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Preliminary Communications

A comparative assessment of α-lipoic acid *N*-phenylamides as non-steroidal androgen receptor antagonists both on and off gold nanoparticles

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ABSTRACT

A group of α-lipoic acid *N*-phenylamides were synthesized employing a variety of amide coupling protocols utilizing electron deficient anilines. These compounds were then assessed for their ability to block androgen-stimulated proliferation of a human prostate cancer cell line, LNCaP. These structurally simple compounds displayed anti-proliferative activities at, typically, 5–20 μM concentrations and were comparable to a commonly used anti-androgen Bicalutamide®. The inclusion of a disulfide (RS-SR) moiety, serving as an anchor to several metal nanoparticle systems (Au, Ag, Fe_2O_3, etc.), does not impede any biological activity. Conjugation of these compounds to a gold nanoparticle surface resulted in a high degree of cellular toxicity, attributed to the absence of a biocompatible group such as PEG within the organic scaffold.

1. Introduction

Prostate cancer is the most commonly diagnosed cancer in men and a leading cause of death in the male population [1]. As prostate cancer development and disease progression is hormone dependent, blockade of androgen action is the cornerstone of most therapies and can be achieved by inhibiting the androgen receptor (AR). In primary prostate cancer cells, testosterone and dihydrotestosterone (5αDHT) binding to the AR enhances tumor cell proliferation. Prostate cancer therapy originally centered on the modification of the naturally occurring steroidal ligands, but due to poor bioavailability, hepatotoxicity and a lack of tissue specific action has led to their discontinued clinical use [2]. Nevertheless, blockade of the proliferative effect of androgens remains a major focus of prostate cancer treatment and includes development of small molecule AR binding inhibitors to mitigate cellular proliferation.

The most notable examples of AR antagonists are Flutamide® **1** and Bicalutamide® **2**; Bicalutamide has superseded the use of Flutamide. Flutamide **1** is converted to its the active form hydroxyflutamide **1a** *in vivo*. With respect to the aryl amide portion of these compounds they are, by and large, electron deficient aromatic rings commonly bearing a trifluoromethyl group in addition to a severely deactivating group at the *meta*- and *para*-positions, such as a nitro (NO_2) or nitrile (CN) in the cases of **1**/**1a** and **2** respectively.

Our interest in these compounds stems from a nanomedicine perspective, whereby biologically active compounds are tethered to a nanomaterial, for example, inorganic nanoparticle, nanotube, micelle, liposome or nanorod. These 'nanoconjugates' are evaluated for any potential therapeutic benefits *in vivo* compared to the corresponding (untethered) small molecule.

The advent of nanomedicine has accelerated research intensity in this field and can be attributed to the advancement in the ability to control morphology, aspect ratio, surface chemistry and magnetic properties of a given nanoparticle allowing for combined therapies, diagnostics and targeted release strategies for various diseases [3]. Despite the growth of the nanomedicine field there has been little work focusing on therapies applied to prostate cancer and is the goal of this project.

Gold nanoparticles (AuNP) are the most common metal based nanoparticles employed for medicinal application, due to their ease of synthesis, surface functionalization and biocompatibility. Nevertheless other less expensive metal nanoparticles, such as silver, copper and iron oxide (Fe_2O_3), are emerging in medicinal applications [3]. A common feature of all these metals are their ability to be surface functionalized by treatment with compounds bearing a thiol (SH) or disulfide (RS-SR) moiety [4]. Therefore the purpose of this study was to synthesize a small range of compounds bearing structural similarity to the aryl portion of Flutamide® **1** while incorporating a suitable sulfur-based functional group for potential attachment to a range of inorganic nanoparticles (Fig. 2). Investigation of any potential anti-proliferative properties towards hormone responsive prostate cancer cells would provide a

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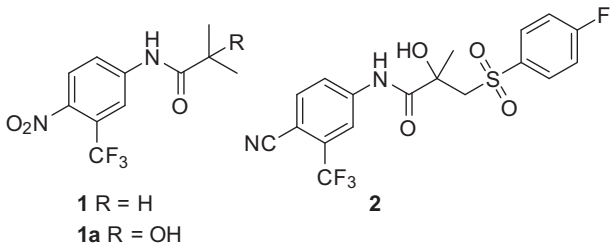


Fig. 1. AR antagonists commonly used for prostate cancer.

