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Neutral analogs of the heat shock protein 70 (Hsp70) inhibitor, JG-98

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ABSTRACT

The heat shock protein 70 (Hsp70) family of molecular chaperones are highly expressed in tumors. Inhibitors containing a pyridinium-modified benzothiazole, such as JG-98, bind to a conserved, allosteric site in Hsp70, showing promising anti-proliferative activity in cancer cells. When bound to Hsp70, the charged pyridinium makes favorable contacts; however, this moiety also increases the inhibitor's fluorescence, giving rise to undesirable interference in biochemical and cell-based assays. Here, we explore whether the pyridinium can be replaced with a neutral pyridine. We report that pyridine-modified benzothiazoles, such as compound **17h** (**JG2-38**), have reduced fluorescence, yet retain promising anti-proliferative activity (EC_{50} values ~0.1 to 0.07 μM) in breast and prostate cancer cell lines. These chemical probes are expected to be useful in exploring the roles of Hsp70s in tumorigenesis and cell survival.

Members of the Hsp70 family of molecular chaperones, including heat shock protein 72 (Hsp72, HSPA1A) and heat shock cognate 70 (Hsc70, HSPA8), are over-expressed in many cancers.[1–3] In tumor cells, these Hsp70s are part of a multi-protein complex that is not present in non-transformed cells,[4] and which seems to stabilize onco-proteins and provide resistance to chemotherapy.[1,5,6] Consistent with this role, knockdown of both Hsp72 and Hsc70 leads to preferential cell death in cancer cells, but not normal epithelia.[7] Interestingly, knockdown of only one family member (*e.g.* Hsp72 or Hsc70) is typically not sufficient to initiate apoptosis, suggesting that pan-inhibitors (hereafter referred to as "Hsp70 inhibitors") may hold particular promise. Fortunately, members of the Hsp70 family are highly conserved, with up to 95% sequence identity.[8,9]

Multiple Hsp70 inhibitors with different mechanisms and binding sites have been explored. For example, VER-155008[10,11] and its analogs[12–14] bind to Hsp70's ATP-binding cleft, while YK5 and its analogs[15–17] bind a nearby allosteric site. Despite their different binding sites, these compounds all seem to limit Hsp70's function by interrupting its nucleotide cycling. Another strategy is to target the protein-protein interactions between Hsp70 and the other factors that are present in cancer-associated, multi-protein complexes. For example, the dihydropyridine, MAL3-101[18,19] and its analogs[20–22], disrupt binding of Hsp70 to J-domain proteins (JDPs).

Whadwa and colleagues identified the benzothiazole, MKT-077 (Fig. 1), as another promising inhibitor of Hsp70s. This compound has anti-proliferative activity in multiple cancer cells, with minimal toxicity in non-transformed cells.[23] MKT-077 progressed to a Phase I clinical trial for solid tumors,[24] however, modest efficacy (~5 μM) and metabolic instability limited its further exploration. Subsequent studies revealed that MKT-077 binds a distinct allosteric site in Hsp70,[25] which is highly conserved amongst the family members.[26] Binding at this allosteric site blocks binding to nucleotide-exchange factors (NEFs), although the pocket does not overlap with the NEF-interaction surface.[27,28] Leveraging this structural knowledge, a series of medicinal chemistry campaigns were designed to improve MKT-077, resulting in analogs such as JG-98 and JG-231 (Fig. 1), with improved anti-proliferative activity and longer lifetimes in rodents.[29–31]

While JG-98 and its analogs have been useful chemical probes, these compounds are fluorescent ($\lambda_{excitation}$ 470 nm/$\lambda_{emission}$ 560 nm). This photochemical property likely originates from the conjugated π electron system that encompasses the rhodacyanine and charged pyridinium. This fluorescence property is not favorable, because it interferes with many assays; for example, these compounds cannot be used in biochemical assays that utilize fluorescence, such as fluorescence polarization (FP). Here, we set out to design neutral, non-fluorescent analogs. Specifically, based on findings with the analog YM-08 (Fig. 1),[32] we reasoned that replacing the rhodacyanine and/or the pyridinium might sufficiently reduce fluorescence and enable use of these chemical probes in a wider range of applications.

