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Discovery of non-peptide, small molecule antagonists of α9α10 nicotinic acetylcholine receptors as novel analgesics for the treatment of neuropathic and tonic inflammatory pain

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ABSTRACT

A series of azaaromatic quaternary ammonium analogs has been discovered as potent and selective α9α10 nicotinic acetylcholine receptor (nAChR) antagonists. The preliminary structure–activity relationships of these analogs suggest that increased rigidity in the linker units results in higher potency in inhibition of α9α10 nAChRs and greater selectivity over α7 nAChRs. These analogs represent a new class of analgesic for the treatment of neuropathic and tonic inflammatory pain.

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channel complexes composed of five (α and/or β) subunits, each spanning the membrane four times and aligning around the central ion channel.[1–3] Nine α (α1–7, α9, α10) and four β (β1–β4) subunits in mammals have been identified.[4] Homomeric or heteromeric combinations of the α and β subunits produce a variety of nAChR subtypes with distinct pharmacological properties.[5]

The α7 and α9 subunits are the only known nAChR subunits that can assemble into a functional homomeric subtype.[6] α9 nAChRs exist as both homomeric α9 and heteromeric α9α10 receptors; the latter is suggested to be the major subtype.[7,8] Mutagenesis studies indicate that the stoichiometry of the major form is α9(2)α10(3).[9] The α10 subunit has only been co-expressed functionally with an α9 subunit.[7] In *Xenopus* oocytes, the co-injection of α9 and α10 subunits boosts functional nAChR expression 100-fold or more compared to injection of α9 subunits alone.[7,8] The pharmacological profiles of the homomeric and heteromeric α9 nAChRs are essentially indistinguishable, and closely resemble those reported for endogenous cholinergic receptors found in vertebrate hair cells.[7] A number of pharmacologically unique features of α9α10 nAChRs have been observed:[10] (i) α9α10 nAChRs can be activated not only by acetylcholine (ACh), but also by the classical muscarinic agonist oxotremorine, indicating a mixed nicotinic/muscarinic pharmacology; (ii) α9α10 nAChRs are antagonized by most classical nAChR agonists, such as nicotine, cytisine, and epibatidine; (iii) the classical muscarinic agonist, muscarine, and the classical antagonist, atropine, both block α9α10 nAChRs; (iv) α-bungarotoxin (α-BTX) and methyllycaconitine (MLA) are antagonists of α9α10 nAChRs; and (v) a number of non-cholinergic antagonists, including strychnine (glycine receptor antagonist), bicuculline (GABA-antagonist) and ICS-205,930 ($5HT_3$ receptor antagonist), also potently block α9α10 nAChRs. Thus, α9α10 nAChRs have a pharmacological profile different from any other nicotinic or muscarinic cholinergic receptor subtype.

The α9 nAChR has been identified in a rich variety, yet restricted set of critical tissues, including lymphocytes and developing thymocytes, bronchial epithelium and airway fibroblasts, sensory organs, nervous tissue (dorsal root ganglia), and sperm, but are not present in brain.[11] To date, with the exception of innervation of the sensory hair cells in the inner ear,[7] the function of α9α10 receptors is still obscure, and research has been hampered by the paucity of selective ligands for probing receptor function. In this regard, chronic constriction of the sciatic nerve increases the number of nAChR-producing lymphocytes at the site of nerve injury,[12]

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and data suggests that locally released ACh from the lymphocytes stimulates α9α10 nAChRs to maintain the immune response and behavioral hypersensitivity.[12] Thus, the lymphocytic cholinergic system may be regulating local immune response associated with nerve injury.

Chronic pain afflicts approximately 20% of the adult population in developed nations.[13] Although there are numerous marketed analgesic drugs, they appear to act through a limited number of molecular mechanisms. Even when these medications are used in combination, substantial pain often persists. Therefore, it is highly desirable to develop new treatments that act through alternative mechanisms of action.[14]