'baseline' cellular activity for comparison to the corresponding nanoparticle conjugates.

In this study α-lipoic acid **3** was chosen as the scaffold to incorporate a disulfide group for nanoparticle conjugation. α-Lipoic acid is a naturally occurring antioxidant that has shown extensive promise in the treatment of several diseases such as: atherosclerosis, type 2 diabetes, Parkinson's disease and has excellent anti-inflammatory properties [5]. Further to this, α-lipoic acid has shown to induce apoptosis in a variety of cancer cell lines and, as a dietary supplement, displays minimal toxicity to healthy cells. Also, flutamide® **1** is known to induce hepatotoxicity during therapy, thus inclusion of the disulfide moiety of α-lipoic acid (known to be hepatoprotective) may mitigate this toxic effect to some extent [5].

Recently Hu et al. [7] synthesized a range of dihydrolipoic acid derivatives and screened their proliferation properties across a broad range of cancer cell lines. Though prostate cancer cells did feature to a small extent in this study, the cell line chosen (PC-3) do not express AR and thus no conclusions regarding antagonistic versus agonistic interactions can be drawn. As such we have synthesized several of these compounds (in addition to several novel compounds) and reevaluated them for their AR binding properties in the hormone dependant prostate cancer cell line (LNCaP) serving as a model for primary tumors of the prostate. In this communication we present the synthesis of several *N*-phenylamide based α-lipoic acid derivatives and their potent anti-proliferative effect on androgen responsive prostate cancer cells.

2. Results and discussion

Our approach was to use a single step peptide coupling as this is the simplest route to the desired compounds. It is well known that amide formation using anilines can be low to moderate yielding as they are not strongly nucleophilic, which in this study, is compounded by the presence of electron withdrawing groups such as CF_3, CN and NO_2. Therefore a short preliminary investigation to various well established peptide formation reactions were undertaken to elucidate which protocol may be most suitable for application to electron deficient anilinic nitrogens. As such we chose the simplest aniline **5a** to undertake this study.

Initially, we chose EDCI mediated coupling (entry 1, Table 1) at room temperature overnight, giving an excellent yield of 81%. In an effort to reduce reaction time we applied microwave irradiation to the EDCI mediated reaction, but unfortunately the yield was reduced (53%, entry 2, Table 1). Exchanging the coupling reagent to Bromo-tris-pyrrolidino phosphoniumhexafluoro phosphate (PyBrop), known to promote difficult amide formations [7], again lead to moderate yield while heated with microwave irradiation (56%, entry 3, Table 1). The reduced yield obtained in entries 2 and 3 was surprising as microwave irradiation is often touted for its ability to promote difficult transformations both efficiently and cleanly [8]. Accessing compound **4a** *via* an α-lipoic acyl chloride intermediate, by treatment with thionyl chloride [6], gave a good yield (72%, entry 4, Table 1), but was still inferior to our initial reaction conditions (EDCI, 16 h, room temp). Although these protocols produced varying yields of the desired product **4a**, their collective success in furnishing the desired *N*-phenylamide confirmed each set of conditions as valid routes of inquiry when dealing with more problematic electron withdrawn anilines.

Our attention turned now to the synthesis of various *N*-aryl lipoic amides bearing a range of electron withdrawing functional groups commonly employed for androgen receptor antagonist design. Our focus on electron withdrawing substituents on the aryl ring was due to literature precedence indicating that these groups (e.g. CF_3, CN, Cl, etc.) were crucial to affect AR binding [9].

