we will evaluate chlorins for their potential as molecurlarly targeted probes in biomedical imaging.

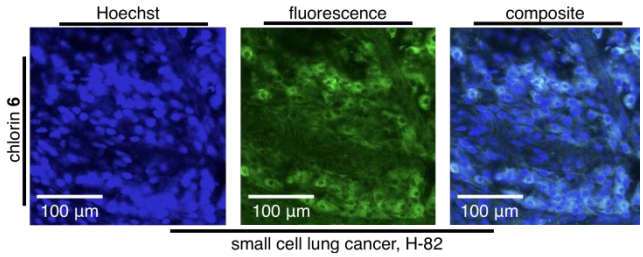

Fig. 4 Fluorescence of chlorin in SCLC. H-82 incubated with 6 (green, 67 nmol, 0.7 mM in 100 µL PBS) for 2 h at 37°C and Hoechst (blue, 20 µM, 1 nmol in 50 µL PBS), fluorescence was observed.

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Conflicts of interest

T.R. and J.S.L are shareholders of Summit Biomedical Imaging, LLC. T.R. is a paid consultant for Theragnostics.

Notes and references

1. N. Bhupathiraju, W. Rizvi, J. D. Batteas and C. M. Drain, *Org Biomol Chem*, 2016, **14**, 389-408.
2. S. K. Bisland, D. Singh and J. Gariepy, *Bioconjug Chem*, 1999, **10**, 982-992.
3. F. Ogata, T. Nagaya, Y. Maruoka, J. Akhigbe, A. Meares, M. Y. Lucero, A. Satraitis, D. Fujimura, R. Okada, F. Inagaki, P. L. Choyke, M. Ptaszek and H. Kobayashi, *Bioconjug Chem*, 2019, **30**, 169-183.
4. R. Bonnett, P. Charlesworth, B. D. Djelal, S. Foley, D. J. McGarvey and T. G. Truscott, *Journal of the Chemical Society, Perkin Transactions 2*, 1999, DOI: 10.1039/a805328f, 325-328.
5. M. Wainwright, *International Journal of Antimicrobial Agents*, 2003, **21**, 510-520.
6. K. O'Riordan, O. E. Akilov and T. Hasan, *Photodiagnosis and Photodynamic Therapy*, 2005, **2**, 247-262.
7. J. Gonzales, G. Pirovano, C. Y. Chow, P. D. de Souza Franca, L. M. Carter, J. K. Klint, N. Guru, J. S. Lewis, G. F. King and T. Reiner, *EJNMMI Res*, 2020, **10**, 49.
8. C. P. Sabino, M. Wainwright, M. S. Ribeiro, F. P. Sellera, C. Dos Anjos, M. D. S. Baptista and N. Lincopan, *J Photochem Photobiol B*, 2020, **208**, 111893.
9. Y. Zhang and J. F. Lovell, *Theranostics*, 2012, **2**, 905-915.
10. R. B. Woodward, *Angewandte Chemie*, 1960, **72**, 651-662.
11. N. S. Kuzmina, V. F. Otvagin, L. V. Krylova, A. V. Nyuchev, Y. V. Romanenko, O. I. Koifman, I. V. Balalaeva and A. Y. Fedorov, *Mendeleev Communications*, 2020, **30**, 159-161.
12. J. Shi, T. W. Liu, J. Chen, D. Green, D. Jaffray, B. C. Wilson, F. Wang and G. Zheng, *Theranostics*, 2011, **1**, 363-370.
13. J. Gonzales, N. V. Bhupathiraju, W. Perea, H. Chu, N. Berisha, V. Bueno, N. Dodic, J. Rozenberg, N. L. Greenbaum and C. M. Drain, *Chem Commun (Camb)*, 2017, **53**, 3773-3776.
14. J. P. Strachan, D. F. O'Shea, T. Balasubramanian and J. S. Lindsey, *J Org Chem*, 2000, **65**, 3160-3172.
15. J. Kralova, T. Briza, I. Moserova, B. Dolensky, P. Vasek, P. Pouckova, Z. Kejik, R. Kaplanek, P. Martasek, M. Dvorak and V. Kral, *J Med Chem*, 2008, **51**, 5964-5973.
16. S. Singh, A. Aggarwal, S. Thompson, J. P. Tome, X. Zhu, D. Samaroo, M. Vinodu, R. Gao and C. M. Drain, *Bioconjug Chem*, 2010, **21**, 2136-2146.
17. S. L. Rakestraw, R. G. Tompkins and M. L. Yarmush, *Proc Natl Acad Sci U S A*, 1990, **87**, 4217-4221.
18. S.-i. Ogura, K. Tabata, K. Fukushima, T. Kamachi and I. Okura, *Journal of Porphyrins and Phthalocyanines*, 2012, **10**, 1116-1124.
19. J. Gonzales, N. Bhupathiraju, D. Hart, M. Yuen, M. P. Sifuentes, B. Samarxhiu, M. Maranan, N. Berisha, J. Batteas and C. M. Drain, *J Org Chem*, 2018, **83**, 6307-6314.
20. J. Gonzales, P. Demetrio De Souza Franca, Y. Jiang, G. Pirovano, S. Kossatz, N. Guru, D. Yarilin, A. J. Agwa, C. I. Schroeder, S. G. Patel, I. Ganly, G. F. King and T. Reiner, *Bioconjugate Chemistry*, 2019, **30**, 2879-2888.
21. Y. Rahimi, A. Goulding, S. Shrestha, S. Mirpuri and S. K. Deo, *Biochem Biophys Res Commun*, 2008, **370**, 57-61.