A growing body of evidence indicates that α9α10 nAChR antagonism may represent a novel mechanism to produce analgesia.[10,15,16] α-Conotoxins Vc1.1 and RgIA are antagonists of α9α10 nAChRs.[12] The subcutaneous or intramuscular administration of Vc1.1 and RgIA acutely alleviates pain resulting from traumatic, inflammatory, and metabolic neuronal injury.[15] Intriguingly, analgesia appears to continue for days or weeks after administration of the conotoxin is discontinued, indicating that blockade of α9α10 nAChRs with these peptides may have disease-modifying effects.[15] Thus, non-peptide, small molecule antagonists of α9α10 nAChRs on immune cell membranes may have the ability to mediate restorative effects rather than just symptomatic management of nerve injury, and thus, represent novel, potential therapeutics for modulating neuropathic and tonic inflammatory chronic pain.[10,12,15,16]

During the course of our search for potent and selective small molecule nAChR antagonists, we generated a diverse library of novel quaternary ammonium analogs.[17–25] Recently, a number of these analogs were found to be highly potent α7 nAChR antagonists.[26] Since the classic α7 antagonists α-BTX and MLA are also potent α9α10 antagonists,[7] we hypothesized that these quaternary ammonium analogs might also be antagonists at α9α10 nAChRs. Thus, in the present work, we evaluated a family of bis-, tris-, and tetrakis-azaaromatic quaternary ammonium analogs (general structures **1–8**, Fig. 1; **9** and **10**, Fig. 2; and **11** and **12**, Fig. 2, respectively) for their ability to inhibit α9α10 nAChR-mediated responses in *Xenopus* oocytes.

Bis-quaternary ammonium analogs of general structures **1–8** (Fig. 1 and Table 1) were prepared by coupling of the linker precursors **13–20** with the appropriate azaaromatic head-group precur-

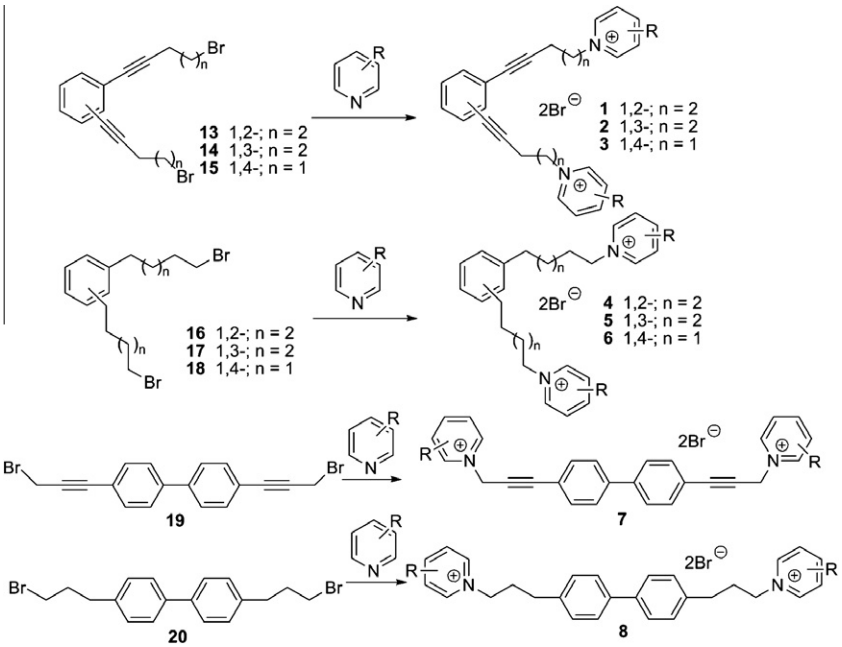


Figure 1. General synthesis and structures of the bis-azaaromatic quaternary ammonium analogs.

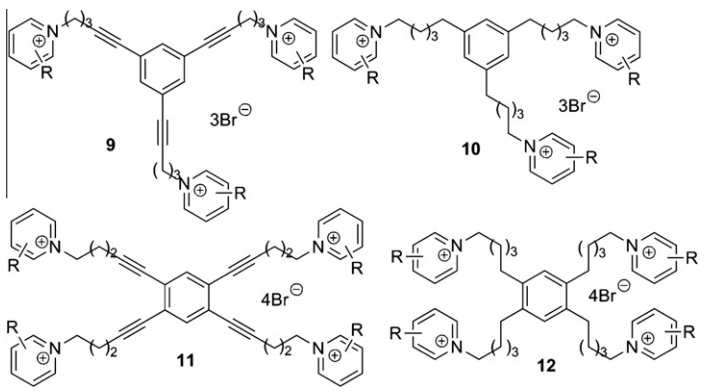


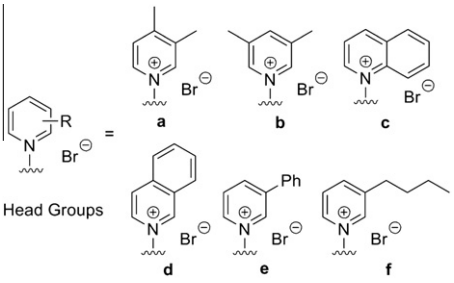
Figure 2. General structures of the tris- and tetrakis-azaaromatic quaternary ammonium analogs.