Coupling chloroaniline **5b** to lipoic acid **3** under the reaction conditions specified in method A gave a poor yield of 27% (entry 1, Table 2) while under microwave irradiation for a much shorter duration (method B) gave a higher and acceptable yield (71%. entry 2, Table 2). Surprisingly the formation of **4c** [11] using method A was higher yielding (44%, entry 3, Table 2) than **4b** under the same conditions despite the deactivating presence of a trifluoromethyl at the *meta*-position on the aniline reactant. Given the moderate yield of 44%, we conducted the same coupling using methods B and C with the highest yield of **5c** being obtained for method B (75%, entry 4, Table 2). The synthesis of compound **4d** has been reported previously giving good yield using method D [5], but in our hands this protocol was not optimal. Indeed, synthesizing **4d** proved very problematic and complex mixtures of products were isolated from the crude reaction mixture for all methods investigated. Analysis of these crude products by ^{1}H NMR showed only method C to have an appreciable amount of **4d**, but still gave a moderately low yield of 36% (entry 7, Table 2). Finally, employing trifluoromethyl cyanoaniline **5e**, despite bearing a very deactivating cyano group at the *para*-position, performed better than **5d** which possesses a nitro group at this position. In our hands we found that either method B or C was optimal, though the yields of **4e** [11] were still moderate (46%, entries 9 and 10, Table 2).

With a range of these compounds in hand our attention turned to investigating their AR interaction properties. The data in Fig. 1 show cell proliferation rates after 6 days in culture with the target compounds (**4a–e**) and are normalized against dihydrotestosterone (5αDHT) while using Bicalutamide® **2** as a positive control. In this study a significant anti-proliferative effect was observed when these compounds, and several novel α-lipoic derivatives, were incubated in the presence of androgen responsive prostate cancer cell lines (LNCaP). The development of compounds which

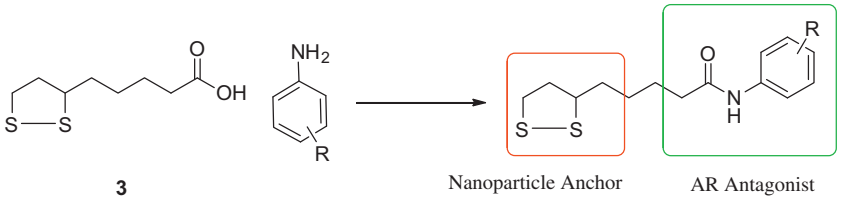
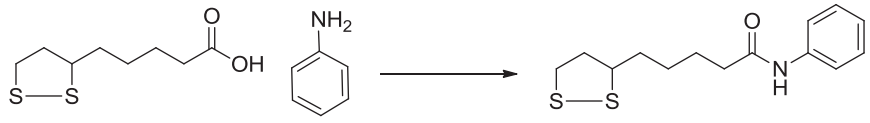


Fig. 2. General structure of desired compounds.

Table 1
Validation of synthetic protocols to synthesize *N*-phenylamides (see supp info).

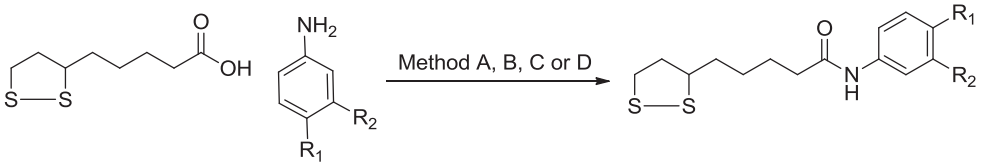


3 **5a** **4a**

Entry	Reagent	Solvent	Time (h)	Temp (°C)	Yield (%)[a]
1	EDCI	$CHCl_3$	16	r.t.	81
2	EDCI	$CHCl_3$	0.5	100	53
3	PyBrop	MeCN	1	100	56
4	$SOCl_2$	CH_2Cl_2	7	50	72

[a] Isolated yield.

Table 2
Methods to generate *N*-phenyl α-lipoic acid derived amides using electron deficient anilines [10].



3

5b R_1 = Cl, R_2 = H
5c R_1 = Cl, R_2 = CF_3
5d R_1 = NO_2, R_2 = CF_3
5e R_1 = CN, R_2 = CF_3

4b R_1 = Cl, R_2 = H
4c R_1 = Cl, R_2 = CF_3
4d R_1 = NO_2, R_2 = CF_3
4e R_1 = CN, R_2 = CF_3

Entry	Compound	Method	R_1	R_2	Yield (%)
1	**4b**	A	Cl	H	27
2	**4b**	B	Cl	H	71
3	**4c**	A	Cl	CF_3	44
4	**4c**	B	Cl	CF_3	75
5	**4c**	C	Cl	CF_3	55
6	**4d**	B	NO_2	CF_3	22
7	**4d**	C	NO_2	CF_3	36
8	**4d**	D	NO_2	CF_3	24
9	**4e**	B	CN	CF_3	46
10	**4e**	C	CN	CF_3	46