Towards this goal, we first replaced the central rhodacyanine with either a benzene (compound **4a-b**) or thiazole (compound **8a-b**). Based on structural information obtained with JG-98, the central rhodacyanine is relatively solvent exposed, yet it sets the position of the other ring systems (*i.e.* the benzothiazole) to optimally interact with two deep

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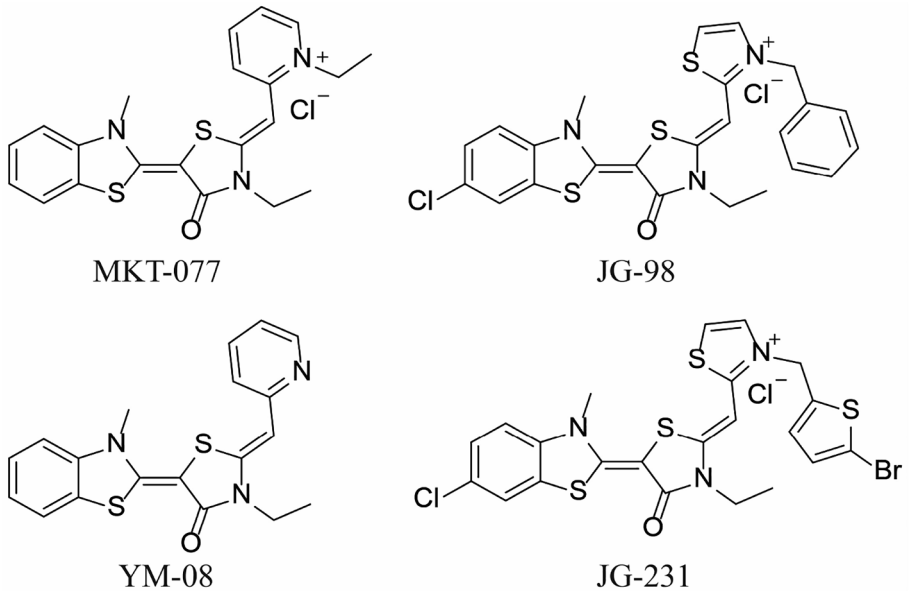
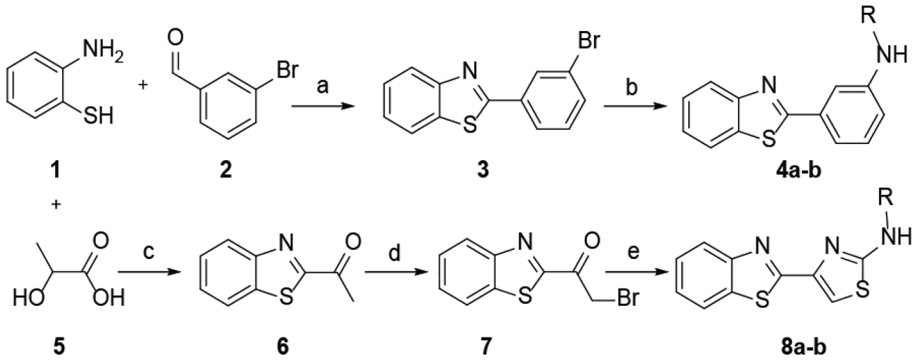


Fig. 1. Previously reported benzothiazole rhodacyanine probes.



Scheme 1. Reagents and conditions: (a) PTSA, H_2O, 70 °C, 12 h, 60%; (b) $Pd(OAc)_2$ (10 mol%), (±)BINAP (10 mol%), Cs_2CO_3 (2 mol), toluene, 25–34%; (c) (i) 4 N HCl aq., reflux, 24 h, 94%; (ii) 10 eq. MnO_2, $CHC1_3$, 72%; (d) Cu(II)Br, $CHC1_3$ + EtOAc, reflux, 12 h, 78%; (e) Substituted thiourea, EtOH, reflux, 2 h, 52–60%.