sors (Table 1, Fig. 1). Dibromide **13**–**18**, linker precursors for the synthesis of analogs **1**–**6**, have been synthesized previously.[23] Dibromides **19** and **20**, linker precursors for the synthesis of analogs **7** and **8**, were prepared utilizing similar procedures as those used for the synthesis of **13**–**18**, via Sonogashira coupling of 4,4′-dibromobiphenyl with propargyl alcohol. The synthesis of the tris- and tetrakis-quaternary ammonium analogs **9**–**12** has been described previously.[24,25]

The analogs were tested initially at a probe concentration of 100 nM on cloned α9α10 nAChRs heterologously expressed in *Xenopus* oocytes for their ability to block ACh-gated currents. Data represent percentile inhibitory responses during the co-application of ACh and analog, and are normalized to the responses to ACh alone (Table 1).[27]

The preliminary screen (Table 1) identified a number of analogs that potently inhibited α9α10 ACh-gated currents, from which tetrakis-analogs **11b**, **11e**, and **12b** exhibited nearly complete inhibition at the probe concentration. In addition, analogs containing three or four quaternary ammonium head-groups were more effective in inhibiting α9α10 receptors at the probe concentration when compared with analogs within the bis-, tris-, and tetrakis-series. Furthermore, analogs bearing bulky, more hydrophobic cationic head-groups generally exhibited greater inhibition of α9α10 nAChRs, with the exception of the tetrakis-analog series, in which the 3,4-lutidinium analogs **11b** and **12b** were more potent than the isoquinolinium analogs **11d** and **12d**.

Table 1
ACh-gated current responses at α9α10 nAChRs after co-application of 100 nM of azaaromatic quaternary ammonium analogs



Compound[a]	α9α10 nAChRs (% response)[b]	Compound[a]	α9α10 nAChRs (% response)[b]
1a	31.3 ± 5.8	**4a**	72.0 ± 6.9
1b	67.6 ± 16.9	**4b**	124.4 ± 10.2
		4c	10.9 ± 1.4
1d	28.03 ± 3.87	**4d**	31.15 ± 8.65
2a	76.9 ± 11.9	**5a**	62.7 ± 7.7
2c	41.6 ± 6.7	**5c**	42.6 ± 9.5
2d	39.7 ± 11.4	**5d**	37.5 ± 6.6
2e	23.2 ± 3.8		
3a	10.6 ± 4.6	**6a**	36.7 ± 11.3
3b	76.2 ± 8.3	**6b**	97.1 ± 9.9
7a	8.1 ± 6.1	**8a**	89.3 ± 10.8
		8b	113.8 ± 4.7
9a	61.12 ± 6.95	**10a**	62.74 ± 7.85
9b	66 ± 12.61	**10b**	79.77 ± 9.71
9c	10.46 ± 3.48	**10c**	15.9 ± 3.5
		10d	6.9 ± 4.71
9e	5.5 ± 2.6	**10e**	3.84 ± 1.95
9f	36.15 ± 7.23	**10f**	16.1 ± 9.3
11a	47.4 ± 8.2	**12a**	11.0 ± 3.1
11b	0.3 ± 0.07	**12b**	0.6 ± 0.4
11d	11.6 ± 3.1	**12d**	17.9 ± 3.3
11e	0.3 ± 0.09	**12e**	6.5 ± 2.9

[a] The number in the compound designation corresponds to the type of linker as shown in Figure 1. The letter corresponds to the head groups.
[b] Inhibition of α9α10-mediated responses to 10 μM ACh in the presence of 100 nM of analog, the data represent % of response; Each value represents data from 3 to 8 independent experiments.