Method A: EDCI (1.2 equivalents), $CHCl_3$, α-lipoic acid, r.t., 16 h.
Method B: EDCI (1.2 equivalents), microwave irradiation, $CHCl_3$, α-lipoic acid, 100 °C, 0.5 h.
Method C: PyBrop (1.2 equivalents), microwave irradiation, $CHCl_3$, α-lipoic acid, 100 °C, 1 h.
Method D: (i) α-lipoic acid, $SOCl_2$, 50 °C, 3 h, reduced to dryness; (ii) NEt_3, aniline, r.t., 6 h.

display anti-proliferative behavior on LNCaP cells is important as these serve as potential leads for the treatment of primary prostate tumors in men. As mentioned above, Hu et al. [7] has evaluated **4a**, **4b** and **4e** against hormone independent prostate cancer cell line (PC-3) for their anti-proliferative properties. These compounds very mildly suppressed cancer cell growth (8–13% and 14–15% suppression for **4a** and **4b** respectively). A similar result was observed for compound **4e** which demonstrated very poor cancer growth inhibition (approximately 5%) [6].

As can be seen from Fig. 3, compound **4a** displayed a significant decrease in cell proliferation at 10 μM but not 20 μM while **4b**, bearing a chlorine substituent at the 4-position on the aromatic ring, showed excellent anti-proliferative activity at both 10 and 20 μM. Unfortunately **4c** failed to reduce cell proliferation to any statistical significance. This result is very interesting given the structural similarity between **4b** and **4c** and the persistence of trifluoromethyl groups at the *meta*-position in potent AR antagonists reported in the literature and used in the clinic [9,11].

Compounds **4d** and **4e** bear the identical aryl portion to Flutamide® **1** and Bicalutamide® **2** respectively, and as such were examined for their anti-proliferative properties at an additional concentration of 5 μM. The dose response behavior for **4d** was excellent with significant reduction in cell growth demonstrated at both 20 and 10 μM while a statistically non-significant reduction was observed at 5 μM concentration. By far the best result was obtained with compound **4e** which displayed significant cellular growth depression at 20, 10 and 5 μM. Comparison of bicalutamide and **4e** at 5 μM shows the amount of cellular growth inhibition to be within 10% of each other, which is significant when considering comparative molecular and synthetic complexity.

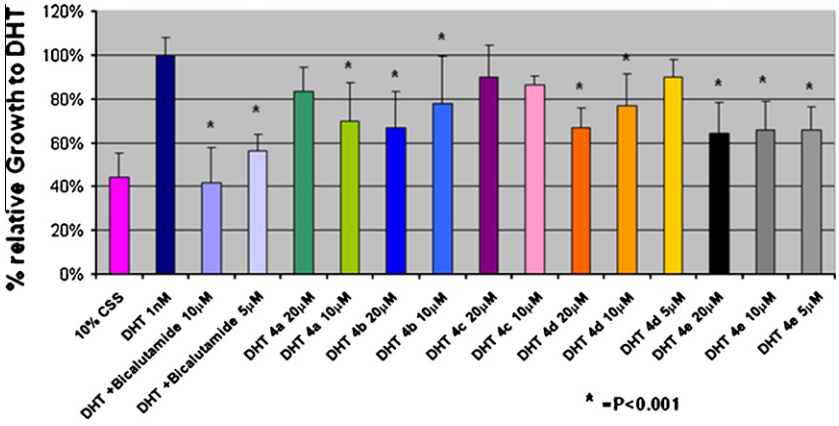


Fig. 3. Cell proliferation data for compounds **4a–e** day six after administration of compounds.