pockets.[26] Thus, compounds **4a-b** and **8a-b** were created to understand whether the rhodacyanine might be replaced without interfering with these contacts. Briefly, the synthesis of compounds **4a-b** started from cyclization of 2-aminothiophenol and 3-bromobenzaldehyde,[33] followed by Buchwald-Hartwig amination with anilines (Scheme 1). In contrast, compounds **8a-b** were synthesized by reacting 2-aminothiophenol with lactic acid, leading to the alcohol which was then oxidized to ketone **6** with manganese dioxide. Intermediate **6** was brominated with copper(II) bromide and then reacted with substituted thiourea to obtain the final products **8a-b**. The purified compounds (> 95% HPLC) were then tested in anti-proliferative assays using cancer cells from breast (MCF7) and prostate (22Rv1 and PC3). However, we found that **4a-b** and **8a-b** tended to have worse activity than JG-98 (Table 1), with IC_{50} values between ~ 0.7 and 13 μM. Based on this result, we decided to retain the rhodacyanine in subsequent analogs and turn our attention to the pyridinium (see Table 2).

Specifically, we assembled analogs (compounds **17a-o**) in which the pyridinium was replaced with a neutral pyridine, using a previously reported route (Scheme 2).[26,29] Briefly, substituted anilines **9** were treated with potassium ethyl xanthate, followed by methylation with iodomethane under basic conditions. The resulting benzothiazoles **10** were first reacted with methyl p-toluenesulfonate and then coupled with 3-ethylrhodanine to obtain intermediate **12**. Intermediate **12** was further activated by methyl *p*-toluenesulfonate, followed by condensation with 1-((1,3-dioxoisoindolin-2-yl)methyl)-2-methylpyridin-1-ium bromide (**14**) to yield compound **15**. Deprotection of **15** with aqueous ammonium hydroxide yielded **16**, which was coupled with anilines to obtain the final products **17a-o**. These products were purified by flash

Table 1
Antiproliferative activities of compounds **4a-b** and **8a-b**.

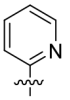
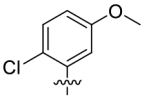
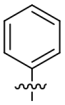
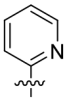
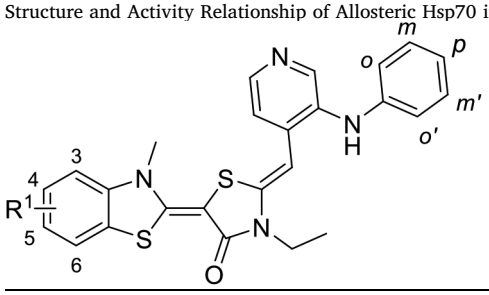
Compd	R	MCF-7 IC_{50}/μM	22RV1 IC_{50}/μM	PC3 IC_{50}/μM
JG-98	–	0.71 ± 0.22	–	–
4a		13 ± 0.90	13 ± 2.0	4.8 ± 1.3
4b		0.71 ± 0.20	2.1 ± 0.15	2.2 ± 0.43
8a		7.6 ± 0.67	6.9 ± 1.4	5.5 ± 0.74
8b		3.6 ± 0.60	3.2 ± 0.28	6.7 ± 0.76

Table 2
Structure and Activity Relationship of Allosteric Hsp70 inhibitors.