Selected compounds, including **7a**, **8a**, **9e**, **10c**, **10d**, **10e**, **11e**, and **12e**,[28] were evaluated further in full concentration–response studies for inhibition of ACh-gated currents (Table 2). Analog **11e**, a tetrakis-quaternary ammonium compound with four 3-phenylpyridium head-groups, exhibited the highest potency in this series, with an IC_{50} value of 0.56 nM. Notably, analogs containing the more rigid triple bond linker units, that is, **7a** (IC_{50} = 16 nM), **9e** (IC_{50} = 1.7 nM), and **11e** (IC_{50} = 0.56 nM), were more potent than their corresponding alkyl linked analogs **8a** (IC_{50} = 670 nM), **10e** (IC_{50} = 3.7 nM), and **12e** (IC_{50} = 5.4 nM), respectively, indicating that a conformationally rigid central core scaffold appears to be critical for optimal inhibitory activity at α9α10 nAChRs.

The above quaternary ammonium analogs are expected to have very limited brain bioavailability due to the highly polar nature of their cationic head-groups, which makes such compounds more attractive as antagonists for α9α10 nAChRs, due to the absence of this receptor in brain. On the other hand, in the design of antagonists of α9α10 nAChRs for chronic pain, their selectivity over peripheral α7 nAChRs is critical, due to the fact that antagonism of these two peripheral nAChRs produces diametrically opposite pharmacodynamic effects.[15] Thus, the lead analogs in Table 2 also were evaluated for their ability to block ACh-gated currents in α7 nAChRs heterologously expressed in *Xenopus* oocytes (Table 2). Interestingly, the presence of a rigid central core scaffold appears to be important in affording selectivity for the α9α10 subtype over the α7 subtype. Bis-analogs **7a** and **9e**, which incorporate conformationally restricted linker units in their structures, were 75-fold and 109-fold more selective, respectively, for the α9α10 subtype versus the α7 subtype. Their corresponding alkyl linker counterparts, **8a** and **10e**, were only 9- and 25-fold more selective, respectively, for the α9α10 versus α7 subtypes. However, conformational restriction of the linker units in the tetrakis-series of analogs appears to have no significant effect on selectivity, since analogs **11e** and **12e** were equipotent, at α9α10 and α7 nAChRs. Interestingly, the nature of the head-group appears to have greater influence on selectivity than potency. For example, IC_{50} values of the tris-analogs **10c**, **10d**, and **10e** at α9α10 are similar, whereas selectivity for the α9α10 subtype versus the α7 subtype is 360-, 35-, and 25-fold, respectively, for these three analogs. Thus, analog **11e** was the most potent α9α10 antagonist (IC_{50} = 560 pM), but was less selective vs α7 when compared to the slightly less potent analog **10c**.

In preliminary proof-of-concept studies, the most potent α9α10 antagonists, compounds **7a**, **10c**, and **11e**, have recently been evaluated in in vivo rat models of pain.[16,29] These three analogs were found to be ineffective as analgesics in the rat tail-flick test, but demonstrated an analgesic effect in the rat formalin model of tonic inflammatory pain (phase 2) and reversed mechanical hyperalgesia in a dose-related manner in the rat chronic constriction nerve injury (CCI) model of neuropathic pain. Furthermore, tolerance to the antihyperalgesic effect was not observed after repeated dosing (seven days) of drug treatment. In addition, efficacy was achieved at doses well below those that produced toxicity in the motor func-

Table 2
IC_{50} values for the azaaromatic quaternary ammonium analog inhibition of α9α10 and α7 nAChRs

Compounds	α9α10 nAChR (IC_{50}, nM)	α7 nAChR (IC_{50}, nM)
7a	16	1190
8a	670	6010
9e	1.7	185
10c	4.2	1510
10d	4.8	170
10e	3.7	91
11e	0.56	8.8
12e	5.4	99

tion rotarod test. The above analogs were 10 to 5000-fold less potent as antagonists at other nAChRs, including α1β1δε, α2β2, α2β4, α3β2, α3β4, α4β2, α4β4, α6α3β2β3, and α6α3β4 subtypes; The most potent analog **11e** is a selective inhibitor of α9α10 nAChRs, having relatively poor inhibitory potency against α4β2 (IC_{50} = 3.8 μM), α3β4 (IC_{50} = 0.8 μM), and muscle (IC_{50} = 0.12 μM) nAChRs.[16] We have also shown that analog **11e** does not interact with $GABA_A$ or $GABA_B$ receptors, and has several orders of magnitude lower affinity for $5HT_3$ and κ-opioid receptors compared to α9α10 nAChRs (unpublished data). More recently, the bis-analog **7a**, was shown to be effective in alleviating vincristin-induced neuropathic pain in the rat CCI pain model.[30] These collective results provide support for the contention that α9α10 nAChRs are a new target for studying pain modulation, and suggest that further development of small, non-peptide α9α10 antagonists as treatments for chronic pain may lead to novel, clinically useful, non-opioid analgesics.