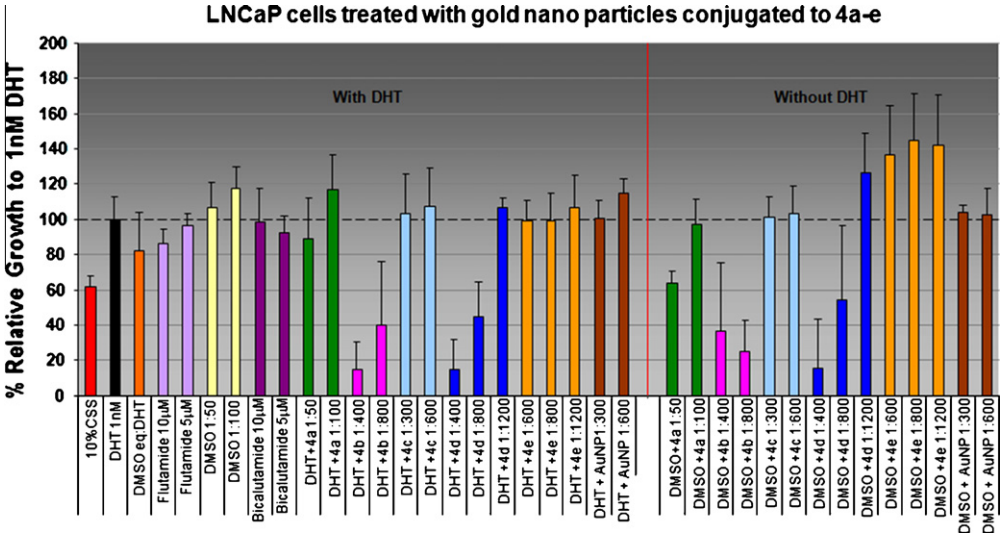


Fig. 4. LNCaP cells treated with fucntionalized gold nanoparticles.

Given these positive results we were interested in how conjugation to an inorganic nanoparticle would affect the biological efficacy of these compounds. Given that these compounds already possessed a disulfide due to the α-lipoic acid moiety, thus the use of gold nanoparticles (AuNP) was determined to be the best choice. Gold nanoparticles were synthesized according to a modified Brust–Schiffrin protocol [12], and were found to be very uniformly dispersed around 6.5 nm using dynamic light scattering [10].

The functionalization of gold nanoparticles is a straight forward process whereby tetraoctaneammonium bromide (TOAB) used in the synthesis of the nanoparticles is relatively weakly bound to the gold nanoparticle surface. This is easily displaced by the favorable Au-S bond which is thought to occur almost instantaneously. To ensure an ordered self-assembled monolayer (SAM) we left the α-lipoic amides stirring in the presence of the AuNPs overnight. A simple procedure of precipitation, centrifugation and washing ensured the removal of excess TOAB [10]. With these AuNP conjugates in hand our attention turned to reassessment of their ability to block the androgen-stimulated growth of LNCaP cells, as before Bicalutamide® was used as the positive control.

Initially, the AuNP conjugates were dissolved in DMSO at a concentration of 1 mg/mL and serially diluted to find a concentration at which these conjugates are not toxic, thus allow the determination of anti-proliferative properties. It was found that concentrations diluted from a 1 mg/mL stock solution to a range of 1:300–1:1200 suitable for assessment using LNCaP cells. These dilutions correspond to concentrations of 3.5 μg/mL to 0.88 μg/mL, respectively of the nanoparticle conjugates. As before, these compounds were assessed using Bicalutamide® as the positive control.

Two separate inprotocols were undertaken using the functionalized gold nanoparticles, this consisted of one proliferation assay conducted in the absence of 1 nM DHT (Fig. 4 – right) to assess potential toxicity of these compounds and another carried out under identical conditions but in the presence of 1 nM DHT to determine agonist activity.

By comparing the gold nanoparticle conjugates with and without DHT it is possible to elucidate any antagonistic activity shown by these gold conjugates. In most cases high levels of cell toxicity were observed at lower dilutions (1:300 and 1:400) while no anti-proliferative properties of these compounds were observed at higher dilutions (1:800 and 1:1200). Thus it seems that there is a serious tradeoff between toxicity and anti-proliferative activity, whereby dilutions which are not toxic to the cells are so dilute that there is no effect on cellular proliferation. The reason for both of these effects is more than likely due to the absence of biocompatible polymers (such as polyethylene glycol, PEG) being

incorporated into the AR antatonist molecular design. It has been shown that the use of PEG spacers between the AuNP surface and the small molecular drug has profound influence on cellular and systemic toxicity [13]. The use of a large spacer between AuNP and the small molecule has another benefit to the system whereby this spacer ensures that the biologically active molecule conjugated to the gold core protrudes from the surface a sufficient amount to allow interaction with the cellular surface. As such these compounds, while effective on their own require a slight redesign and synthesis to allow for a biocompatible polymer to be incorporated into the scaffold to by-pass the cellular toxicity observed in this case.