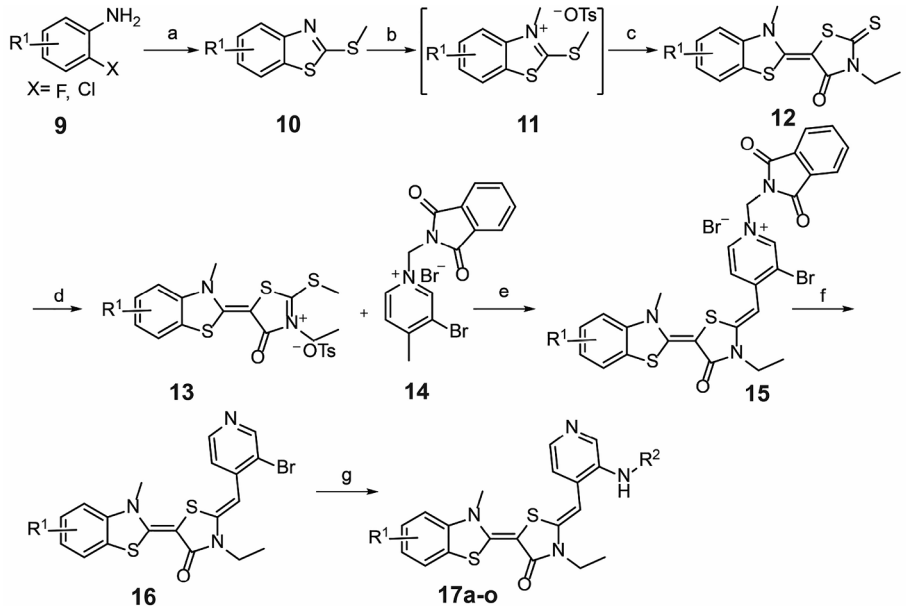


Compd	R_1	R_2	MCF-7 IC_{50}/μM	22RV1 IC_{50}/μM	PC3 IC_{50}/μM
17a	H	*o,m'*-Me	1.7 ± 0.05	1.8 ± 0.43	2.0 ± 0.60
17b	H	*o, m'*-F	1.5 ± 0.49	1.1 ± 0.55	1.2 ± 0.23
17c	H	*o*-Me	0.99 ± 0.01	0.47 ± 0.20	1.2 ± 0.11
17d	H	*o*-Cl	0.75 ± 0.11	0.46 ± 0.25	0.78 ± 0.07
17e	H	*o*-F	0.33 ± 0.06	0.30 ± 0.04	0.36 ± 0.02
17f	4-Me	*o*-Me	0.40 ± 0.10	0.48 ± 0.15	0.33 ± 0.02
17g	4-Me	*o*-Cl	0.65 ± 0.01	0.35 ± 0.05	0.50 ± 0.10
17h (JG2-38)	4-Me	*o*-F	0.10 ± 0.01	0.15 ± 0.02	0.07 ± 0.01
17i	4-OCH_3	*o*-Me	0.42 ± 0.03	0.41 ± 0.08	0.37 ± 0.03
17j	4-OCH_3	*o*-Cl	0.71 ± 0.30	0.50 ± 0.08	0.76 ± 0.01
17k	4-OCH_3	*o*-F	0.13 ± 0.01	0.19 ± 0.02	0.10 ± 0.01
17l	5-F	*o*-Me	1.3 ± 0.35	2.3 ± 0.94	2.3 ± 0.31
17m	5-F	*o*-Cl	1.3 ± 0.15	0.49 ± 0.03	1.2 ± 0.31
17n	5-F	*o*-F	0.23 ± 0.04	0.15 ± 0.07	0.26 ± 0.06
17o	5-ethyl	*o*-F	1.2 ± 0.02	1.2 ± 0.10	2.2 ± 0.01

chromatography (> 95% HPLC) and characterized by LC-MS/MS and ^{1}H NMR (see Supporting Information).

A key goal of this synthetic effort was to reduce the compound's intrinsic fluorescence. To specifically test this idea, compounds **17a-o** (50 μM) were dissolved in aqueous buffer (100 mM Tris buffer with 10% DMSO, pH 7.4), and the emission spectra of these solutions measured (($\lambda_{excitation}$ 470 nm/$\lambda_{emission}$ 520 to 750 nm). We found that all of the neutral analogs were significantly less fluorescent than JG-98 (Fig. 2).

With these compounds in-hand, we measured their anti-proliferative activity against the breast (MCF7) and prostate cancer (22Rv1 and PC3) cell lines. Our previous report shows that ortho- and meta-modification of the benzene ring in JG-98 can increase potency,[29] so we initially tested compounds with dimethyl (**17a**) or difluoro (**17b**) substituents at the o- and m'- positions. Indeed, both had modest activity against all three cancer cell lines, with IC_{50} values in the range of ~1 to 2 μM. Removal of the methyl group at the meta position (**17c**) further improved activity, especially against 22Rv1 cells (IC_{50} ~ 0.5 μM). Accordingly, we tested ortho-substituted analogs with either electron withdrawing or donating groups at the ortho-position



Scheme 2. Reagents and conditions: (a) (i) Potassium ethylxanthate, DMF, 140 °C, 4 h; (ii) MeI, NEt_3, EtOH, 80 °C, 1 h; 40–80%; (b) *p*-Ts0Me, anisole, 125 °C, 4 h, used directly for next step; (c) 3-Ethyl rhodanine, Net_3, MeCN, 25 °C, 4 h, 30–75%; (d) *p*-Ts0Me, DMF, 135 °C, 3 h, 50–85%; (e) Net_3, MeCN, 70 °C, 3 h, 70–83%; (f) DCM/MeOH (1:3), aq. NH_3, r.t., l h, 79–87%; (g) anilines, $Pd_2(dba)_3$/BINAP/*t*-BuONa (0.05:0.15:2), toluene, 100 °C, overnight, 38–55%.