In conclusion, a series of azaaromatic quaternary ammonium analogs has been discovered that are potent and selective antagonists of α9α10 nAChRs. These first-in-class compounds represent excellent leads for the subsequent development of highly potent and subtype-selective small molecule antagonists at α9α10 nAChRs as treatments for chronic pain.

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27. In the voltage clamp electrophysiology assays (Tables 1 and 2), *Xenopus* oocytes were voltage-clamped and exposed to ACh and test compounds as previously described.[11] Briefly, the oocyte chamber consisting of a cylindrical well (~30 μL in volume) was gravity perfused at a rate of ~2 mL/min with ND96 containing 0.01% (wt/vol) BSA and 1 μM atropine to block potential contaminating signal from endogenous muscarinic receptors. For experiments involving α7 and α9α10, atropine was excluded from the perfusion solution because it has been shown to block these receptor subtypes. Oocytes were exposed once a minute to 1 s pulses of ACh. ACh concentrations used were 200 μM for α7, 10 μM for α1β1δε and α9α10 and 100 μM for all other subtypes. Compounds were applied at the beginning of a five min static bath incubation. The % block was calculated as a% of ND96 control (no compound) response. Concentration–response data were fit to the equation $Y = 100/(1 + 10^{((\log IC_{50} - \log[toxin])*Hill\ slope)})$ by nonlinear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA). Data points in the concentration–response represent the mean ± SEM from at least three oocytes.
28. *Spectra data of selective compounds*: compound **7a**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.01 (s, 2H), 8.97 (d, *J* = 6.3 Hz, 2H), 7.99 (d, *J* = 6.3 Hz, 2H), 7.76 (dd, *J* = 6.6, 2.1 Hz, 4H), 7.61 (dd, *J* = 6.6, 2.1 Hz, 4H), 5.81 (s, 4H), 2.52 (s, 6H), 2.40 (s, 6H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 159.6, 143.1, 142.0, 140.4, 138.7, 133.1, 128.9, 127.7, 120.9, 88.8, 83.1, 50.4, 20.7, 17.2 ppm; **8a**, ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.82 (s, 2H), 8.73 (d, *J* = 6.3 Hz, 2H), 7.78 (d, *J* = 6.3 Hz, 2H), 7.46 (d, *J* = 8.4 Hz, 4H), 7.29 (d, *J* = 8.4 Hz, 4H), 4.64 (t, *J* = 7.2 Hz, 4H), 2.79 (t, *J* = 7.2 Hz, 4H), 2.47 (s, 6H), 2.39 (s, 6H), 2.32–2.42 (m, 4H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.4, 143.0, 141.4, 139.6, 138.5, 138.3, 129.0, 128.3, 126.6, 60.6, 32.6, 32.0, 19.4, 16.1 ppm; **9e**, ^{1}H NMR (300 MHz, CD_3OD) δ 9.50 (s, 3H), 9.09 (d, *J* = 6.0 Hz, 3H), 8.78 (d, *J* = 8.1 Hz, 3H), 8.16 (dd, *J* = 8.1, 6.0 Hz, 3H), 7.75–7.87 (m, 6H), 7.42–7.65 (m, 9H), 7.17 (s, 3H), 4.96 (t, *J* = 6.9 Hz, 6H), 2.69 (t, *J* = 6.3 Hz, 6H), 2.42 (m, 6H) ppm; ^{13}C NMR (75 MHz, CD_3OD) δ 144.4, 144.3, 144.2, 142.6, 134.9, 134.5, 131.5, 130.8, 129.5, 128.7, 125.1, 90.0, 81.5, 62.6, 30.8, 17.4 ppm; **10c**, ^{1}H NMR (300 MHz, CD_3OD) δ 9.46 (dd, *J* = 6.0, 1.5 Hz, 3H), 9.22 (d, *J* = 8.4 Hz, 3H), 8.57 (d, *J* = 9.0 Hz, 3H), 8.45 (dd, *J* = 8.4, 1.5 Hz, 3H), 8.30 (m, 3H), 8.02–8.14 (m, 6H), 6.80 (s, 3H), 5.11 (t, *J* = 7.5 Hz, 6H), 2.56 (t, *J* = 7.5 Hz, 6H), 2.14 (m, 6H), 1.69 (m, 6H), 1.52 (m, 6H) ppm; ^{13}C NMR (75 MHz, CD_3OD) δ 150.3, 148.9, 143.4, 139.4, 137.3, 132.2, 131.8, 131.4, 127.2, 123.1, 119.9, 59.4, 36.7, 32.3, 31.1, 27.3 ppm; **10d**, ^{1}H NMR (300 MHz, CD_3OD) δ 9.99 (s, 3H), 8.69 (dd, *J* = 6.9, 1.5 Hz, 3H), 8.47–8.54 (m, 6H), 8.22–8.36 (m, 6H), 8.07 (m, 3H), 6.78 (s, 3H), 4.78 (t, *J* = 7.5 Hz, 6H), 2.53 (t, *J* = 7.5 Hz, 6H), 2.15 (m, 6H), 1.67 (m, 6H), 1.44 (m, 6H) ppm; ^{13}C NMR (75 MHz, CD_3OD) δ 150.8, 143.2, 138.8, 138.2, 135.8, 132.5, 131.5, 129.0, 128.5, 127.5, 127.1, 62.8, 36.5, 32.3, 32.0, 26.8 ppm; **10e**, ^{1}H NMR (300 MHz, CD_3OD) δ 9.39 (s, 3H), 8.98 (d, *J* = 6.0 Hz, 3H), 8.85 (ddd, *J* = 6.0, 1.8, 1.2 Hz, 3H), 8.15 (dd, *J* = 8.1, 6.0 Hz, 3H), 7.78–7.90 (m, 6H), 7.50–7.65 (m, 9H), 6.82 (s, 3H), 4.74 (t, *J* = 7.8 Hz, 6H), 2.55 (t, *J* = 7.5 Hz, 6H), 2.11 (m, 6H), 1.69 (m, 6H), 1.45 (m, 6H) ppm; ^{13}C NMR (75 MHz, CD_3OD) δ 144.2, 143.9, 143.4, 142.8, 134.6, 131.5, 130.8, 129.4, 128.7, 127.2, 63.3, 36.7, 32.7, 32.2, 27.0 ppm; **11e**, ^{1}H NMR (300 MHz, CD_3OD) δ 9.50 (s, 4H), 9.09 (d, *J* = 6.0 Hz, 4H), 8.74 (dd, *J* = 8.4, 1.2 Hz, 4H), 8.14 (dd, *J* = 8.1, 6.0 Hz, 4H), 7.70–7.85 (m, 8H), 7.45–7.62 (m, 12H), 7.23 (s, 2H), 4.95 (t, *J* = 6.9 Hz, 8H), 2.71 (t, *J* = 6.6 Hz, 8H), 2.30–2.47 (m, 8H) ppm; ^{13}C NMR (75 MHz, CD_3OD) δ 144.2, 142.6, 136.2, 134.5, 131.5, 130.8, 129.6, 128.7, 126.1, 94.8, 81.0, 62.6, 31.1, 17.7 ppm; **12e**, ^{1}H NMR (300 MHz, CD_3OD) δ 9.48 (s, 4H), 9.05 (d, *J* = 6.0 Hz, 4H), 8.83 (d, *J* = 8.4 Hz, 4H), 8.14 (dd, *J* = 7.8, 6.3 Hz, 4H), 7.89 (dd, *J* = 7.8, 1.2 Hz, 8H), 7.40–7.65 (m, 12H), 6.85 (s, 2H) 4.80 (t, *J* = 7.5 Hz, 8H), 2.52 (t, *J* = 6.9 Hz, 8H), 2.11 (t, *J* = 6.6 Hz, 8H), 1.37–1.69 (m, 16H) ppm; ^{13}C NMR (75 MHz, CD_3OD) δ 142.9, 142.8, 142.6, 141.2, 137.3, 133.3, 130.3, 130.1, 129.6, 128.4, 127.6, 62.1, 32.0, 31.7, 31.0, 26.2 ppm.
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