3. Summary

In summary we have synthesized a range of α-lipoic acid *N*-phenylamides **4a–e** bearing a representative range of electronic substituents and these compounds were then assessed for their ability to block androgen-stimulated proliferation of a human prostate cancer cell line, LNCaP. Various synthetic protocols were used for the synthesis of these compounds showing that no one method is optimal for both electron withdrawn and electron neutral anilines. Additionally these compounds generally displayed excellent anti-proliferative activities in the 20–5 μM range with compound **4e** showing by far the best cancer cell growth inhibition.

Conjugation of these compounds a gold nanoparticle and reassessment of their anti-proliferative potential has shown that cellular toxicity shown by the nanoparticle conjugates in a limiting factor before any further studies can take place.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bioorg.2011.11.007.

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EDCI = N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride; PyBrop = bromotripyrrolidinophosphonium hexafluorophosphate.

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[10] See Electronic Supplementary Information for Representative Procedures for Each Method A–D, and for the Biological Evaluation Process, Gold Nanoparticle Synthesis and Dynamic Light Scattering Analysis.
[11] Characterisation data for compounds 4c and 4e: 4c ^{1}H NMR ($CDCl_3$, 270 MHz): δ 7.84 (1H, s, Ar-H) 7.70–7.68 (1H, d, J = 8 Hz, Ar-H) 7.42–7.39 (1H, d, J = 12 Hz, Ar-H) 3.58–3.53 (1H, m, CH) 3.17–3.11 (2H, m, CH_2) 2.39–2.36 (4H, m, CH_2) 1.93–1.87 (2H, m, CH_2) 1.77–1.64 (2H, m, CH_2) 1.51–1.46 (2H, m, CH_2) (NH is not observed); ^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 171.8, 132.0, 128.6 (q, $^{2}J_{C-F}$ = 32 Hz), 126.9, 122.6 (q, $^{1}J_{C-F}$ = 272 Hz), 120.6, 118.9 (q, $^{3}J_{C-F}$ = 6 Hz), 116.6, 56.5, 40.4, 38.6, 37.3, 34.7, 28.9, 25.2; ^{19}F NMR ($CDCl_3$, 270 MHz) −62.67; HRMS (ESI m/z): Calcd. For $[C_{15}H_{17}F_3N_2OS_2\ Na^+]$ 406.02844, found 406.02763; 4e ^{1}H NMR ($CDCl_3$, 270 MHz): δ 8.4 (1H, s, NH) 8.04–8.03 (1H, d, J = 1.6 Hz, Ar-H) 7.93 (1H, dd, J = 1.89, 8.64 Hz, Ar-H) 7.74–7.71 (1H, d, J = 8.64 Hz, Ar-H) 3.59–3.49 (1H, qui, J = 6.48 Hz, CH) 3.20–3.03 (2H, m, CH_2) 2.50–2.32 (4H, m, CH_2) 1.97–1.77 (2H, m, CH_2) 1.72–1.62 (2H, m, CH_2) 1.59–1.30 (2H, m, CH_2); ^{13}C NMR ($CDCl_3$, 67.5 MHz): δ 172.1, 142.7, 136.9, 134.0 (q, $^{2}J_{C-F}$ = 32.5 Hz), 121.8122.2 (q, $^{1}J_{C-F}$ = 272 Hz), 117.3 (q, $^{3}J_{C-F}$ = 5.5 Hz), 117.2, 103.6, 56.4, 40.3, 38.6, 37.4, 34.6, 28.8, 24.9; ^{19}F NMR ($CDCl_3$, 270 MHz) −62.05; HRMS (ESI m/z): Calcd. For $[C_{16}H_{117}F_3N_2OS_2\ Na]^+$ 397.06266, found 397.06197.
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