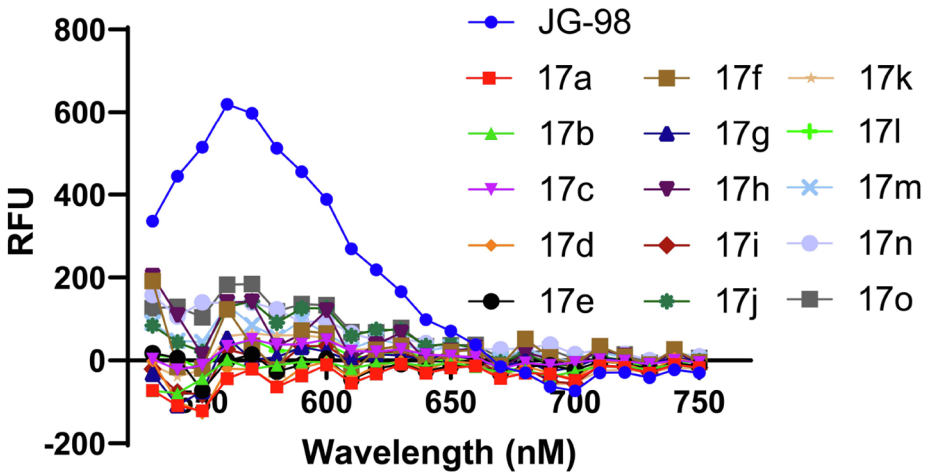


Fig. 2. Compounds **17a-o** are less fluorescent than **JG-98**. The emission spectrum of compounds **17a-o** when excited at 470 nM.

(**17c-e**), revealing that the *o*-fluorine compound **17e** was the most potent of these analogs, with IC_{50} values ~0.3 μM.

Using these ortho-substituents at the benzene ring, we turned our attention to modifications of the benzothiazole. In JG-98, small alkyl and halogen substitutions have been reported to increase activity.[26] Similarly, we found that a methyl group at the 4-position (**17f-h**) resulted in approximately 2-fold improvements. For example, compound **17h** (**JG2-38**) had IC_{50} values of 0.1 μM against MCF7 cells, 0.15 μM in 22Rv1 cells and 0.07 μM for PC3 cells. These values represent a ~7 to 10-fold improvement on JG-98. Replacement of methyl with methoxy (**17i-k**) resulted in similar trends, with the *o*-fluoro analog **17k** again having the best activity (IC_{50} values ~ 0.1 to 0.19 μM). In JG-98 analogs, the benzothiazole substitution is tolerated on either the 4- or 5-position.[26] Accordingly, we tested the effects of replacing the 4-methoxy of **17i-k** with 5-fluoro groups (**17l-n**). However, this modification generally decreased activity (IC_{50} values 2.3 to 0.15 μM). Similarly, larger substitutions, such as 5-ethyl (**17o**), did not improve activity (IC_{50} values ~2.1 to 0.18 μM).

Based on these results, we selected compound **17h** (**JG2-38**) for further exploration. First, we studied its possible binding pose, using molecular docking in Schrodinger, as previously described.[29] In these studies, we used the structure of a truncated form of human Hsc70 (PDB code 3HSC). In the best-predicted models (Fig. 3), compound **17h** (**JG2-38**) was anchored in a deep pocket formed by residues R72, K71, T13, F150, P147 and T204. In this orientation, the pyridine ring of **17h** (**JG2-38**) was largely solvent-exposed, but it positioned the benzyl group into an adjacent hydrophobic pocket. This binding pose might explain the preference for only small, ortho-substitutions, because this group is nestled against Y149 and the β-sheet composed of residues 222–226.

Finally, it is known that treatment of cancer cells with Hsp70 inhibitors leads to degradation of pro-survival proteins, likely because chaperone function is needed to maintain their folding. To confirm whether **17h** (**JG2-38**) might share this activity on Hsp70 biomarkers, we treated 22Rv1 cells for 24 h and then performed western blots for oncogenic kinases, Akt1, c-Raf-1 and CDK4, and the translation factor HuR (Fig. 4). The levels of each protein were reduced by ~50%, consistent with Hsp70 inhibition. Moreover, consistent with previous findings on other Hsp70 inhibitors,[29] we found that treatment with **17h** (**JG2-38**) did not cause a stress response, as measured by the constant levels of Hsp72 and Hsp90 (Fig. 4). This result is important because compensatory stress responses have been hypothesized to reduce sensitivity to chaperone inhibitors.

In summary, we identified **17h** (**JG2-38**) as a less fluorescent Hsp70 inhibitor, which retains anti-proliferative activity (IC_{50} ~ 0.1 μM) and the expected effects on Hsp70 "client" proteins, such as oncogenic

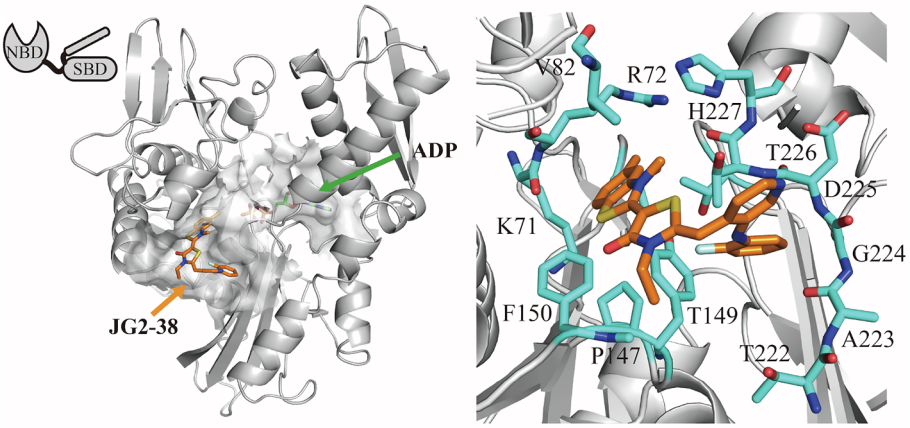


Fig. 3. Compound **17h** (**JG2-38**) binds to an allosteric site in the nucleotide binding domain of Hsp70. Compound **17h** (**JG2-38**) and the residues within 5 angs are shown as stick representation. Carbon atoms of the protein and **17h** (**JG2-38**) are shown in gray and orange, respectively. Oxygen, nitrogen and sulfur are shown in red, blue and yellow, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

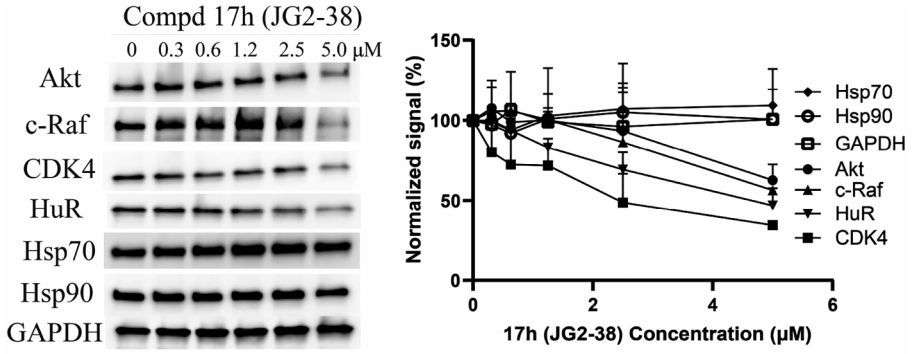


Fig. 4. Compound **17h** (**JG2-38**) led to degradation of oncoproteins without eliciting a heat shock response. 22-RV1 cells were treated with **17h** (**JG2-38**) for 24 h at the indicated concentration, lysed and western blots performed. Results are representative of at least two independent experiments. Quantifications shown are averages of at least two independent experiments and the error bars represent S.E.

kinases. Further work will be needed to optimize the metabolic stability and solubility of this analog for use in animal models, but its improved photochemical properties are expected to be beneficial in a range of applications.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.126954.

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